THE INTERPLAY BETWEEN IMMUNE ACTIVATION AND CARDIOVASCULAR DISEASE DURING INFECTION, AUTOIMMUNITY AND AGING: THE ROLE OF T CELLS

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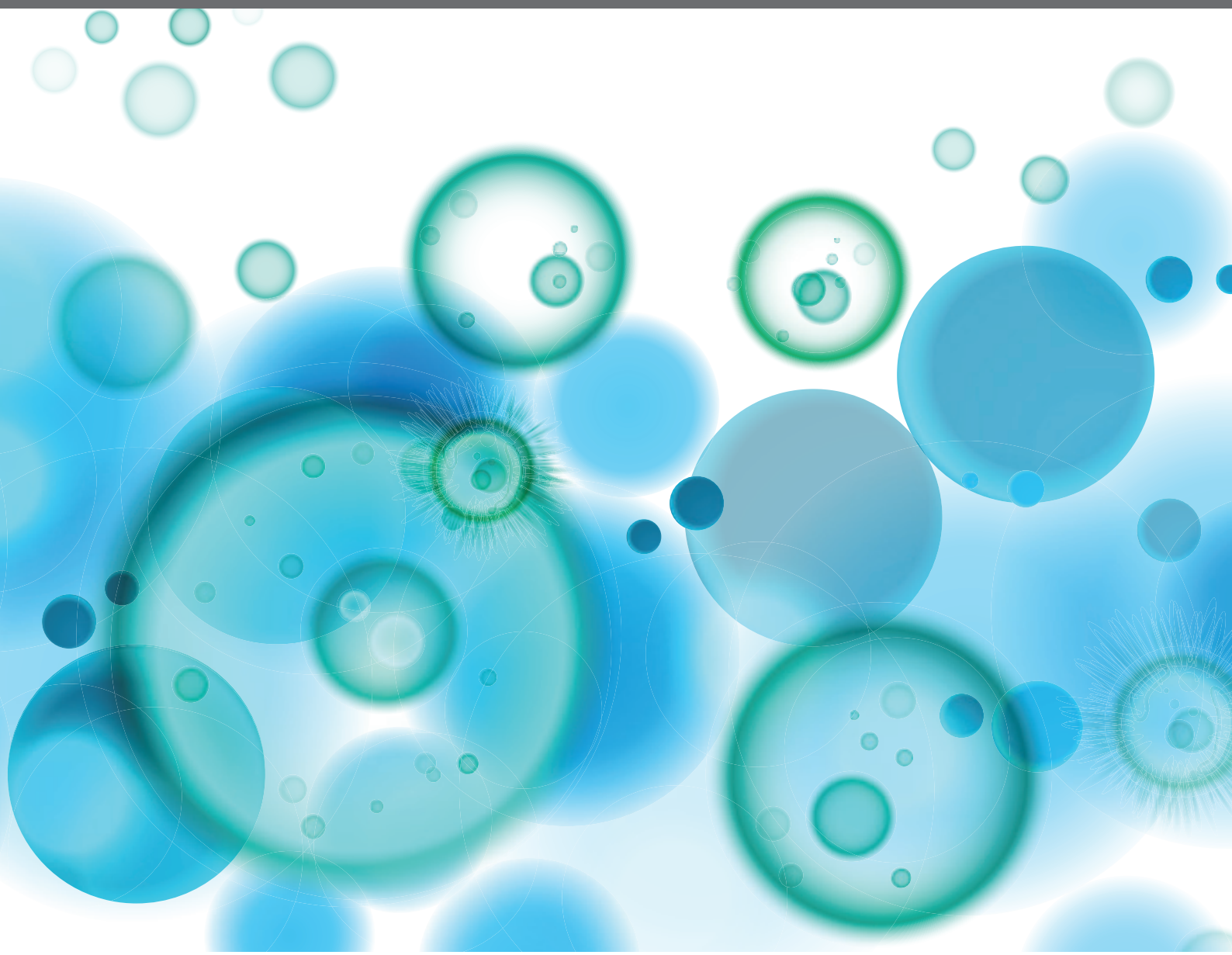

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THE INTERPLAY BETWEEN IMMUNE ACTIVATION AND CARDIOVASCULAR DISEASE DURING INFECTION, AUTOIMMUNITY AND AGING: THE ROLE OF T CELLS

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EDITORIAL
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Editorial: The Interplay Between Immune Activation and Cardiovascular Disease During Infection, Autoimmunity and Aging: The Role of T Cells

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Editorial on the Research Topic

The Interplay Between Immune Activation and Cardiovascular Disease During Infection, Autoimmunity and Aging: The Role of T Cells

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INTRODUCTION

Chronic activation of cells of the immune system including T cells and systemic inflammation are well known risk factors for cardiovascular disease (CVD). Many human pathological conditions including viral infections, autoimmune diseases and aging are recognized drivers of increased risk of CVD. Among viral infections, Cytomegalovirus (CMV) infection is a contributing risk element to the existing traditional risk factors of atherogenesis; Influenza infection is correlated with increased the risk of cardiovascular events leading to deaths and HIV infection is an independent predictor of cardiovascular risk. The pandemic of SARS-COV2 infection showed that the severe presentation of the disease manifests with vascular damage and cardiovascular events. Autoimmune and chronic inflammatory diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and psoriatic disease are also associated with cardiovascular disease. Lastly, in adults over 65 years, the accumulation of age-related phenotypic and functional alterations in immune cells parallels with a decline of the cardiovascular system with an increased incidence of cardiovascular disease.

The mechanisms behind are not well defined, and while the role of innate immune cells has been established, the involvement of T cells in promoting vascular pathology and cardiovascular disease has emerged more recently (1). Chronic systemic inflammation and increased circulating levels of cytokines and chemokines can indeed contribute to vascular damage by promoting endothelial cell activation and oxidative stress thus linking to the increased risk of CVD (2, 3). Activation of endothelial cells promotes recruitment of circulating immune cells including T cells that will be activated and differentiate into distinct effector cells contributing to the pathology of the disease (4–6).

Endothelial cells in this context have also been proposed to act as "semiprofessional" antigen presenting cells (APC) presenting antigens and providing several costimulatory signals to T cells leading to T cell activation especially at sites defined as endothelium-dependent microvascular

reactivity sites by Laser Doppler Flowmetry Assessment (7). A milestone in the understanding the role of T cells in promoting vascular inflammation has been reached with the characterization of the immune cell infiltrate in human atherosclerotic plaque by scRNA-seq technology which defined the main subsets of T cells in atherosclerosis (8). This data paved the way for further investigations about the role of T cells as a putative mechanistic link in pathologies associated with an increased risk of CVD. The proposed mechanisms by which T cells contribute to the pathology of the disease include dysregulated T helper and CD8 T cell function, expansion of terminally differentiated cytotoxic effectors $CD4^+CD28^-$ T cells and impaired Tregs function.

This Research Topic has the aim to provide an overview of the latest advances in the study of the role of T cell activation and endothelial inflammation in cardiovascular risk and disease in the context of infection, autoimmunity and aging. The Research Topic highlights the emerging common and distinctive features of the putative immune mechanistic links between the pathophysiological conditions and the associated cardiovascular disease. The Research Topic comprises 11 articles, original research articles, 5 review and one systematic review and was divided into 3 sections.

1. Endothelial inflammation and T cell activation in CVD associated with infection.
2. T cell mechanisms involved in CVD associated with autoimmune diseases.
3. The immunology of cardiovascular disease during aging.

T CELL ACTIVATION, ENDOTHELIAL INFLAMMATION IN THE SETTING OF INFECTION AND ITS IMPACT IN CVD RISK

Platelets play an important role in the immunity against pathogens, and in the setting of chronic inflammation they can promote vascular damage, atherogenesis, and thrombosis. In this review by Zamora et al. the authors focus the attention in the role of platelets (PLTs), systemic inflammation and CVD. This article highlights the dual role of platelets in promoting both pro-and anti-inflammatory processes and underlines their capability to interact with leukocytes and endothelial cells. They discuss that this mechanism contributes to systemic inflammation by favoring the arrest of leukocytes on endothelium, the production of inflammatory cytokines and NETs formation. On the other hand, the binding of PLTs to leukocytes decreases the inflammatory response, participating in the resolution of thrombo-inflammation. The authors also mention the use of platelet to lymphocyte ratio as an emerging biomarker of systemic inflammation and cardiovascular outcomes.

In this complex interaction between cells of the immune system and the vasculature, Ferrari et al. illustrate the importance of purinergic signaling in modulating pro- and anti-atherogenic responses, particularly in T cells and macrophages. Extracellular nucleotides (ATP, ADP, UTP and UDP) and nucleosides such as adenosine are known to be involved in the onset of pathologic states including blood hypercoagulability, thrombosis and atherosclerosis. Nucleotides and nucleosides signal through P2 and P1 receptors respectively modulating a wide array of cell and tissue functions. Particularly, extracellular ATP is an important activator of NLRP3 inflammasome, and the lack of the purinergic receptor P2X7 (P2 receptor) signaling in mouse model of atherosclerosis showed decreased disease development. Therefore, these data strengh the importance of investigating purinergic signaling in inflammasome activation and atherosclerosis.

In the setting of chronic infections immune activation and systemic inflammation is also associated with increased cardiovascular risk. People with HIV (PWH) infection have twice the risk of cardiovascular disease compared to the general population (9). Endothelial inflammation and injury play a critical role in the pathogenesis of cardiovascular risk/disease. The homeostasis, maintenance and repair of endothelial cells is mediated in part by bone marrow-derived endothelial cell progenitors and other cell progenitors from the hematopoietic origin participate in this process. In addition, a subset of T cells, angiogenic T cells (T_{angs}) can support the proliferation and differentiation of endothelial progenitor cells. In this manuscript, Zhu et al. investigated the role of HIV driven immune activation in the process of endothelial repair. They found two subsets of circulating progenitors $LIN4^-CD45^-CD34^+$ and $LIN4^-CD45^{dim}CD34^+$ in PWH, and showed that the phenotype but not frequencies was associated with biomarkers of inflammation. Importantly, CD8 T_{ang} cells express the chemokine receptor CX3CR1 suggesting the recruitment of pro-inflammatory T cells to the sites of endothelial injury. This data provides a new tool to better address the impact of HIV infection in endothelial inflammation and repair and the players involved in the process.

In the review by Gopal R et al., the authors discuss in depth the increased risk of cardiovascular morbidity and mortality during influenza infection. They examine the immune mechanisms driving the pathogenesis and cardiovascular risk, and they provide striking similarities and differences between the epidemiological and pathogenic mechanisms involved in cardiovascular events associated with coronavirus disease 2019 (COVID-19) and influenza infection. The authors highlight the pathology induced by either direct viral infection or indirect tissue damage due to the inflammatory cytokine storm in these infections, and the impact of these factors when combined with underlying conditions such as atherosclerosis. Interestingly, while influenza virus triggers type I and type II IFN responses, the induction of IFNs in the setting of SARS-CoV2 infection, in respiratory epithelial cells is low and this may contribute to the increase viral replication and immunopathology in the lung. Lastly, the authors discuss the potential role of the dysregulation of the immune responses and the increased risk of cardiovascular events.

The original article by Huaman et al. investigates the role of *Mycobacterium tuberculosis* infection and risk of cardiovascular disease. Studies had indicated that people with a history of tuberculosis disease, and latent tuberculosis infection have higher risk of coronary artery stenosis and myocardial infarction.

To address the mechanisms behind these associations, they use the *Ldlr*$^{-/-}$ mice, a murine model of atherosclerosis. They evaluate the impact of *M. bovis* BCG infection in exacerbating disease. They found that despite no significant differences in plasma cholesterol or triglyceride levels between the infected and uninfected mice fed with western diet, infection induced immunological changes in T cells (increase CD4/CD8 ratio) and monocytes (Ly6C low non-classical monocytes) that were associated with increased atherosclerotic lesion formation in the aorta. These data propose a mechanistic model in which mycobacterial infection is capable of enhancing atherosclerosis development.

T CELL MECHANISMS INVOLVED IN CVD ASSOCIATED WITH AUTOIMMUNE DISEASES

The role T cells in CVD associated with autoimmune and chronic inflammatory diseases is comprehensively summarized by the review article of this Research Topic by Schwartz et al. focused on evidence indicating that T cells are drivers of vascular inflammation in autoimmunity associated CVD. In particular it is considered the role of T cells in primary vasculitis and on three systemic autoimmune diseases: such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and psoriasis.

The authors first underline the role of T cells dysregulation in systemic autoimmunity supported by the association between early onset autoimmunity and mutations in FOXP3 and CTLA4 genes and single nucleotide polymorphisms (SNPs) in genes involved in T cell functions. They point out that autoimmune diseases are often paralleled by the presence of autoreactive T cells opening the concept that self-reactive T cells may represent the link between autoimmune disease and the risk of CVD in patients with RA, SLE and psoriasis. Accordingly, in rheumatoid arthritis, CD4 and CD8 T_{EM} cells are expanded and correlate with coronary artery calcification. In addition, expansion of $CD4^+CD28^-$ T cells are observed in these patients with preclinical atherosclerosis. Th17 subset on the other hand, is likely to play a major role in linking CVD and atherosclerosis in psoriasis. In addition, CD4 expressing CXCR3 have been shown to play a role in the migration to the arterial wall and induction of vascular inflammation in the setting of SLE. Interestingly Abatacept treatment in SLE patients decreases the expansion $CD4^+CD28^-$ T cells.

The role of T cells in vasculitis is further discussed in the review presented by Watanabe et al. focusing on large vessels vasculitis such as Giant Cell Arteritis (GCA) and Takayasu arteritis (TAK) that are characterized by an aggressive inflammation of arteries leading to hemorrhage and occlusions of the vessels. T cells play a major role in the pathogenesis of these two diseases which have long been considered largely overlapping. The authors underline the substantial differences between giant cells arthritis and Takayasu arteritis in both genetic association and the contribution of CD4 and CD8 T cell subsets in the pathology of the disease. Moreover, the authors discuss the considerable resistance to treatment underlining the importance to evaluate signaling pathways involved and their redundancy to therapeutically target the most relevant mechanism in each disease/patients. These include the sustained activation of mTORC1 and the JAK STAT pathway, that leads to a sustained immune responses in arterial wall, an immune privileged site that is intolerant to damage.

The involvement of T cells in CVD is further discussed in the manuscript by Bortoluzzi et al. Specifically, the study is focused on angiogenic T cells and $CD4^+$ and $CD8^+CD28^-$ T cell subsets in patients with Systemic Lupus Erythematosus and CVD. Angiogenic T (T_{ang}) cells are a subset of T cells ($CD3^+CD31^+CXCR4^+$) that promotes vasculogenesis and their characterization represents a promising field of research in cardiovascular medicine. Through the secretion of proangiogenic factors T_{ang} cells exert a critical role in the formation of colonies of endothelial progenitor cells (EPCs) as well as in their differentiation and function with a resulting protective role in the context of CVD. Within T_{ang} subset, in SLE patients, there was a significant expansion of a subpopulation with immunosenescent characteristics such as the loss of the costimulatory molecule CD28, required for T cell activation, survival and proliferation. This exhausted subset of T_{ang} correlated with SLE disease activity index- and was inversely related to T_{ang} cells percentage. The data of this study definitely point out the importance of reinforcing the knowledge of loss of CD28 in the T_{ang} compartment in relation to cardiovascular risk and lower percentage of endothelial precursor cells in SLE patients.

Further Chernomordik et al. analyze the role of autoreactive T cells in CVD. They analyzed the presence of reactive T cells to the cathelicidin antimicrobial peptide LL-37 in patients with acute coronary syndrome. In a murine model of atherosclerosis ($ApoE^{-/-}$) immunized with the mouse orthologue of LL-37, mCRAMP, they present evidence of role of LL-37 reactive T cells in the pathology of the disease. LL-37 is a major driver in the initial phase of psoriasis pathogenesis and a target of self-reactive T cells identified in patients. LL-37 is also present in human atherosclerotic plaques, and it associates with platelet activation and induction of thrombosis. Therefore, the authors investigated these molecules as a potential linker between the innate immunity activation in chronic inflammation with autoreactive T cell generation and cardiovascular disease. The study shows that LL-37 stimulation of PBMCs from patients with acute coronary syndrome induced the persistence of CD8 T_{EM} cell response compared to PBMCs from patients with stable coronary artery disease. However, in ApoE deficient mouse, adoptive transfer of T cells from mice immunized with mouse orthologue of LL-37 mCRAMP was associated with smaller atherosclerotic aortic plaque area and absence of aortic sinus plaque calcification. This work represents a starting point of an interesting future perspective that could reveal key aspects of the mechanistic link between DAMP-mediated priming of a chronic inflammatory/autoreactive response and cardiovascular events.

The study by Zhao R. et al. analyzes the expression of Aryl hydrocarbon receptor (AHR) in peripheral blood mononuclear cells (PBMCs) from patients with Type-2 diabetes (T2D) and metabolically healthy obesity (MHO). AHR is a ligand-activated transcription factor regulated by small molecules derived from

diet, metabolism or xenobiotics and critical transcription factor determining the lineage commitment of pro-inflammatory Th17 and Th22. Its ligands have been epidemiologically linked to obesity and type-2 diabetes. The results reported in this research article indicate that the expression of AHR mRNA in PBMCs was increased in patients compared to healthy subjects and correlated with plasma levels of IL-17 and IL-22 in metabolically healthy obese and T2D patients. Correlation was also observed with serum hsCRP levels and with the index of insulin resistance. The authors highlight a potential role of AHR in the interplay between metabolism, inflammatory status and the development of obesity and T2D, and hypothesize a role for AHR as a sensor involved in metabolic stress response.

THE IMMUNOLOGY OF CARDIOVASCULAR DISEASE DURING AGING

Aging involves highly variable age-related changes in all organs, tissues and cells, and confers vulnerability to different stressors and diseases. Of particular interest are the age-related changes in the immune and cardiovascular systems. While aging of the cardiovascular system is associated with highly prevalent age-related diseases, a growing body of knowledge has shown that the aging of the immune system plays an active role of cardiovascular health.

In this Research Topic, Delgobo et al. analyze the relationship between the age-related accumulation of terminally differentiated CD4 T cells and myocardial inflammation. Using a xenograft mouse model this study elegantly shows that human naïve CD4 T cells, when transferred to immune-depleted mice, undergo homeostatic proliferation and differentiation of effector cells. Together with the accumulation of differentiated and terminally differentiated CD4 T cells, the authors observed an increase of effector CD4 T cell infiltration, monocytes, macrophages and dendritic cells infiltrating the myocardium, a phenotype that recapitulates age-related changes in elderly individuals. This interesting study suggests that the age-related accumulation of terminally differentiated CD4 T cells could play a critical role in promoting myocardial alterations in the aging human heart.

However, while trying to understand the interconnection and influence of the age-related changes of the immune system with the incidence and prevalence of cardiovascular diseases in the elderly, we should be mindful of the high heterogeneity that exists among all studies focusing on immune system aging. To better understand this limitation, Rodriguez et al. performed a meta-analysis to determine the best set of markers to define immunosenescent cells. Strikingly, they observed that the lack of consensus not only on markers, but also on techniques and the high risk of bias did not allow for the identification of this "validated" phenotype. This result strongly suggests that active efforts towards identifying standard operating procedures (SOPs) are needed if we ever want to understand such a complex process as it is the aging of the immune system and its crucial role in the aging process of all other systems, including cardiovascular aging.

CONCLUDING REMARKS

This Research Topic highlights T cell mediated mechanisms involved in the interplay between a dysregulated immune T cell activation such as those observed in the setting of an acute, chronic infection, autoimmune diseases, aging and cardiovascular risk/disease. While the pathogenesis of these human diseases is unique, the integrated view provided in this collection of manuscripts delineate common features linking T cell activation and increased CVD risk. Several CD4 and CD8 T cell subsets seems to be involved participating in the disease pathology promoting endothelial inflammation and injury, progression of atherosclerotic plaques and infiltration of myocardium. Distinct subsets of T cells including CD4 T_{EM} cells with Th1 function and those with a $CD4^{+}CD28_{null}$ T cell phenotype have been described. T cells producing IFNγ and pro-inflammatory cytokines, and cytotoxic function are shown to be expanded in patients with CVD in lupus and other diseases and correlated with disease severity. The CD8 T cell subset is also involved and its role appears to be relevant in advanced phases of CVD such as atherosclerosis, in which CD8 T cells are present mainly in fibrous cap areas at higher number than CD4 T cells. These T cells have cytotoxic activity and secrete pro-inflammatory cytokines driving the progression and instability of the lesions. Finally, the emerging role of T cells in vasculogenesis highlights another important function of T cells. This angiogenic T cell subset or T_{ang} subpopulation is enlightened as it is involved in vascular repair processes promoting new vessel formation, and new insights are provided into their role in pathogenesis or vascular disease. In addition, T helper cells expressing AHR, a putative sensor for environmental and metabolic stressors emerge as a potential subset that could link metabolic dysregulation with systemic chronic systemic inflammation in patients with Type-2 diabetes (T2D) and metabolically healthy obesity (MHO).

Overall, this Research Topic highlights the role of T cell immune activation in cardiovascular disease suggesting an important role of T cells in the homeostasis of the vascular and cardiac system, and when dysregulated can contribute to the pathology of the disease. These emerging concepts offer new interesting avenues of investigation of the role of T cell activation in health and disease, and identify novel diagnostic and prognostic biomarkers of cardiovascular risk in pathologies characterized by a dysregulated immune T cell activation.

AUTHOR CONTRIBUTIONS

ER, SFM and MC wrote the article. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Increased AHR Transcripts Correlate With Pro-inflammatory T-Helper Lymphocytes Polarization in Both Metabolically Healthy Obesity and Type 2 Diabetic Patients

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Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor whose transcription activity is regulated by small compounds provided by diet, xenobiotics, and metabolism. It has been proven to be involved in energy homeostasis and inflammation in most recent years. Epidemiologically, exposure to xenobiotic AHR ligands contributes to obesity and type 2 diabetes (T2D). AHR is also the critical transcription factor determining the lineage commitment of pro-inflammatory Th17 and Th22 cells from naïve CD4+ T lymphocytes. It has been well-illustrated in animal models that IL-22, the major effector cytokine of Th17 and Th22 cells, played a major role in the interaction of metabolism and gut microbiota. But there were still missing links between gut microbiota, IL-22, and metabolism in humans. Our previous findings indicated that elevated circulating levels of IL-22 and frequencies of Th22 cells were associated with insulin resistance in both patients with obesity and T2D. Additionally, the hyperactive Th17 and Th22 cells phenotype also correlate with islets β-cell dysfunction in T2D. In this study, we made efforts to determine AHR expressions in peripheral blood mononuclear cells (PBMCs) from patients with T2D and metabolically healthy obesity (MHO). Correlation analyses were conducted to assess the possible link between AHR and the metabolic and inflammatory context. We revealed that mRNA expression of AHR was up-regulated and correlated with the percentage of Th17, Th22 as well as Th1 cells. Elevated plasma levels of IL-22 and IL-17 also correlated with increased AHR transcripts in PBMCs from both MHO and T2D patients. The transcription factor AHR may thus have a plausible role in the interaction between metabolism and pro-inflammatory status of patients in the development of obesity and T2D.

Keywords: aryl hydrocarbon receptor, metabolically healthy obesity, type 2 diabetes, CD4+ T cells, metabolic inflammation, gut microbiota

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INTRODUCTION

The prevalence of obesity, in parallel with type 2 diabetes (T2D), has expanded immensely in recent years and has risen as a global epidemic concern (1). Epidemiological and clinical insights offer substantive clues in our understanding of molecular pathways and physiologic systems underlying the regulation of metabolic balance (2). Beside the long list of metabolic comorbidities, obesity, and T2D have also been linked to chronic low-grade systemic inflammation (meta-inflammation), which is postulated to be causative in the development of both insulin resistance and progression to diabetic complications (3–6). The study of how metabolic stress impact the regulation of immune homeostasis and how the immune imbalance triggers the meta-inflammation in return are now topics of intensive investigation. Previous works revealed that T cell subsets were important regulators of meta-inflammation in both animal models and patients with obesity and T2D (7). These studies identified an significant elevation in the Th17 and Th1 subsets together with a decrease in the Treg subset (3, 4). Pro-inflammatory polarization of T helper (Th) lymphocytes, with hyperactivated Interferon-γ (IFN-γ) and interleukin-17 (IL-17) producing Th subsets (Th1 and Th17) and/or impaired regulatory T cells (Tregs), could directly trigger the activation of the innate immunity, and subsequently lead to meta-inflammation and insulin resistance in obesity and T2D (8). We as well as other independent teams have even identified Th22 as a novel potent participant in the development of obesity and diabetes (9–11). Our previous finding indicated that elevated serum IL-22 levels and Th22 frequencies were associated with insulin resistance in both obesity and T2D patients (9, 12). Additionally, the hyperactive Th17 and Th22 cell phenotype also correlate with islets β-cell dysfunction in T2D (9). The notable correlation of pro-inflammatory Th subsets with clinical parameters in patients implicate that the immunologic disturbance may play more determinant roles in both insulin resistance and β-cells dysfunction (9, 12–14). However, up until recently, the specific immunologic sensors involved in response to metabolic stress to produce such a state of immunologic disturbance were not identified.

The aryl hydrocarbon receptor (AHR) is an evolutionarily conservative ligand-activated transcription factor who was first identified by its role in modulating the organism's response to xenobiotics (e.g., toxicants such as polycyclic aromatic hydrocarbons and other environmental pollutants), and physiological molecules such as dietary indoles (15). It can be bound and activated by a wide range of small compounds provided by intrinsic metabolites, commensal microbiota, diet, and the environment (16). Upon ligand binding, AHR translocates to the nucleus, prompts protein–protein interactions with type 2 basic helix–loop–helix PER-ARNT-SIM (bHLH-PAS) proteins (i.e., Arnt) and regulates the subsequent transcriptional activity involved in detoxification (i.e., Cyp1a1), NF-κB regulation, and immune modulation (17). In the past decades, AHR has also been increasingly established as an important modulator of disease, especially for its emerging role in regulating immune responses and inflammation (18). AHR has been proved to be the determinant transcription factor driving the lineage commitment of Th22 from naïve CD4+ T cells (19). It has also been shown to regulate the developmental programs of Tregs and Th17 cells (20). Moreover, there is a reciprocal relationship between the activated AHR and the molecular circadian clock activation (21). Epidemiological evidence also revealed that exposure to xenobiotic AHR ligands such as polycyclic aromatic hydrocarbons could contribute to the incidence of obesity and T2D (22, 23). Taken together, the multiplicity of AHR signaling may shed light on the understanding of the integrated network of environmental factors, immune response, and energy metabolism.

Up to now, there is a growing body of evidence concerning the role of AHR signaling in obesity and T2D (21, 24). It has been well-illustrated in animal models that IL-22, the major effector cytokine of Th17,Th22, and type 3 innate lymphoid cells (ILC3), plays a critical role in the interaction of gut microbiota, mucosal immunity and metabolism (25–27). Our previous data add evidence to the pro-inflammatory Th polarization in obese and T2D patients (9). However, to our knowledge, there was no study present concerning the expression of the AHR gene in patients with obesity or T2D. Since Th17 lineage, driven by Retinoic acid receptor related orphan receptor gamma (RORC), represents another source of IL-22, we made efforts to investigate the expression of the transcription factor AHR, as well as RORC, in peripheral blood mononuclear cells (PBMCs) from patients with T2D and metabolically healthy obesity (MHO) subjects. Correlation analyses were further conducted to assess the possible link between the key transcription factors and the metabolic and inflammatory parameters in different groups of subjects.

MATERIALS AND METHODS

Subjects

Enrollment of participants took place from April, 2013 to December, 2015 in Qilu Hospital, Shandong University, China. Participants were generally arranged into three groups. The T2D group was selected from clinically definite T2D inpatients in the Department of Endocrinology & Metabolism, Qilu Hospital. The age of the disease onset was above 35 year-old for all participants. Serum levels of autoantibodies including anti-islet cell antibody, anti-insulin antibody and anti-glutamic acid decarboxylase antibody as well as anti-thyroid peroxidases antibody were all negative. Detailed clinical records were kept for all patient including the disease history, physical, and laboratory findings, to exclude Type 1 diabetes and other autoimmune disorders (e.g., autoimmune thyroid disease). Two control groups were selected. The first included healthy subjects with a normal Body Mass Index (BMI) referred to as the healthy control (CTL) (BMI: 19–23.9 kg/m^2), and the second included subjects that fell into the category of metabolically healthy obesity [MHO; which was defined as patients with a BMI $\geq$ 30 kg/m^2 and normal glucose tolerance and lipid profiles as previously described (9)]. Subjects from both groups were selected from age-matched healthy volunteers from local neighborhood communities and hospital staff. All subjects from both control groups were given a general medical examination including

history of disease, physical, and laboratory examinations, to exclude hyperglycemia, hyperlipidemia, hypertension, and diagnosed autoimmune disorders. A total number of 80 participants, including twenty healthy control (CTL), thirty MHO and thirty T2D patients, were consecutively enrolled to guarantee the sufficient power (>0.80) for the study. Exclusion criteria included any clues of other autoimmune diseases, acute, and chronic infections (within the past 4 weeks), fever of unknown origin, tumors, or significant elevation in erythrocyte sedimentation rate etc. Participants should not use immunomodulatory or immunosuppressive agents in the past 12 months prior to sampling. Our research was approved by the Medical Ethical Committee of Qilu Hospital of Shandong University [No.2013(046)]. The investigation was performed in a blinded manner. Consent forms were obtained from all participants.

Sample Preparation

Samples for flow cytometric analysis were prepared as previously reported (9). Briefly, fasting peripheral blood were obtained from each donor at seven to nine a.m. Fresh samples were processed within 2 h after collection. Serum or plasma were isolated, and sent for biochemical tests or stored at −80°C for ELISA analysis immediately. Four hundred microliter of the heparinized peripheral blood was diluted with 400 μL of RPMI 1640 (containing 50 ng/mL of phorbol-12-myristate-13-acetate, 2 μg/mL of ionomycin, and 3.4 μg/ml Monensin) and incubated for 4.5 h at 37°C, 5% CO_2 (all from Sigma, Saint Louis, USA) as described before (9).

The rest of heparinized blood was diluted 1:1 with saline at room temperature and centrifuged on Ficoll-Paque gradients (TBD,Tianjin, China) at 450 g for 20 min. PBMCs from the interface were collected and washed twice in cold phosphate buffer saline, and resuspended using 500 μL TRIzol® Reagent (Invitrogen, CA, USA) for total RNA isolation.

Flow Cytometric Assay

Stimulated blood samples was further stained to determine the cytokine-producing cells by flow cytometry. Generally, after stimulation, whole blood samples were stained with PerCP-Cy5.5 conjugated anti-human CD4 monoclonal antibodies (Cat No. 85-45-0048-42) at room temperature for 20 min, and then incubated with an equal volume of Fix-perm reagent A for 15 min. After washing, Fix-perm reagent B was added. The sample was subsequently incubated with FITC-conjugated anti-IL-17A monoclonal antibodies (Cat No.85-11-7179-42), PE-conjugated anti-IFN- monoclonal antibodies (Cat No. 85-11-7319-82), and eFluro680-conjugated anti-IL22 monoclonal antibodies (Cat No. 85-50-7229-42) for 20 min at room temperature. Cat No.Cat No.Cat No.Antibodies above were all purchased from eBioscience, CA, USA. Fix-perm reagents were purchased from Invitrogen (CA, USA). Samples were washed and immediately detected by BD AccuriC6 Flow Cytometer. Isotopes were applied as negative controls and. Data were analyzed using FlowJo 7.6. Gating strategy could be referred to as previously reported (9).

Extraction of Total RNA and Building of cDNA Library by Reverse Transcription

Isolated PBMCs were resuspended using TRIzol® Reagent as described previously. 0.2 ml of chloroform (Sigma-Aldrich, USA) was added to a 500 μL homogenized sample and followed by vigorous vortexing. The samples were centrifuged at 13,000 rpm for 10 min. The total RNA was then extracted from the upper aqueous phase. Total RNA was washed and purified for each sample (purity > 1.75) and synthesized immediately into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the instructions. Two microliter of freshly isolated RNA was reverse transcribed in a 20 μL system with 5 × Reaction Buffer, 100 mM dNTP Mix, RevertAid Reverse Transcriptase, oligo(dT)18 primerand RiboLock RNase inhibitor. The reaction was performed in the iCycler thermocycler (Bio-rad, Germany) at 42°C for 1 h and then at 70°C for 5 min.

Relative Quantification of the Expression of AHR and RORC by Real-Time Quantitative PCR

Real-time quantitative PCR was performed using an ABI Prism 7500 Real-time PCR system (Applied Biosystems, CA, USA). The final volume of 20 μl system contained 1.8 μL of cDNA sample, 10 μl of 2 × SYBR Green Master Mix (Toyobo, Japan), 0.2 μl of Taq polymerase, 7 μl of ddH_2O and 0.5 μl of the forward and reverse primers, respectively. The sequences of primers were as follows, respectively: AHR Forward 5′-CAA ATC CTT CCA AGC GGC ATA-3′, Reverse 5′-CGC TGA GCC TAA GAA CTG AAA G-3′. RORC Forward 5′-CAA TGG AAG TGG TGC TGG TTA G-3′, Reverse 5′-GGG AGT GGG AGA AGT CAA AGA T-3′; All tests were conducted in triplicates with the following protocol: denaturation at 95°C for 15 s, annealing at 62°C for 15 s, extension at 72°C for 45 s. The PCR primers and protocols were primarily testified by agarose gel electrophoresis to determine product sizes and to confirm that no by-product was formed before real-time quantification. And products were also analyzed by melt curve analysis in the Real-time PCR system. The result was expressed as a ratio relative to the number of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts used as an internal control. GAPDH was analyzed using the following primers: Forward 5′-AAG GTG AAG GTC GGA GTC AAC-3′, Reverse 5′-GGG GTC ATT GAT GGC AAC AAT A-3′. Relative amounts of gene transcripts normalized to endogenous reference gene was calculated using the comparative Ct method formula $2^{-\Delta Ct}$.

Quantification of Plasma Cytokines by ELISA

The plasma levels of Th1/Th17/Th22 cells predominantly effector cytokines IFN-γ, IL-17A, and IL-22 This was determined with quantitative sandwich enzyme linked immunoassay e

as per the manufacturer's recommendations (Multisciences, CN). The minimum detectable concentrations of IFN-γ, IL-17A, IL-22were 0.30, 0.55, 2.56 pg/ml, respectively. Leptin levels were determined using a Leptin Human ELISA Kit (Thermo Scientific, US) with the minimum detectable limit of 15.60 pg/mL. The plasma levels of hypersensitive C-reactive protein (hsCRP) were detected using a hypersensitive latex-enhanced immunoturbidimetric assay by Roche Cobas Integra 800 full-automated analyzer (Roche Diagnostics, North America). The intra-assay and inter-assay coefficients of variation were < 5 and 10%, respectively. The lower detecting limit was 0.1 mg/L.

Statistical Analysis

All data were represented as the mean, ± SD or median (range) according to the data distribution. Differences among 3 groups were determined by one-way ANOVA, and differences between two groups were further determined using the Newman–Keuls multiple comparison tests. Non-normal data were analyzed using the Kruskal–Wallis test and the Dunn test. Correlation was analyzed using Pearson or Spearman correlation test depending on data distribution. Partial correlation test was performed to eliminate interference from age and sex. And multiple linear regression analysis was conducted to further evaluate the relationship between parameters. All tests were conducted by SPSS 18.0 or GraphPad Prism 8.0 system. A $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of Subjects

Significant differences were found in BMI, fasting insulin, and IL-22 levels within each of the three cohorts as well as between each group. The homeostasis model of assessment for insulin resistance index (HOMA-IR) has been established as an indicator of insulin resistance. Its nature logarithmic value Ln (HOMA-IR) is an even more reliable parameter and was used as a parameter reflecting basal insulin-secreting function of β-cell in this study (28). Although we did not observe significantly higher fasting glucose in MHO participants, there were significant elevations in Ln (HOMA-IR) and serum levels of leptin, insulin, TNFα, IL-22, and hsCRP compared with CTLs. This is inconsistent with previous publications, and the data indicated that obesity, either metabolically healthy or not, was accompanied with hyperinsulinemia, leptinemia, and low-grade inflammation. Ln (HOMA-IR) and serum levels of IL-17, IL-22 were even significantly higher in T2D patients compared with the MHO group, indicating a decompensating inflammatory status. Differences in age, sex, IFN-γ, etc., might show a remarkable tendency but did not show statistical significance in our study. Since we did not ask our patients to stop necessary hypoglycemic treatment (especially insulin preparations and secretagogues) before sampling, the hypoglycemic treatment had potential neglectable influences in insulin levels. The insulin levels in these patients could not reflect the real β cell function level. Therefore, the differences of fasting insulin in T2D patients compared with CTLs and MHOs did not have scientific value. The detailed parameters are demonstrated in **Table 1**.

Elevations of AHR mRNA Expressions Were More Remarkable in PBMCs From T2D Patients

Previously, we reported an significant increase in peripheral Th22 cells together with Th1 and Th17 cells in MHO and T2D patients. There were also significant higher levels of predominant cytokines in these subjects. Correlation analysis was also performed in all subcohorts of plasma donors and the agreement of elevated Th17, Th22 frequencies with IL-17 and IL-22 was further verified (9). It is well-known that AHR acts as the determinant transcription factor which drives the lineage commitment of Th22 from naïve CD4+ T cells and RORC for Th17. Theoretically, increased peripheral Th22 and Th17 cells could be promoted both/either/neither by AHR and/or RORC signaling in T2D and/or obesity. However, there were no relevant clinical data before. We thus tested whether there were significances of both AHR and RORC expressions (relative to GAPDH) in our case-controlled study. The relative mRNA expressions of AHR (ratio normalized to endogenous control gene) in PBMCs were significantly different among the three subcohorts (***$P < 0.0001$), as shown in **Figure 1A**. Compared with CTLs (0.0195 ± 0.0106, $n = 20$), the relative mRNA expressions of AHR (compared to GAPDH, same below) was significantly increased, by 2.02- and 3.06-fold respectively, in MHO (0.0394 ± 0.0202, $n = 30$) (*$P = 0.0145$) and T2D (0.0596

TABLE 1 | Demographic characteristics of participants.

	CTL	MHO	T2D
Number	20	30	30
BMI (kg/m2)	21.20 ± 1.64	32.62 ± 1.99*	24.87 ± 3.87*#
Age (year)	47.10 ± 8.53	46.07 ± 8.66	50.37 ± 6.92
Sex (female/male)	9/11	13/17	12/18
FBG (mM)	4.76 ± 0.31	5.29 ± 0.41	8.32 ± 3.18*#
FINS (IU/mL)	5.20 ± 1.20	11.54 ± 4.19*	15.81 ± 8.35*#
Duration of illness (year)	n.a.	n.a.	6.34 ± 3.63
IFN-γ (pg/mL)	0.91 ± 0.89	1.84 ± 2.32	1.58 ± 1.36
IL-17 (pg/mL)	2.11 ± 0.60	2.87 ± 2.07	4.77 ± 1.95*#
IL-22 (pg/mL)	35.83 ± 2.69	42.78 ± 7.93*	51.55 ± 9.73*#
TNFα (pg/mL)	12.20 (1.50–16.62)	30.38 (18.17–48.65)*	49.16 (26.77–91.81)*
IL-6 (pg/mL)	7.355 (4.66–14.52)	10.86 (7.76–17.49)	16.95 (7.92–24.01)*
Leptin (pg/mL)	3090 (1961–4873)	6078 (5230–9117)*	3338 (1979–4877)#
hsCRP (mg/L)	0.320 (0.01–1.30)	1.620 (0.70–3.26)*	1.255 (0.85–4.36)*
Ln(HOMA-IR)	0.1290 ± 0.1363	0.9413 ± 0.3273*	1.562 ± 0.5009*#
Ln(HOMA-β)	4.420 ± 0.3978	4.835 ± 0.4444	4.273 ± 1.094#

*FBG, Fasting blood glucose; FINS, fasting insulin level; HOMA-IR, The homeostasis model of assessment for insulin resistance index; HOMA-β, The homeostasis model of assessment for β-cell function; n.am not applicable; *P < 0.05 compared with CTL; #P < 0.05 compared with MHO.*

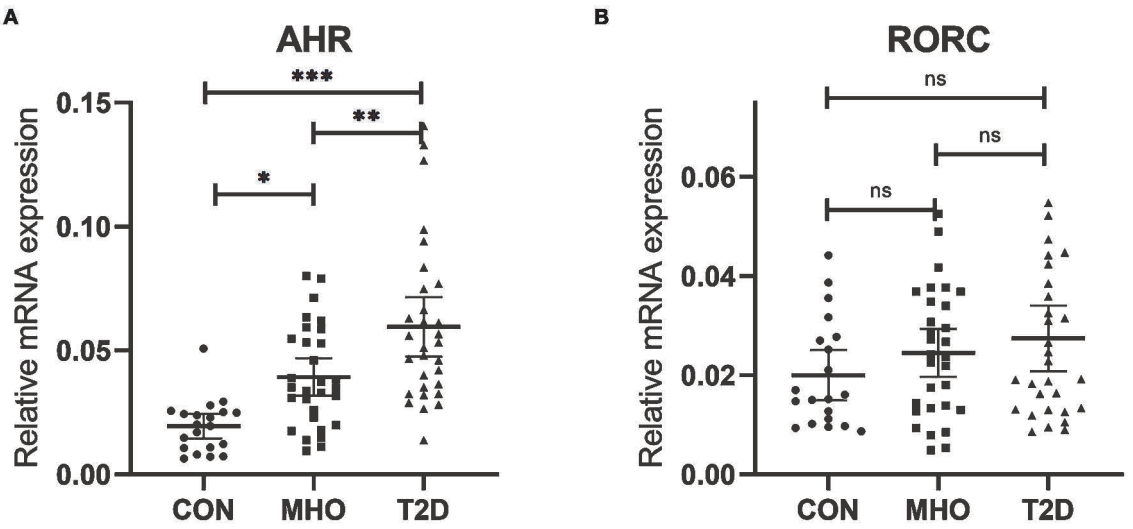

FIGURE 1 | Comparison of Relative mRNA expressions of AHR and RORC. Relative mRNA expressions of AHR **(A)** and RORC **(B)** genes in PBMC (expressed as ratio to GAPDH transcripts). Statistical analysis of the differences between each two groups was performed by Newman–Keuls multiple comparison tests (q-test). $P < 0.05$ were considered significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

$\pm$ 0.0322, $n = 30$) ($^{***}P < 0.0001$) patients. An even higher mRNA expressions of AHR were observed in T2D compared with MHO subjects ($^{**}P = 0.0045$).

However, no significant difference was found in the relative mRNA expressions of RORC among the three subcohorts ($P = 0.2138$). The average relative mRNA expressions of RORC seemed higher in PBMCs of MHO (0.0246 $\pm$ 0.0108, $n = 30$) and T2D patients (0.0275 $\pm$ 0.0129, $n = 30$) compared with CTLs (0.0201 $\pm$ 0.0177, $n = 20$). But our present research failed to show statistically-significant difference (**Figure 1B**).

Increased AHR mRNA Expressions Correlated With BMI in PBMCs From Non-diabetic Participants

There were no data concerning whether demographic characteristics e.g., age, sex, BMI, and fasting glucose had an influence on the level of AHR expression. Bivariate correlation analysis was performed to check whether the relative AHR expression level had any statistic connection with age, BMI, and fasting blood glucose (FBG). As shown in **Table 2**, a significantly positive correlation of relative AHR expression was only seen with FBG ($r = 0.5015$, $^{*}P < 0.0001$, $n = 80$, Spearman analysis) in all the subcohorts. In our present study, however, AHR mRNA expression level showed no statistical correlation with age or BMI. Likewise, RORC mRNA expression level did not have statistical correlation with age, BMI or FBG (shown in **Table 3**).

Since the confounding factors such as glucose control and hypoglycemic treatment affect body weight and the FBG level directly, inclusion of T2D subcohort may exert more misunderstanding rather than objective information. We carried out the analyses excluding data from T2D patients (seen in **Table 2**. BMI1 and FBG1). Not surprisingly, the results were quite opposite. This time the relative AHR mRNA expression level showed no statistical correlation with FBG ($r = 0.1868$, $P = 0.1940$, $n = 50$, Pearson analysis) but had significant and remarkable positive correlation with BMI ($r = 0.4783$, $^{***}P = 0.0004$, $n = 50$, Pearson analysis). The relative RORC mRNA expression level did not have statistical correlation with age or BMI, but did correlate positively with FBG ($r = 0.3486$, $^{*}P = 0.0131$, $n = 50$, Pearson analysis) in PBMCs from non-diabetic participants (shown in **Table 3**). In an attempt to identify the significance and dependency/independence of the association, multiple linear regression analysis was conducted. The well-established inflammatory marker, CRP, was specified as dependent variable in the model. According to preliminary correlation analyses, AHR, together with BMI, TNFα, IL-17, IL-22, and Leptin were entered as independent variables. AHR transcript (relative to that of GAPDH) turned out to be the only independent risk factor of increased CRP levels [β Coefficient: 55.04(18.65–91.43), $P = 0.005^{**}$], even after adjusting sex, age, disease status (diabetes duration, and medications).

Increased Expressions of AHR Gene Correlated With Pro-inflammatory Polarization of Th Lymphocytes in Obesity and T2D Patients

The plausible links of increased expressions of AHR and RORC with pro-inflammatory Th subsets were verified by previous *in vitro* studies or data from animal models. However, there is still a lack of clinical data regarding whether expressions of the

TABLE 2 | Bivariate correlation analysis of AHR expression with demographic characteristics.

AHR	Age	BMI	FBG	BMI1	FBG1
n	80	80	80	50	50
r value	0.0875	0.02768	0.5015	0.4783	0.1868
95% confidence interval	−0.1348–0.3014	−0.1932–0.2459	0.3167–0.6496	0.2306–0.6677	−0.0966–0.4422
P value (two-tailed)	0.4403	0.807	<0.0001***	0.0004***	0.1940

*BMI1 and FBG1 represent analyses conducted in a subcohort of non-diabetic participants (n = 50) in our study. *P < 0.05, **P < 0.01, ***P < 0.001.*

TABLE 3 | Bivariate correlation analysis of RORC expression with demographic characteristics.

RORC	Age	BMI	FBG	BMI1	FBG1
n	80	80	80	50	50
r-value	−0.0704	−0.0660	0.1133	0.1638	0.3486
95% confidence interval	−0.2857–0.1517	−0.2816–0.1560	−0.1092–0.3249	−0.1200–0.4229	0.07777–0.5715
P-value (two-tailed)	0.5350	0.5610	0.3172	0.2556	0.0131*

*BMI1 and FBG1 represent analyses conducted in a subcohort of non-diabetic participants (n = 50) in our study. *P < 0.05, **P < 0.01, ***P < 0.001.*

transcription factors correlate with frequencies of corresponding Th subsets in obesity and T2D patients. Notably, AHR also played a role in Th17 differentiation (20). For further analysis of the relationship between the expression of transcription factors and corresponding Th cells frequencies, a correlation study was performed in all subcohorts of participants (n = 80). In this study, both AHR and RORC transcript ratios correlated significantly with their corresponding Th subsets. Increased expressions of AHR mRNA matched significantly with elevated Th22 frequency (r = 0.4995, Pearson analysis, $^{***}P < 0.0001$) (**Figure 2C**). Increased RORC transcripts also matched significantly with Th17 frequency (r = 0.2592, Pearson analysis, $^{*}P = 0.0203$) (**Figure 2E**). What's more, unanticipated correlations of AHR transcripts with Th1 (r = 0.3573, Pearson analysis, $^{**}P = 0.0011$) and Th17(r = 0.3780, Pearson analysis, $^{**}P = 0.0005$) (**Figures 2A,B**) were revealed in our study. Although previous studies have indicated a role of AHR signaling in Th17 differentiation, there was no data showing its clinical relevancy with peripheral Th17 frequency in diabetes or obesity. Even fewer studies demonstrated the role of AHR in Th1 polarization. Our data indicated that a general increase in expression of the AHR gene in PBMC not only correlated with the corresponding Th22 lineage, but also correlated with overall pro-inflammatory polarization of other Th subsets in obesity and T2D patients as well. In contrast, correlation of RORC transcripts with Th1 and Th22 might show a remarkable tendency but did not have statistical significance in this study (shown in **Figures 2D,F**).

Increased Expressions of AHR Gene Correlated With Elevated Serum Pro-inflammatory Cytokines in Obesity and T2D Patients

To further evaluate the potential role of AHR or RORC in the aberrant Th function, we also assessed the relationship between AHR or RORC transcripts and the downstream pro-inflammatory cytokines in all subcohorts of participants (n = 80). Correlation analysis revealed significant relevancies of AHR transcripts with levels of pro-inflammatory cytokines, including IL-17 (r = 0.3804, $^{**}P = 0.0005$, spearman analysis) IL-22 (r = 0.4256, $^{***}P < 0.0001$, spearman analysis) as well as TNFα (r = 0.4266, $^{***}P < 0.0001$, spearman analysis) and IL-6 (r = 0.3396, $^{*}P = 0.0021$, spearman analysis) levels. There was also a remarkable, though statistically non-significant, relevancy with IFN-γ levels (r = 0.1853, P = 0.0999, spearman analysis) (shown in **Figure 3** and **Supplementary Table 1**). hsCRP is a well-established marker for the meta-inflammatory status, especially in obesity and diabetes. Here we also found a significantly positive correlation of AHR transcripts with serum hsCRP levels (r = 0.4244, $^{***}P < 0.0001$, spearman analysis). To note, Th17, whose differentiation is driven by ROR signaling, represents another important source of IL-22. However, we failed to find statistically significant relevance between expression of RORC with above mentioned cytokines or markers (shown in **Figure 3**). Together with the correlation analysis of Th lymphocyte polarization, our data indicate that AHR, rather than ROR signaling, might have closer ties with the pro-inflammatory status in T2D and obesity.

Among the emerging mediators of inflammation, leptin, a key controller of metabolic function, has been extensively implicated in modulating immune responses and promoting chronic inflammatory responses in peripheral tissues. In our cohort, serum leptin levels also correlated significantly with BMI (r = 0.5887, $^{***}P < 0.0001$, spearman analysis), FBG (r = 0.2862, $^{*}P = 0.0439$, spearman analysis) (n = 50, as T2D group was excluded concerning BMI and FBG as mentioned above) and hsCRP (r = 0.2912, $^{*}P = 0.0088$, spearman analysis). However, the present data did not establish significant correlation of AHR (r = 0.0709, P = 0.5321, spearman analysis) or RORC transcripts (r = 0.01201, P = 0.9157, spearman analysis) with serum leptin levels (shown in **Figure 3**).

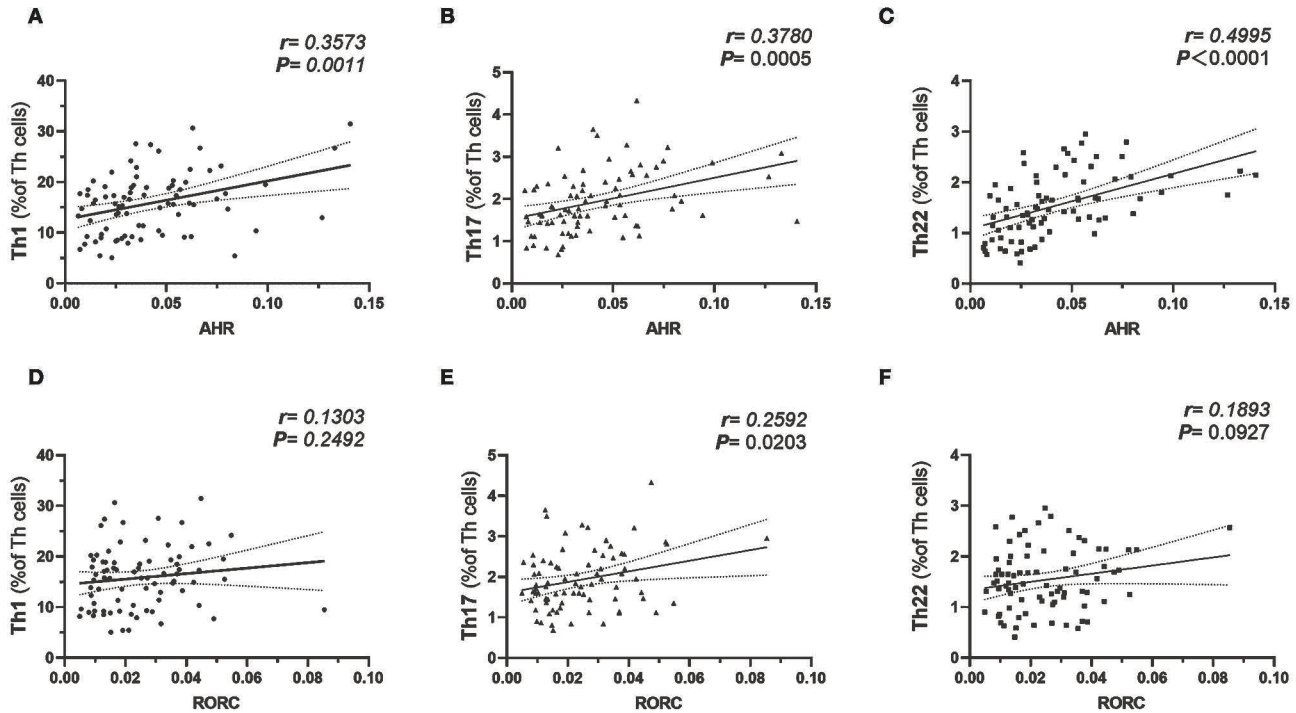

FIGURE 2 | Pearson's correlation analysis between AHR Transcripts (relative to GAPDH) and corresponding peripheral frequencies of Th1 **(A)**, Th17 **(B)**, Th22 **(C)** subsets. Pearson's correlation analysis between RORC Transcripts (relative to GAPDH) and corresponding peripheral frequencies of Th1 **(D)**, Th17 **(E)**, Th22 **(F)** subsets. *r* represents Pearson's correlation coefficient; *P* represents statistical significance.

Elevated AHR Expression Positively Correlated With Both Insulin Resistance and Islets β-Cell Function

We further studied the potential role of elevated AHR mRNA expression in the development of insulin resistance. In our study, the correlation analysis revealed notable relevance of both elevated AHR ($r = 0.6188$, $^{***}P < 0.0001$, Pearson analysis) and RORC ($r = 0.2541$, $^{*}P = 0.0289$, Pearson analysis) (relative to GAPDH) mRNA expression to increased insulin resistance (**Figure 4**). We further conducted multiple linear regression analysis to rule out potential confounders in association with HOMA index for insulin resistance. AHR, together with RORC, BMI, TNFα, IL-6, IL-17, IL-22, and CRP were entered as independent variables. AHR transcript (relative to that of GAPDH) turned out to be an independent risk factor of increased CRP levels [β Coefficient: 9.25(0.46–18.03), $P = 0.040^{*}$], even after adjusting sex, age, disease status (diabetes duration and medications).

As an even more significant increase of AHR transcripts was found in T2D patients compared with MHO, we tested the assumption that whether the increase of AHR transcripts in PBMCs correlated with the compensation of islet β-cell function. Since we did not request our patients to stop necessary hypoglycemic treatment (especially insulin preparations and secretagogues) before sampling, the treatment had potential neglectable influences in insulin levels. The insulin levels in these patients could not reflect the intrinsic β-cell function. Therefore, the data from T2D groups were excluded in this analysis. In subcohorts of CTL and MHO ($n = 50$), our research demonstrated an unexpected significant positive correlation between AHR transcripts and Ln (HOMA-β) values ($r = 0.4765$, $^{**}P = 0.0005$, Pearson analysis) (see **Figure 4**). No significant correlation was identified between Ln (HOMA-β) and that of RORC ($r = -0.1182$, $P = 0.4138$, Pearson analysis). Moreover, in a multiple linear regression model with AHR, BMI, TNFα, IL-17, IL-22, leptin, and CRP included as independent variables, AHR transcript (relative to that of GAPDH) also represented as an independent predictive factor of increased Ln (HOMA-β) (β Coefficient: 9.25(0.46–18.03), $P = 0.040^{*}$) in non-diabetic participants, even after adjusting age and sex.

DISCUSSION

Obese individuals are typically accompanied by deleterious metabolic profiles, especially Type 2 diabetes. But various epidemiological studies have identified a subset of patients with a normal metabolic phenotype referred to as the metabolically healthy obesity (MHO) (29). MHO is a novel concept that stratifies obese individuals according to their metabolic status (30). Studies into MHO provide important implications for both understanding of the disease progression and the targeted

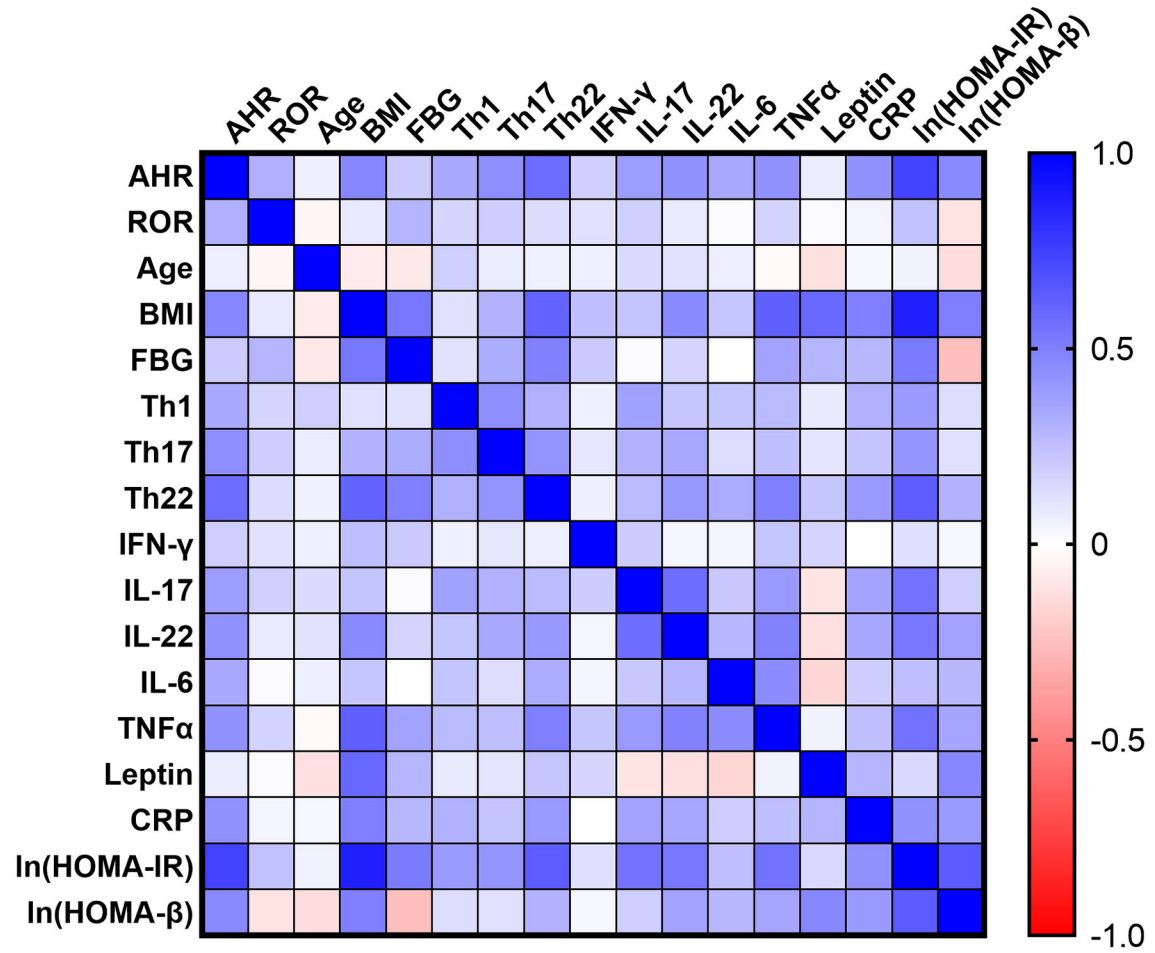

FIGURE 3 | Correlation Matrix: Correlation Coefficients between Each Pair of Parameters. Spearman correlation coefficients between two variables are shown in the heat-map. The correlation coefficients are represented in terms of change of the intensity of red (for negative correlation)/blue (for positive correlation) color, as shown in the color bar. The Spearman correlation coefficients and significances are listed in **Supplementary Table 1**, and the partial correlation coefficients and significances adjusted by age and sex are also listed in **Supplementary Table 2**.

prevention of obesity related metabolic disorders (31). However, there are conflicting evidences in the literature on this topic regarding its risk profile. But generally, MHO show a substantially increased risk of developing T2D compared with metabolically healthy normal-weight adults (CTL) (32). Insulin resistance is well-known as the dominant initiating and persisting factor in obesity-associated metabolic disorders, and decompensated β-cell function is the determinant process distinguishing T2D from normoglycemic obesity (33). However, the culprits for the deterioration and decompensation have not been identified. In the past two decades, the chronic low-grade inflammation, referred to as meta-inflammation, has been established as the central link between obesity and T2D (3–5). Moreover, emerging data revealed that meta-inflammation was also present in MHO so that the "metabolically healthy" might not represent a persistent favorable condition (4, 9, 29). However, more studies are needed to evaluate the meta-inflammation in obesity from two separate subcohorts (34). This study was designed to include both T2D patients and MHO subjects, and thus demonstrated more similarities as well as distinguishment between MHO and T2D.

Our study revealed significant elevation in Ln (HOMA-IR) and serum levels of leptin, insulin, TNFα, IL-22, and hsCRP in MHO compared with CTLs. The data indicated that obesity, either metabolically healthy or not, was accompanied with hyperinsulinemia, leptinemia, and low-grade inflammation. Serum levels of pro-inflammatory cytokines including IL-17, IL-22, and IL-6 were even significantly higher in T2D patients compared with the MHO group, indicating an aggravated inflammatory status. The persistent and progressive status of meta-inflammation accompanies the whole trajectory of the two diseases, from its pathogenesis to complication development (35). Diabetic patients show an dysregulated number and function of immune cells, of both innate and adaptive immunity (4). Accumulating evidence established that immune cells especially T lymphocyte alterations preceded the loss of insulin sensitivity in adipose tissue and contribute to the general pro-inflammatory drift observed in obesity and T2D

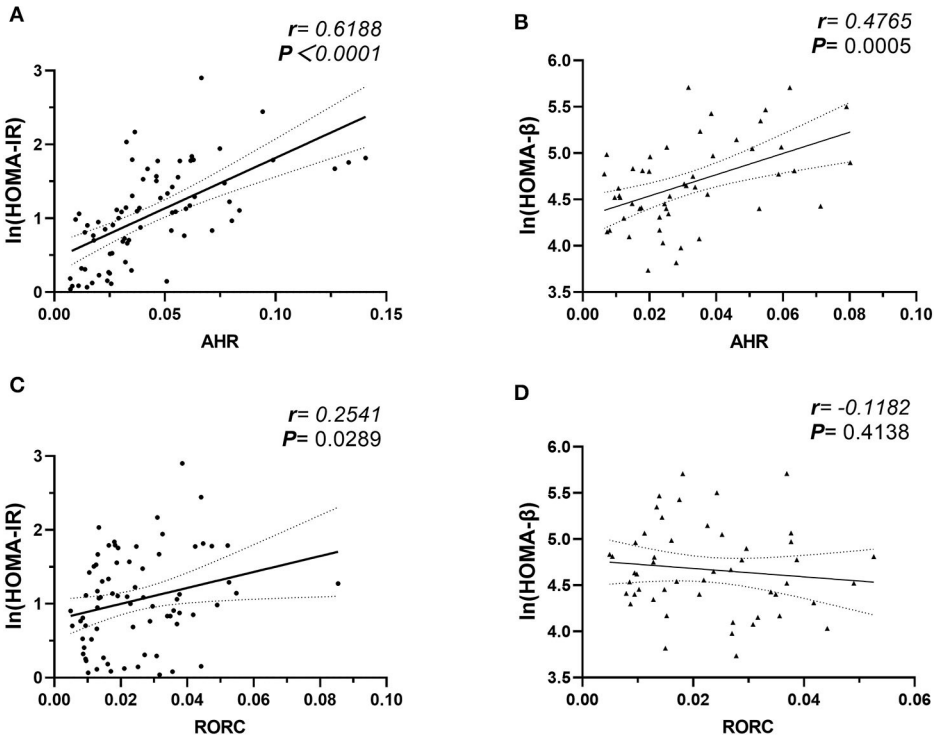

FIGURE 4 | Correlation Analysis of AHR and RORC Transcripts with Ln (HOMA-IR) and Ln (HOMA-β). Pearson's correlation analysis between AHR and RORC Transcripts (relative to GAPDH) and Ln (HOMA) parameters. Note that correlation of AHR **(A)** and RORC **(C)** transcripts with Ln (HOMA-IR) values were conducted in all participants ($n = 80$), while correlation of AHR **(B)** and RORC **(D)** transcripts with Ln (HOMA-β) values were conducted in subcohorts of CTL and MHO ($n = 50$), respectively.

(4, 7). Accumulating evidence identified an elevation of pro-inflammatory subsets together with a significant decrease in Tregs, which may directly evoke hyperactivation of innate immunity and then meta-inflammation, which contributes greatly to both insulin resistance and β-cell damage (10–14). More thorough investigations are disclosing the possible "triggers" of inflammatory responses, most of which are at the crossroad of environmental signaling, metabolic sensing and immune modulation, e.g., leptin (36). More recently, data on how pro-inflammatory Th cells and/or effector cytokines affect energy metabolism have been piling up. However, the primary adapter which senses the environmental signals (e.g., dietary components (37), daily schedule, gut microbiota, environmental pollutants and endogenous metabolites etc.) and decodes them into metabolic and immunologic disturbance still remains ill-defined.

A biological entity that tightly links genes and the environment is AHR, which is a nuclear receptor best known for its role in xenobiotic metabolism. AHR is an ancient transcription factor which was first identified with its role in modulating the organism's response to xenobiotics, e.g., polycyclic aromatic hydrocarbons (15). Previous work establishes AHR as a pivotal environmental modifier that integrates signals from chemical exposure in the regulation of lipid and energy metabolism (16). With advances in the knowledge of AHR physiology, constitutive AHR signaling driven by both environmental signals and endogenous metabolites has been deemed to be of crucial importance for the maintenance of AHR-governed cellular processes, including hormone levels, circadian clock, gastrointestinal homeostasis, immune regulation and cell proliferation (16, 17). Upon ligand binding, the AHR translocates to the nucleus, where it binds to forms a complex with the AHR nuclear translocator (ARNT). The AHR/ARNT heterodimer regulates the transcription of genes in the cytochrome P450 Cyp1 family and thousands of other genes (24) including other nuclear receptors relevant to obesity (38).

Moreover, in the past decade, AHR has been increasingly recognized as an important modulator in immune and inflammatory responses (18). AHR has been proved to be the determinant transcription factor driving the development of Th22 (19). It has also been shown to regulate the differential programs of Tregs and Th17 cells (20). Moreover, AHR signaling in other immune cell populations, e.g., monocytes, also contributed to priming CD4+ T cells to skewed differentiation (39). Our previous work added weight to the evidence that Th cell polarization contributed to the pathogenesis of obesity and T2D. Apart from evidence from animal models, correlation between pro-inflammatory Th cells and metabolic parameters implied that the imbalance of T cell subsets was responsible for the pathogenesis of obesity and T2D in human (9, 14). We and others also identified significantly elevated Th22 frequencies from peripheral blood of both MHO and T2D patients, suggesting the possible role of Th22 subsets in disease progression (9, 11).

However, there was no human study present concerning the potential triggers driving the pro-inflammatory polarization of Th subsets. In this study, the relative amount of transcripts of the transcription factors AHR and RORC in PBMCs responsible for Th17 and Th22 differentiation were determined in obesity and T2D patients. Our study first identified that AHR transcripts (relative to GAPDH) were notably increased in the PBMCs from both obesity and T2D patients compared with lean healthy subjects with a normal BMI. Elevation of AHR mRNA expression was even more remarkable in PBMCs of patients with T2D compared to MHO patients. However, no significant difference was found in the transcripts of RORC among the three subcohorts. We further evaluated whether there was correlation between the two transcription factors with the demographic or basic clinical parameters. AHR transcripts only showed a significant and remarkable positive correlation with BMI in the subcohorts of non-diabetic participants. RORC transcripts did not have statistical correlation with age or BMI but did correlate positively with FBG in PBMCs from non-diabetic participants. These data primarily established a link of AHR and RORC expression with obesity and glucose intolerance. And the negative findings between demographic parameters and AHR and RORC expression also helped to distinguish the confounding factors in the further analysis between subcohorts. Both AHR and RORC transcripts correlated significantly with their corresponding Th subsets, verifying the agreement of expressions of transcription factors in PBMCs with corresponding Th cells frequencies. Since AHR is well-established as the major driver of Th22 commitment, our data explained at least partially for the increased Th22 cells in obesity and T2D. Moreover, unanticipated correlations among AHR transcripts and Th1 and Th17 cells were revealed in our further analysis, indicating that increased expressions of AHR gene may contribute to the overall pro-inflammatory polarization of Th lymphocytes in obesity and T2D patients. This observational study only revealed statistical correlations but could not support a causal relationship. However, considering its well-established transcription activity, AHR would more likely to be an important regulator of immune response (18), rather than consequence of Th polarization in MHO and T2D (40). AHR expression in PBMCs would more likely to be a causative factor in Th polarization, even though the intermediate links and mechanisms are currently unknown. It is important to note that the expressions of the transcription factors were determined in PBMCs but not isolated T lymphocytes in this study. Changes in the expressions of transcription factors in PBMCs may be not only associated with changes in Th cells, since other cell subpopulations, such as monocytes, also demonstrate considerable AHR expression. Given that AHR may not be involved in Th1 polarization, it is quite possible that Th polarization in obesity and T2D may be regulated, at least partially, by expressions of AHR in other cell populations (e.g., monocyte) other than CD4+ T lymphocyte itself. In contrast, correlation of RORC transcripts with Th1 and Th22 might show a remarkable tendency but did not have statistical significance in our study. To further evaluate the potential role of AHR or RORC in the aberrant Th cell function, we also assessed the relationship between AHR or RORC transcripts and the corresponding cytokines in plasma of participants of all subcohorts. The present data revealed remarkable relevancies of AHR transcripts with not only IL-22, but also IFN-γ, IL-17 as well as TNFα and IL-6 levels. To note, Th17, whose differentiation is driven by ROR signaling, represents another important source of IL-22 (19). However, we failed to find statistically significant relevance between expressions of RORC with relevant cytokines or markers. Taken together, our data indicate that AHR, rather than ROR signaling, might have a more important role in the pro-inflammatory status in individuals with T2D and obesity. With a significant positively correlation with serum hsCRP levels, AHR transcripts in PBMCs may represent as a novel mediator of the immune disturbance and meta-inflammation in obesity and T2D patients, independent of leptin, the previously-known key metabolic controller and immune modulator (36). What's more, it is quite possible that the overall pro-inflammatory status in T2D and MHO may be associated to a general increase in AHR levels in PBMCs, which is at least partially, independent from Th cell polarization.

Up to now, there is a growing body of evidence concerning the role of the AHR signaling in obesity and T2D. Studies have shown that the toxicant-activated AHR may disrupt fat metabolism and contribute to obesity. Adipocyte AHR has been proved responsible for pollutants (e.g., biphenyls) induced adipose inflammation and impairment of glucose homeostasis in mice (41). AHR overactivation directly promotes obesity, hepatic steatosis and insulin resistance under HFD exposure (21, 24). More recently, AHR was even proven to be the major adapter integrating signals from gut microbiota alternation (42) and circadian clock disruption (21) with metabolic dysfunction. Taken together, AHR signaling established a critical role in obesity and glucose intolerance in animal models. Our work provided clinical data verifying the alternations in AHR expression in PBMCs of both patients with obesity and T2D patients. We further studied the possible role of elevated AHR transcripts in the pathogenesis of insulin resistance. The present data showed a remarkable linear correlation of AHR transcripts with Ln (HOMA-IR), indicating that AHR signaling may contribute greatly to insulin resistance. What's more, an unanticipated positive relevancy of AHR transcripts with basal β-cell function was revealed. Different with previous work demonstrating a remarkable negative correlation between Th22 frequencies and Ln (HOMA-β) in drug-naïve T2D patients (9), the present data showed a positive correlation of AHR transcripts with Ln (HOMA-β) in non-diabetic participants. Previous study indicated peripheral Th22 cell frequencies might be associated with decompensation of β cell function in T2D patients. However, in this study, the correlation of AHR transcripts with Ln (HOMA-β) may indicated a possible role of compensated insulin secretion in health donors and more especially in MHO individuals. These findings may not support the contention that the possible influence of AHR expressions in PBMC on β cell compensation or dysfunction in obesity and T2D depend totally on Th22 cells. Moreover, IL-22 secreting ILC3s, whose differentiation was also driven by AHR transcription activity, were proved more recently to be potentially protective in maintaining immunologic and metabolic homeostasis (43, 44). However, circulating IL-22 producing Th subsets or even neutrophils had deteriorating role in immunologic and metabolic

disturbance (45). As reported in animal models, AHR signaling, together with IL-22, may exert a double-sided effect in β-cell dysfunction and compensation (44, 46). Therefore, intrinsic increase in AHR expression, together with environmental factors influencing AHR signaling (such as endogenous or exogenous ligands), may exert multifaceted influence in the pathophysiology of the metabolic disorders.

Collectively, we identified for the first time significant elevated AHR transcripts from peripheral blood of both MHO and T2D patients. Increased expressions of the AHR gene may contribute to the overall pro-inflammatory polarization of Th lymphocytes in obesity and T2D patients. Our data preliminarily suggest that AHR signaling might have a dual role in both development of insulin resistance and compensated β-cells function. In context of the emerging multidisciplinary research about AHR signaling, further understanding of the mechanisms underlying may provide a novel therapeutic target for obesity and its related metabolic disorders.

DATA AVAILABILITY STATEMENT

Access to more primary data will be considered by the author upon request (rusingstar@163.com). The authors will make the primary data available under the premise of privacy protection, restricted usage, and duplication.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of Qilu Hospital of Shandong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RZ, XH, and LC contributed conception and design of the study. RZ, SS, QH, JQ, PL, and YS conducted the investigation. RZ, SS, QH, and LS organized the database. RZ, LS, and JS performed the statistical analysis. RZ wrote the first draft of the manuscript. SS, JQ, QH, YS, and PL wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01644/full#supplementary-material

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REVIEW
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Cellular Signaling Pathways in Medium and Large Vessel Vasculitis

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Autoimmune and autoinflammatory diseases of the medium and large arteries, including the aorta, cause life-threatening complications due to vessel wall destruction but also by wall remodeling, such as the formation of wall-penetrating microvessels and lumen-stenosing neointima. The two most frequent large vessel vasculitides, giant cell arteritis (GCA) and Takayasu arteritis (TAK), are HLA-associated diseases, strongly suggestive for a critical role of T cells and antigen recognition in disease pathogenesis. Recent studies have revealed a growing spectrum of effector functions through which T cells participate in the immunopathology of GCA and TAK; causing the disease-specific patterning of pathology and clinical outcome. Core pathogenic features of disease-relevant T cells rely on the interaction with endothelial cells, dendritic cells and macrophages and lead to vessel wall invasion, formation of tissue-damaging granulomatous infiltrates and induction of the name-giving multinucleated giant cells. Besides antigen, pathogenic T cells encounter danger signals in their immediate microenvironment that they translate into disease-relevant effector functions. Decisive signaling pathways, such as the AKT pathway, the NOTCH pathway, and the JAK/STAT pathway modify antigen-induced T cell activation and emerge as promising therapeutic targets to halt disease progression and, eventually, reset the immune system to reestablish the immune privilege of the arterial wall.

Keywords: giant cell arteritis, Takayasu arteritis, large vessel vasculitis, T cells, macrophages, NOTCH, co-stimulation, immune checkpoint

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INTRODUCTION

Arterial blood vessels are categorized according to their diameter into large, medium, and small vessels. Large vessels are the aorta and its major branches, medium vessels are the main visceral arteries and the arteries supplying blood to the brain, and small vessels are intraparenchymal arteries (1). Common elements are the lumen-lining endothelial cells (intimal layer) and the smooth muscle cells enabling dynamic change of diameter and resistance (medial layer). The outermost layer (adventitial layer) contains connective tissue, nerves, and vasa vasorum networks to supply nutrients and blood to the wall (2). Notably, each category of arteries is subject to different disease processes. Atherosclerotic disease, now recognized as a smoldering inflammatory process triggered by subendothelial lipid deposits that spans multiple decades of life, is a major cause of morbidity and mortality (3–5). In contrast, autoimmune inflammation of arteries is a more aggressive process, complicated by hemorrhage, vessel rupture and vessel occlusion. Vasculitides

share immunopathologic features with other autoimmune diseases but have specifying immune abnormalities and clinical manifestations that are related to the life-sustaining role of arteries (1).

The two most frequent autoimmune diseases affecting the aorta and its branch vessels are giant cell arteritis (GCA) and Takayasu arteritis (TAK), two vasculitides manifesting with aortitis and wall inflammation in the carotid, subclavian, mesenteric and more peripheral arteries (6–8). In both diseases, $CD4^+$ T cells and macrophages form granulomatous infiltrates in the vessel wall leading to wall vascularization, loss of medial smooth muscle cells, destruction of elastic membranous lamellae and elastin fibers in the medial layer and growth of lumen-stenosing neointima (6–8) (**Figures 1**, **2**). Damage patterns are similar in GCA and TAK, but the individuals at risk are clearly distinct based on geographic distribution and age at disease onset. Recent advances in non-invasive imaging techniques have demonstrated that the blood vessels targeted by GCA and TAK overlap, giving rise to the ongoing debate whether the two diseases are separate or within the same disease spectrum (9–12). Molecular studies have emphasized that pathogenic events rely on cellular signal transduction pathways that are common in the two diseases, particularly when it comes to upstream pathologic effector functions of $CD4^+$ T cells (13–17). However, significant differences mediating disease-relevant processes and more detailed analyses of participating immune cells have supported the proposition that disease-specific activation pathways are potential therapeutic targets (18–20). Here, we will review recent progress in understanding the particular contributions of T cells in disease pathogenesis, how they arrive in the tissue microenvironment of a blood vessel wall, how they function as signal-sending and signal-receiving cells and how their reliance on activating signaling pathways might be exploited therapeutically.

GIANT CELL ARTERITIS

Earlier studies gave rise to the notion that T cells are clonally expanded within the vascular lesions and that human leukocyte antigen (HLA)-DRB1 alleles are genetic risk factors for GCA (21–24). More recent genetic analyses have confirmed the strong correlation between HLA class I and II molecules and susceptibility to GCA (25, 26). These data provide compelling support to the concept that GCA is a disease in which $CD4^+$ T cells react to antigen presented within polymorphic HLA molecules and that antigen recognition and expansion of $CD4^+$ T cells are critical disease mechanisms. Here we review different aspects of T cell biology in GCA, with the intent to utilize that knowledge to design more effective therapeutic interventions.

Effector $CD4^+$ T Cells

Multiple studies have shown that Th1 cells that produce interferon (IFN)-γ and Th17 cells that produce IL-17 participate in the vasculitic infiltrates (27–31). An expansion of Th1 and Th17 cells has also been reported for peripheral blood of GCA patients (27, 32). Interferons (IFNs) have a key role in antiviral immunity. IFN-γ, the sole type II IFN, has weaker antiviral effects than type I IFNs, such as IFN-α and IFN-β, but is a potent regulator of various cell types such as endothelial cells, stromal cells, dendritic cells and macrophages (33). IFN-γ vigorously increases major histocompatibility complex expression, increases antigen presentation and amplifies chemokine production, while suppressing cell proliferation (33). IFN-γ is the prototypic macrophage-activating factor that promotes cytokine and chemokine production, phagocytosis, and intracellular killing of microbial pathogens. By releasing IFN-γ, vasculitogenic T cells can effectively activate macrophages and direct their multiple effector functions. In GCA-affected arteries, stimulated macrophages release vascular endothelial growth factor (VEGF), thus fostering neoangiogenesis (34). A spectrum of macrophage functions depends directly on activating signals deriving from IFN-γ-producing $CD4^+$ T cells, assigning a key role to these T helper cells in granulomatous vasculitis.

The T cell cytokine IL-17 serves a complimentary role in the disease process. Th17 cells utilize the master transcription factor RAR-related orphan receptor gamma (RORγt) and require IL-23 for lineage differentiation and commitment (35). Besides the classical IFN-γ-supplying Th1 lineage, the so-called "IL-23-IL-17" axis makes critical contributions to autoimmune disease (36). IL-17A has been implicated in barrier and surface protection, functions in neutrophil recruitment and contributes to tissue repair. The "IL-23-IL-17" axis appears to be particularly important in psoriasis and spondyloarthritis (37–39). How IL-17 affects the disease process in GCA is not entirely understood.

Another T cell effector cytokine linked to granulomatous vasculitis is IL-21 (32). IL-21 is predominantly produced by follicular helper T (Tfh) and also by Th17 cells. IL-21 balances helper T cell subsets and induces B cell generation and differentiation into plasma cells, thus enhancing the production of immunoglobulins (40, 41).

IL-9-producing Th9 cells are also enriched in GCA lesions (42). IL-9 is believed to be involved in type 2 inflammation, induces activation of T helper cells and affects the function of various tissue resident cells such as mast cells and epithelial cells in the mucosa. T helper cells make a commitment to the Th9 lineage when stimulated with transforming growth factor β and IL-4 (43). IL-4 is distinctly low in GCA lesions and it is unclear whether Th9 cells are recruited as precursors or fully differentiated. Also, the precise role of IL-9 in GCA lesion remains unknown.

Increased expression of IL-22 in temporal artery biopsies from GCA patients has been reported (44). IL-22 is produced by both innate and adaptive immune cells, including innate lymphoid cells, and natural killer (NK) cells as well as T lymphocytes (Th17 and Th22) (45). IL-22 acts synergistically with TNF-α, IL-1β, and IL-17, and overall has pro-inflammatory effects (46). IL-22 is considered as potential therapeutic target in several autoimmune disease (47–49), but its precise role and drug-ability in GCA requires further investigation.

$CD4^+$ Regulatory T Cells

Regulatory T (Treg) cells, characterized by the expression of the lineage-determining transcription factor FOXP3, have

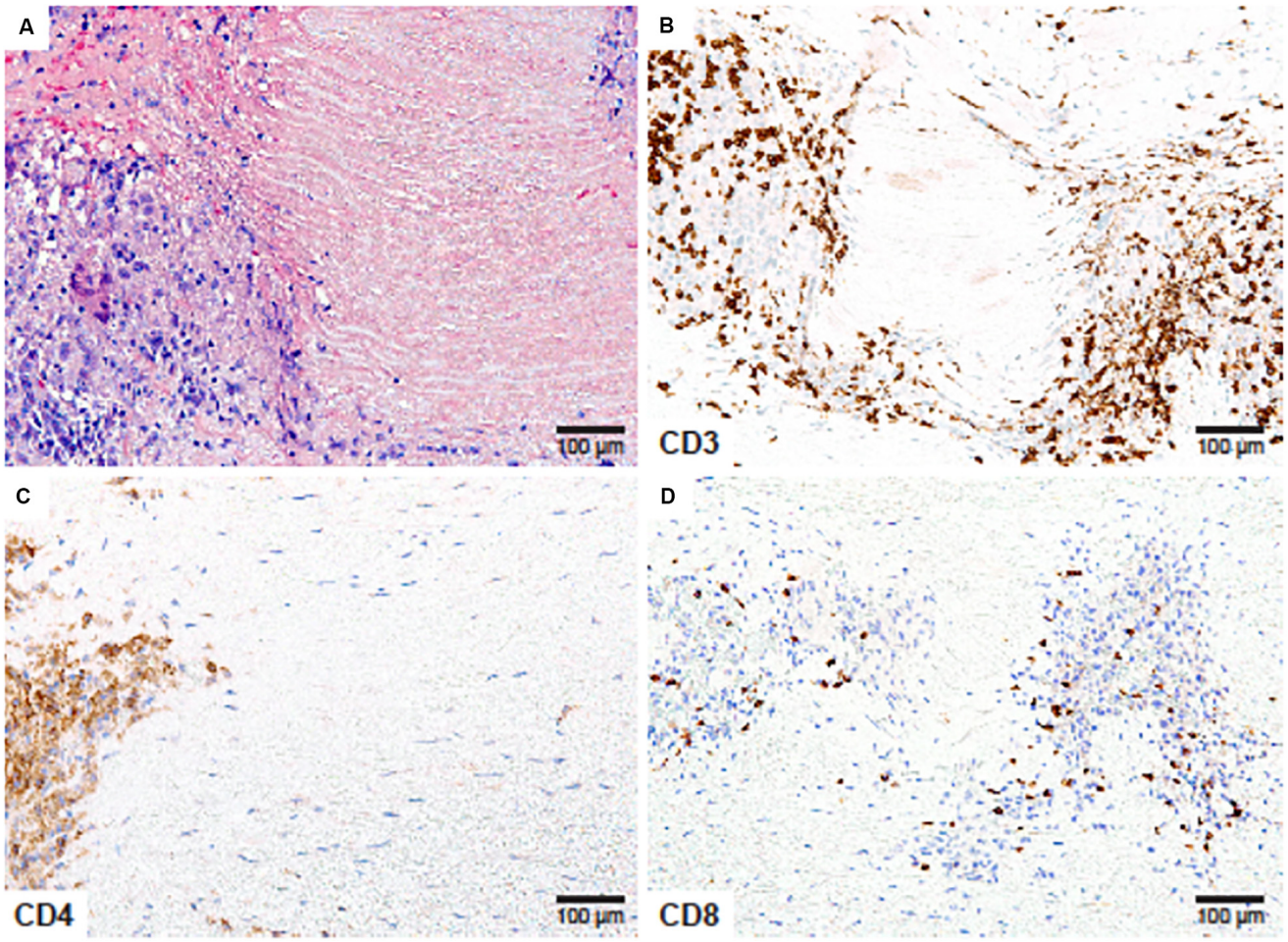

FIGURE 1 | $CD4^+$ and $CD8^+$ T cells in Giant Cell Arteritis. Tissue sections from an ascending aortic aneurysm repair in a 66-year-old woman presenting with back pain in the mid-thoracic region. **(A)** High power magnification of the medial layer showing granulomatous inflammation surrounding depleted smooth muscle cells and necrotic elastic fibers (Hematoxylin and eosin ×200). **(B)** CD3 immunostaining showing numerous T cells within the medial infiltrates (×200). **(C)** CD4 immunostaining of T-helper cells at the edge of the granuloma (×200). **(D)** CD8 staining showing scarce T-cytotoxic cells within the T-cell rich inflammatory lesions (×200).

a critical role in the maintenance of immune homeostasis and prevention of autoimmunity (50, 51). Patients with GCA are believed to have insufficient $CD4^+$ Treg cell function, eventually resulting in a peripheral tolerance defect (32). Frequencies of $CD4^+FOXP3^+CD25^{high}CD127^-$Tregs have been reported to be around 3% in GCA patients, as compared to 4–5% in age-matched controls (32). How this reduction in $CD4^+$ Treg cells leads to vasculitis is unresolved. Additional evidence for insufficient Treg cell function came from studies describing enrichment of dysfunctional Treg cells in active GCA patients (52). Specifically, such patients had higher frequencies of IL-17-secreting Tregs, characterized by the expression of an hypofunctional isoform of FOXP3 that lacks exon 2 (52). It has been proposed that tocilizumab, an antibody blocking the IL-6 receptor, may be able to improve the function of Tregs (52). Mechanistic studies, measuring recruitment, stability and suppressive functions of bona fide $CD4^+$ Treg cells in the vasculitic lesions are needed to get a better understanding of $FOXP3^+CD4^+$ T cells in GCA.

$CD8^+$ T Cells in GCA

While there is agreement that $CD4^+$ T cells are key drivers of both GCA and TAK, $CD8^+$ T cells appear to play a disease-specific role. The contribution of effector $CD8^+$ T cells to the pathogenesis of GCA is considered to be minor, based on the low number of $CD8^+$ T cells in GCA-affected arteries and a marked decrease of circulating $CD8^+$ T cells in active GCA patients (53, 54). $CD8^+$ T cells in GCA patients have been described to be clonally expanded and to use a restricted T cell repertoire (55, 56). Through which mechanisms effector $CD8^+$ T cells may have an impact on pathogenic events in GCA is unresolved. Recent immunohistochemical analyses have confirmed that overall $CD8^+$ T cells were lower in GCA-affected vascular lesions compared to Takayasu's arteritis (57).

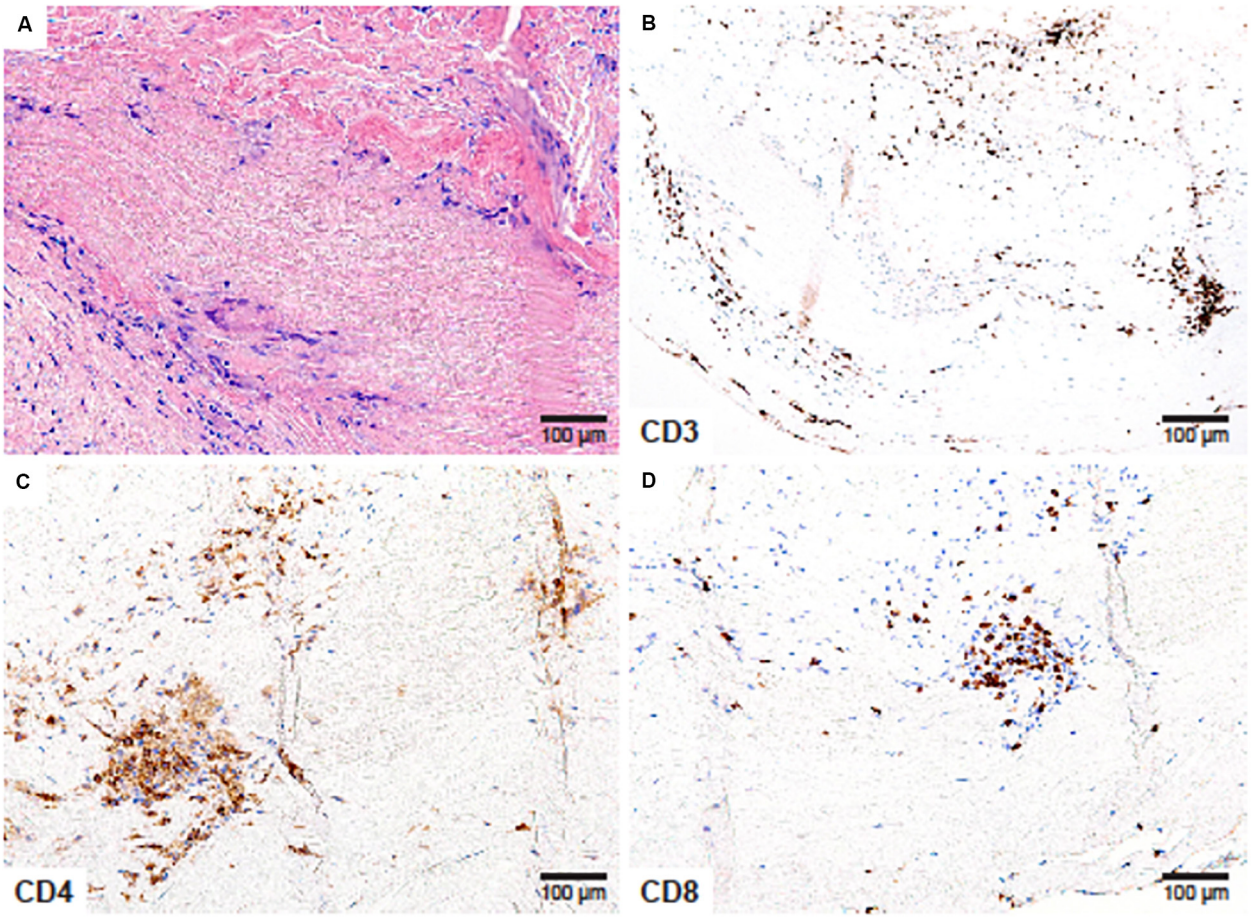

FIGURE 2 | $CD4^+$ and $CD8^+$ T cells in Takayasu arteritis. Aneurysm of the aortic root and the aortic arch, complicated by aortic regurgitation in a 41-year-old female. **(A)** Necrotizing granulomatous inflammation composed of lymphocytes and multinucleated giant cells surrounding a zone of necrotic medial tissue (H&E ×200); **(B)** $CD3^+$ T cells create an inflammatory collar around the zone of necrosis (×200); **(C)** $CD4^+$ T-helper cells (×200) and **(D)** $CD8^+$ T-cytotoxic cells (×200) comprise the majority of tissue-residing T-cells.

One specialized $CD8^+$ T cell subset, $CD8^+FOXP3^+$ Treg cells, have been assigned a critical role in the breakdown of the vessel wall immune privilege in GCA (58). Like $CD4^+$ Tregs, $CD8^+CD45RA^+CCR7^+FOXP3^+$ regulatory T cells have immunosuppressive potential and can be induced *ex vivo* from naïve $CD8^+$ T cells by low-affinity T cell receptor signaling combined with IL-15 (59). $CD8^+$ Treg cells localize to secondary lymphoid organs in young, healthy individuals, and suppress effector $CD4^+$ T cells by inhibiting phosphorylation of ZAP-70, a proximal adaptor molecule in the T cell receptor activation cascade (**Figure 3**) (58). However, in older individuals and in patients with GCA, $CD8^+$ Treg cells are low in numbers and diminished in function. $CD8^+$ Treg cells function by releasing NADHP oxidase 2 (NOX2)-containing exosomes, that transfer reactive oxygen species (ROS) into recipient $CD4^+$ T cells. Inability to secrete NOX-2-containing exosomes has been identified as the underlying defect of $CD8^+$ Treg cells in the old and in the GCA patient (58). Therapeutic targeting of $CD8^+$ Tregs, such as increasing functional $CD8^+$ Treg numbers or restoring NOX2 production in $CD8^+$ Tregs, may control not only GCA but also age-related inflammation or "inflammaging" (58, 60–62).

T Cell-Macrophage Interactions

Macrophages are immune cells of hematopoietic origin that provide fast immune defense (63). They are equipped to sense and respond to danger signals, usually released from dead and dying cells attacked by infectious microorganisms or other noxious stimuli (64, 65). In GCA, macrophages are unequivocal disease drivers and, together with $CD4^+$ T cells, form the pathognomonic granulomatous lesions. They not only produce cytokines (IL-1β, IL-6, and TNF-α) and chemokines (CXCL9, 10, 11, CCL5, and CCL 18) but also contribute to phagocytosis and antigen presentation, and provide co-stimulatory ligands regulating *in situ* T cell activation and survival (66). In the vasculitic lesions, they differentiate into tissue-destructive effector cells by releasing collagenases and matrix metalloproteases (MMP-2, 7, and 9) (67–69). Notably, MMP-9 is almost exclusively produced by $CD68^+$ macrophages and controls T cell entry into the vessel wall

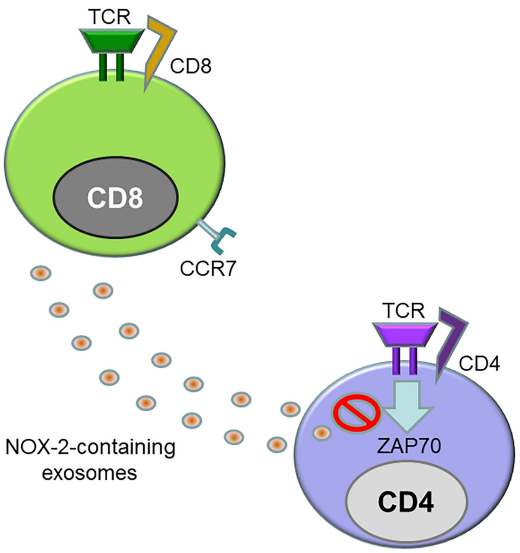

FIGURE 3 | Defective $CD8^+$ regulatory T cells in giant cell arteritis. Like $CD4^+$ regulatory T cells, $CD8^+$ regulatory T cells ($CD8^+$ Treg) express the transcription factor FOXP3 and act as a suppressor of immune responses. $CD8^+$ $CCR7^+$ Tregs inhibit immunity by releasing exosomes that contain the enzyme NADPH oxidase 2 (NOX2). These exosomes are integrated into the membrane of neighboring $CD4^+$ T cells, where they disrupt proximal signaling events, including the phosphorylation of ZAP70. $CD8^+$ Tregs from patients with GCA are reduced in number and are functionally defective.

by digesting the structural integrity of the external basement membrane (68). Blocking MMP-9 efficiently suppressed T cell infiltration into the artery and abrogated the remodeling of the vessel wall, including neointima formation and adventitial neovascularization (68). Essentially, $CD4^+$ T cell require MMP-9-releasing macrophages to enter the immune-privileged tissue site and cause vascular inflammation.

While patient-derived macrophages in GCA patients have many features of pro-inflammatory effector cells, their metabolic signature is surprisingly insipid. Expressions of glucose transporters, glycolytic enzymes and transcription factors regulating glycolysis have been described to be indistinguishable from macrophages generated from healthy individuals (66), with healthy and GCA macrophages utilizing glucose as their main substrate. As GCA macrophages enter the tissue microenvironment, they may have access to additional non-glucose energy sources, supporting longevity in the tissue niche. By providing fuel sources adapted to the needs of tissue-invading monocytes and macrophages, the tissue microenvironment attacked by vasculitic immune responses may make a critical contribution to disease pathogenesis.

T Cell-Dendritic Cell Interactions

Dendritic cells (DCs) are defined as cells with a stellate morphology that can efficiently present antigens on MHC molecules and activate naïve T cells (70). DCs initiate and shape both innate and adaptive immune responses. Considering the reactivity to autoantigen, DCs have long been considered important players in the loss of tolerance leading to autoimmunity, but in recent years their quintessential role in anti-tumor immunity has also been recognized (71, 72). Three-layered human arteries contain a population of DCs, so-called vascular DCs, localized at the adventitia-media border, where they can interact with T cells entering the vessel wall from the adventitial vasa vasorum (73). Vascular DCs sense danger-associated molecular patterns released by pathogens and Toll-like receptor (TLR)-stimulated vascular DCs break self-tolerance and induce T- cell recruitment and activation via co-stimulatory molecule expressions and chemokine release (74, 75). Notably, TLR profiles expressed by vascular DCs are unique for each vascular territory (76).

In addition to co-stimulatory molecules, co-inhibitory molecules are expressed on DCs (77), enabling the DCs to control the initiation, duration and robustness of an immune response. Interactions between programmed cell death protein-1 (PD-1) on activated T cells and its ligand (PD-L1) on DCs have attracted much attention recently since blockade of this axis has anticancer potential (78–81). Our recent work has identified a deficiency of PD-L1 expression on vascular DCs in GCA (82). PD-L1 expression on GCA monocyte-derived DCs was diminished compared to healthy monocyte-derived DCs, even under optimal stimulatory conditions with IFN-γ and LPS. This deficiency leads to overactivation of $CD4^+$ T cells, and production of IFN-γ, IL-17, and IL-21 (82). Using a large-vessel vasculitis model, we showed that PD-L1 blockade exacerbates vascular inflammation, promoting infiltration of activated T cells into the arterial wall and wall remodeling with neointima formation and adventitial neovascularization (82). These data provide unequivocal evidence that activated T cells play a central role in vascular remodeling and that anti-tumor therapy with checkpoint inhibitors blocking the PD-1-PD-L1 axis threatens the immune protection of the aorta and its major branches (83). In support of these data, case reports and observational studies have confirmed that patients treated with checkpoint inhibitors are at risk for therapy-induced vasculitis (84, 85). Autoimmunity and tumor immunity emerge as two sides of the same coin. If PD-L1 expression is reduced on DCs, risk for autoimmunity is high, but anti-tumor immunity is effective. In fact, it has been reported that the frequency of malignancy is relatively high in rheumatoid arthritis, Sjogren's syndrome, inflammatory myositis (86–89); however, the overall risk for cancer in GCA is not increased compared to healthy individuals (90). Understanding why GCA patients have a defect in PD-L1 expression should yield important insights. PD-L1 expression is dependent on glucose uptake and intracellular glycolytic activity (66, 91, 92). Large epidemiological studies demonstrate that body mass index and fasting blood glucose levels were negatively associated with the development of GCA (93, 94), supporting the concept that low glycolytic activity promotes low PD-L1 expression, enabling uncontrolled autoimmunity.

Additional co-inhibitory pathways appear to also be less functional in GCA patients. V-domain immunoglobulin-containing suppressor of T cell activation (VISTA) has been identified as a novel inhibitory receptor expressed on myeloid cells and T cells (95). Expression of VISTA on $CD4^+$ T

cell from GCA patients has been reported to be decreased, thereby facilitating T cell differentiation toward Th1, Th17, and Tfh (96). Expression of V-set and immunoglobulin domain containing 3 (VSIG-3), a ligand of VISTA (97), has not been explored in GCA lesions.

Signal Transduction Pathways in GCA CD4$^+$ T Cells

CD28-AKT-mTORC1 Axis

Activation of T cells requires at least two signals; one delivered by the T cell receptor complex and one provided by engagement of co-stimulatory receptors, such as CD28 (98, 99). T cell activation initiates a metabolic program required for cell growth, proliferation and differentiation (100). The demand for glucose uptake and glycolysis during T cell activation is known as the Warburg effect (101, 102). CD28 co-stimulation signals the activation of the phosphatidylinositol 3-kinase (PI3K)-AKT axis and maximizes glycolytic flux (103, 104). The mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase and forms part of mTOR complex 1 (mTORC1) (105). mTORC1 is targeted by the PI3K-AKT axis, and integrates a variety of environmental cues to regulate cell growth and tissue repair (106). mTORC1 promotes glycolysis through upregulation of the hypoxia inducible factor (HIF) 1α (105). mTORC1 is recognized as a signaling hub in multiple pathological conditions, such as cancer, obesity, neurodegeneration and the aging process (107). The PI3K-AKT-mTORC1 pathway is frequently overactivated in various human cancers (108). Sustained activation of mTORC1 is a signature abnormality of CD4$^+$ T cells in GCA (**Figure 4**). In the humanized mouse model of GCA, blockade of CD28 co-stimulation by anti-CD28 antibody was highly effective in dampening vascular inflammation. This therapeutic effect depended on inhibiting mTORC1 activity and constraining glycolytic flux in CD4$^+$ T cells (109). Blocking CD28 signaling and preventing mTORC1 activation curtailed mitochondrial respiration and, subsequently, cytokine production (109). Thus, the CD28-AKT-mTORC1 pathway is essential for vasculitic activity and emerges as a promising therapeutic target (13, 15, 109).

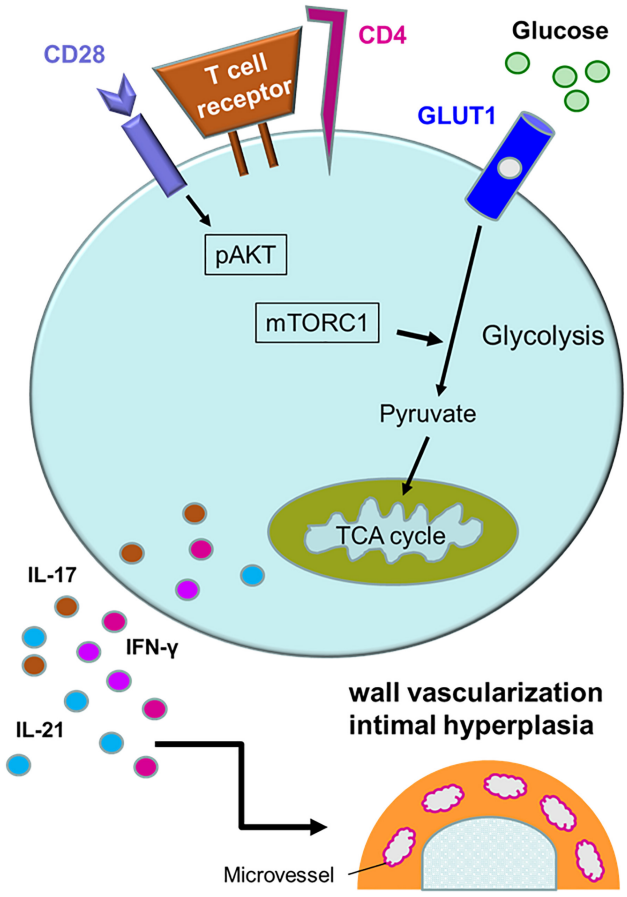

FIGURE 4 | Activation pathways in pathogenic CD4$^+$ T cells. Multiple signaling pathways contribute to the activation of pathogenic CD4$^+$ T cells in giant cell arteritis. Antigen recognition by the T cell receptor triggers rapid division, differentiation and lineage commitment. CD28 receptor engagement co-stimulates, mediated through AKT phosphorylation and downstream activation of the mechanistic target of rapamycin complex 1 (mTORC1). Additional stimuli derive from metabolic signals. Glucose uptake is controlled through the expression of the glucose transporter 1 (GLUT1). Glycolytic breakdown generates pyruvate, which functions as a critical energy carrier for mitochondria, sustaining ATP production and the release of metabolic intermediates. Antigen-stimulated, metabolically active CD4$^+$ T cells differentiate into effector cells secreting IFN-γ, IL-17, and IL-21 and sustain a vessel wall remodeling program resulting in wall vascularization and lumen-occlusive intimal hyperplasia.

NOTCH-mTORC1 Axis

"Classical" mTOR inputs are growth factors, nutrients and cellular energy, whereas "non-classical" mTOR inputs include WNT signaling and NOTCH signaling (110). NOTCH signaling primarily regulates cell proliferation, differentiation and cell fate decisions (111). Binding of cell-surface-bound ligands (Delta and JAGGED) to NOTCH receptors on neighboring cells initiates a biochemical cascade that results in of cleavages of the NOTCH receptor. The NOTCH intracellular domain translocates into the nucleus and acts as a transcriptional co-activator that promotes gene expression (110, 111). Aberrant expression of the NOTCH1 receptor is a signature abnormality in CD4$^+$ T cells of GCA patients (15). Transcriptomic analysis of biopsy material form GCA patients led to the discovery that the NOTCH1 ligand, JAGGED1, is expressed on microvascular endothelial cells, specifically on the vasa vasorum. VEGF, circulating in high amounts in the blood of GCA patients, was identified as an inducer of JAGGED1 on the endothelial cells (15). Activation of the NOTCH1 pathway resulted in the elevation of the HES1 protein, a potent activator of gene transcription (**Figure 5**) (15). Taken together, not only the CD28-PI3K-AKT axis but also the JAGGED1-NOTCH1 pathway contribute to high mTORC1 activity, leading to Th1 and Th17 differentiation and to equipping lesional T cells with functionality of disease orchestrators. Recognizing the NOTCH pathway as a driving force in medium and large vessel vasculitis will provide new opportunities for immunomodulation (112).

JAK-STAT Pathway

Cytokines represent soluble factors with essential roles in immune response and employ diverse intracellular pathways.

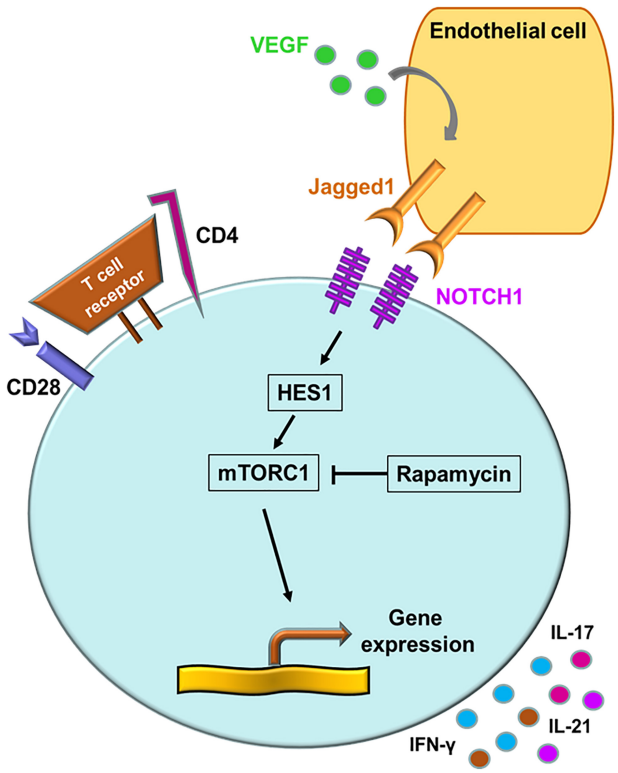

FIGURE 5 | The NOTCH signaling pathway in giant cell arteritis. A signature abnormality of $CD4^+$ T cells in GCA is the aberrant expression of the NOTCH1 receptor. $NOTCH1^+$ $CD4^+$ T cells engage the JAGGED1 ligand on the surface of microvascular endothelial cells. Vascular endothelial growth factor (VEGF) in the circulation functions as the JAGGED1 inducer. Via HES1, the NOTCH signaling pathway activates the mechanistic target of rapamycin complex 1 (mTORC1). Persistent NOTCH signaling promotes T cell proliferation, invasion of $CD4^+$ T cells into the vessel wall, upregulation of the metabolic program and differentiation into cytokine-producing effector cells.

A subset of cytokines that bind type I and type II cytokine receptors utilizes the Janus kinase-signal transducer of activators of transcription (JAK-STAT) pathway (113, 114). They include IL-6, common, chain cytokines (IL-2, 4, 7, 9, 13, and 15), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-10, IFN-α, IFN-β, and IFN-γ (115). Since genome-wide association studies have revealed this pathway as highly relevant to human autoimmunity, pharmacological inhibitors of JAK or JAKinibs have created a new paradigm for the treatment of rheumatic disease (115). In the autoimmune disease rheumatoid arthritis, JAK inhibitors are as effective as biological disease-modifying antirheumatic disease (116, 117), indicating that key disease pathways rely on JAK-STAT signaling. JAK-STAT signaling appears to be exceedingly relevant in vasculitis as well, as recent work in GCA-affected tissue lesions and in patients' T cells suggests (**Figure 6**) (16). In human artery-SCID chimera mice, tofacitinib, an inhibitor that primarily targets JAK3 and JAK1, efficiently suppressed vasculitis by restraining the activation of T cells and macrophages (16). Analysis of T cells in the vasculitic infiltrates identified a population of highly proliferative tissue-resident memory T cells, that was dependent on JAK-STAT signaling and sensitive to tofacitinib-mediated inhibition. Such tissue-resident T cells are now emerging as the source of self-renewing T cells, that maintain the granulomatous formations in the vessel wall. In a clinical study, re-biopsy of patients after 3, 6, 9, and 12 months of corticosteroid therapy demonstrated that the vascular inflammatory lesions were unexpectedly stable and tissue-occupying T cells persisted in the majority of patients for more than 1 year (118). These "tissue-resident memory T cells" are now recognizes as a specialized T cell subset, that can adapt to the tissue environment and retain the capacity to locally replenish the infiltrate. These T cells express CD103 and CD69 as a phenotypic surface marker (119–121). In a *trans*-engraftment model, in which human arteries with vasculitis lesions are engrafted into "empty" immunodeficient mice, the survival strategy of human T cells can be analyzed. In these *trans*-engrafted arteries, tissue-resident memory T cells expand autonomously and their expansion is druggable with JAKinib (16). Tofacitinib was sufficient to diminish cell proliferation and cytokine production from activated $CD4^+$ T cells (16), demonstrating that JAK-STAT signaling contributes to disease-relevant processes in GCA.

Type I IFN Signature

Interferons (IFN) are cytokines that have antiviral, antiproliferative, and immunomodulatory effects (122). Inborn errors or impaired function of IFN-mediated immunity confer predisposition to viral and mycobacterial infection (123). There are two main classes of IFN: IFN-γ is the only type II IFN, and type I IFNs include IFN-α, IFN-β, and others that bind a common cell-surface receptor (122). Type I IFNs activate the JAK-STAT signaling pathway to induce expression of interferon-stimulated genes, called "interferon signature" (**Figure 6**) (33). Interferons also induce an "interferon epigenomic signature" by activating latent enhancers and chromatin (33). The type I IFN signature is upregulated in several autoimmune diseases, identifying this cellular activation pathway as a prime candidate for immunosuppressive therapy (124, 125). Transcriptome analysis of biopsy material from patients with GCA has demonstrated robust induction of type I and II IFN signatures (16), indicating that both type I and type II IFNs are abundant within the vasculitic lesions. The source of type I IFNs in GCA has not been defined, providing an opportunity to identify key cellular drivers. In the autoimmune disease systemic lupus erythematosus, plasmacytoid DCs are the main source of type I IFN (126, 127). There is no evidence, to date, that plasmacytoid DC are present in the inflamed vessel wall. The effect that IFNs have on T cells is complex, involving direct and indirect interference in T cell functionality (128). Type I IFN receptors are broadly expressed in many tissues and by many cell types, both innate as well as adaptive immune cells are regulated by this cytokine family. Receptors for type II IFN are mostly encountered on granulocytes, monocytes and macrophages, Type II IFN is a product mainly of T cells and NK cells, placing the T cell-monocyte/macrophage axis at center stage in GCA.

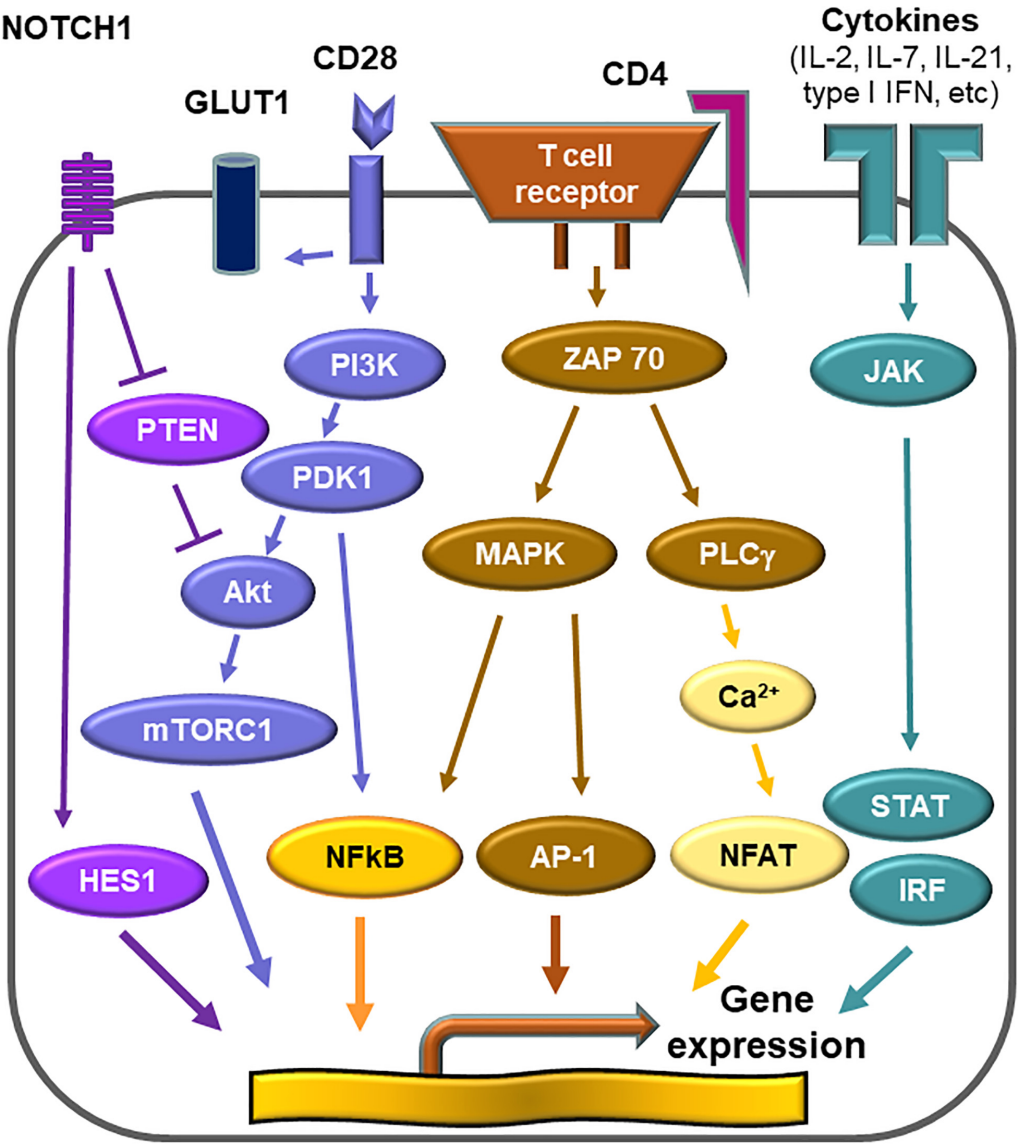

FIGURE 6 | Druggable signaling cascades in vasculitogenic T cells. Numerous signals converge to shape the activation patterns of T cells. In the case of vasculitis-inducing T cells, vasculitogenic antigens trigger the T cell receptor activation cascade. Several co-stimulatory signals adjust signal strength and duration and thus determine differentiation, effector functions and longevity of the T cell. CD28-dependent signaling regulates the metabolic program of vasculitogenic T cells. CD28-mediated signals activate mTORC1, thus determining T cell proliferation and lineage assignment. Persistent NOTCH signaling is a signature abnormality of vasculitogenic $CD4^+$ T cells and regulates tissue invasiveness. Cytokines modulating T cell function utilize the JAK-STAT signaling pathway. Disease-relevant cytokine signals derive from IL-2, IL-7, and, possibly, from type 1 interferon.

TAKAYASU'S ARTERITIS

Unlike GCA, that has the highest incidence in elderly women of Northern European descent (8, 129), Takayasu's arteritis (TAK) is more prevalent in young Asian women (130–132). HLA-B52 is recognized as a susceptibility locus in TAK (20, 133–135). A subset of TAK patients have pre-existent or co-existent inflammatory bowel disease, connecting this vasculitis to systemic immune abnormalities (136, 137). HLA-B52 is detected in a high rate of patients with TAK complicated with ulcerative colitis (138). The vascular lesions of TAK resemble those encountered in GCA and are composed of highly activated T cells and macrophages, arranged in granulomatous formations (6). In the past decade, TAK and GCA have often been considered to represent a spectrum of one disease, supported by similarities in target blood vessel patterning and histopathologic findings (9–12). However, genome-wide associated studies and immunophenotyping of immune cells in the peripheral blood and in the vascular lesions have revealed substantial differences (18–20, 57). Particularly, immune cells other than $CD4^+$ T cells and macrophages have shown to be critical in the pathogenesis of TAK (19, 20, 139, 140). Here, we review the current knowledge

implicating $CD4^+$ T cells, macrophages, $CD8^+$ T cells, natural killer (NK) cells as critical players in the disease process leading to TAK. New data demonstrating the production of autoantibodies against endothelial cells in TAK patients shed new light on pathogenic adaptive immunity (141). Special emphasis has been placed on cellular signaling pathways that are active in TAK and GCA, with the goal to define common and disease-specific pathogenic mechanisms.

$CD4^+$ Effector T Cell

Giant cell arteritis and Takayasu arteritis share the predominance of Th1 cells and the participation of Th17 cells in the inflammatory process (141). IFN-γ and IL-17 are markedly increased in the peripheral blood and in the affected aortic tissue (141). The entire signaling pathways mediating the induction of Th1 cells appears to be upregulated. *IL-12B* is well established as a susceptibility gene in TAK (134), and plasma IL-12 levels are elevated (142), biasing T cell differentiation toward the Th1 lineage (143). IL-23 is also increased in the serum of patients with TAK (141), and IL-23 promotes IL-17 production by $CD4^+$ T cells (144). In essence, the cytokine environment enables the emergence of Th1 and Th17 cells, promoting immune responses associated with granulomatous inflammation.

Differential responses to glucocorticoid therapy have been reported for GCA and TAK patients. In GCA, Th17 cells are susceptible, while Th1 cells are resistant to glucocorticoid therapy; whereas steroid treatment is able to suppress Th1 cytokines, but left Th17 cytokines unaffected in TAK (27, 141). The reason for this strikingly different responsiveness remains unclear, but these data strongly support the hypothesis that the cytokine environment may ultimately be very different in these two disorders.

A shared feature of $CD4^+$ T cells in both GCA and TAK is the strong upregulation of mTORC1 activity (13, 14). mTOR is a signaling hub in T cell fate decisions and mTORC1 activation biases T cell differentiation toward the Th1 and Th17 lineage. Strong evidence for mTORC1's role as a key decision maker comes from studies using rapamycin to guide T cell differentiation. The mTORC1 blocker was able to prevent differentiation into Th1 and Th17 cells (13, 14). The cause of persistent mTORC1 activation in GCA and TAK T cells is not understood but may be an independent factor in directing disease-relevant immunity. Upregulation of the mTORC1 pathway has also been reported for endothelial cells and vascular smooth muscle cells in the aorta (13, 145), suggesting that the mTORC1 pathway is universally activated in TAK.

In a recent study, a group of French scientists explored whether IFN signatures are activated in isolated $CD4^+$ and $CD8^+$ T cells from patients with active TAK (17). Transcriptome analysis demonstrated that 248 genes were dysregulated in $CD4^+$ T cells and 432 genes in $CD8^+$ T cells. Pathway enrichment analysis identified type I and type II IFN signatures and cytokine/chemokine signaling as highly enriched in both $CD4^+$ and $CD8^+$ T cells from TAK patients. Further analysis pointed toward active signaling in the STAT5 pathway, suggesting that the patients' T cells might be exposed to elevated levels of the T cell growth factor IL-2. Treatment of two TAK patients with a JAK-STAT inhibitor resulted in a measurable decreased of Th1 and Th17 cell frequencies (17), supporting the premise that T cells activation in TAK is ultimately controlled by cytokines and growths factors utilizing the JAK-STAT pathway. This conclusion is supported by a series of case reports showing the efficacy of JAK inhibitors in refractory TAK patients (146, 147). These studies suggest a potential role of JAK-STAT pathway blockade as a promising approach to dampen disease activity in TAK (148).

$CD4^+$ Regulatory T Cells

Common to most autoimmune diseases, Treg cell dysfunction has been proposed to underlie the chronic immune stimulation in TAK. Recent work has shown a decrease in the function of peripheral blood $CD4^+$ Treg cells in TAK patients (149). Plasticity of $CD4^+$ Tregs acquiring effector cell function has been described (150, 151), and the authors proposed that Th2-like transformed Tregs that secret IL-4 and IL-13 contribute to the development of TAK (149). Th2 cytokines are rarely encountered in GCA (152), and the appearance of this class of cytokines may indeed be a distinguishing features between TAK and GCA. Selective therapeutic manipulation of $CD4^+$ Tregs in autoimmunity has long been an objective (51) and the above-mentioned study reported that the blockade of the JAK-STAT pathway can restore $CD4^+$ Treg cells and increase the ratio of $CD4^+$Treg/$CD4^+$ effector T cells (17). Correction of the aberrantly activated JAK-STAT pathway may, therefore, be effective in modifying several pathogenic domains in TAK. However, this form of treatment in TAK raises a number of safety concerns, all of which need to be addressed before new therapies can be introduced into standard management; including the age of the patients, the chronicity of disease and the risk of immunosuppression during the pandemic spread of new viral agents, promotion of neoplasia, etc.

$CD8^+$ T Cells

Detailed analyses of the inflammatory burden in TAK have demonstrated $CD8^+$ T cells accounting for approximately 15% of the wall-infiltrating cells in TAK-affected aortas (153). $CD8^+$ T cells are able to release perforin directly onto the surface of aortic vascular cells, thus causing direct tissue damage (153). Immunophenotypic studies of peripheral blood immune cells using multiparametric fluorescent techniques have provided evidence that the numbers of total $CD8^+$ T cells and $CD8^+$ effector T cells are both higher in TAK than in GCA (19). Notably, memory $CD8^+$ T cells remained high even during clinical remission and the number of total $CD8^+$ T cells was correlated with TAK, but not GCA relapse (19). In TAK patients, $CD8^+$ T cells are increased not only in the circulation but also in the vessel wall infiltrates (57). Immunohistochemical analysis has shown that, compared with GCA-affected arteries, vascular surgical specimens in TAK had increased proportions of $CD8^+$ T cells and the CD4/CD8 ratio differentiated the two vasculitides (57). A recent GWAS study also supported the critical role of $CD8^+$ T cells in the pathogenesis of TAK (20). Taken together, these results indicate that GCA and TAK have critical differences

TABLE 1 | $CD4^+$ and $CD8^+$ T cells in giant cell arteritis and in Takayasu arteritis.

		Giant cell arteritis	Takayasu arteritis
$CD4^+$	Contribution to the disease	Key driver	Key driver
	Dominant helper T cell subset	Th1, Th17, Th21, Th9	Th1, Th17, Th21, Th9
	Dominant effector molecules	IFN-γ, IL-17, IL-9, IL-21, IL-22	IFN-γ, IL-17, IL-9, IL-21
	$CD4^+$ Treg	Dysfunctional	Decreased
	mTORC1	Highly activated	Highly activated
	NOTCH pathway	Highly activated	??
	JAK-STAT pathway	Highly activated	Highly activated
	Type I Interferon signaling	Activated	Activated
$CD8^+$	Contribution to the disease	Minimal	Substantial
	Dominant effector molecule	??	Perforin
	$CD8^+$ Treg	Decreased and dysfunctional	??
	mTORC1	??	Highly activated
	JAK-STAT pathway	??	Highly activated
	Type I Interferon signature	??	Activated

in immune cell composition in the peripheral blood and within the vasculitic lesions.

$CD4^+$ T cells and $CD8^+$ T cells in TAK patients share sustained mTORC1 activity (14, 154, 155). Upstream signals that drive activation of the mTORC1 pathway have not yet been defined and our understanding of the functional consequences for protective and pathogenic immunity in these patients is limited. Continuous activation of central cellular signaling pathways appears to be an overall theme in patients affected by TAK. Besides chronic mTORC1 activation, T cells from TAK patients have ongoing activation of the JAK-STAT pathway, inducing type I and type II IFN gene expression signatures (17). Comparative studies of $CD4^+$ and $CD8^+$ T cells, which are responsible for fundamentally different effector pathways, may shed light on the state of chronic stimulation of the adaptive immune system in this form of vasculitis.

Natural Killer (NK) Cells

Seko et al., were the first to report that NK cells have a prominent position in the vascular injury leading to TAK (153). $CD16^+$ NK cells account for 20% of the immune cells in the aortic wall infiltrates (153). Like $CD8^+$ T cells, NK cells were positive for perforin immunostaining, providing strong support for a role in inducing cellular damage. Further support for the critical contribution of NK cells has come from the GWAS study by Terao et al., defining NK cells as the most promising target in the pathophysiology of TAK (20). NK cells are regulated by accessory molecules, such as the major histocompatibility complex (MHC) class I chain-related gene (MIC) family, and the leukocyte immunoglobulin-like receptor (LILR) family (139). Overexpression of MIC-related A (MICA) and its receptor natural killer group 2 member D (NKG2D) in aortic tissue from TAK cases has been reported (156). Expression of MHC and MICA on aortic vascular cells allows NK cells to recognize them through the NKG2D receptor and, in turn, attack them (139). LILR family members (LILR A1 to A6 and LILR B1 to B5) are widely expressed on hematopoietic cells and mediate activation as well as inhibition of immune cell function (157–159). Among them, *LILRA3* was identified as a novel susceptibility loci in the TAK GWAS study (20). How precisely LILRA3 regulates immune response in TAK remains largely unknown, but it has been proposed that LILRA3 may bind to the major TAK susceptibility molecule HLA-B52 (20, 139). So far, NK-directed therapies are unavailable but TAK may become the signature disease to tap into novel opportunities of immune modulation and preventing immune cell-dependent cellular injury.

Autoantibodies Against Endothelial Cells (ECs)

A recent report on anti-endothelial cell autoantibodies has raised the question of the potential role that B cells participate in TAK disease pathogenesis. B cells are infrequent in the vasculitic infiltrates, which are typically cell admixtures of T cells and macrophages. However, B cells may play a critical role in the breakdown of immune tolerance that precedes the invasion of the vessel wall by inflammatory cells. Anti-endothelial cell antibodies (AECA) are a heterogenous group of autoantibodies against ECs (160). AECA have been detected across the spectrum of systemic vasculitides, ranging from small-vessel vasculitides to medium- and large-vessel vasculitides (161). Classically, AECA bind to ECs and induce apoptosis through direct complement-dependent cytotoxicity or through indirect antibody-dependent cytotoxicity (161), identifying antibodies as regulators of EC survival. In TAK, IgG deposits have been reported in the intima (162) and autoantibodies recognizing ECs have been evaluated as disease activity markers (163, 164). As expected, identification and quantification of AECA varies based on the technique used, e.g., indirect immunofluorescence, enzyme-linked immunosorbent assay, fluorescence-activated cell sorting, and immunoblot assays (160). Accordingly, measurement of AECA has not found its way into routine diagnostic schemes. This may change as a recent publication has defined autoantigens recognized by AECA. Mutoh et al., have identified two autoantigens expressed on ECs by applying an elegant serological identification system based on a retroviral expression system and flow cytometry (165). In this system, a cDNA library of ECs was retrovirally

transfected into a rat myeloma cell line and AECA-positive clones were sorted by flow cytometry (165, 166). This yielded endothelial protein C receptor (EPCR) and scavenger receptor class B type 1 (SR-BI) as bona fide autoantigens (165). In a cohort study, the authors found that approximately 1 in 3 patients with TAK produce autoantibodies against either antigen. Remarkably, both molecules that are recognized by autoantibodies negatively regulate endothelial cell function, undermining the protective role of the endothelial layer (165). The authors propose that the autoantibodies disrupt the barrier function of ECs, opening the intimal surface to immune cell infiltration. Autoantibodies recognizing antigen specifically expressed by ECs appears to accompany chronic inflammatory disease (167, 168), but, interestingly, the autoantigens display a disease-specific pattern. Understanding the timing of events such as the emergence of AECA may be informative in assigning AECA-specific pathogenic determinants.

CONCLUSION

The inaccessibility of the body's major arteries to tissue sampling have complicated the diagnosis and pathogenic understanding of autoimmunity in blood vessels. With the advent of non-invasive imaging techniques, vasculitis of the aorta and its major branch vessels can now be assessed, classified, and monitored. Comprehensive analysis using DNA, RNA, proteins, cell surface markers, transcription factors, and signaling pathways have greatly contributed to our understanding of the pathophysiology of large vessel vasculitis. A major insight has been that LVV has two principle disease components, the systemic inflammatory response, and the granulomatous vasculitis in the vessel wall. Traditionally, it has been assumed that patients with GCA and TAK develop autoimmunity against vascular antigens, which induces granulomatous vasculitis and, as a spill over, systemic inflammation. Much improved conceptualization of autoimmunity is beginning to question the validity of this traditional disease concept.

Patients with GCA and TAK are born with genetic risk factors that render them susceptible to a disease that will present clinically 2–6 decades later. Genes within and outside of the HLA complex have been identified as risk determinants, but a consistent theme of the association studies is the connection of genetic risk factors with immune cell function. With solid evidence that GCA and TAK are essentially immune-mediated diseases, multiple immune cell types are now recognized as critical disease players, including $CD4^+$ and $CD8^+$ T cells, monocytes and macrophages, NK cells and autoantibody-producing B cells. Emerging data indicate that $CD8^+$ T cells may be more important in TAK than in GCA, opening opportunities to implicate different immune cells in different aspects of pathogenesis (**Table 1**). Pathogenic studies in TAK have been complicated by multiple hurdles, such as access to diseased tissue, the lack of reliable animal models and the low disease prevalence. Therefore, data on disease mechanisms in TAK have remained less robust. In both, GCA and TAK, immune cell-mediated injury to vascular cells may lie upstream of the chronic granulomatous reaction typifying these autoimmune diseases.

As in other autoimmune diseases there is now recognition that pathogenic events leading to GCA and TAK may involve multiple disease components, that are not always coordinated and that require specialized therapeutic interventions. Specifically, the extravascular and the vascular component of LVV seem to follow different trajectories, rely on different mechanisms, and respond differently to current treatments. Extravascular GCA and TAK are characterized by intense systemic inflammation and are measured by elevation of acute phase reactants, such as C-reactive protein and erythrocyte sedimentation rate. They are simple to measure in the peripheral blood and are easy to suppress with corticosteroids or by blocking IL-6 signaling (169, 170). Much more challenging is the vascular component of TAK and GCA; a persistent, refractory granulomatous inflammation positioned in the vessel wall (69, 171). Vascular GCA and TAK are difficult to treat; 50% of patients have persistent vasculitis despite intense therapy for 1 year (118). The resistance to standard immunosuppression is corroborated by recent reports of ongoing disease activity in patients treated with anti-IL-6 therapy (172–176). Underlying molecular mechanisms are those of sustained activation of innate and adaptive immune cells through a plethora of signaling pathways. Most significant are the enduring activation of the mTOR pathway, the NOTCH signaling pathway and the JAK-STAT pathway (**Table 1** and **Figure 6**). In combination, these essential cellular signaling pathways drive lasting immune responses in a tissue site that is intolerant to damage. The unparalleled effectiveness of corticosteroids in treating large vessel vasculitis may well reflect their imprecision in suppressing cellular activation pathways.

AUTHOR CONTRIBUTIONS

RW, JG, and CW wrote the manuscript. GB contributed the tissue images. DL participated in the concept development. All authors contributed to the article and approved the submitted version.

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The Role of T Cells Reactive to the Cathelicidin Antimicrobial Peptide LL-37 in Acute Coronary Syndrome and Plaque Calcification

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The human cationic anti-microbial peptide LL-37 is a T cell self-antigen in patients with psoriasis, who have increased risk of cardiovascular events. However, the role of LL-37 as a T cell self-antigen in the context of atherosclerosis remains unclear. The objective of this study was to test for the presence of T cells reactive to LL-37 in patients with acute coronary syndrome (ACS). Furthermore, the role of T cells reactive to LL-37 in atherosclerosis was assessed using apoE−/− mice immunized with the LL-37 mouse ortholog, mCRAMP. Peripheral blood mononuclear cells (PBMCs) from patients with ACS were stimulated with LL-37. PBMCs from stable coronary artery disease (CAD) patients or self-reported subjects served as controls. T cell memory responses were analyzed with flow cytometry. Stimulation of PBMCs with LL-37 reduced CD8+ effector T cell responses in controls and patients with stable CAD but not in ACS and was associated with reduced programmed cell death protein 1 (PDCD1) mRNA expression. For the mouse studies, donor apoE−/− mice were immunized with mCRAMP or adjuvant as controls, then T cells were isolated and adoptively transferred into recipient apoE−/− mice fed a Western diet. Recipient mice were euthanized after 5 weeks. Whole aortas and hearts were collected for analysis of atherosclerotic plaques. Spleens were collected for flow cytometric and mRNA expression analysis. Adoptive transfer experiments in apoE−/− mice showed a 28% reduction in aortic plaque area in mCRAMP T cell recipient mice ($P < 0.05$). Fifty six percent of adjuvant T cell recipient mice showed calcification in atherosclerotic plaques, compared to none in the mCRAMP T cell recipient mice (Fisher's exact test $P = 0.003$). Recipients of T cells from mice immunized with mCRAMP had increased IL-10 and IFN-γ expression in CD8+ T cells compared to controls. In conclusion, the persistence of CD8+ effector T cell response in PBMCs from patients with ACS stimulated with LL-37 suggests that LL-37-reactive T cells may be involved in the acute event. Furthermore, studies in apoE−/− mice suggest that T cells reactive to mCRAMP are functionally active in atherosclerosis and may be involved in modulating plaque calcification.

Keywords: acute coronary syndrome, T cells, atherosclerosis, cathelicidin, LL-37, mCRAMP

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INTRODUCTION

Adaptive immunity has a major role in atherosclerosis (1), the underlying cause of coronary artery disease (CAD), associated with a variety of antigens that have been described (2). However, other potential self-antigens remain unknown (3). It has been proposed that in the chronic inflammatory state present in atherosclerosis, tolerance to self-antigens could be broken through several potential mechanisms, implicating an autoimmune component in atherosclerosis (4). This is supported by the reported correlation between atherosclerosis and T Effector Memory cells (5). Furthermore, T Effector Memory cell density is associated with atherosclerotic plaque stage in humans (6). Rupture of the atherosclerotic plaque is the most common cause of acute coronary syndromes (ACS).

Antimicrobial peptides are an important part of the innate immune system and they are active against pathogens directly through their antimicrobial properties, or through their immunomodulatory effects (7). The human antimicrobial peptide LL-37, a cleavage product of the cathelicidin proprotein hCAP-18, is present in human atherosclerotic plaques (8), and is associated with platelet activation and induction of thrombosis (9), with higher serum levels in coronary circulation compared to systemic levels in patients with ST elevation myocardial infarction (STEMI) (10). Moreover, LL-37 is present in neutrophil extracellular traps (NETs), which have been implicated in atherogenesis (11). They are associated with self-immunity as self-DNA/LL-37 complexes that aggravate atherogenesis (12). Interestingly, NET burden was associated with infarct size and negatively associated with ST segment resolution in patients with STEMI (13). Furthermore, LL-37 induced differentiation of human mononuclear cells into bone-forming cells (14), suggesting a potential role in calcific mineralization. LL-37 is a T cell self-antigen in patients with psoriasis (15), who have increased risk of cardiovascular morbidity and mortality (16, 17). It is not known if there is a self-reactive T cell response to LL-37 as a self-antigen in the context of atherosclerosis and if so, whether it is beneficial or pathogenic.

CRAMP is the mouse homolog of the human cathelicidin proprotein hCAP-18, which like CRAMP, is proteolytically cleaved to generate a cathelin-like domain and the cationic anti-microbial domain LL-37, or mCRAMP in mice (**Figure 1**). Similar to LL-37 in humans, the proprotein CRAMP has been linked to atherogenesis in apoE−/− mice as suggested by experiments showing that Cramp−/− apoE−/− mice have less atherosclerosis compared to apoE−/− mice (18). Our group has previously shown that immunization with a low dose of the murine proprotein CRAMP was associated with decreased atherosclerosis in apoE−/− mice (19).

The objective of this study was to test the role of LL-37 as a potential T cell self-antigen in patients with ACS. Furthermore, the role of T cells reactive to LL-37 in atherosclerosis was assessed using apoE−/− mice immunized with the LL-37 mouse ortholog, mCRAMP.

MATERIALS AND METHODS

Human PBMC

The protocols were approved by the Cedars-Sinai Institutional Review Board (IRB). Peripheral blood mononuclear cells (PBMCs) were isolated from blood collected from 10 patients with ACS within 72 h of admission to the Cedars-Sinai Cardiac Intensive Care Unit. Patients were consented under the approved IRB protocol Pro48880. Exclusions were inability to give informed consent, age less than 18 years old, active cancer treated with chemotherapy or radiation, patients taking immune-suppressive drugs, and pregnant women. PBMCs from 10 stable CAD patients were isolated from blood collected on the day of coronary angiography, consented under the approved IRB protocol Pro50839 with the same exclusions. Consented data use was limited to age, sex, LDL levels, and use/non-use of cholesterol-lowering medication. PBMCs were isolated using Ficoll density gradient centrifugation and cryo-preserved in commercially available cryogenic solution (Immunospot) in liquid nitrogen. Cryo-preserved PBMCs from self-reported controls (N = 15) were purchased from a commercial source (Immunospot).

Peptide Stimulation of Human PBMC

Cryo-preserved PBMCs were thawed, rinsed in anti-aggregation solution (Immunospot), and seeded in culture plates at a density of 3×10^6cells per ml of RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum and 1× antibiotic/antimycotic. Peptides (LifeTein) used for stimulation corresponded to LL-37 and the truncated cathelin domain of hCAP-18 [cat-hCAP-18 (aa 39-136)]. Cells were stimulated with one of the following: 20 μg/ml LL-37 or cat-hCAP-18 peptide, 0.5× T cell stimulation cocktail containing PMA and ionomycin (Thermo Fisher). Culture medium was added at $^1/_3$ of the starting volume 48 h later to replenish the nutrients in the medium. Cells were harvested 72 h after seeding, stained for viability (LIVE/DEAD Fixable Aqua Dead Stain Kit, Thermo Fisher), and subjected to cell surface staining for flow cytometry using the following antibodies: CD3, CD4, CD8, CD45RA, CD45RO, CD62L, and CD197 (CCR7). Isotypes were used as staining control. CD4+ or CD8+ T Effector cells were gated on CD45RO+CD62L(−)CD197(−). T Effector Memory cells were CD45RO+CD45RA(−) CD62L(−)CD197(−), T Effector Memory RA+ cells were CD45RO+CD45RA(+)CD62L(−)CD197(−). Results were tabulated as Response Index using the following calculation (20):

$$\frac{(\%\ peptide\ stimulation\ -\ \%\ no\ stimulation)}{(\%\ cocktail\ stimulation)} \times\ 100$$

The results are expressed as Response Index to account for inherent variations introduced by culturing cells in vitro over time, controlled for by assessing response relative to baseline cell phenotype (% no stimulation) and maximal stimulation (% cocktail stimulation) for each subject PBMC. Each data point represents one subject.

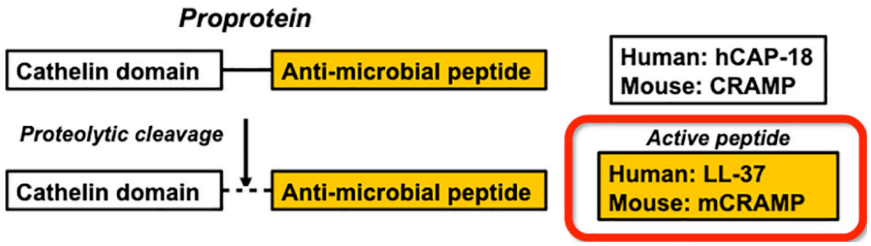

FIGURE 1 | Enzymatic processing of the human antimicrobial peptide LL-37 and the mouse ortholog mCRAMP.

PDCD1 mRNA Expression

In samples with sufficient cell numbers, stimulated PBMCs cultured for 72 h were collected for RNA extraction using Trizol (Thermo Fisher) and subjected to qRT-PCR with SYBR green and a primer pair for human PDCD1. Cyclophilin A served as the reference gene. Results were expressed as fold-change relative to non-treated cells of each sample using the $Ct_{\Delta\Delta}$ method.

IFN-γ ELISA

Conditioned medium was collected after 72 h of LL-37 stimulation of PBMCs and IFN-γ was measured using a commercially available ELISA kit (Abcam) according to manufacturer's instructions.

MHC Blocking

PBMCs from control samples were stimulated with LL-37 alone or in the presence of either anti-human HLA-A, B, C (clone W632) or anti-human HLA-DR (clone L243) monoclonal antibody (Biolegend; 30 μg/ml) for 72 h and stained for flow cytometry as described above.

Animal Experiments

The studies were approved by the Cedars-Sinai Institutional Animal Care and Use Committee. Male apoE−/−mice were purchased from Jackson Laboratory at 6 weeks of age and housed in a specific pathogen-free facility, kept on a 12-h day/night cycle, and had unrestricted access to water and food.

mCRAMP Immunization

The mCRAMP peptide was purchased (AnaSpec) with >95% purity according to the manufacturer. Donor mice were subcutaneously immunized with either 100 μg mCRAMP in adjuvant [Adju-Phos (Brenntag, 12.5 μl of a 2% solution) and monophosphoryl lipid A (MPLA-SM VacciGrade, InvivoGen, 10 μg)] or adjuvant alone in a final volume of 200 μl using PBS as vehicle. One group of mice was fed normal chow and immunized at 7, 10, and 12 weeks of age. Mice were then euthanized and splenocytes isolated to assess T cell response at 13 weeks of age using flow cytometry. Another group of mice was fed normal chow and immunized at 7, 10, and 12 weeks of age. The mice were then euthanized as T cell donors at 13 weeks of age. Recipient mice were fed high fat diet consisting of 21% fat, 0.15% cholesterol (TD.88137, Envigo) starting at 7 weeks of age until euthanasia at 23 weeks of age.

Isolation and Transfer of T Cells

After collection of donor mouse splenocytes, red blood cells were lysed using RBC lysis buffer (BioLegend), washed with PBS, and cells from the same group were pooled. Splenocytes were enriched for T cells using a commercially available mouse T cell magnetic isolation kit (ThermoFisher) according to the manufacturer's protocol. T cells were enriched to ∼90% assessed by flow cytometry. After counting the isolated T cells, a suspension of 2 $x10^6$ T cells in PBS was injected by tail vein injection into 18 week-old recipient mice fed with high fat diet for 11 weeks. High fat diet feeding in recipient mice continued for an additional 5 weeks and mice were euthanized at 23 weeks of age.

Tissue Harvesting

At 23 weeks of age, recipient mice were euthanized and spleens, aortas and hearts were collected. Serum was collected for cholesterol level measurement. Spleens were collected for flow cytometric staining and analysis. A small portion of the spleen was used for RNA extraction. The aortas were dissected free of connective tissue and fat, and stained with Oil Red O for en face lipid staining (19). Aortic size and plaque content were quantified by a blinded observer using image analysis software (ImagePro Plus version 4.0, Media Cybernetics Inc., Rockville, Maryland). Heart bases were imbedded in OCT (Optimum Cutting Temperature, Tissue-Tek) and frozen for cryo-sectioning. Ten-micron cryosections of the aortic sinus were collected. Lipid was stained using Oil-Red-O. Staining for macrophage was performed using CD68 antibody. Collagen area was assessed using Masson trichrome stain. Three slides of approximately 0.1-millimeter intervals for each animal were used for each stain and averaged. Image analysis was performed using ImagePro. Tissue calcification in aortic sinus plaques was confirmed by Alizarin Red-S stain.

Flow Cytometry

Splenocytes were stained for viability and surface markers CD3, CD4, CD8, CD44, and CD62L. Intracellular staining for FoxP3 was performed after surface marker staining followed by fixation and permeabilization (eBioscience). For intracellular cytokine staining, cells were resuspended in 10% heat inactivated FBS-RPMI medium containing 1× Monensin (Invitrogen) and

cultured at 37°C and 5% CO_2 for 4 h. After viability and surface marker staining, cell fixation and permeabilization was performed followed by intracellular staining for IL-10 and IFN-γ for flow cytometry.

CD107a

For degranulation assay to measure CD8+ T cell cytolytic activity, splenocytes were incubated with 2.5 μg/ml fluorescent conjugated CD107a in RPMI for 1 h followed by the addition of Monensin and incubation for another 4 h. Cells were collected and stained for surface markers for flow cytometry.

Splenic mRNA Expression

Splenic RNA was isolated using Trizol and subjected to qRT-PCR using primer pairs for mouse IL-1β, Pdcd1, Ctla4, Wnt10b, Runx2, RANKL, and osteocalcin. GAPDH was used as reference gene. Data were analyzed using the $Ct_{\Delta\Delta}$ method with one Adjuvant sample as calibrator. Results are expressed as fold change relative to Adjuvant.

ELISA

Mouse serum levels of soluble RANKL and undercarboxylated osteocalcin, the active form of osteocalcin, were measured using commercially available ELISA kits (ThermoFisher and MyBioSource, respectively) according to manufacturers' instructions.

Statistical Analysis

Statistical analysis was performed using R software version 3.5 (R Core Team, 2018) and GraphPad Prism version 7 (GraphPad Software, La Jolla, California). Data are presented as mean ± standard deviation. For multiple group analysis, significance for normally distributed samples was tested using ANOVA followed by Holm-Sidak's multiple comparisons test. Significance for non-normally distributed samples was tested using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Correlation was analyzed using the Spearman test with a two-tailed *P*-value. For two-group analysis, following normality testing, differences between groups were performed using *t*-test or Wilcoxon test, accordingly. A P-value < 0.05 was considered significant, but data trends were also noted.

RESULTS

T Cell Immune-Reactivity to the Human Cationic Antimicrobial Peptide LL-37 in Acute Coronary Syndrome Patients

Given the role of anti-microbial peptides as potential self-antigens in atherosclerosis, and the possible association with acute events, we tested if the cleaved fragment of hCAP-18, the cationic antimicrobial peptide LL-37, would induce differential T cell immune responses in patients with ACS. Peripheral blood mononuclear cells (PBMCs) from self-reported healthy controls (Controls), patients with stable CAD (Stable) or ACS were stimulated with LL-37 for 72 h. PBMCs stimulated with the cathelin domain of hCAP-18 (cat-hCAP-18) served as control. Baseline characteristics of the subjects are detailed in **Table 1**. Stimulation with LL-37 resulted in reduced CD8+ Effector T cell response, in both T Effector Memory (TEM) and T Effector Memory RA+ (TEMRA) cells in PBMCs from controls and patients with stable CAD, while PBMCs from patients with ACS were resistant to this reduction (**Supplementary Figure 1** and **Figures 2A–C**). CD4+ Effector T cell responses were trending similar to CD8+ Effector T cells (**Figures 2D–F**). There was no significant difference in CD8+ Effector T cell response when cells were stimulated with cat-hCAP-18 (**Figure 3**). There was reduced expression of programmed cell death protein 1 (PDCD1) mRNA in PBMCs from patients with ACS stimulated with LL-37 compared to PBMCs from controls (**Figure 4A**), but no difference was noted between groups in PDCD1 mRNA expression when cells were stimulated with cat-hCAP-18 (**Figure 4B**).

The nature of the self-reactive responses to cat-hCAP-18 and LL-37 were investigated further by testing the relationship between the T cell response to both antigens in each subject. There was significant correlation in CD4+ Effector T cell response (**Figure 4D**) to cat-hCAP-18 and LL-37, but not in CD8+ Effector response (**Figure 4C**). The results suggest that there is potentially shared antigenic reactivity to cat-hCAP-18 and LL-37 in CD4+ T cell responses. IFN-γ measured in conditioned medium from PBMCs stimulated with LL-37 showed qualitative difference in ACS compared to control and stable CAD patients (**Supplementary Figure 2**). The results suggest that LL-37 reactive T cells may be involved in the acute event. Additionally, Effector T cell responses in control PBMCs stimulated with LL-37 was partially reversed by blocking with HLA Class-I antibody (**Supplementary Figure 3**) suggesting HLA Class-I mediated response.

The T cell response in ACS patients was further subdivided into patients with their first ACS event and those who had a recurrent event. The CD8+ T Effector response to LL-37 was consistent between first and recurrent ACS patients, compared to patients with Stable CAD (**Supplementary Figures 4A–C**). On the other hand, CD4+ T Effector response was significantly higher only in the recurrent ACS patients but not in the first ACS event compared to Stable patients (**Supplementary**

TABLE 1 | Characteristics of human subjects.

	Control (*N* = 15)	Stable CAD (*N* = 10)	ACS (*N* = 10)
Mean age	58.7 ± 10.2	75.6 ± 9.0	59.3 ± 16.4
Male sex	70%	80%	70%
Mean LDL cholesterol (mg/dL)	N/A	86.62 ± 40.22*	105.96 ± 44.85†
Use of cholesterol-lowering medications on admission	N/A	90%	40%

*ACS: acute coronary syndrome; CAD: coronary artery disease; LDL: low density lipoprotein; N/A: not available. *Based on chart review. †At enrollment.*

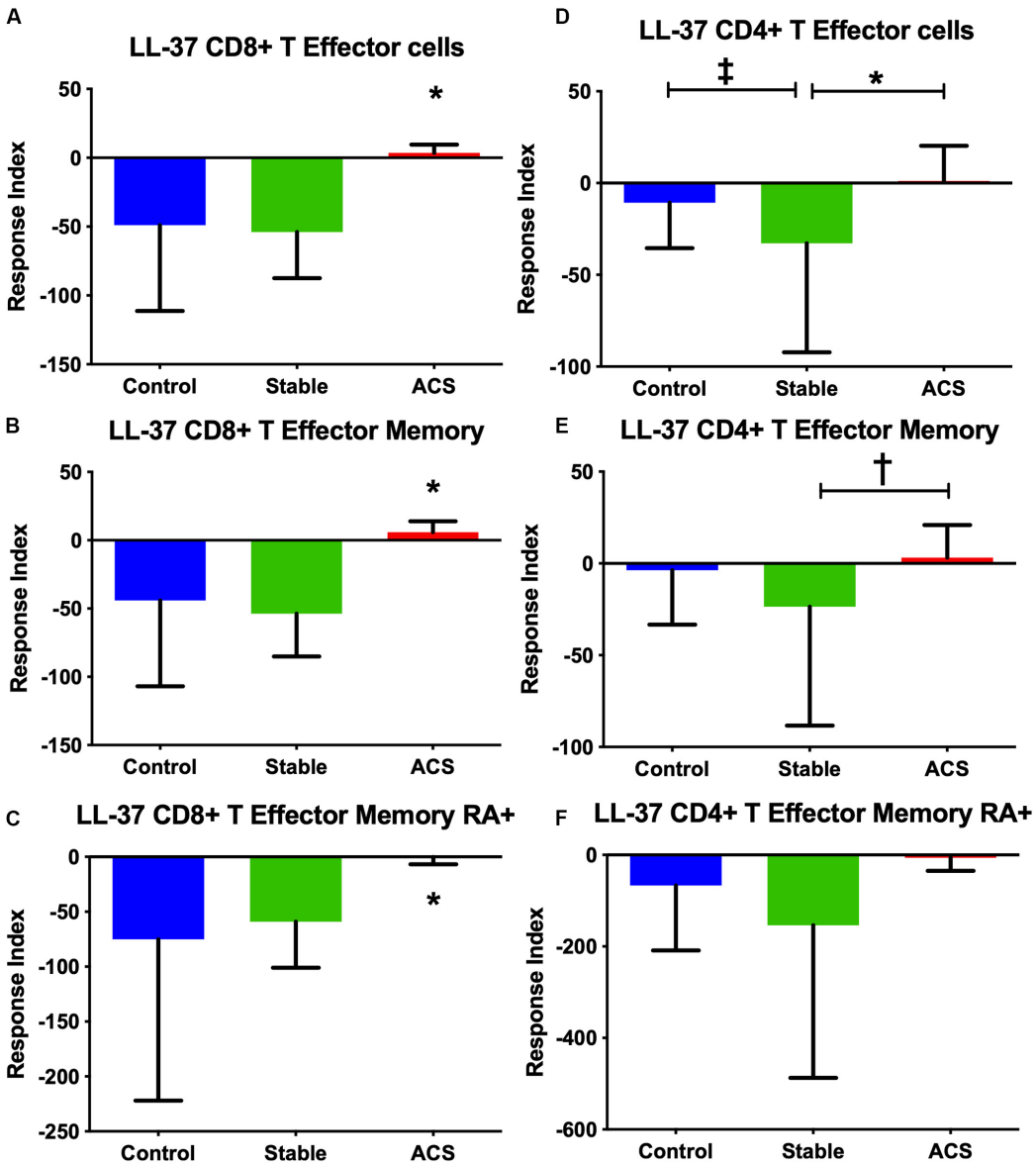

FIGURE 2 | PBMC T Effector cell response to stimulation with the human antimicrobial peptide LL-37. CD8+ **(A–C)** and CD4+ **(D–F)** Memory T cell responses to stimulation of peripheral blood mononuclear cells from self-reported controls (Control), stable coronary artery disease (Stable), and acute coronary syndrome (ACS) patients. T Effector Memory **(B,E)** and T Effector Memory RA+ **(C,F)** were based on CD45RO/CD45RA flow cytometric stain as detailed in the gating scheme described in **Supplementary Figure 1**. Control $N = 15$; Stable N = 10; ACS $N = 10$. $^{*}P < 0.05$ ACS vs Control or Stable CAD **(A–C)** and Stable vs ACS **(D)**; $^{‡}P = 0.053$ Control vs Stable; $^{†}P = 0.055$ Stable vs ACS. Kruskal-Wallis and Dunn's multiple comparisons test.

Figure 4D). This suggests that CD8+ T cell response to LL-37 persists in immunologic memory and is a common response in both first event ACS and recurrent ACS. CD4+ T cell Effector response on the other hand seems to have evolved and is more prominent in the recurrent ACS. No significant differences were observed in cat-hCAP-18 response between first ACS event and recurrent ACS patients in CD8+ T Effector (**Supplementary Figures 5A–C**) and CD4+ T Effector (**Supplementary Figures 5D–F**) responses.

To investigate the potential role of T cells that are self-reactive to the cationic antimicrobial peptide in atherosclerosis, we used the apoE−/− mouse model of atherosclerosis and immunization with the mouse ortholog of LL-37 called mCRAMP.

Immunization Provokes a Self-Reactive T Cell Response to mCRAMP in apoE−/− Mice

To investigate the potential role of T cells reactive to the mouse cationic antimicrobial peptide mCRAMP in the male apoE−/− mouse model of atherosclerosis, we first tested if T cells were reactive to mCRAMP. ApoE−/− mice fed

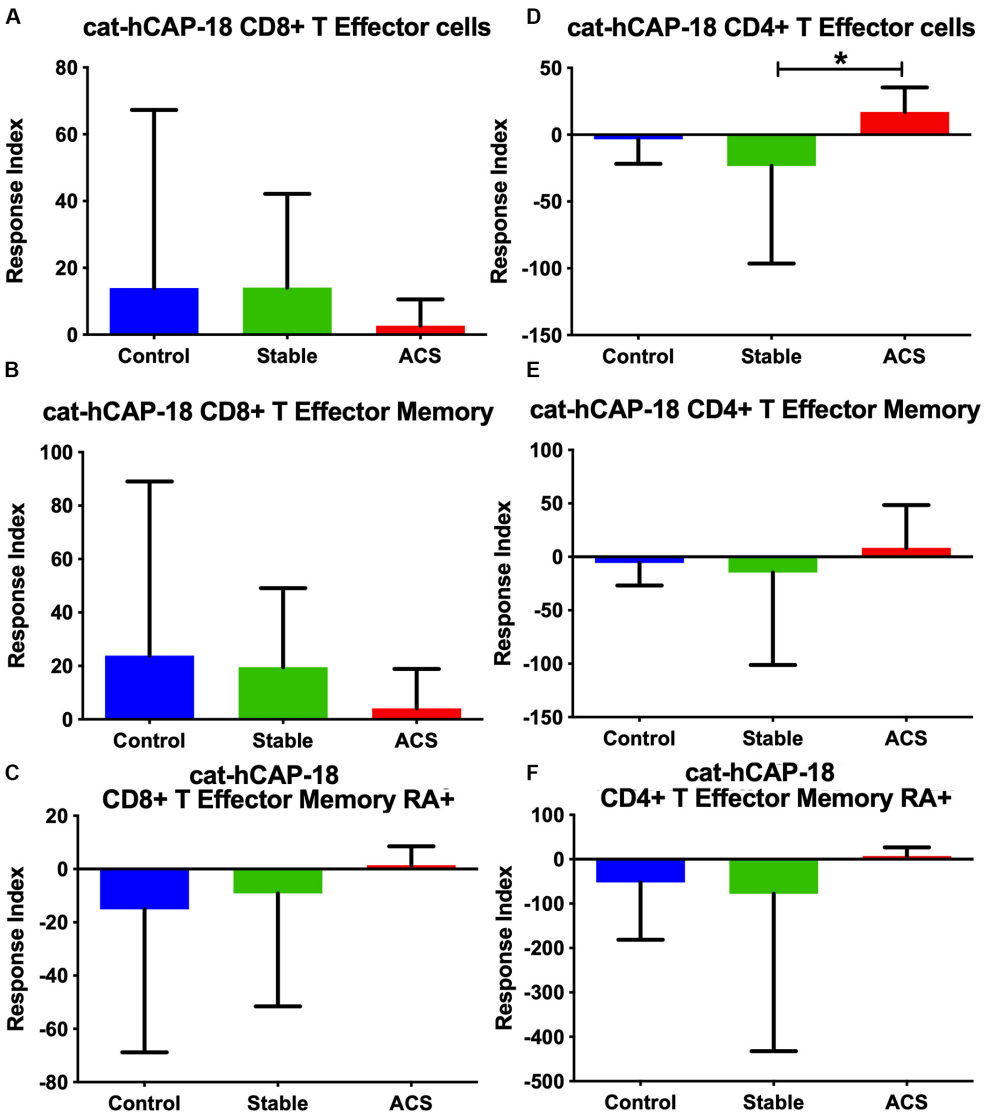

FIGURE 3 | PBMC T Effector cell response to stimulation with the cathelin domain of the human proprotein hCAP-18, cat-hCAP-18. CD8+ **(A–C)** and CD4+ **(D–F)** Memory T cell responses to stimulation of peripheral blood mononuclear cells from self-reported controls (Control), stable coronary artery disease (Stable), and acute coronary syndrome patients (ACS). T Effector Memory **(B,E)** and T Effector Memory RA+ **(C,F)** were based on CD45RO/CD45RA flow cytometric stain as detailed in the gating scheme described in **Supplementary Figure 1**. Control $N = 15$; Stable CAD $N = 10$; ACS $N = 9$; $^*P < 0.05$ Stable vs ACS. Kruskal-Wallis and Dunn's multiple comparisons test.

with normal chow were immunized with mCRAMP at 7, 10, and 12 weeks of age and euthanized 1 week later for assessment of T cell response. Mice injected with adjuvant alone served as control. There was no difference in CD8+ Effector Memory T cells (**Supplementary Figure 6** and **Figure 5A**) but a significant increase in CD8+ Central Memory (CM) T cells (**Figure 5B**) in splenocytes from apoE−/− mice immunized with mCRAMP. Additionally, there was decreased CD8+FoxP3+ cells (**Supplementary Figure 6** and **Figure 5C**) and increased cytolytic activity, assessed by CD8+CD107a+ T cells (**Supplementary Figure 7** and **Figure 5D**), in splenocytes of mice immunized with mCRAMP compared to adjuvant. No differences were observed in CD4+ memory T cell subsets or in CD4+FoxP3+ Treg cells (**Figures 5E–G**).

mCRAMP-Primed T Cells in Atherosclerosis

To assess whether mCRAMP-primed T cells are functionally involved in modifying atherosclerosis, adoptive transfer of donor T cells from apoE−/− mice immunized with mCRAMP or adjuvant alone was performed on apoE−/− recipient mice that had been fed high fat diet for 11 weeks prior to transfer. The 11-week feeding with high fat diet assured that the recipient mice were already primed for atherosclerosis. Recipient mice were euthanized 5 weeks after cell transfer.

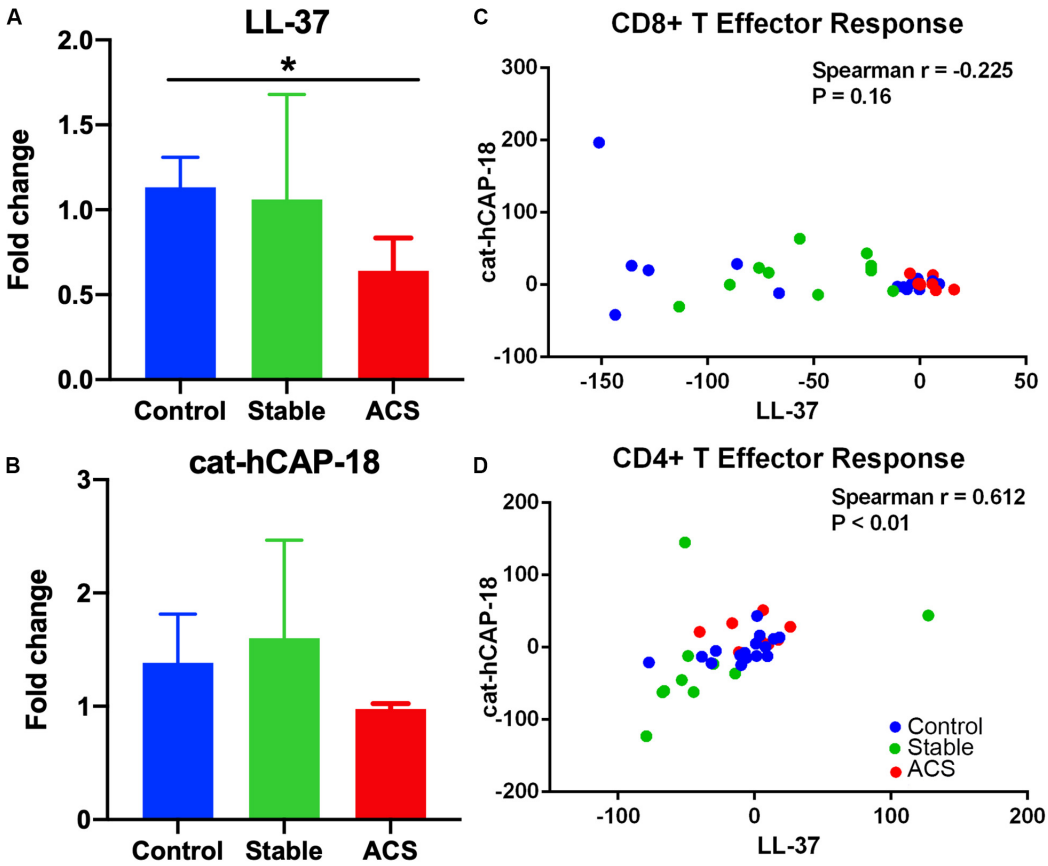

FIGURE 4 | Immune checkpoint PDCD1 mRNA expression and correlation in T Effector response to LL-37 and cat-hCAP-18. Programmed cell death protein 1 (PDCD1) mRNA expression in peripheral blood mononuclear cells stimulated with the human antimicrobial peptide LL-37 **(A)** or the cathelin domain of the proprotein hCAP-18, cat-hCAP-18 **(B)**. Control $N = 6$; Stable = 5; ACS = 5. $^*P < 0.05$ (ACS vs Control; Kruskal-Wallis and Dunn's multiple comparisons test). Correlation plot between CD8+ T Effector response to LL-37 and cat-hCAP-18 **(C)**. Correlation plot between CD4+ T Effector response to LL-37 and cat-hCAP-18 **(D)**.

Recipients of T cells from mCRAMP immunized mice had significantly reduced aortic plaque area compared to recipients of T cells from adjuvant mice (28% reduction, $P < 0.05$, **Figures 6A,B**). There was no significant difference between groups in mean body weight (mCRAMP = 42 ± 6 gr; Adjuvant = 43 ± 6 gr) or mean serum cholesterol (mCRAMP = 1500 ± 294 mg/dL; Adjuvant = 1270 ± 366 mg/dL). Thus, atherosclerosis progression was reduced in the mCRAMP T cell recipient mice without differences in weight or serum cholesterol compared to adjuvant T cell recipient mice.

Aortic sinus plaque size, lipid content (Oil Red O staining; **Figures 7A–C**), macrophage (CD68 staining; **Figures 7D,E**), and collagen area (Masson's trichrome staining; **Figures 7F,G**) were not different between the T cell recipient groups. There were also no differences in IL-1β (**Figure 8A**), PDCD1 (**Figure 8B**) or cytotoxic T-lymphocyte-associated protein 4 (Ctla4, **Figure 8C**) splenic mRNA expression between the T cell recipient groups.

Plaque Calcification in T Cell Recipient Mice

Focal staining of hematoxylin was observed in several aortic sinus sections from recipient mice, suggesting calcification in the plaque. The presence of plaque calcification was assessed with the calcium-specific Alizarin Red S staining. Calcification in atherosclerotic plaques occurred in 56% of adjuvant T cell recipient control mice, compared to none in the mCRAMP T cell recipient mice (**Figures 9A,B** and **Table 2**; Fisher's exact test $P = 0.003$).

Immune pathways associated with T cell mediated tissue calcification were then investigated further. Recipients of T cells from mCRAMP immunized mice had increased IL-10 and IFN-γ expression in splenic CD8+ T cells (**Supplementary Figure 8** and **Figures 9C,D**), but not in CD4+ T cells (**Figures 9E,F**), compared to adjuvant T cell recipient control mice. There was increased expression of Wnt10b mRNA in splenocytes of mCRAMP T cell recipients, when compared to adjuvant T cell recipient control mice (**Figure 10A**). However, there was no difference in the expression of Runx2 mRNA (**Figure 10B**). Furthermore, splenocytes from mCRAMP T cell recipients had increased RANKL and osteocalcin mRNA expression when compared to adjuvant (**Figures 10C,D**). However, no difference between groups was noted in serum RANKL and undercarboxylated osteocalcin concentration (**Figures 10E,F**), which is the known active state of osteocalcin.

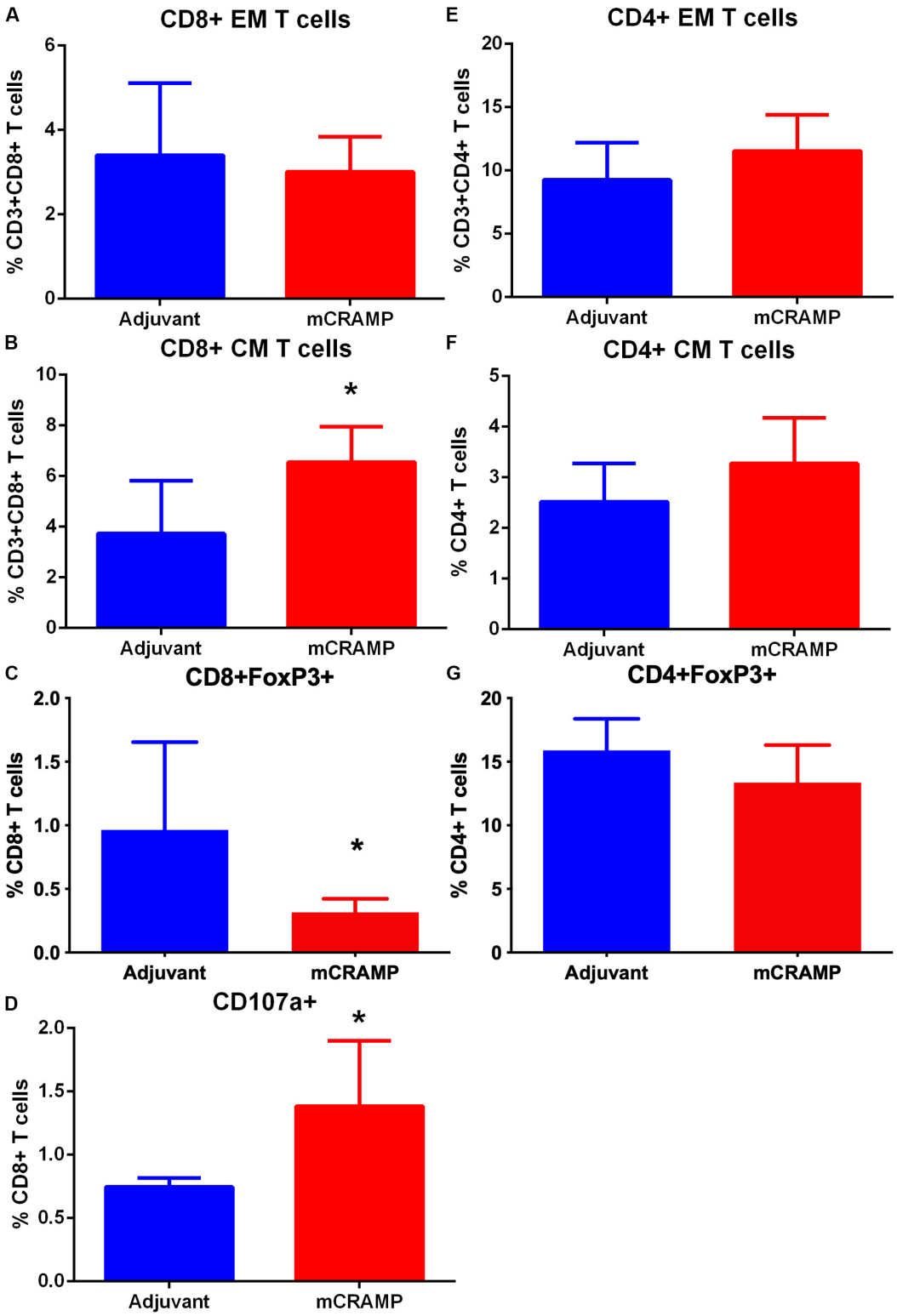

FIGURE 5 | Memory T cell response of splenocytes to immunization of apoE–/– mice with the murine antimicrobial peptide mCRAMP. CD8+ Effector Memory (EM) T cells **(A)**, Central Memory (CM) T cells **(B)**, CD8+FoxP3+ T cells **(C)** and CD8+CD107a+ T cells **(D)** in mice immunized with mCRAMP compared to adjuvant. $N = 5$ each. CD4+EM T cells **(E)**, CD4+ CM T cells **(F)**, and CD4+FoxP3+ T cells **(G)** in mice immunized with mCRAMP compared to Adjuvant. $N = 5$ each; $^*P < 0.05$, *t*-test. Gating scheme for memory T cells and FoxP3+ T cells shown in **Supplementary Figure 6**. Gating scheme for CD8+CD107a+ T cells shown in **Supplementary Figure 7**.

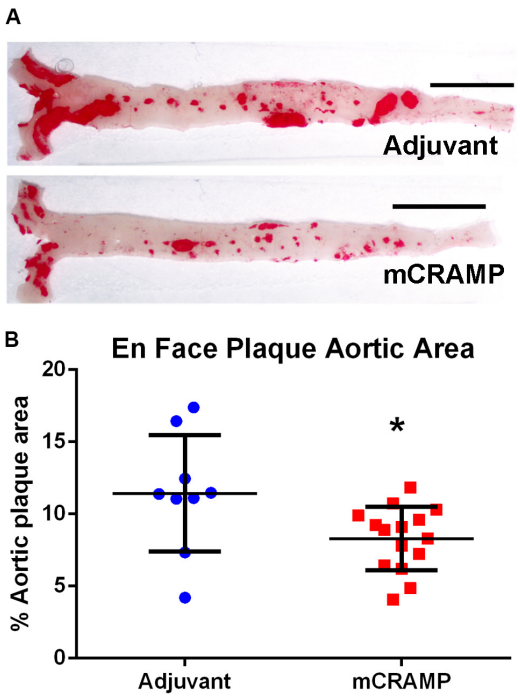

FIGURE 6 | Aortic atherosclerosis plaque burden in T cell recipient apoE–/– mice. Representative photographs of en face aortas stained with Oil red O **(A)** from T cell recipients of adjuvant and mCRAMP immunized mice. Bar = 0.5 cm. Atherosclerotic burden was assessed by measuring Oil Red O stained plaque area **(B)**. Adjuvant $N = 9$; mCRAMP $N = 15$; $^*P < 0.05$, *t*-test.

DISCUSSION

In this study, we showed that: (a) LL-37 stimulation of PBMCs from patients with ACS induced the persistence of CD8+ TEM cell response compared to patients with stable CAD or self-reported controls; (b) Immunization of apoE−/− mice with mCRAMP, the cationic fragment of CRAMP, increased CD8 CM T cell activation and cytolytic activity; and (c) Adoptive transfer of T cells from mice immunized with mCRAMP was associated with smaller atherosclerotic aortic plaque area, and absence of aortic sinus plaque calcification.

Our findings suggest that LL-37 self-reactive T cells may be important in an acute coronary event in the context of atherosclerotic disease. LL-37 is a T cell self-antigen in patients with psoriasis (15), who have increased risk of early cardiovascular disease (16, 17). It has been proposed that psoriasis and atherosclerosis, both chronic inflammatory conditions, share a common inflammatory pathogenic basis (21, 22) that can potentially explain the increased risk of atherosclerosis and its complications associated with this condition. TEM cells have rapid effector function upon re-exposure to the antigen, but a limited proliferative potential (23). Memory T cells correlate well with atherosclerosis in both mice and humans (5, 24), and with vulnerable and ruptured plaques in humans (6).

LL-37 was reported to decrease T cell proliferation in resting human PBMCs with increased cell viability without changes in CD4+FoxP3+ T_{reg} cell percentage suggesting that in the steady-state, LL-37 treatment results in a degree of immune-modulation apparently independent of increased T_{regs} (25). In the same report, T cells increased proliferation when co-activated with phytohaemagglutinin without affecting cell viability and with increased T_{reg} cells. Increased T cell proliferation coupled with increased T_{reg} cells in their report suggests that T_{regs} may be compensating for the increased proliferation, or that T_{regs} may not have a significant role in LL-37 reactive T cells. Our findings extend their report by demonstrating that T cell memory response to LL-37 was reduced in the control subjects and stable CAD but persisted in samples from ACS patients. This is supported by the qualitative difference in the IFN-γ secretion among the groups. These differences were associated with reduced immune checkpoint PDCD1 mRNA expression in PBMCs from ACS patients. The reduction in T cell response to LL-37 in the control PBMC was blocked by HLA Class-I antibody suggesting that the intrinsic response is at least partially MHC-I dependent. However, our results cannot rule out the possibility that adjuvant effects attributed to LL-37 are also involved (15). The observed blocking of complementary CD4+ T cell memory response by anti-HLA Class-I antibody is consistent with the report by Lande et al. (15) suggesting complementarity of T cell subset responses to LL-37. Whether the complementary T cell response to LL-37 in the controls also extend to ACS as was reported for some psoriasis patients (15) remains to be determined. Thus, combined with other reports, our results suggest that the intrinsic T cell response to LL-37 is down-modulation but in the presence of co-activating factors such as those in the ACS patients the T cell memory response persists that may be due in part to reduced checkpoint PDCD1 expression. These findings have potentially important clinical implications with the reported cases of ACS associated with immune checkpoint inhibitor treatment (26).

The results in our report may be a response to the acute event but adaptive immune memory to neoantigens develop over a longer time frame than that in our study (within 72 h of admission for the acute event). It is notable that CD8+ TEM response to LL-37 is consistent in patients whether it was their first ACS event or a recurrent event suggesting the continued presence of memory T cells. It cannot be determined at this time whether the persistent T cell response in ACS is pathogenic or a compensatory response in the acute stage of the disease. On the other hand, the CD4+ T Effector response was altered in patients that had a recurrent event suggesting that the T cell response in ACS evolved as the recurrent patients remained at risk. These observations are consistent with the notion of underlying inflammation in patients who remain at risk for a recurrent event.

Our results further show a correlation between the CD4+ T cell response to LL-37 and cat-hCAP-18. This finding may be a manifestation of expanding antigenic determinants reported in antigen spreading, wherein the original reactive antigen determinant spreads to other regions of the same protein (27), in this case hCAP-18. Although speculative, it is interesting that

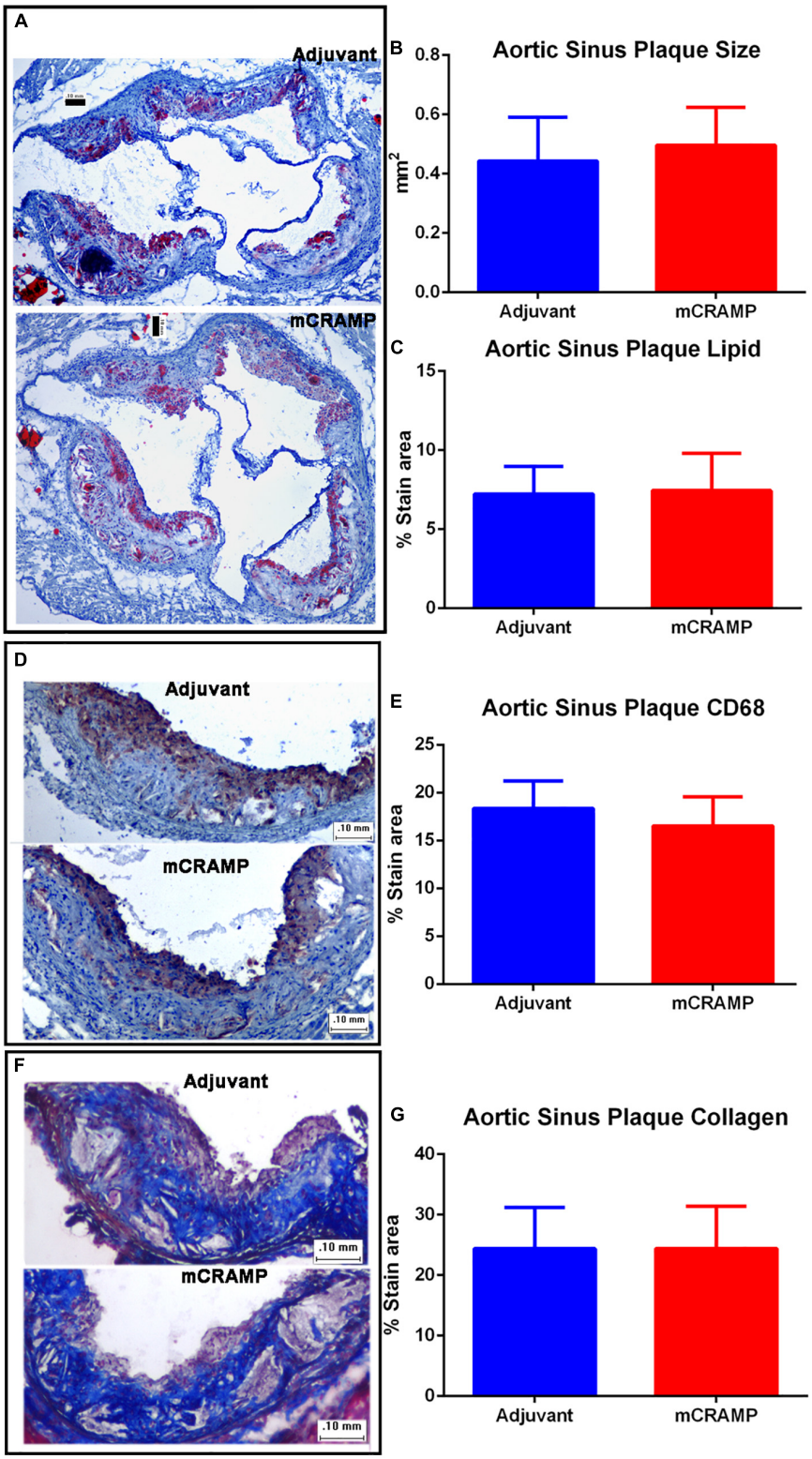

FIGURE 7 | Aortic sinus plaque composition in T cell recipient apoE–/– mice. Representative photos of Oil Red O stain for lipids **(A)**. Aortic sinus plaque size (**B**; Adjuvant *N* = 9, mCRAMP *N* = 12) and lipid area (**C**; Oil Red O staining; Adjuvant *N* = 9; mCRAMP *N* = 12). Representative photos of CD68 stain for macrophage **(D)** and macrophage area (**E**; *N* = 6 each). Representative photos of Masson's trichrome stain **(F)** and collagen stain area (**G**; Adjuvant *N* = 9; mCRAMP *N* = 15). Bar = 0.1 mm.

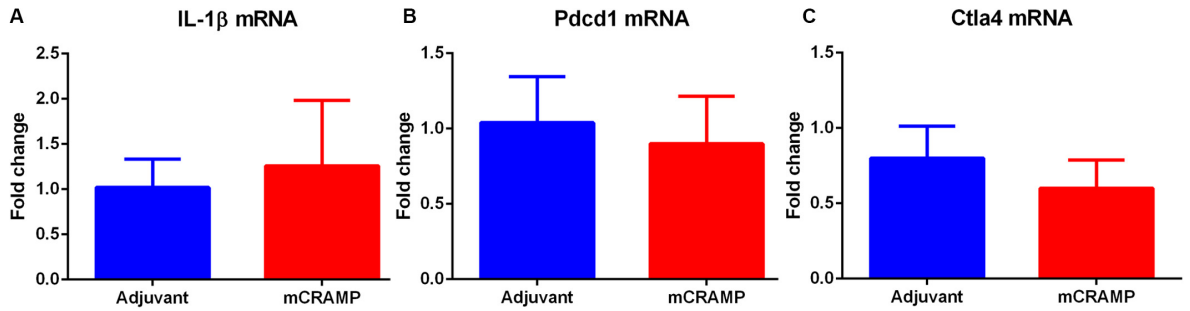

FIGURE 8 | Analysis of key immune regulatory pathways in T cell recipient mice. Splenic mRNA expression of IL-1β **(A)**, Pdcd1 **(B)** or Ctla4 **(C)** between mCRAMP or adjuvant T cell recipient mice. *N* = 5 each.

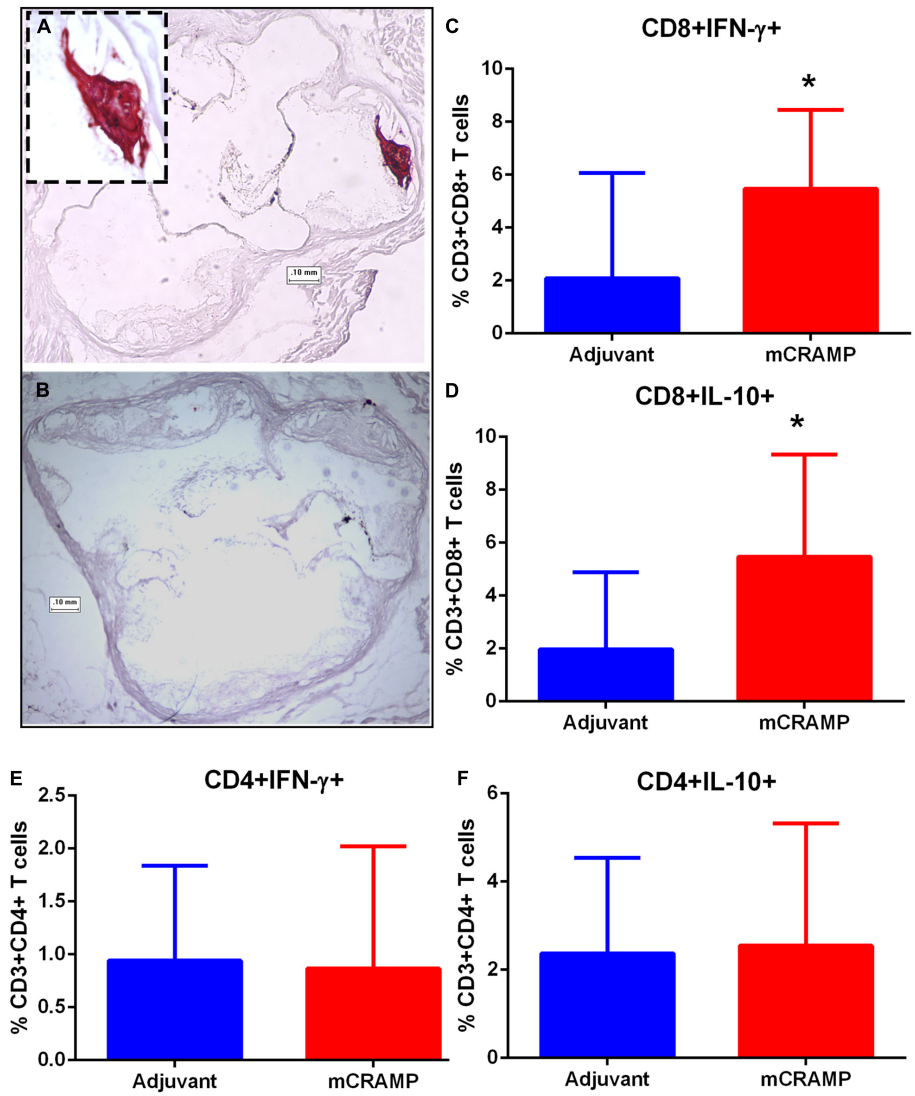

FIGURE 9 | Atherosclerotic plaque calcification in T cell recipient mice. Representative photographs of aortic sinuses stained with the calcium specific Alizarin Red S in adjuvant (**A**, inset 20× magnification) or mCRAMP **(B)** T cell recipient mice. Bar = 0.1 mm. Intracellular staining for splenic CD8+INF-γ+ T cells **(C)** and CD8+IL-10+ T cells **(D)**. Intracellular staining for splenic CD4+INF-γ+ T cells **(E)** and CD4+IL-10+ T cells **(F)**. Adjuvant *N* = 8; mCRAMP *N* = 14. *$P < 0.05$, Mann-Whitney test. Gating scheme for T cell intracellular staining shown in **Supplementary Figure 8**.

TABLE 2 | Fisher's Exact test of aortic sinus plaque calcification prevalence in T cell recipient mice (two-sided $P = 0.003$).

	Number of mice with plaque calcification	Number of mice without plaque calcification	Total
Adjuvant	5	4	9
mCRAMP	0	15	15
Total	5	19	24

this was not observed in CD8+ T cell response. It remains to be determined what the nature of the involvement of the TEM response to LL-37 is in the acute event.

Studies were performed in apoE−/− mice to investigate the role of T cells reactive to the cationic antimicrobial peptide in atherosclerosis. Although the cathelin domain of the proprotein hCAP-18/CRAMP is reported to be active (28), functional activity is mostly attributed to the cationic antimicrobial peptide domain. Our results show increased CD8+ CM T cells and CD8+ T cell cytotoxic activity in mice immunized with the self-peptide mCRAMP coupled with decreased CD8+FoxP3+ T cells. These extend our previous report where immunization with the proprotein CRAMP resulted in increased CD8+ T cells and cytolytic activity, and reduced atherosclerosis (19). CD8+ T_{regs} have an important role in self-tolerance, and lower levels CD8+

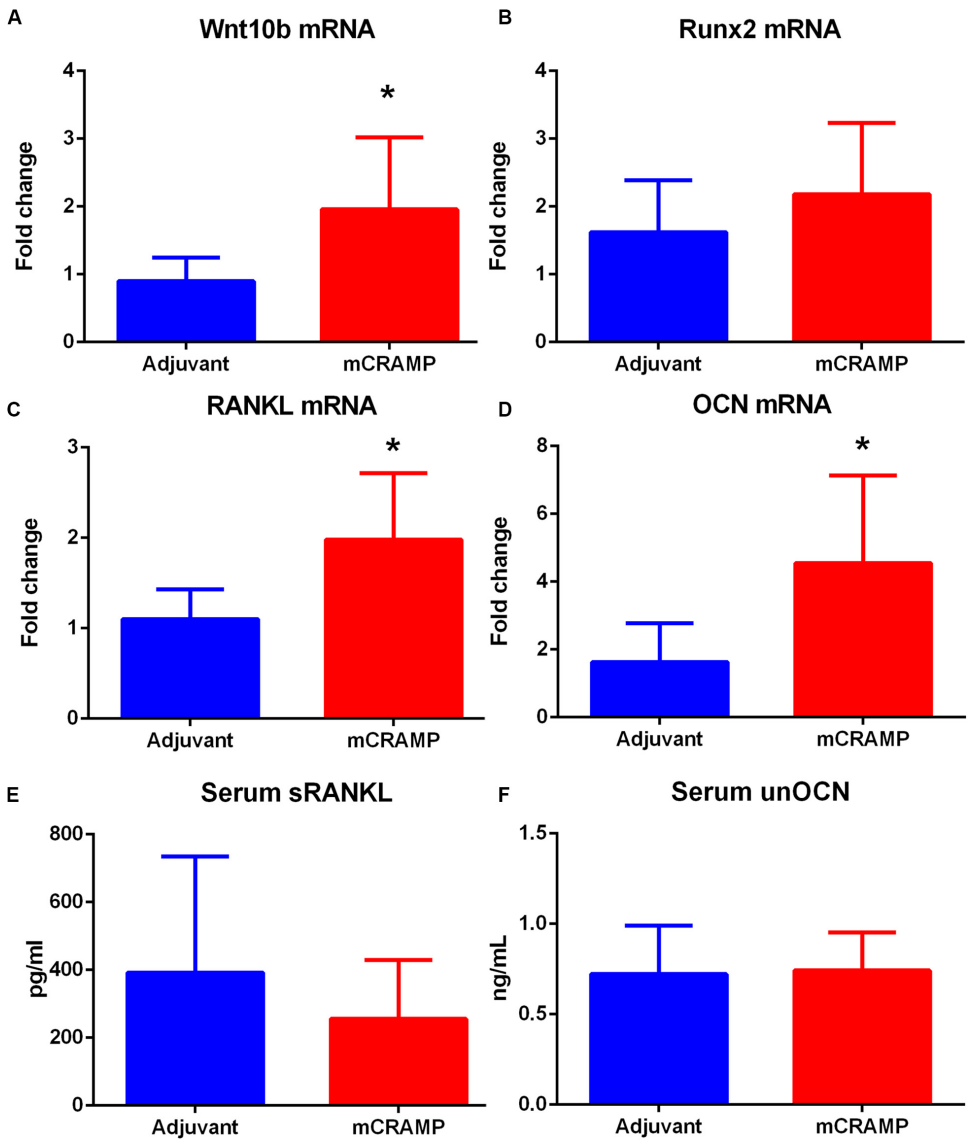

FIGURE 10 | Pathways of tissue calcification potentially mediated by T cells. **(A)** Splenic Wnt10b mRNA expression in mCRAMP compared to adjuvant T cell recipient mice at 23 weeks of age (**A**; $N = 7$ each group). Splenic Runx2 mRNA (**B**; $N = 5$ each group), RANKL mRNA **(C)**, and osteocalcin (OCN) mRNA **(D)** expression in mCRAMP T cell recipient mice compared to adjuvant T cell recipients ($N = 5$–7 each). Serum RANKL (**E**; Adjuvant $N = 9$; mCRAMP $N = 14$) and serum undercarboxylated osteocalcin (unOCN) levels (F; Adjuvant $N = 9$; mCRAMP $N = 14$). *$P < 0.05$; **(A,C)**, t-test; **(D)**, Mann-Whitney test.

T_{regs} have been associated with increased immune activity (29–31).

We confirmed that T cells reactive to mCRAMP are functionally involved in atherosclerosis by the adoptive transfer of enriched T cells from mCRAMP-primed apoE−/− mice into recipient apoE−/− mice. To the best of our knowledge, this is the first report of reduced atherosclerosis attributed to T cells primed with mCRAMP. However, no differences were found in aortic sinus plaques consistent with the report of site-specificity for atherosclerosis in murine models of atherosclerosis (32).

Some key signaling pathways involved in tissue calcification potentially regulated by T cells in the recipient mice were investigated. Wnt10b, a member of the Wnt family signaling pathway, is secreted by T lymphocytes (33, 34) and activates signal transduction cascades that regulate Runx2, a transcription factor needed for osteoblast differentiation (35). RANKL activates osteoclasts and bone remodeling in adult mice (36). T cell expression of RANKL (37) is involved in the regulation of bone metabolism (38). Osteocalcin is secreted by osteoblasts associated with bone formation. Splenocytes expressing osteocalcin induce atherosclerosis and vascular calcification in apoE−/− mice (39). Although the dysregulation of calcification pathways is manifested in differential mRNA expression of specific genes involved, the systemic levels detected in serum seemed to remain in equilibrium. This is further observed in the increase in both IL-10 and IFN-γ expressing CD8+ T cells. The results suggest the involvement of factors that remain to be characterized.

Nevertheless, these observations suggest dysregulated tissue calcification pathways in atherosclerosis potentially mediated by memory T cells. Interestingly, LL-37 is a T cell antigen in psoriatic disease (15) and altered bone remodeling through osteoblast–osteoclast uncoupling has been proposed as an explanation for the concomitant dysregulated processes of both pathological bone formation and resorption usually found in patients with psoriatic arthritis (40). In our study, plaque calcification was absent in mice that were recipient of T cells primed with mCRAMP suggesting a role in regulating the process.

Coronary artery calcification is a marker of atherosclerosis and its role in CAD is nuanced (41). On one hand, the widely used coronary artery calcium score is a strong independent risk factor of major adverse cardiovascular and cerebrovascular events (42, 43). On the other hand, calcified atherosclerotic plaques may be more stable than non-calcified plaques (44). The use of statins has been associated with progression of plaque calcification, in spite of their protective role in atherosclerosis (45). The association found in our animal experiments between recipients of mCRAMP-primed T cells and the lack of plaque calcification is intriguing, but its significance remains to be determined. The role of T cells reactive to LL-37 in humans, and their potential influence in the pathways of atherosclerotic plaque calcification need to be investigated further.

Limitations of the study include the potential of LL-37 to be a "promiscuous" HLA-binding peptide or to have intrinsic adjuvant properties (15) which coupled with the reduced PDCD1 expression in ACS may explain the observed persistence of CD8+ Effector cells. Although anti-HLA Class-I antibody blocked the response to LL-37 in controls, our study cannot completely exclude these possibilities. The mouse studies demonstrating the involvement of T cells reactive to mCRAMP as a self-antigen in atherosclerosis is consistent with the observed TEM cell response to LL-37 in ACS patients, supporting the presence of a self-reactive TEM cell population involved in atherosclerosis. However, the persistence of TEM response to LL-37 in patients who suffered an acute event is not in complete alignment with the results of adoptive transfer of mCRAMP-primed T cells in our mouse studies. One might speculate that the controlled nature of immune priming in the mice skewed the response to be protective against atherosclerosis, even as the physiologic role of reduced calcification of mouse plaques remains to be clarified. It is also possible that the persistence of LL-37 reactive TEM response in ACS patients is a compensatory response to the inflammatory milieu that subsequently proved inadequate. These remain speculative handicapped by several limitations, including the lack of a reliable mouse model of spontaneous coronary artery plaque rupture which is the major cause of ACS in humans.

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 studies involving human participants were reviewed and approved by Cedars-Sinai IRB. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Cedars-Sinai Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

FC, BC, WL, PM, and PD contributed to conception and design of the study. FC, WL, JY, PM, XZ, JZ, and PD contributed to the data acquisition and analysis. BC and RH contributed to patient recruitment. FC, BC, WL, K-YC, PS, and PD contributed to the interpretation of the data, drafting, and revising the manuscript. 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/fimmu.2020.575577/full#supplementary-material

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MINI REVIEW
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T Cells in Autoimmunity-Associated Cardiovascular Diseases

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T cells are indisputably critical mediators of atherosclerotic cardiovascular disease (CVD), where they secrete pro-inflammatory cytokines that promote vascular pathology. Equally well-established is the fact that autoimmune diseases, which are mediated by autoreactive T cells, substantially increase the risk of developing CVD. Indeed, as immunomodulatory treatments have become more effective at treating end-organ pathology, CVD has become a leading cause of death in patients with autoimmune diseases. Despite this, investigators have only recently begun to probe the mechanisms by which autoreactive T cells promote CVD in the context of autoimmune diseases. T cells are best-studied in the pathogenesis of systemic vasculitides, where they react to self-antigen in the vessel wall. However, newer studies indicate that T cells also contribute to the increased CVD risk associated with lupus and rheumatoid arthritis. Given the central role of T-cell-derived cytokines in the pathogenesis of psoriasis, the role of these factors in psoriatic CVD is also under investigation. In the future, T cells are likely to represent major targets for the prevention and treatment of CVD in patients with autoimmune diseases.

Keywords: T cells, autoimmunity, cardiovascular, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, vasculitis

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INTRODUCTION

Atherosclerotic cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality in the United States and globally (1, 2). Over the last several decades, inflammation has emerged as a key driver of atherosclerotic CVD, as well as a major therapeutic target (3, 4). In particular, a large body of preclinical and clinical studies implicate CD4+ and CD8+ T cells in the pathogenesis of atherosclerotic CVD (2). T cells are enriched in atherosclerotic plaque, where they recognize lipid- and endothelial-derived antigenic peptides and secrete proinflammatory cytokines (2, 5–7). Moreover, adoptive transfer of effector T cells promotes atherogenesis in murine models, whereas transfer of regulatory T (Treg) cells is protective (8–10). Taken together, these and other studies clearly establish that T cell-mediated immunity is a major modulatory of atherosclerotic CVD pathogenesis (2, 11–13).

Systemic autoimmune diseases are characterized by aberrant adaptive immune responses to autoantigens. Autoreactive T cells play a central role in the pathogenesis of autoimmunity. Severe early-

onset autoimmunity is a prominent feature of immune dysregulation syndromes caused by mutations in T cell specific genes such as *FOXP3* and *CTLA4*. Moreover, common autoimmune diseases are strongly associated with polymorphisms in genes that are preferentially expressed in T cells (14). A large body of human and murine studies has established multiple mechanisms by which T cell dysfunction promotes systemic autoimmunity in a variety of common rheumatic diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), myositis, psoriasis/psoriatic arthritis, and vasculitis (15).

Considering the major pathogenic role of T cells in both atherosclerosis and systemic autoimmunity, it is perhaps unsurprising that autoimmune diseases represent a major risk factor for CVD (15, 16). Furthermore, CV risk is reduced in patients with rheumatic diseases who achieve clinical remission. This observation has led to multiple studies testing the efficacy of anti-inflammatory therapies as a primary prevention strategy for CVD in patients with autoimmune disease (17–21). In order to select the most promising therapeutic targets, it is critical to understand the specific mechanisms by which T cells interact with other dysregulated populations to promote CVD in patients with autoimmunity. This review will focus on the mechanistic evidence implicating T cells as drivers of vascular inflammation, starting with primary vasculitides and then focusing on three prototypic systemic autoimmune diseases: RA, SLE, and psoriasis. We will also briefly review the efficacy of T-cell-directed therapies in the treatment of autoimmunity-associated vascular dysfunction.

OVERVIEW OF T CELLS IN PRIMARY VASCULITIDES

A review of T-cell mediated inflammation in autoimmunity-associated CVD would be incomplete without a discussion of T cells in the context of primary vasculitides. Vasculitides are a group of heterogenous disorders classified according the size of the vessel they predominantly affect: small-, medium-, and large vessel (22). Vasculitis can develop as secondary to various underlying medical conditions or constitute a primary autoimmune disease, where the vasculature is the target of immune-mediated pathology. The etiology and pathogenesis of primary vasculitis are not completely understood, but accumulating evidence has suggested a pathogenic role for T cells. This role has been most extensively explored in two prototypical vasculitic disorders that will be the focus of this review: the small-vessel disease antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) and the large-vessel vasculitis giant cell arteritis (GCA).

T CELLS IN ANCA-ASSOCIATED VASCULITIS

The AAV comprise three clinical syndromes: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA). Because T cells are critical orchestrators of antigen-specific autoimmunity, T cell dysfunction in the context of AAV is thought to directly promote disease (**Figure 1A**) (23). CD4+ T cells are considered particularly important to disease pathogenesis, since effector memory CD4+ T (T_{EM}) cells are persistently expanded in AAV (24, 25). Indeed, T_{EM} cells migrate from the peripheral circulation into inflamed tissues during recurrent disease, indicating that they may drive disease relapse (26–28). Moreover, AAV-associated T_{EM} cells express natural killer group 2D (NKG2D) receptor, giving them the capacity to mediate vascular injury through cytotoxicity (29, 30). Taken together, this suggests a central role for CD4+ T cells in AAV-associated vascular inflammation. As in atherosclerotic CVD, CD4+ T cell dysfunction AAV can occur through three broad mechanisms: dysregulated T helper (Th) differentiation, $CD4^+CD28-$ T cell expansion, and impaired regulatory T cell (Treg) function.

CD4+ T cells differentiate into various effector subsets (Th1, Th2, Th17, Th9, Th22, T follicular helper or Tfh), each of which mediates a discrete immunological response through the secretion of subset-specific effector cytokines (31). Studies have revealed a shift toward Th2 response in patients with generalized GPA with systemic vasculitis, whereas a Th1 response is seen in localized GPA with predominantly nasal lesions (32, 33). GPA is also associated with Tfh expansion, which may contribute to ANCA autoantibody production, whereas Th2 and Th17 expansion have been observed in EGPA (34–37). Proteinase-3 (PR3), the key pathogenic antigen associated with GPA, can itself modulate Th differentiation: PR3-expressing apoptotic cells promote a Th2/Th9 response, while PR3-ANCA promotes Th17 differentiation (38).

Expansion of the proinflammatory and cytotoxic $CD4^+CD28-$ T cell subset has been consistently reported in GPA (39–43). CD4+CD28− T cell expansion is associated with latent cytomegalovirus (CMV) infection and confers a poor prognosis (39, 40). However, it is not yet apparent whether CD4+CD28− T cells contribute to AAV-associated vascular inflammation, or whether they worsen outcomes through other mechanisms (41, 44). For example, CD4+CD28− T cell expansion is associated with impaired immunological responses to vaccination, which could increase infection-related morbidity (39, 40).

In contrast to T effector cells, Tregs are key negative regulators of inflammation that promote immune tolerance (45). Several studies have described reduced Treg frequency in AAV, but others have reported increased numbers, possibly due to the different methodologies of identifying human Tregs (36, 46–50). Moreover, functional Treg impairment is seen in active AAV and improves during disease remission (46–50). Treg impairment may arise from utilization of a hypofunctional isoform of the Treg-associated master transcription factor Forkhead box P3 (FoxP3), or from enhanced conversion into pathogenic Th17 effector cells (47, 51).

A limited body of data suggests that CD8+ T cells may also play a role in AAV. CD8+ T cells promote glomerular injury in murine MPA, and circulating CD8+CD28− T cells are expanded in GPA (52, 53). A subset of circulating T cells expressing both CD4 and CD8 has also been described in the context of human

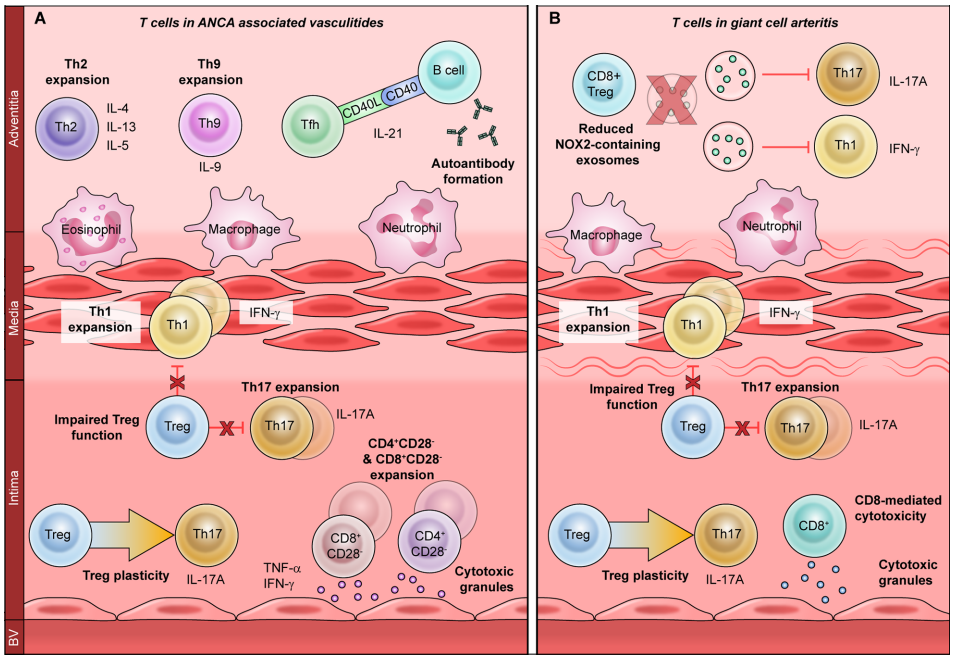

FIGURE 1 | The role of T cells in primary vasculitides. T cells promote vascular inflammation in primary vasculitides through a variety of mechanisms. Expansion of proinflammatory T helper (Th)-1 and Th17 subsets is associated with both ANCA-associated vasculitis **(A)** and giant cell arteritis **(B)**. Regulatory T cells are also hypofunctional in both vasculitides and display increased plasticity, or conversion to Th17 cells. Th17 cells recruit neutrophils and macrophages to promote acute vascular inflammation, whereas Th1 cells regulate macrophages to promote chronic damage. In ANCA-associated vasculitis **(A)**, T follicular helper (Tfh) cells promote the production of anti-neutrophil cytoplasmic antibodies (ANCA), which induce vascular inflammation. Th2 and Th9 cells produce IL-4, IL-13, IL-5, and IL-9, which promote eosinophilic vascular inflammation. CD4+CD28– and CD8+CD28– cells produce atherogenic cytokines such as TNF-α and IFN-γ, as well as directly damaging the vasculature by releasing cytotoxic molecules. Mechanisms of T cell dysfunction specific to giant cell arteritis **(B)** include enhanced CD8-mediated cytotoxicity and reduced anti-inflammatory function of CD8+ Tregs. This is due to reduced production of NOX2-containing exosomes, which inhibit the proliferation of Th1 and Th17 cells.

disease, although the function of this subset is incompletely characterized (54). CD4+CD8+ double-positive T cells are expanded in GPA and exhibit a memory phenotype, with co-expression of CD28 and NKG2D (53). Future investigations will be needed to define the role CD4+CD8+ double-positive cells in the pathogenesis of AAV.

T CELLS IN GIANT CELL ARTERITIS

GCA is a large-vessel vasculitis of unknown etiology that occurs mainly in individuals over age 50 (55). The pathological hallmark of GCA is granulomatous arterial wall inflammation, with infiltration of T lymphocytes, macrophages, dendritic cells (DCs) and multinucleated giant cells (56). While the pathogenesis of GCA is incompletely understood, over two decades of work implicate CD4+ T helper cells as major drivers of the pathological immune response (**Figure 1B**) (57–60).

GCA patients have marked expansions of Th1 and Th17 cells, which are thought to differentiate from a common precursor but promote two discrete pathologies (60, 61). Th17 cells promote neutrophil and macrophage recruitment, and Th17 expansion correlates strongly with signs of active inflammation. Th17 expansion also normalizes promptly with corticosteroid treatment, implying that Th17 cells primarily induce acute vessel inflammation (60). Conversely, Th1 expansion is associated with chronic persistent inflammation and vascular remodeling (60, 62). The Th1 effector cytokine IFN-γ activates macrophages and promotes giant cell formation (60). IFN-γ-stimulated macrophages also secrete platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which induce vascular hyperplasia and neoangiogenesis, ultimately causing luminal occlusion and ischemia (63–65). Notably, the Th1 responses in GCA are resistant to corticosteroid treatment which may explain why even patients in remission are at a high risk of subsequent vascular events (62, 66).

Reduced Treg frequency and Treg dysfunction have also been reported in GCA, though these findings are complicated by the different methodologies used to identify human Tregs in various studies (62, 67). As in AAV, Tregs derived from GCA patients have impaired suppressive ability and utilize the hypofunctional FoxP3 isoform (68). Treg plasticity has also been implicated in GCA pathogenesis, as FoxP3+T cells expressing the Th17-associated cytokine IL-17A have been identified in temporal artery biopsies (69, 70). Unexpectedly, temporal artery expression of IL-17A is associated with a favorable prognosis,

indicating that IL-17A+ Tregs may retain at least some suppressive capacity (71).

Although the role of CD8+ T cells in large vessel vasculitis is less clearly defined, CD8+ dysfunction and CD8+-specific transcriptomic changes have been reported in association with GCA (72, 73). CD8+ cells can also function as regulatory cells and promote immune tolerance, like their CD4+ counterparts (74). CD8+ Treg function is impaired in elderly individuals, with the highest degree of impairment seen in elderly individuals with GCA (75). This is thought to result from reduced production of NADPH oxidase 2 (NOX2), which CD8+ Tregs release in exosomes to dampen CD4+ proliferation and resultant autoimmunity (75).

T CELLS IN CVD ASSOCIATED WITH RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis (RA) is an autoimmune disease with a United States prevalence of 0.5 to 1 (76). Although joint destruction is the hallmark of RA, almost 50% of patients develop devastating extra-articular manifestations, including CVD (77). The association between RA and CVD is extremely well-established, with multiple studies demonstrating a 1.5-fold increased risk of CVD in RA patients (17, 78, 79). Traditional CV risk factors clearly contribute to CVD in RA patients, including hyperlipidemia, obesity, and smoking. However, traditional CV risk factors do not fully account for the increased CVD risk burden in RA, RA disease severity correlates with CVD, and immunomodulatory treatments reduce the risk of CVD in RA patients (17, 78, 79). Observational studies suggest that abatacept, a T cell immunomodulator, is more effective at preventing CVD in RA patients than TNF inhibitors, which act on multiple immune cell populations (17). Taken together, these data strongly implicate primary immune dysregulation, including T cell dysfunction, as a central driver of CVD in RA patients (**Figure 2A**).

T cells are central drivers of RA disease pathogenesis, promoting joint destruction through various mechanisms including secretion of proinflammatory cytokines, B cell activation, regulatory T cell dysfunction, and direct cytotoxicity – many of the same mechanisms implicated in CVD pathogenesis (2, 80, 81). Terminally differentiated T_{EM} CD4+ and CD8+ T cells are expanded and correlate significantly with coronary artery calcifications in RA patients, suggesting a pathogenic role (82). RA is also characterized by CD4+CD28− cell expansion, which is closely tied to the development of atherosclerotic CVD (83, 84). Accordingly, the frequency of circulating CD4+CD28− cells significantly correlates with preclinical atherosclerosis in RA patients, indicating that these cells may be major inducers of RA-associated CVD (84, 85). CD8+CD28− cells have also been described in association with RA-associated CVD, although the role of this subset is not as clearly defined (86).

Like primary vasculitides, RA is characterized by expanded proinflammatory Th1 and Th17 cells (81). In murine autoimmune arthritis models, pathogenic Th17 cells interact with vascular endothelial cells to promote both angiogenesis and joint destruction through production of placental growth factor, which correlates with IL-17A levels in RA patients (87). This provides strong mechanistic evidence that Th17 cells can promote RA-associated vascular injury. A subset of angiogenic T cells, characterized by coexpression of CD3/CD31/CXCR4, can also attenuate vascular injury by promoting endothelial repair (88). Two studies have analyzed the frequency of angiogenic T cells in RA patients, with discrepant results (36, 88). This could be related to differences in patient populations: one study focused on European patients with a high risk of CVD whereas the other investigated Asian patients with very few CV risk factors. This would be consistent with prior observations that RA-associated CVD is driven by complex interactions between traditional CV risk factors and systemic inflammatory mediators (89).

T CELLS IN CVD ASSOCIATED WITH PSORIASIS

Psoriasis is a T-cell-mediated autoimmune disease whose hallmark symptom is chronic skin inflammation. Psoriasis has a prevalence of 2% to 3% and causes extracutaneous disease in up to 30% of patients (90). Psoriasis is associated with a number of comorbid conditions that increase the risk of atherosclerotic CVD, including metabolic syndrome and chronic kidney disease. As in other autoimmune conditions, CVD risk in psoriasis patients correlates with disease severity and improves with immunomodulatory therapy (91, 92). An extensive body of work over the last several decades has shown that psoriasis is a T-cell-mediated disease, with Th17 cells emerging as the central drivers of cutaneous pathology (90). Accordingly, blockade of Th17-derived IL-17A and the Th17-inducing cytokine IL-23A are both highly efficacious for skin disease in most patients with psoriasis (93).

Given the centrality of Th17 cells to both atherosclerotic CVD and psoriatic skin disease, it is reasonable to conclude that Th17 cells link psoriatic immunopathology and inflammatory CVD (**Figure 2B**). Accordingly, Th17 cells from murine psoriatic skin lesions migrate to the arterial wall, where they promote atherogenesis by regulating high density lipoprotein (HDL) trafficking and collagen accumulation (94). Moreover, blocking IL-17A and IL-23 prevented psoriasis-related thrombosis in preclinical studies (95, 96). Subsequently, a number of late phase clinical trials tested the effects of blocking IL-17A and IL-23 on aortic vascular inflammation in patients with psoriasis. Although the immunomodulatory treatments caused transient improvements in inflammation, these changes were not sustained (20, 21, 92). This may be due to the role of other T helper subsets in psoriatic CVD, or because these large studies evaluated aortic inflammation instead of a more sensitive primary outcome measure such as coronary artery plaque burden. Indeed, more recent data has shown that biologic therapy reduces coronary plaque and coronary inflammation over a 1-year period of treatment (18, 97). Additional studies are ongoing that will use a variety of outcome measures, including aortic inflammation and carotid artery pulse wave velocity (NCT02144857, NCT03478280).

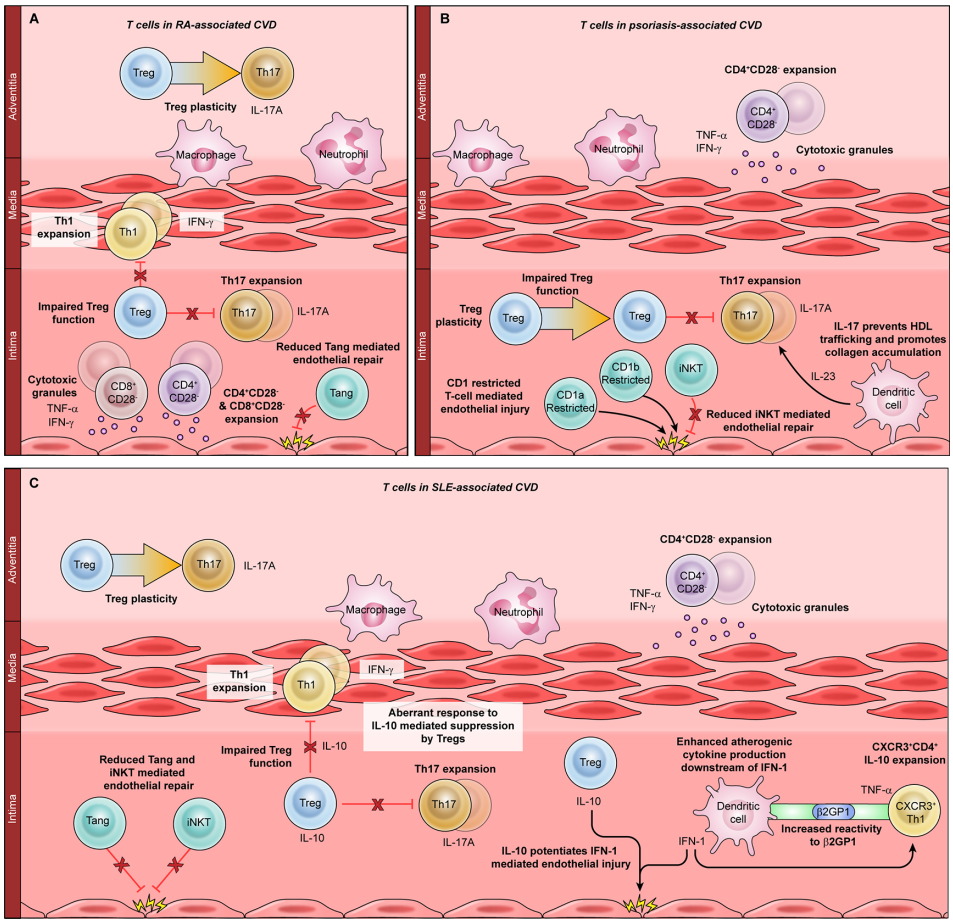

FIGURE 2 | The role of T cells in autoimmunity-associated cardiovascular disease (CVD). T cell dysfunction has been implicated in CVD associated with rheumatoid arthritis (RA, **A**), psoriasis **(B)**, and systemic lupus erythematosus (SLE, **C**). Mechanisms common to all three autoimmune diseases include expansion of CD4+CD28 – cells, which produce atherogenic cytokines such as TNF-α and IFN-γ, and release cytotoxic molecules that damage the vasculature. Proinflammatory T helper (Th)-1 cells are expanded in RA **(A)** and in SLE **(C)**; Th1-mediated atherogenesis is enhanced by the SLE-associated cytokine IFN-1. Th17 cells are expanded in all three autoimmune diseases and are particularly important for psoriatic CVD. Th17 differentiation is enhanced by the psoriasis-associated cytokine IL-23 and inhibited by regulatory T cells (Tregs). Treg dysfunction and plasticity, or conversion to Th17 cells, are implicated in CVD associated with RA, SLE, and psoriasis. In SLE, the Treg-derived cytokine IL-10 synergizes with the dendritic cell-derived cytokine IFN-1 to promote atherogenesis. Angiogenic T cells and CD1-restricted T cells such as invariant natural killer T (iNKT) cells can directly mediate endothelial damage and repair. Dysfunction of these subsets is seen in RA, SLE, and psoriasis.

Due to the prominent role of Th17 cells in psoriasis, most mechanistic studies of psoriatic CVD have focused on the Th17 lineage and its associated cytokines. However, other T cell subsets have also emerged as potential modulators of atherogenesis in patients with psoriasis (**Figure 2B**). As for many other immunological disorders, several of these studies have focused on the role of CD4+CD28− cells. Circulating and skin-resident CD4 +CD28− cells have been identified in patients with psoriasis, but their functions have not yet been defined in this population (98, 99). iNKT cells, which respond to lipids presented by the CD1d family of antigen-presenting molecules, have also been identified in psoriatic skin (100). In addition to iNKT cells, CD1-restricted cells comprise multiple other subtypes with various specialized immunological functions (101). Autoreactive CD1a-restricted T cells recognize lipid autoantigens in patients with psoriasis, providing a potential link between skin inflammation and CVD (102). CD1b-autoreactive cells promote murine psoriatic skin inflammation but have not been found to induce atherogenesis (103). As more information emerges about the roles of CD1-restricted T cells in human immunity, these cells may emerge as major links between cutaneous disease and atherogenesis in patients with psoriasis.

T CELLS IN CVD ASSOCIATED WITH SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease with a prevalence of 30 to 50 per 100,000

(104). SLE is typified by a combination of innate and adaptive immune dysregulation, which act in concert to promote disease pathogenesis (104). T cells have an essential role in SLE pathogenesis, with T effectors directly promoting SLE and Tregs attenuating end-organ pathology (105–107). Like RA, SLE is associated with a significantly increased risk of CVD not entirely explained by traditional risk factors (104).

An emerging body of evidence implicates T cell dysfunction as a key cause of atherogenesis in patients with SLE (**Figure 2C**). Aberrant T cell activation is a prominent feature of SLE-associated CVD, and adoptively transferred CD4+ T cells are sufficient to induce murine SLE-associated atherogenesis (108, 109). Additionally, T cell immunomodulation is an effective therapeutic strategy for CVD in SLE models and is even superior to lipid lowering therapy (110, 111). Atherogenic CD4+ T cells that express the Th1 marker CXCR3 are expanded in SLE, where they migrate to the arterial wall and directly induce vascular pathology. This process is enhanced by Type 1 interferon (IFN-I) signaling, which is a hallmark feature of immune dysregulation in SLE (112). IFN-I derives primarily from plasmacytoid dendritic cells, demonstrating that innate immune dysregulation and T-cell-driven atherogenesis are closely linked in patients with SLE (112). T cells from SLE patients also display enhanced reactivity to plasma β2 glycoprotein I, leading to immune-mediated hypercoagulability, endothelial cell dysfunction, and subclinical atherosclerosis (113).

As in other autoimmune diseases, both Treg dysfunction and abnormal T effector differentiation have been implicated in SLE-associated CVD. Th17 expansion correlates with both disease activity and atherosclerosis in SLE, whereas Tregs are reduced in SLE-associated CVD (108, 114, 115). In murine SLE-associated atherogenesis, pathogenic T effector cells are also resistant to Treg suppression, possibly due to reduced expression of IL-10 receptor (108). IL-10 is a Treg-derived cytokine with anti-inflammatory properties that suppresses T cell proliferation. Intriguingly, IL-10 is elevated in SLE patients, and IL-10 potentiates IFN-I-induced endothelial dysfunction (116). This suggests another link between CD4+ T cell dysfunction, innate immune dysregulation, and atherogenesis. SLE is also characterized by development of high titer autoantibodies, a process mediated by autoreactive B cells and Tfh cells (104). Atherogenesis promotes the differentiation of Tfh cells in lupus-prone mice, augmenting systemic autoimmunity and providing another link between SLE disease activity and atherogenesis (117).

A limited body of data also suggests a role for other T cell subsets in SLE-related CVD. CD4+CD28− T cells are expanded in SLE, but their relationship to atherogenesis is not well defined (118). Angiogenic T cells have also been described in the context of SLE; in contrast to RA, SLE is typified by expansion of angiogenic CD8+ cells but not angiogenic CD4+ cells. However, angiogenic CD8+ T cells do not correlate with SLE-related disease activity, and their role in CVD is indeterminate (119). Invariant natural killer T (iNKT) cells are an innate-like subset of T cells that can rapidly produce proinflammatory or anti-inflammatory cytokines in response to lipid antigens. In SLE patients, iNKT cells with an anti-inflammatory phenotype are atheroprotective, and their loss confers an increased risk of CV events (120). Future studies will be needed to dissect the roles of these and other non-CD4+ T cell subsets in the pathogenesis of autoimmunity-related CVD.

COMMON AND DISEASE-SPECIFIC MECHANISMS OF AUTOIMMUNITY-RELATED CVD

While this review has focused on a selected group of representative systemic autoimmune diseases, the risk of CVD is elevated in multiple organ-specific and systemic autoimmune disorders (121–123). It is impossible to comprehensively address every study linking autoimmunity to the development of CVD, but many of the mechanisms implicated are the same ones identified for vasculitis, RA, SLE, and psoriasis. This is perhaps unsurprising, as many genetic variants that predispose individuals to autoimmunity are shared between multiple autoimmune diseases, including polymorphisms in genes critical for T cell differentiation and function, like *HLA-DRB1*, *PTPN22*, and *CD25* (14). Common T-cell-dependent mechanisms of autoimmunity-associated CVD include CD4+CD28− expansion, CD8+CD28− expansion, Treg dysfunction, and proinflammatory cytokine production by T effector cells (Th1, Th17). By contrast, several T cell subsets are thought to promote CVD in the context of specific autoimmune diseases, including angiogenic T cells (SLE, RA), iNKT cells (psoriasis, SLE), and Tfh cells (AAV, SLE). However, it is important to acknowledge that many T-cell-dependent mechanisms have not yet been studied across multiple autoimmune conditions and could be more broadly shared. For example, IFN-1 is best studied in the context of SLE. Accordingly, IFN-1 is described to enhance Th1-mediated vascular damage in SLE but not in other diseases (112). However, IFN-1 is also implicated in the pathogenesis of RA and psoriasis (80, 124); therefore, IFN-1- may enhance T cell-mediated CVD in RA and psoriasis. Similarly, direct immune-mediated destruction of the vasculature is the hallmark of the primary vasculitides but can also be seen in secondary vasculitides related to underlying SLE or RA. Further investigations are needed to differentiate common and disease-specific T-cell-dependent mechanisms underlying CVD in various autoimmune conditions.

THERAPEUTIC MODULATION OF T CELLS IN AUTOIMMUNITY-RELATED CVD

Although T cells are clearly central to the pathogenesis of autoimmunity-related CVD, other cell types also play a major pathogenic role. These include dendritic cells, B cells, monocytes, neutrophils, and platelets (80, 125, 126). Of note, many of these cells directly interact with T cells to promote autoreactivity or induce endothelial injury downstream of T cell dysfunction.

Thus, various proinflammatory cytokines and factors can be targeted both to directly repress dysfunctional T cells and to prevent crosstalk between T cells and other critical effectors. Most conventional disease-modifying antirheumatic drugs (DMARDs) modulate the function of multiple immune cell subsets, including T cells. Methotrexate, which improves CVD in RA, psoriasis, and vasculitis, inhibits T cell activation and promotes Treg differentiation (127, 128). Calcineurin inhibitors, which potently block T-cell-receptor signaling, reduce markers of atherosclerotic CVD in SLE (129, 130). Mycophenolate mofetil also represses dysfunctional T cells and has attenuated CVD in murine models of SLE-related atherogenesis (111). Hydroxychloroquine, which reduces subclinical atherosclerosis in SLE, inhibits T cells by blocking the AP-1 transcription factor downstream of T cell receptor activation (131, 132).

T cells can also be efficiently targeted using biological and targeted synthetic DMARDs. Tumor necrosis factor (TNF) inhibitors, IL-6 receptor inhibitors, and JAK inhibitors all inhibit multiple immune subsets, including pathogenic T cells; these agents are all associated with reduced markers of CVD in patients with systemic autoimmunity (80, 126, 128, 133, 134). Biological DMARDs can also block T-cell-derived factors: as noted previously, blockade of Th17-derived IL-17A may ameliorate CVD in psoriasis, although further studies are needed (18, 19, 21, 97). Finally, the biological DMARD abatacept, which is FDA-approved for RA and psoriatic arthritis, directly targets T cell activation by blocking costimulation. Abatacept lowers the frequency of CD28− T cells and reduces CVD risk in RA, with a larger effect than TNF inhibitors and B-cell-directed therapies (135–140). Abatacept did not prove effective in clinical trials for SLE (141); therefore its effects on SLE-associated CVD is unknown. Early-phase clinical trials suggest that abatacept may also be efficacious for LVV and AAV (142, 143), with phase 3 trials ongoing (NCT02108860, NCT04474847). Taken together, these studies demonstrate that targeting dysfunctional T cells is a safe and effective therapeutic strategy for the prevention and treatment of autoimmunity-related CVD and vascular inflammation.

GENERALIZABILITY TO ATHEROSCLEROTIC CVD IN PATIENTS WITHOUT SYSTEMIC AUTOIMMUNITY

In addition to their role in autoimmunity-related CVD, T cells have an indisputable role in the pathogenesis of atherosclerotic CVD in patients without underlying autoimmunity. Although the focus of this review does not concern T-cell-dependent CVD in the general population, it is worth noting that many mechanisms implicated in autoimmunity-related CVD also promote atherogenesis in the general population. These include Treg dysfunction/instability, production of proatherogenic cytokines by effector T cells, and T-cell-mediated cytotoxicity (2). The presence of these shared mechanisms suggests that therapies efficacious for autoimmunity-related CVD might also be used to treat patients with atherosclerotic CVD. Indeed, T cell modulation with mycophenolate mofetil may be beneficial in atherosclerotic CVD (144); and clinical trials are ongoing or planned for hydroxychloroquine (NCT02648464, NCT04161339, NCT03636152), temsirolimus (NCT03942601, NCT04433572), tocilizumab (NCT03004703), and abatacept (NCT04344873). However, it must also be recognized that patients with systemic autoimmunity develop more inflammation and T cell autoreactivity than patients with atherosclerosis (125). Accordingly, some disease modifying antirheumatic drugs, such as methotrexate, prevent CVD in patients with systemic autoimmunity but not in patients with atherosclerosis (128, 145). Another T-cell-directed strategy involves the use of tolerogenic vaccinations or low-dose IL-2 to induce atheroprotective Tregs (2). Early phase clinical trials are underway to evaluate the potential efficacy of these strategies for CVD in the general population (NCT01284582, NCT03113773, NCT03042741, NCT02508896) but thus far these methods remain untested. Future studies are warranted to determine the generalizability of T-cell-mediated mechanisms of autoimmunity-related CVD to the general population, and the efficacy of T cell immunomodulation for CVD in patients without underlying autoimmunity.

CONCLUSIONS AND FUTURE DIRECTIONS

Over the last several decades, T cells have emerged as major mediators of atherosclerotic cardiovascular disease. The centrality of T cell dysfunction to human autoimmune diseases, and the increased risk of CVD in patients with autoimmunity, has sparked intense interest in the role of T cell dysfunction in autoimmunity-related vascular inflammation. A large body of evidence has established that T cells are central mediators of vascular inflammation in patients with systemic autoimmune diseases, suggesting that they underlie the increased risk of CVD associated with these disorders.

Several broad mechanisms of T cell dysfunction promote autoimmunity-associated CVD. Aberrant T helper differentiation leads to expansion of Th1 and Th17 cells, which migrate to the arterial wall and promote atherogenesis. This proinflammatory cytokine secretion is potentiated by Treg dysfunction, as well as reduced capacity of effector T cells to respond to Treg-derived cytokines. Cytotoxic CD4+CD28− cells also promote atherogenesis by inducing endothelial damage through various mechanisms. Finally, a potential role has emerged for other T cell lineages in autoimmunity-associated CVD; these include angiogenic T cells and CD1-restricted lipid responsive T cell subsets.

Because CVD is a major cause of morbidity and mortality in patients with systemic autoimmunity, targeting the immunologic drivers of vascular inflammation has the potential to substantially improve the quality of life of these individuals (78, 92, 113). Investigating the mechanisms of T-cell-mediated CVD in psoriasis has already culminated in late phase clinical trials, with additional studies ongoing (18, 20, 21). Ongoing investigations into the mechanisms by which T cell cells promote autoimmunity-related CVD will uncover additional therapeutic targets, allowing a more sophisticated approach to preventing and treating CVD in these cohorts. As systemic autoimmune diseases are present in up to

10% of the global population, these insights are likely to have a major public health impact (14). Ultimately, these findings may also have broader translational relevance to atherosclerotic CVD, where T cell dysfunction is also a major driver of vascular pathology.

AUTHOR CONTRIBUTIONS

DS, AB, MK, and YL: literature review, manuscript preparation, and generation of figures. DS and NM: oversight, editing, and planning. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immune Mechanisms in Cardiovascular Diseases Associated With Viral Infection

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Influenza virus infection causes 3–5 million cases of severe illness and 250,000–500,000 deaths worldwide annually. Although pneumonia is the most common complication associated with influenza, there are several reports demonstrating increased risk for cardiovascular diseases. Several clinical case reports, as well as both prospective and retrospective studies, have shown that influenza can trigger cardiovascular events including myocardial infarction (MI), myocarditis, ventricular arrhythmia, and heart failure. A recent study has demonstrated that influenza-infected patients are at highest risk of having MI during the first seven days of diagnosis. Influenza virus infection induces a variety of pro-inflammatory cytokines and chemokines and recruitment of immune cells as part of the host immune response. Understanding the cellular and molecular mechanisms involved in influenza-associated cardiovascular diseases will help to improve treatment plans. This review discusses the direct and indirect effects of influenza virus infection on triggering cardiovascular events. Further, we discussed the similarities and differences in epidemiological and pathogenic mechanisms involved in cardiovascular events associated with coronavirus disease 2019 (COVID-19) compared to influenza infection.

Keywords: influenza, heart, immune mechanism, myocardial infarction, atherosclerosis, myocarditis, SARS-CoV-2, COVID-19

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INTRODUCTION

Influenza virus infection causes approximately 36,000 deaths and 200,000 hospitalizations each year in the United States. During influenza epidemics, research focuses on lung disease as the main cause of death. However, epidemiological studies reported significant mortality associated with cardiovascular diseases during influenza virus infection (1, 2). Influenza vaccination reduces cardiovascular events associated with influenza virus infection (3–5). Pandemic outbreaks of novel, highly virulent influenza strains can have an even larger impact on healthcare settings. Pandemics increase both the cardiovascular morbidity and mortality rates compared to those associated with seasonal influenza. During the recent H1N1 influenza pandemic, mortality associated with heart disease was higher in children and young adults than adults (6). Dawood et al. estimated that the 2009 influenza A H1N1 pandemic caused 201,200 respiratory deaths worldwide, with an additional 83,300 deaths associated with cardiovascular complications. 80% of these fatalities were in people younger than 65 (7).

During viral infection, the innate and adaptive immune systems activate a variety of signaling pathways that induce type I (IFNα/β), type II (IFNγ), and type III (IFNλ) interferons (IFNs), and a large number of inflammatory cytokines and chemokines (8–11). These IFNs and inflammatory mediators recruit monocytes, neutrophils, and macrophages to the lungs for viral control. However, an excessive influx of innate immune cells and the dysregulated production of inflammatory cytokines results in host-mediated pathological responses during viral infection (8, 11–14).

In the heart, influenza-associated injury can occur either directly by viral entry or indirectly through induction of inflammatory mediators, acute phase proteins, and coagulation factors. Atherosclerosis is a common cause of coronary artery disease (CAD), including MI, stroke, and heart failure. The innate and adaptive immune responses to modified lipids in subendothelial space cause a series of events that result in plaque formation in medium- to large-sized arteries. If the inflammation continues, plaques become vulnerable to rupture, leading to myocardial infarction (MI). A variety of cells, including vascular endothelial cells (VE), macrophages, T cells, and vascular smooth cells play a significant role in atherosclerosis. Understanding the impact of influenza infection on these cells will help to identify therapeutic targets. In this review, we analyzed the direct and indirect effects of influenza infection on these cells in the aspects of atherosclerotic progression, plaque rupture, and thrombosis that subsequently cause acute coronary events. We further discussed the potential mechanism involved in influenza associated myocarditis, ventricular arrhythmia, and heart failure.

COVID-19, caused by severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV-2), has emerged as a global pandemic and has caused significant mortality and morbidity worldwide. SARS-CoV-2 is a highly contagious virus that enters the respiratory epithelium through angiotensin-converting enzyme II (ACE2) receptor and causes pneumonia. The effects of SARS-CoV-2 infection vary from mild asymptomatic infection to lethal disease. Clinical presentation in severely infected patients includes acute respiratory distress syndrome, acute cardiac injury, and secondary illness (13). Studies have shown that COVID-19 patients with one or more underlying conditions, including diabetes, hypertension, and cardiovascular diseases, are more likely to be severely ill (13, 15, 16). COVID-19 also contributes to cardiovascular events such as myocarditis, acute coronary syndrome, cardiomyopathy, and arrhythmias. Influenza virus infection and SARS-CoV-2 infection have similarities in pulmonary immune responses, cellular recruitment, and inflammatory cytokine production. Unlike influenza infection, SARS-CoV-2 causes an abnormal vascular coagulopathy in severely infected COVID-19 patients and multi-systemic inflammatory syndrome with cardiac damage in children. In this review, we discuss the possible mechanisms involved in cardiovascular events associated with COVID-19 in comparison with influenza-associated cardiovascular diseases.

ASSOCIATION BETWEEN INFLUENZA VIRUS INFECTION AND CARDIOVASCULAR DISEASES

Several studies have shown that influenza virus infection can trigger detrimental cardiovascular events (3, 15–21). The association between influenza virus infection and non-respiratory causes of death was first identified in the 1930s (22). A case series analysis from 1959 to 1999 showed that mortality from ischemic heart disease (IHD), cerebrovascular disease, and diabetes was highly correlated with influenza and pneumonia cases (23). Another study collected autopsy data between 1993 and 2000 in patients who died from MI and IHD and identified that the odds for MI (1.3, 95% confidence interval (Cl): 1.08–1.56) and chronic IHD (1.10 (95% CI; 0.97–1.26) were increased during influenza seasons (1). A time-series analysis has shown that seasonal influenza virus infection-associated emergency visits correlated with an increase in MI-related mortality, especially in individuals 65 and older (24). Further, a recent study has confirmed that the risk of MI is six times higher during the acute phase (days 1–7) of laboratory-confirmed influenza virus infection (2). These data show the association between influenza infection and MI.

Several groups have analyzed antibody and cellular responses to influenza virus infection in MI patients. Guan et al. found a positive association between IgG and influenza A/B in patients with MI when compared to patients without MI (18, 25). A recent study has shown that influenza-associated cardiovascular excess mortality, including ischemic heart disease, is higher with influenza B virus infection than pandemic influenza A strains (H1N1 and H3N2) (26).

Multiple studies have analyzed whether seasonality impact influenza-associated cardiovascular diseases. A report from central Bohemia has shown that increased influenza epidemics in February positively correlated with a peak in MI incidence (16). A time-series study from 1998 to 2008 has shown that the increase in MI cases associated with influenza virus infection is similar in both temperate and subtropical climates (27).

Studies have also analyzed whether influenza or other respiratory viral infections have a similar impact on triggering cardiovascular diseases. Kwong et al. have shown that the incidence ratio for MI is higher with influenza virus than with respiratory syncytial virus and other viral infections (2). Warren-Gosh et al. (28) have shown that, when compared to other viral infections, influenza has a stronger correlation with triggered MI (28). These studies suggest that the effect of influenza virus infection on triggering cardiovascular events is greater than that of other viral infections.

ST-segment myocardial infarction (STEMI) is a severe condition when the coronary artery is completely blocked in which the patient requires immediate reperfusion therapy and percutaneous coronary intervention (PCI). A non-STEMI (NSTEMI) presentation of MI is due to partial blockage of the coronary artery. Vejpongsa et al., 2019 observed STEMI (9.7%) and NSTEMI (90.3%) cases among MI patients with influenza viral infection. Another study analyzed cardiac injury markers in

143 veterans who were positive for influenza within the previous 30 days showing that 25% of patients had NSTEMI, and 24% had probable STEMI. These studies suggest that both STEMI and NSTEMI presentations of MI are present among influenza-infected MI patients (29).

Influenza infection can trigger myocarditis (29–31), ventricular arrhythmia (32) or heart failure (33). The frequency and damage of myocardium caused by pandemic H1N1 is higher than the seasonal influenza infection (29). ECG reports from H1N1 influenza-infected patients has shown the ventricular dysfunction is associated with influenza infection (34). Another study has shown abnormal ECG findings on days 1, 4, 11, and 28 days after the influenza disease presentation in young adults (35). Some groups also have shown an association between influenza infection and ventricular arrhythmias and hospitalizations of heart failure (32, 33, 36, 37).

Studies have shown elevation of cardiac injury markers and acute phase proteins are indicators of cardiovascular events associated with influenza virus infection (38, 39). Myocardial injury can be determined by serum biomarkers including the MB form of creatine kinase (CK-MB), lactate dehydrogenase (LDH), and troponin (TnT) (40, 41). Cardiac injury markers are shown to be elevated in influenza-positive veterans within 30 days of laboratory confirmation (42). Acute phase proteins are also potential biomarkers for cardiovascular diseases. B-type natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP) are the biomarkers in diagnosis of heart failure (40, 41). Increased levels of C-reactive protein (CRP) and NT-proBNP, along with increased leukocyte numbers, correlated with mortality rate in elderly patients with 2009 H1N1 infections and cardiovascular diseases (43).

Several clinical reports have shown that influenza vaccination reduces influenza-associated cardiovascular events (4, 36, 44–53). Gwini et al. identified that the influenza vaccine-induced protective effect is greater in those receiving the vaccine before mid-November (49). In another study, Hung et al. found that dual pneumococcal and influenza vaccination reduced respiratory, cardiovascular, and cerebrovascular disease (54). Influenza vaccination has beneficial effects not only against influenza virus infection, but also for other diseases. A study has shown that elderly patients with COPD are protected against acute coronary syndrome if they received influenza vaccination (55). Similarly, influenza vaccination decreased hospitalization rates due to heart failure or acute coronary syndrome in elderly patients with chronic kidney disease (CKD) (56). All these studies suggest a possible link between influenza virus infection, cardiovascular diseases, and a protective role for flu prevention.

PATHOGENIC MECHANISM INVOLVED IN INFLUENZA-ASSOCIATED CARDIOVASCULAR DISEASES

Immune Response to Influenza Virus Infection

Influenza virus enters the lung through airway and alveolar epithelial cells. Viral binding to host cells induces a variety of innate immune signaling, leading to induction of type I and type III IFNs, and pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) and chemokines (CCL2, CCL4, CCL5) (8–11). Type I and type III IFNs bind to their receptors, resulting in activation of Janus kinase (JAK) and signal transducer and activation of transcription (STAT) signaling pathways resulting in the induction of interferon stimulated genes (ISGs), thereby controlling the virus (10, 57, 58).

Pathology during influenza virus infection can be caused by direct viral infection, or indirect damage due to the inflammatory cytokine storm. Influenza virus infection triggers apoptosis or necrosis of alveolar epithelial cells, disrupts tight junction proteins, and damages the endothelium (59–61). Influenza also induces epithelial cell release of a variety of cytokines and chemokines, including TNFα, IL-8, IL-6, CCL2, CCL5, CXCL1, and CXCL10, which attract macrophages and neutrophils to the infection site. These recruited immune cells produce nitric oxide (NO) and reactive oxygen species (ROS) which increase lung injury (62, 63). Further, the inflammatory cytokines may enter the vessel through lung leak or inflammatory cell migration to the circulation (64, 65). Together, these responses increase the accumulation of proteinaceous material in the alveoli, impairing gas exchange and subsequently causing severe respiratory insufficiency (62, 63).

Following the innate immune response, the adaptive immune system plays a role in viral clearance. During influenza virus infection, dendritic cells capture viral antigens and traffic to the draining lymph nodes, presenting antigens to T cells. Antigen presentation occurs on MHC-I and MHC-II molecules to cytotoxic and helper T cells, respectively. Activated effector cytotoxic CD8+ and helper CD4+ T cells migrate from the draining lymph nodes to the lungs and kill viral infected cells. Cytotoxic CD8+ T cells clear the virus or infected cells through induction of IFNγ, release of perforin or granzymes, and triggering of apoptosis by Fas/FasL interactions (66–68). CD4+ T cells facilitate IFNγ production by CD8+ T cells and virus neutralizing antibody production by B cells (69–72). CD4+ T cells differentiate into Th1, Th2, Th17, T regulatory, or T follicular cells based on the polarizing cytokines produced by dendritic cells. These subsets have specific effects on antiviral responses, promoting B cell responses, and regulation of host immune responses during influenza virus infection.

Pathophysiology of Atherosclerosis

Atherosclerosis is the most common cause of acute coronary syndrome. A variety of cells, including vascular endothelial cells, macrophages, T cells, and vascular smooth muscle cells are important in atherosclerosis plaque formation. Systemic inflammatory responses, along with direct viral effects on vascular endothelial cells or atherosclerotic plaques during influenza virus infection, may be possible mechanisms in the progression of atherosclerosis or plaque rupture, which can cause subsequent acute coronary events.

The atherosclerotic process is initiated by endothelial dysfunction and accumulation of low-density lipoprotein (LDL) in the sub-endothelial space (73–77). LDL is oxidized

(to ox-LDL) by myeloperoxidases and lipoxygenases from immune cells (78). Ox-LDL stimulates the vascular endothelium to increase the expression of adhesion molecules and chemokines that recruit macrophages and T cells into the sub-endothelial space (79–82). Macrophages increase their expression of scavenger receptors, engulf ox-LDL, and become foam cells (83–88). Over time, foam cells undergo apoptosis or necrosis, thus leading to the accumulation of cell debris and the formation of a necrotic core within the intima. Smooth muscle cells then synthesize collagen and elastin to form the fibrous cap that covers the necrotic core. If the fibrous cap is fragile, it may rupture and cause coronary artery disease including MI, stroke, and heart failure (89, 90).

Direct Effect of Influenza on Atherosclerosis

Influenza-associated effects on atherosclerosis can occur directly by infection of vascular endothelial cells or atherosclerotic plaques, or indirectly through systemic inflammatory responses. Studies have shown the presence of influenza viral antigens in the aorta by PCR and immunohistochemistry (91). Influenza virus has been shown to induce host cell proteases, such as trypsin and matrix metalloprotease 9 (MMP-9), in various organs. This may be a possible mechanism for increased vascular permeability and viral entry in different organs (92). Animal models, including atherosclerotic Apoe$^{-/-}$ mice infected intranasally with influenza virus, have shown antigen localization and influenza viral activity in the aorta (91). However, the cells that potentially carry the virus from the lungs to the aorta are unknown.

Normal vascular endothelial homeostasis is maintained by nitric oxide (NO)-induced relaxing and contracting factors. In normal vascular homeostasis, NO prevents adhesion of leukocytes to the endothelium. In vascular endothelial dysfunction, increased expression of adhesion molecules favors leukocyte binding. It has been shown that HL-60 cells adhere to influenza-infected human umbilical vein endothelial cells (HUVEC) in a viral dose dependent manner (93). Further, adherence depends on the surface hemagglutinin (HA) protein from influenza virus (93).

Systemic and endothelium-induced inflammatory mediators play a role in interrupting endothelial homeostasis. Studies have shown that influenza virus infection increases expression of the chemokines CCL2, CCL5, and IL-8, and the adhesion molecules ICAM1, VCAM-1 and E-selectin in human coronary endothelial cells (HAEC) (91) and increases CXCL10 and CXCL9 in HUVEC cells (94). Another study has shown that, similar to live virus, viral particles also upregulate the expression of chemokine transcripts (95). These data suggest that both whole virus and viral particles contribute to increased antiviral and inflammatory mediators, thereby potentially increasing atherosclerosis.

Accumulation of oxidized LDL (ox-LDL) in the sub-endothelial space is the crucial factor in the development of atherosclerosis. Ox-LDL synergistically increases the expression of pro-inflammatory molecules such as IL-1β, IL-6, TNFα, and MMP-9 in response to H1N1 PDM 2009 influenza in HUVEC cells (96). Both influenza and ox-LDL have been shown to increase apoptosis in vascular endothelial cells, the latter through caspase-9 and caspase-3 cascades (87–91). One study has shown that influenza virus infection synergistically increases ox-LDL-induced apoptosis when compared to apoptosis caused by influenza or ox-LDL alone (97). These influenza-induced effects are possible mechanisms involved in atherosclerotic progression (**Figure 1**).

Indirect Effect of Influenza Virus Infection on Atherosclerosis

The indirect effect of influenza virus infection on atherosclerosis is likely through systemic inflammatory mediators and cell trafficking induced by the virus.

Influenza-Induced Inflammatory Mediators and Atherosclerosis

Influenza virus infection induces interferons and a variety of inflammatory cytokines both systemically and locally. Type I, type II, and type III IFNs play an indispensable role in controlling influenza virus (9, 10, 57). In mouse models of atherosclerosis, influenza virus infection increases the expression of ISGs including Mx1 and OAS in the aorta (91). These studies suggest that the influenza viral infection in aorta that induces IFN response.

Studies have shown that type I IFNs promote atherosclerotic plaques by inducing chemotactic factors such as CCL2, CCL3, CCL4, and CCL5, resulting in macrophage accumulation (98), foam cell formation in macrophages (99), and proliferation of smooth muscle cells (100). Type II IFN promotes atherosclerosis by multiple mechanisms (101, 102). IFNγ promotes atherosclerosis by inducing inflammatory mediators such as CCL2, CXCL9, CXCL10, CXCL12, CXCL16, and VCAM1 in vascular endothelial cells, increasing inflammatory cytokines production by macrophages and T cells, promoting foam cell formation by increasing scavenger receptor expression on macrophages, and increasing smooth muscle migration [reviewed in (102)]. Also, IFNγ induces MMP production from macrophages and vascular smooth muscle cells, which favors plaque rupture (102).

In addition to interferons, influenza virus infection induces a variety of inflammatory cytokines and chemokines. Pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) have been shown to play a pro-atherogenic role by increasing vascular endothelial adhesion and chemokine production (103). IL-17 promotes plaque formation by either driving type 1 responses or increasing the levels of IL-6, G-CSF, CXCL1, and CCL2 (104–107). In contrast, some studies have shown that IL-17 has a protective role in atherosclerosis by increasing IL-10 (108). Chemokines (CCL2, CCL3 and CCL5) play proatherogenic roles by increasing cellular recruitment and vascular endothelial adhesion [reviewed in (109–112)].

In the Apoe$^{-/-}$ mouse model of atherosclerosis, influenza virus infection increases the levels of IL-1β, IL-6, G-CSF, GM-CSF, CCL2, CCL3, and CCL5 in the serum (91). Also, it has been shown that levels of IL-2, IL-6, IL-18, TNFα, IFNγ, ET-1,

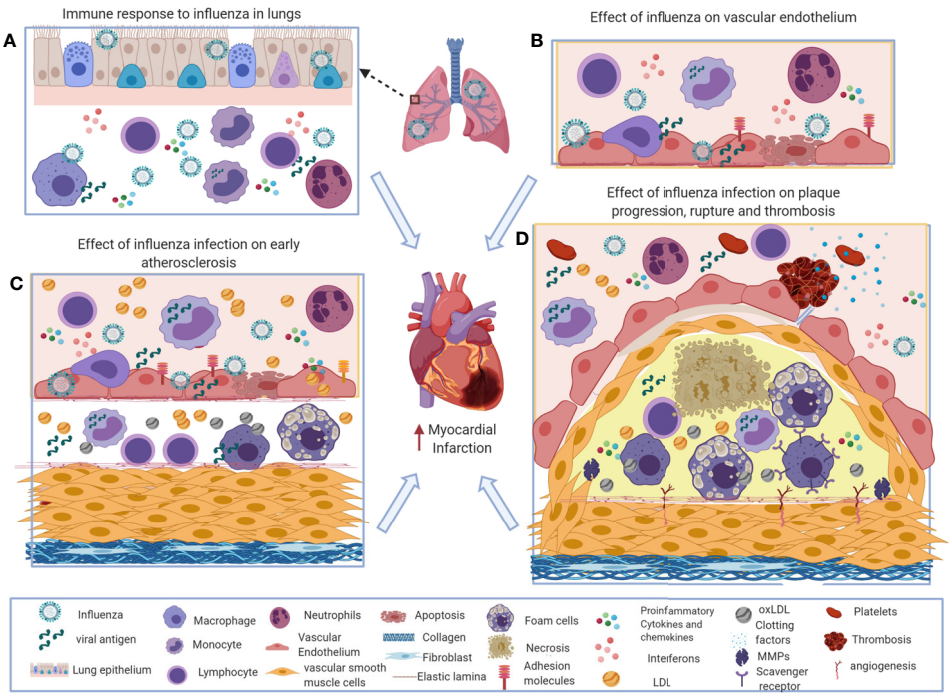

FIGURE 1 | Potential immune mechanisms of influenza-induced exacerbation of atherosclerosis. **(A)** During influenza virus infection, the innate and adaptive immune systems induce interferons and a variety of inflammatory mediators to recruit macrophages, neutrophils, and natural killer (NK) cells to the site of infection to control the virus. Excess influx of innate immune cells and dysregulated production of inflammatory cytokines and chemokines results in pathological responses during influenza virus infection. **(B)** Systemic and local interferons and pro-inflammatory cytokines increase chemotactic factors and adhesion molecules on vascular endothelial cells that increase inflammatory cell recruitment in atherosclerosis. **(C, D)**. Influenza-induced inflammatory mediators increase foam cell formation, activate smooth muscle proliferation, plaque rupture, and thrombosis that exacerbates atherosclerosis and subsequently can cause acute myocardial infarction. This figure was made in ©BioRender—biorender.com.

sICAM-1, and sVCAM-1 are increased in influenza-infected MI patients (25). Collectively, these studies indicate that soluble inflammatory mediators from influenza virus infection may favor atherosclerotic plaque progression (**Figure 1**).

Effect of Cellular Trafficking During Influenza Virus Infection on Atherosclerosis

In atherosclerosis, endothelial dysfunction increases adhesion molecules, causing monocytes to migrate into the sub-endothelial space and differentiate into macrophages. Monocytes, especially Ly6C^{hi} monocytes, play an important role in atherosclerotic lesion progression (113, 114). During this process, CCR2, CCR5, and CX3CR1 assist in the recruitment of Ly6C^{hi} monocytes in the lesion (113, 114). Ly6C^{hi} subsets express high levels of CCR2 and resemble inflammatory macrophages, whereas Ly6C^{low} monocytes express high levels of CX3CR1 and resemble tissue repair or resolving-type macrophages. In acute inflammatory conditions, such as MI, increased accumulation of Ly6C^{hi} monocytes accelerates atherosclerosis (115). Ly6C^{hi} monocytes are a key mechanistic player involved in lung pathology during influenza virus infection (116, 117). Due to the high trafficking rates of these cells, it may be possible that Ly6C^{hi} monocytes carry the influenza virus or viral antigen from the lungs to the aorta and favor atherosclerotic lesion progression.

Once the monocytes enter the sub-endothelium, they differentiate into macrophages with the help of growth factors. Further, macrophages increase scavenger receptor expression and engulf ox-LDL becoming foam cells. The local environment is a crucial determinant of the inflammatory (M1) or resolving (M2) macrophage phenotype. IFNγ and LPS favor macrophage differentiation into M1 macrophages, while IL-4 drives macrophages towards the M2 phenotype. It has been shown that influenza virus infection in Apoe$^{-/-}$ mice increases macrophage infiltration in the sub-endothelium (118). Another study has shown that influenza virus infection in LDLR$^{-/-}$ mice increases macrophage infiltration into the aortic arch (119). Based on these results, it is possible that an increased proportion of inflammatory (M1) type macrophages favor atherosclerotic lesion progression.

T cells also play a crucial role in the outcome of atherosclerosis. Driven by antigen-specific responses, T cells differentiate into inflammatory effector T (Teff) cells or anti-inflammatory regulatory T (Treg) cells. One study has shown influenza-specific proliferative responses in T cells isolated from atherosclerotic plaques in patients undergoing endarterectomies, suggesting that

influenza viral antigens may increase T cell activation and subsequent exacerbation of atherosclerosis (**Figure 1**).

Effect of Influenza Virus Infection on Vascular Smooth Muscle Cells

In normal healthy conditions, vascular smooth muscle cells (VSMCs) maintain a contractile or quiescent form and express smooth muscle actin (ACTA2), tangelin (TAGLN), smooth muscle myosin heavy chain (MYH11), and smoothelin markers [reviewed in (86, 120–122)]. In atherosclerosis, inflammatory mediators induced by immune cells and vascular endothelial cells transform these contractile VSMCs into a synthetic or dedifferentiated form. The synthetic form of VSMCs acquires the capacity to proliferate and migrate from media to intima and produce extracellular matrix proteins collagen and elastin, which form the fibrous cap that covers the necrotic core [reviewed in (86, 120–122)]. The transition of VSMC phenotypes may be due to the induction of growth factors, such as platelet derived growth factors, fibroblast growth factors, and matrix metalloproteinases by macrophages and vascular endothelial cells [reviewed in (86, 120–122)]. The dedifferentiated VSMCs can also induce pro-inflammatory cytokines and chemokines (91). In an $Apoe^{-/-}$ mouse model of atherosclerosis, influenza virus infection increased VSMC infiltration into the sub-endothelium (118). Also, an *in vitro* study using human coronary smooth muscle cells has shown that influenza virus infection increases the expression of adhesion molecules (VCAM1 and ICAM1) and production of chemokines (CCL2, CCL5, and IL-8) (91). These studies suggest that influenza virus infection increases smooth muscle cell migration and induction of inflammatory chemokines and adhesion molecules in VSMCs (**Figure 1**).

Effect of Influenza Virus Infection on Plaque Rupture

The transition of fatty streak to fibro atheroma occurs with VSMC migration and proliferation (122). The formation of stable or unstable plaques depends on the pro- or anti-inflammatory status of the plaque. Studies have shown that increased levels of IL-4 and IL-10 are associated with stable plaques (123). In contrast, increased levels of IFN$\gamma$and TNFα are associated with unstable plaques that are highly prone to rupture (102, 124, 125). Increased accumulation of dead macrophages and smooth muscle cells, along with increased matrix degradation products, results in an enlarged necrotic core. These products weaken the fibrous cap, favoring plaque rupture leading to MI (102).

MMPs are known to be among the factors that increase fibrin degradation (MMP1, MMP3, MMP7, MMP9, MMP13, and MMP14) (126–128). An *in vitro* study using HUVEC cells has shown that influenza virus infection increases the expression of MMP9. Another study has shown that the expression of MMP-13 is increased in the atherosclerotic plaques of $Apoe^{-/-}$ mice infected with influenza A virus (129). These studies suggest that influenza virus infection-induced inflammatory mediators may increase plaque destabilization and rupture, leading to MI. One study has shown that the risk of MI is six times higher during the acute phase (days 1–7) of influenza virus infection (2). These results correlate with the excessive inflammatory response during the acute phase of influenza virus infection in the lung, which may increase the chances of plaque rupture and subsequent triggering of MI. Accordingly, a study has shown that influenza vaccination induces stable atherosclerotic lesions in $Apoe^{-/-}$ mice (130). Also, the study showed that influenza vaccination reduces the levels of IFNγ, IL-2, and TNFα production and increases the levels of IL-4 in serum (130). These results positively correlate with anti-influenza IgG production from vaccination suggesting that flu prevention by vaccination may limit indirect atherosclerotic damage induced by infection (130) (**Figure 1**).

Role of Influenza Virus Infection in Activation of Thrombosis

Plaque rupture releases necrotic components, rich in lipid-laden macrophages, tissue factor, and collagen, into the circulation triggering thrombus formation and leading to acute coronary events. Various mechanisms, including coagulant and anticoagulant factor dysregulation, increased fibrinolysis protease inhibitors, and inflammatory cytokine responses due to vascular infection or injury, increase intravascular coagulation.

The clotting mechanism is initiated once tissue factor and collagen are exposed in the bloodstream and release von Willebrand factor (vWF) [reviewed in (131)]. Tissue factor forms a complex with coagulation factor VII, which in turn activates the extrinsic pathway, whereby collagen release in the blood initiates the intrinsic pathway (131). Activation of both pathways resulting in fibrin deposition and subsequent thrombus formation [reviewed in (131)]. It has been shown that influenza virus infection increases tissue factor and vWF expression in the vascular endothelium (132, 133). Pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) were shown to increase tissue factor in endothelial cells (134). Hypoxia also increases tissue factor expression in the vascular space (135, 136). All these studies suggest that influenza infection directly or indirectly induces clotting factors that may enhance thrombus formation in atherosclerosis.

In contrast, dysregulation of anticoagulant factors such as protein C, antithrombin, and tissue factor pathway inhibitor (TFPI) also enhance thrombus formation. The expression of protein C is activated through a cell surface receptor, thrombomodulin (TM). Studies have shown that influenza virus infection decreases protein C activity, thereby inducing the clotting cascade (137, 138).

Plasminogen activator inhibitor (PAI), a serine protease inhibitor, regulates fibrinolysis and enhances clot formation. Pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) are known to increase PAI-1 activation (139, 140). Influenza virus infection has been shown to increase PAI-1 levels in plasma (137). D-dimer, one of the commonly used markers of fibrin degradation, is also used in the diagnosis of venous thromboembolism. Wang et al. has shown that increases in D-dimer levels correlate with hypoxemia during influenza virus infection (141).

Immunothrombosis, a mechanism involved in the interaction of leukocytes with platelets increases the clot formation. Inflammatory cytokines in the circulation increase receptors on vascular endothelial cells for platelet binding and activation. The activated platelets interact with neutrophils to form neutrophil extracellular traps (NETs) to kill microbes. These neutrophil-platelet aggregates in the circulation may also increase thrombosis (142). Influenza infection has been shown to increase NETs that may favor thrombus formation (143, 144). These studies collectively suggest that influenza virus-induced coagulation factors, fibrinolysis protease inhibitors, and pro-inflammatory immune responses increase thrombosis that subsequently increases the possibility of coronary heart diseases (**Figure 1**).

Pathogenic Mechanism Involved in Influenza-Associated Myocarditis, Ventricular Arrhythmia, and Heart Failure

Influenza can trigger myocarditis, ventricular arrhythmia and heart failure through systemic and local inflammatory mediators (29, 145–153). Pan et al. have shown induction of trypsin during influenza virus infection as a mechanism to explain the presence of virus in the heart. They have also shown that influenza virus infection up regulates IL-6, IL-1β, TNFα, and MMPs in the myocardium, and trypsin inhibitors alleviate these effects (146). Kotaka et al. have shown macrophage and lymphocytic infiltration in cardiomyocytes (145). A recent study has shown influenza viral replication in cardiomyocytes and purkinje cells in mice (154). Further, Kenny et al. have shown that interferon-induced transmembrane protein-3 (IFITM3) is crucial in controlling influenza viral replication in the heart (155). These studies suggest that influenza-induced pro-inflammatory cytokines and proteases are a possible mechanism in infection-associated myocarditis. Inflammatory cytokines and chemokines, acute phase proteins, and coagulation factors are shown to be possible mechanisms involved in MI that can cause subsequent heart failure and/or ventricular arrhythmias (147–150, 152, 153). All these studies suggest that there is a direct and indirect effect of influenza virus infection on triggering cardiovascular events.

COMPARISON OF CARDIOVASCULAR CONDITIONS ASSOCIATED WITH SARS-COV-2 AND INFLUENZA INFECTION

Several prospective and retrospective analyses have shown the association between influenza and MI. However, information regarding the link between the SARS-CoV-2 infection and MI is limited. A retrospective case series analysis from COVID-19 patients with STEMI during the initial period of pandemics in New York has shown that out of 18 patients, eight patients had an obstructive coronary artery lesion, and ten patients had a non-obstructive myocardial injury (156). Similarly, a study of 28 COVID-19 patients with STEMI from Italy has shown that 39.3% of patients did not show an obstructive lesion (157). Another report of 79 patients with COVID-19 and STEMI from four hospitals from Italy, Lithuania, Spain, and Iraq from February to April 2020 has shown that patients had stent thrombosis, and they were managed with fibrinolytic and PCI therapy (158). These studies suggest that there is an association between COVID-19 patients and MI. However, the sample sizes are small, and the observation period is too short to draw finite conclusions.

Similar to 2009 pandemic H1N1 influenza infection; several cases of myocarditis have been reported in COVID-19 patients. A meta summary analyzed 31 studies with a total of 51 myocarditis cases (159). Out of these, 12 patients were diagnosed based on cardiac magnetic resonance imaging (MRI) or histopathology, and 39 patients were diagnoses based on the inflammatory markers and electrocardiogram (ECG) (159). Also, comparable to 2009 H1N1 influenza infection, several fulminant myocarditis cases were observed in COVID-19 patients (160–168). These data suggest that there are similarities in pandemic H1N1 influenza infection and SARS-CoV-2 infection in triggering myocarditis. However, the incidence of myocarditis due to seasonal influenza is rare and for SARS-CoV-2 unknown.

Unlike influenza infection, SARS-CoV-2 infected children sometimes present with a condition called a multisystemic inflammatory syndrome (MIS-C). In April 2020, the first few cases were observed in the United Kingdom, and later a case in the United States was observed with Kawasaki disease with concurrent COVID-19 (169, 170). Further, a study has shown that 76% of 21 children with Kawasaki disease show evidence of myocarditis (171). Also, a case series analysis of 58 hospitalized children with SARS-CoV-2 has shown 22% with Kawasaki disease and 14% coronary artery dilatations (172). Another systemic review from 39 observational studies from 662 patients showed that the patients presented with gastrointestinal symptoms and abnormal inflammatory, coagulation, cardiac markers, and ECG with decreased heart ejection fraction (173). These data demonstrate aberrant systemic inflammatory responses in children due to MIS-C with COVID-19.

Similar to influenza, studies have shown the association between COVID-19 and cardiac arrhythmias (174–177). Early reports from Wuhan have reported that out of 138 patients, 16.7% patients had arrhythmia, and 44% of these patients were transferred to an intensive care unit (ICU) due to arrhythmia (178). Another study has observed ventricular fibrillation in 5.9% of COVID-19 patients (174). A retrospective case series analysis of five COVID-19 patients with ARDS has shown that ventricular arrhythmia was a primary cause of death of these patients (179). These studies suggest that cardiac arrhythmia is among the most common complications in severely ill COVID-19 patients.

Comparable to influenza infection, reports have also shown heart failure is associated with COVID-19. Zhou et al., 2020 has shown that 23% of 191 COVID-19 patients had heart failure (14). Takotsubo cardiomyopathy, a reversible condition, occurs

due to physical and emotional stress that affects the left ventricle. Several reports have shown Takotsubo cardiomyopathy in COVID-19 patients (180–185). Correspondingly, this condition is also reported in influenza-infected patients (186–189). These reports suggest that cardiomyopathy, especially stress-induced cardiomyopathy is reported in influenza viral infected and COVID-19 patients.

Like influenza infection, cardiac injury markers and acute-phase proteins are elevated in critically-ill patients with COVID-19 (174, 190). Shi et al. have shown that out of 416 hospitalized patients, 82 (19.7%) had increased levels of cardiac injury markers, including CRP, procalcitonin, CK-MB, myohemoglobin, troponin (TnT), and N-terminal pro-B-type natriuretic peptide (NTproBNP). Increased mortality rate (51.2%) was observed in patients with cardiac injury when compared to those without cardiac injury (4.5%) (190). Guo et al. demonstrated that in fatal cases of COVID-19, TnT levels rose over time from patient admission to shortly before death (174). Further, TnT levels had a significant correlation with CRP and plasma NTproBNP levels. Cao et al. has shown that 11% of the COVID-19 patients had increased TnT levels, and these patients had no preexisting cardiovascular conditions (191). These data suggest that increased cardiac injury markers are likely due to viral-induced cardiac injury.

Coagulopathy is one of the most concerning sequelae in COVID-19 patients. Several reports have shown mortality associated with pulmonary emboli and venous thrombosis in severely ill COVID-19 patients (192–199). Interestingly, a study by Wichmann et al. described the results of 12 mandatory autopsies of COVID-19, PCR confirmed patients. Of these patients, seven had deep venous thrombosis that was not known before the autopsy (200). Further, four patients died as a result of pulmonary embolism (200). Several studies also have shown changes in coagulation parameters. Increased D-dimer levels, CRP, Factor VIII, vWF, fibrin degradation product (FDP), longer PT, and activated partial thromboplastin time (APTT) was observed in critically ill COVID-19 patients (13, 178, 201, 202). A report has shown 69% patients were positive for venous thromboembolism, and 23% were positive for pulmonary embolism out of 26 COVID-19 patients tested from ICU (202). In a study of autopsy samples from 38 patients who died from COVID-19, 86% showed platelet-fibrin thrombi in small arterial vessels in the lung (203). In influenza case series analysis, fewer number (5.9%) of pulmonary thrombosis and embolism cases were only reported when compared to COVID-19 (204). Another study from autopsy samples from COVID-19 patients and H1N1 patients have shown nine times more numbers of alveolar capillary microthrombi in COVID-19 patients when compared to influenza-infected patients (205). These studies suggest that the COVID-19 associated pulmonary vascular thrombosis is more pronounced when compared to influenza infection and that may be a possible mechanism involved in increased cardiovascular events.

The preceding studies demonstrate cardiovascular events during acute COVID-19 infection. However, a recent report has shown the cardiovascular consequence of COVID-19 after the recovery. A study of 100 convalescent patients 64–92 days after COVID-19 diagnosis by cardiac magnetic resonance imaging showed ongoing myocardial inflammation in 78% of the patients, and 60% out of these patients had no preexisting conditions (142). These data suggest that cardiac inflammation brings long term cardiovascular sequelae. Future studies with a large sample size with various time points after recovery will provide valuable information on the long-term effects of COVID-19 on the heart.

The discussed studies show similarities and dissimilarities between cardiovascular complications associated with influenza and SARS-CoV-2 infection. Cardiovascular conditions such as MI, myocarditis, cardiomyopathy, arrhythmia, and thrombosis are present in both influenza and SARS-CoV-2 infection. However, the multisystemic inflammatory syndrome is only present in SARS-CoV2 infection. The morbidity and mortality rate due to microvascular thrombi and vascular occlusion are high in COVID-19 patients compared to influenza-infected patients. Further, the incidence rate is higher in SARS-CoV-2 infection than the influenza virus infection. There are several possible explanations for the differences in the incidence rates among influenza and SARS-CoV-2 infections. The number of cases analyzed in SARS-CoV-2 is small, and the observation period is short. Most of these data from COVID-19 patients were analyzed from severely ill patients. The available reports are also in a population in the absence of vaccine for SARS-CoV-2 infection versus a population with available vaccines for influenza virus infection. The incidence rate in COVID-19 may change when the analysis is carried out with large sample size and vaccine availability.

PATHOGENIC MECHANISM OF CARDIOVASCULAR EVENTS ASSOCIATED WITH COVID-19 IN COMPARISON WITH INFLUENZA VIRUS INFECTION

Direct Effect of SARS-CoV-2 on Vascular Endothelium and Cardiomyocytes

Like influenza, SARS-CoV-2 may increase risks of cardiovascular events through direct infection or systemic inflammatory responses. A recent report has shown ACE2 receptor expression in the lung, heart, kidney, and gastrointestinal tract (206). The presence of ACE2 receptor in vascular endothelial and VSMCs and myocytes may favor direct viral entry (206, 207). A study has shown viral particles, cellular accumulation, and apoptotic cells in vascular tissue sections from autopsy samples from COVID-19 patients (208). Another report has shown viral RNA in the myocardium in autopsied patients who died from COVID-19. The study also shows increased expression of TNFα, IFNγ, CCL-5, IL-6, IL-8, and IL-18 in patients with >1,000 RNA viral copies compared to SARS-CoV-2 negative patients (209). A case report of a child with MIS-C who had cardiac failure demonstrated interstitial and perivascular myocardial cellular

infiltration and cardiomyocyte necrosis (210). Further, electron microscopy analysis has shown viral particles in cardiomyocytes and endocardial endothelial cells (210). An *in vitro* study has shown that SARS-CoV-2 virus enters cardiomyocytes and multiplies and transduces a cytopathic effect (211). These reports show the direct effect of the SARS-CoV-2 virus on myocardium and vascular endothelial cells to induce cardiovascular diseases including MI, myocarditis, arrhythmias and heart failure (**Figure 2**).

Indirect Effect of SARS-CoV-2 Infection on Triggering Cardiovascular Events

In influenza infection, pulmonary induction of type I and type II IFN is a possible mechanism involved in MI. However, in SARS-CoV2 infection, the induction of type I IFN and type III IFNs in respiratory epithelial cells is low (212). The defective IFN responses may lead to an increase in viral multiplication and subsequent increases in inflammatory monocyte accumulation in the lung (213). Also, a study has shown the impaired type I IFN response leads to enhanced pro-inflammatory responses (214). These data suggest that the differences in IFN production and viral control in influenza *versus* SARS-CoV-2 infection may influence the outcome of cardiovascular diseases associated with these viral infections.

During influenza infection, increased production of inflammatory cytokines and increased cellular recruitment may be associated with triggering of cardiovascular diseases. Similarly, Huang et al. have shown increased levels of IL-1β, IL-1RA, IL-7, IL-8, IL-9, IL-10, GCSF, CM-CSF, basic FGF, IFNγ, IP-10, MCP-1, MIP-1a, MIP-1b, PDGF, TNFα, and VEGF in COVID-19 infected patients *versus* healthy controls (13). Also, Qin et al. have shown increased levels of TNFα, IL-2R, IL-6 in serum in severe disease compared to mild cases of COVID-19 (215). Studies have shown higher numbers of leukocytes and neutrophils and fewer lymphocytes in the blood of critically ill COVID-19 patients (13, 215). Another study has shown decreased numbers of CD4+ and CD8+ T cells in the blood of severely infected patients than moderately infected patients (216). These dysregulated and hyper-inflammatory cytokine storms may cause increased vascular permeability, decreased gas exchange, activation of pro-coagulation pathways, and subsequently ARDS. Defective gas exchange may increase myocardial injury due to oxygen supply/demand mismatch (217). Like influenza infection, these inflammatory cytokines may trigger cardiovascular diseases such as MI, myocarditis, arrhythmia, and heart failure. Also, these cytokines and dysregulated inflammatory cellular responses may be a possible mechanism in MIS-C in children. These

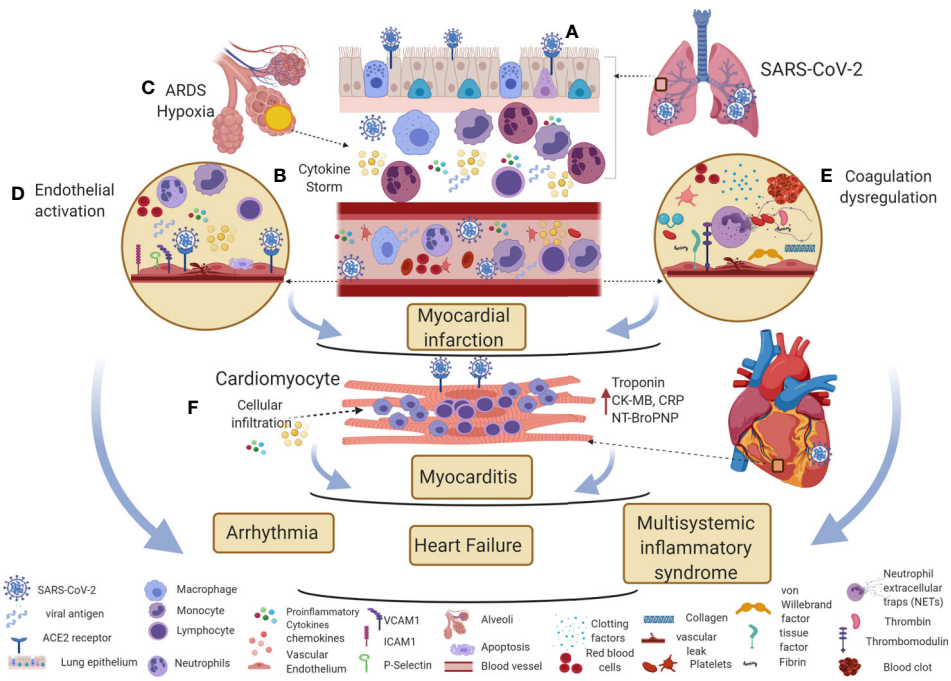

FIGURE 2 | Potential immune mechanisms of COVID-19 associated cardiovascular diseases. **(A)** SARS-CoV-2 enters the respiratory epithelium through the angiotensin-converting enzyme II (ACE2) receptor. The innate immune response induces various cytokines and chemokines to recruit macrophages and neutrophils to control the virus. **(B, C)** The hyperinflammatory cytokine storm increases vascular permeability, decreases gas exchange, stimulates pro-coagulation pathways, and subsequently causes ARDS. **(D, E)** Direct viral entry and inflammatory mediators can activate endothelial adhesion and clotting factors in the vascular space. Inflammatory cytokine storm, oxygen supply/demand mismatch due to hypoxia, endothelial activation, and dysregulation of clotting factors are likely mechanisms involved in triggering myocardial infarction in COVID-19 patients. **(F)** Direct viral entry, along with viral-induced inflammatory mediators increase myocarditis. All these pathological effects lead to arrhythmia, heart failure, and myocardial inflammation in multisystemic inflammatory syndrome. This figure was made in ©BioRender—biorender.com.

studies suggest that, similar to influenza, cytokine storm induced by SARS-CoV-2 infection is possibly involved in triggering cardiovascular sequelae.

Like influenza, SARS-CoV-2 infection also activates clotting factors, fibrinolysis proteases, and immunothrombosis that favor coagulopathy in COVID-19 patients. A study has shown that platelet-monocyte aggregates were observed in severely ill COVID-19 patients, associated with tissue factor induction (218). SARS-CoV-2 viral entry decreases the expression of ACE2 that enhances the levels of Angiotensin II (Ang II). Further, Ang II is shown to increase tissue factor in monocytes (219). The other major clotting factor, vWF antigen was shown to be increased (mean 565%, SD 199) in ICU and (278%, SD 133) non-ICU COVID-19 patients (220). However, during influenza infection, the levels of vWF antigen (123 to 211%) are lower when compared with COVID-19 patients (221, 222). These studies suggested that induction of the clotting factors is comparatively high in COVID-19 patients *versus* influenza virus infected patients.

Similar to influenza virus infection, reports have shown increased levels of PAI1 in COVID-19 patients. Zhou et al. have shown that the presence of at least 1 μg/ml of D-Dimer is associated with 18 times higher mortality rate in COVID-19 patients (14). Out of 172 COVID-19 patients analyzed, 68% patients showed >0.5 μg/ml of D-Dimer levels suggestive of increased mortality rate among these patients (26). However, in 2009 H1N1 influenza-infected patients, a study has shown concentrations of 1.13 ± 1.09 μg/ml of D-Dimer in patients from non-respiratory failure group *versus* 6.74 ± 5.11 μg/ml in patients in the respiratory failure group (141). Another study has shown levels of D-Dimer from 0.3 to 0.5 μg/ml in seasonal influenza-infected patients. These studies suggest that the D-Dimer level-associated mortality risk is different in influenza virus infected and COVID-19 patients. The differences may be due to the effect of other coagulation factors and inflammatory mediator difference between these two infections.

Studies have also shown that endothelial injury due to SARS-CoV-2 infection increases coagulation marker levels. An autopsy report showed the presence of viral particles and apoptotic bodies in the vascular endothelium (208). Studies have shown elevated expression of endothelial and platelet activation markers ICAM1, VCAM1, P-selectin, sCD40L and thrombomodulin in COVID-19 patients when compared to controls (220, 223). These data suggest that the direct SARS-CoV-2 viral infection also induces procoagulant factors that favor vascular thrombosis. Studies also have shown influenza virus infects vascular endothelial cells, but the thrombotic events are more pronounced in COVID-19 patients.

Several reports have also shown that NET-platelet aggregates favor vascular coagulation in COVID-19 patients. A recent report has shown microvascular thrombi associated with platelet-neutrophil aggregates in the lungs, kidney and the heart (224). Another study has shown NETs in the heart by electron microscopy (EM) in an autopsy sample from COVID-19 patient with MIS-C (210). These studies suggest that similar to influenza infection, immunothrombosis is one of the mechanisms involved in vascular thrombosis in COVID-19 (**Figure 2**).

CONCLUSION

Influenza contributes to cardiovascular diseases through a number of different mechanisms. Influenza virus infection increases immune cell recruitment, adhesion, and/or apoptosis, leading to atherosclerotic plaque progression. VSMCs can transform into synthetic VSMCs during the course of infection that then proliferate and migrate into the intima, favoring plaque rupture. The inflammatory response generated during influenza virus infection greatly increases the risks of plaque rupture. Also, influenza-induced coagulation factors may increase thrombosis that can cause acute coronary events. Interactions between influenza-induced pro-inflammatory cytokines and proteases may be a mechanism involved in influenza-induced myocarditis. The highlighted studies illuminate direct and indirect effects of influenza virus infection on triggering or exacerbating cardiovascular diseases.

Based on the presence of ACE2 in various tissues including the lung epithelium, vascular endothelium, and cardiomyocytes, direct SARS-CoV-2 viral-induced effects may trigger cardiovascular events. Several clinical reports from COVID-19 patients show dysregulated production of inflammatory mediators, coagulation factors, and the effect of hypoxia due to ARDS as possible indirect mechanisms involved in triggering cardiovascular events in COVID-19 patients. Epidemiological and pathogenic studies showed similarities and differences between influenza virus and COVID-19 associated cardiovascular diseases. However, the sample sizes analyzed in COVID-19 patients are small, and the observation period is short. The studies are carried out in severely or moderately infected patients. However, identifying the pathogenic mechanisms with the severely ill patients may help to identify therapeutic targets. Animal models will be helpful to understand viral-induced effects on various cells that are involved in triggering cardiovascular events associated with SARS-CoV-2 infection. Potential site and/or cell-specific gene deficient mouse models will help to understand the role of specific cellular responses to pulmonary viral infection on triggering cardiovascular diseases.

AUTHOR CONTRIBUTIONS

RG and MM wrote the manuscript. JA provided critical comments and revised the text. All authors contributed to the article and approved the submitted version.

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The IMMENSE Study: The Interplay Between iMMune and ENdothelial Cells in Mediating Cardiovascular Risk in Systemic Lupus Erythematosus

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Patients with systemic lupus erythematosus (SLE) have a significant increase in cardiovascular (CV) risk although they display a preserved number of circulating angiogenic $CD3^+CD31^+CXCR4^+$ T cells (T_{ang}), a subpopulation of T cells which promotes repair of damaged endothelium. This happens due to the concomitant expansion of a T_{ang} subset with immunosenescent features, such as the loss of CD28. Therefore, the aim of this study was to elucidate the interplay between T_{ang} subpopulations and endothelial cells in a group of young SLE patients without previous cardiovascular events. Twenty SLE female patients and 10 healthy controls (HCs) were recruited. Flow cytometric analysis of endothelial progenitor cells (EPCs) and T_{ang} subsets were performed and serum levels of interleukin (IL)-6, -8, matrix metalloproteinase (MMP)-9 and interferon (IFN)-γ were measured. Human umbilical vein endothelial cells (HUVECs) proliferation and pro-inflammatory phenotype in response to subjects' serum stimulation were also evaluated. Results showed that the percentage of T_{ang} and EPC subsets was reduced in SLE patients compared with HCs, with a marked increase of senescent $CD28^{null}$ cells among T_{ang} subset. SLE disease activity index-2000 (SLEDAI-2K) was inversed related to T_{ang} cells percentage. Furthermore, IL-8 serum levels were directly correlated with the percentage of T_{ang} and inversely related to the $CD28^{null}$ T_{ang} subsets. We indirectly evaluated the role of the T_{ang} subset on the endothelium upon stimulation with serum from subjects with a low percentage of T_{ang} $CD3^+$ cells in HUVECs. HUVECs displayed pro-inflammatory phenotype with up-regulation of mRNA for IL-6, intercellular adhesion molecule (ICAM)-1 and endothelial leukocyte adhesion molecule (ELAM)-1. Cell proliferation rate was directly related to IL-8 serum levels and EPC percentage. In highly selected young SLE patients without previous CV events, we found that the deterioration

of T_{ang} compartment is an early event in disease course, preceding the development of an overt cardiovascular disease and potentially mediated by SLE-specific mechanisms. The overcome of the $CD28^{null}$ subset exerts detrimental role over the T_{ang} phenotype, where T_{ang} could exert an anti-inflammatory effect on endothelial cells and might orchestrate *via* IL-8 the function of EPCs, ultimately modulating endothelial proliferation rate.

Keywords: angiogenic T cells, endothelial progenitor cells, immunosenescence, systemic lupus erythematosus, cardiovascular risk

INTRODUCTION

Systemic lupus erythematosus (SLE) is a polymorphic systemic autoimmune disease, burdened by a significant cardiovascular (CV) risk (1–3). The overall prevalence of vascular events ranges between 10 and 30%, with a 50-fold higher risk of myocardial infarction among young lupus women compared to age-matched controls (4). Patients also display a raised mortality due to vascular disease, and thrombotic events are the strongest predictors of death at five years from diagnosis (5). The increased CV burden manifests early in disease course, being largely attributable to endothelial activation and accelerated atherosclerosis (6). Indeed, patients with SLE have two-fold higher number of atherosclerotic plaques in the femoral arteries; at 5-year follow-up, 32% of SLE patients develop carotid atherosclerosis compared with 4% of controls (7, 8). Vascular damage is likely multifactorial, resulting from a complex interplay between traditional CV risk-factors and SLE-driven inflammation. Framingham risk-factors do not adequately account for cardiovascular disease (CVD) in lupus. Several SLE-associated items have been shown to contribute to the increased CV hazard such as disease activity and duration, renal involvement and steroid treatment (4). The presence of anti-phospholipid antibodies (aPL) represents an additional CV risk-factor in patients with SLE, as aPL not only trigger thrombotic events but also exert a direct role in the atherosclerotic process *via* the induction of endothelial activation (9). Given such important vascular morbidity and mortality, it is essential to investigate the mechanisms responsible for the increased CV burden in SLE.

Angiogenic T (T_{ang}) cells are a subset of T cells ($CD3^+CD31^+CXCR4^+$) that promotes vasculogenesis by orchestrating the function of endothelial progenitor cells (EPCs), and their characterization represents a promising field of research in CV medicine. Through the secretion of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), interleukin (IL)-8 and matrix metalloproteinase (MMP)-9, T_{ang} cells exert a critical role in the formation of EPCs colonies, the differentiation of early EPCs and the potentiation of the function of early EPCs (10).

The pro-angiogenic potential of T_{ang} cells has been confirmed in *in vivo* models and in clinical studies conducted in the general population: the levels of T_{ang} cells are inversely related with age and CV risk-factors and correlate with EPC colony numbers, playing a role as predictive factor of CV events when reduced (10). Scant data are available in SLE where a conserved number of T_{ang} cells compared to healthy controls (HCs) have been found (11). An explanation to such apparent paradox comes from the observation that in SLE patients there is a significant expansion of a subpopulation within T_{ang} subset which displays immunosenescent characteristics with the loss of the co-stimulatory molecule CD28, required for T cell activation, survival and proliferation. Differently from the $CD28^+$ counterpart, which likely represents the subgroup of protective T_{ang} cells, $CD28^{null}$ T_{ang} cells exert detrimental effects on the endothelium (11). In fact, they display a cytotoxic profile, documented by the expression of perforin, granzyme B, CD56, and the secretion of significant amount of interferon (IFN)-γ (11), as previously demonstrated for $CD4^+CD28^{null}$ T cells (12).

Therefore, the aim of the IMMENSE (Interplay between iMMune and ENdothelial cells in mediating cardiovascular risk in Systemic lupus Erythematosus) study was to characterize T_{ang} subpopulations, investigating the crosstalk of T_{ang} with endothelial cells in young lupus patients without previous CV events.

Abbreviations: aCL, anti-cardiolipin antibodies; ACR, American College of Rheumatology; aPL, anti-phospholipid antibodies; BMI, body mass index; cIMT, carotid intima-media thickness; CRP, C-reactive protein; CV, cardiovascular; CVD, cardiovascular disease; ELAM-1, endothelial cell leukocyte adhesion molecule 1; ENA, extractable nuclear antigen; EPCs, endothelial progenitor cells; ESR, erythrocyte sedimentation rate; FMO, fluorescence minus one; HCs, healthy controls; HDL, high-density lipoprotein; HSCs, hematopoietic stem cells; HUVECs, Human umbilical vein endothelial cells; ICAM-1, inter-cellular adhesion molecule 1; IFN-, interferon; IL-, interleukin; IMT, intima-media thickness; IQR, interquartile range; LA, lupus anti-coagulant; LDL, low-density lipoprotein; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SLEDAI-2K, systemic lupus erythematosus disease activity index 2000; T_{ang}, angiogenic T cell; TNF-, tumor necrosis factor; VEGFR2, vascular endothelial growth factor receptor 2; WBCs, white blood cells.

MATERIALS AND METHODS

Patients and Controls

From November 2017 to January 2019, a total of 20 patients aged less than 40 years and with a diagnosis of SLE according to the 1997 American College of Rheumatology (ACR) or the 2012 classification criteria for SLE (13, 14), attending the Rheumatology Unit of two tertiary referral centers for SLE, were recruited.

Exclusion criteria were any history of CVD including coronary heart disease (*i.e.* myocardial infarction, angina, coronary revascularization), cerebrovascular disease (*i.e.* stroke, transient ischemic attack), peripheral arterial disease, diabetes

and chronic kidney disease (creatinine clearance <60 ml/min). Patients were matched for sex and age with 10 healthy controls (HCs) with no history of manifestations suggestive for systemic autoimmune disease and negative autoantibody profile. The study was approved by the Ethics Committee of each participating center (approval numbers 170187 [University of Ferrara], 2793 [University of Brescia] and 2017_10_24_3 [Istituto Auxologico Italiano]), and all patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Demographic features, including age, gender and ethnicity, were recorded. Data on the following CV risk-factors were collected: arterial hypertension (systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg), dyslipidemia (blood total cholesterol, HDL-cholesterol and/or triglycerides outside normal limits), smoking (current or past), and obesity (body mass index >30). In order to investigate the presence of subclinical atherosclerosis, the carotid intima–media thickness (cIMT) was assessed by carotid ultrasound examination in the common carotid artery and the detection of focal plaques in the extracranial carotid tree. A commercially available scanner, (Mylab 70 Esaote, Genoa, Italy), equipped with 7–12 MHz linear transducer and the automated software guided technique radiofrequency "Quality Intima Media Thickness in real-time" (QIMT, Esaote, Maastricht, Netherlands) was used as elsewhere described in patients and controls (15). A cIMT greater than 0.9 mm was considered abnormal, and the presence of a plaque was identified by an IMT equal or greater than 1.5 mm, or by a focal increase in thickness of 0.5 mm or 50% of the surrounding cIMT value (16). Anti-hypertensive and lipid-lowering medications, anti-platelet or anti-coagulant agents were recorded in all patients and controls. Ongoing treatment with antimalarials, disease-modifying antirheumatic drugs or a combination of these agents was recorded together with the daily and the cumulative prednisone equivalent dose. In patients with SLE, the disease activity index-2000 (SLEDAI-2K) and the Systemic Lupus International Collaborating Clinics (SLICC) damage index (SDI) were calculated (17, 18).

Blood Samples

Peripheral venous blood samples, from each patient and control, were collected into BD Vacutainer 6 ml tube containing EDTA for flow cytometry analysis, 3 ml tube containing sodium citrate for lupus anti-coagulant (LA) testing, 3 ml of serum-separating tube for serological assays and *in vitro* experiments (all from BD Biosciences, Franklin Lakes, NJ, USA). Aliquots of serum samples were stored at −20°C until assaying.

Autoantibody Profile and Complement Dosage

All patients and controls were investigated for serum autoantibodies. Anti-nuclear antibodies (ANA) were tested in serum samples (5 µl) at indirect immunofluorescence on HEp-2 cells using the NOVA LiteTM ANA HEp-2 kit (Inova Diagnostics, San Diego, CA, USA) (positivity was defined at a titer ≥1:160) by manual reading with an epifluorescence microscope (Nikon Eclipse E400, Tokyo, Japan). Anti-dsDNA antibodies were detected by indirect immunofluorescence using Kallestad® Crithidia luciliae (Bio-Rad Laboratories, CA, USA) with a cut-off titer of 1:10. Antibodies anti-extractable nuclear antigen (ENA) were detected with ANA Screen 9 Kit (Euroimmun AG, Lübeck, Germany) by ELISA using 1420 Multilabel Counter Victor3TM (PerkinElmer, UK) (positivity was defined at a titer >10 U/ml). The presence of LA was performed according to international guidelines (19). Anti-cardiolipin (aCL) antibodies, antibodies against beta2 glycoprotein I (anti-β2GPI IgG/IgM/IgA) and against β2GPI domain 1 (anti-D1 IgG) were detected in serum (30 µl/test) by a chemiluminescent immunoassay exploiting the BIO-FLASH technology using QUANTA Flash assays (Inova Diagnostics) (20). The cut-off values for aCL and anti-β2GPI IgG/IgM/IgA and anti-D1 IgG positivity were set at 20 chemiluminescent units (CUs), as recommended by the manufacturer.

C3 and C4 were measured by nephelometry; hypo-complementemia was defined by local laboratory reference values (C3 < 90 and C4 < 11 mg/dl detected in at least two separate occasions). CRP and ESR were considered as increased when above the cut-off defined by local routine laboratory.

IL-6, MMP-9, IL-8, and IFN-γ Serum Levels

Serum levels of IL-6, IL-8, MMP-9, and IFN-γ were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The optical density (OD) values were evaluated at 450 nm using 1420 Multilabel Counter Victor3TM (PerkinElmer).

Flow Cytometric Analysis

Peripheral venous blood samples were collected as above described and White Blood Cells (WBCs) were isolated for the analysis of flow cytometry using homemade red blood cells lysis buffer. Briefly, 1:1 part of blood:PBS (5 ml of blood; 5 ml of PBS) was added to nine parts (90 ml) of the homemade NH_4Cl lysis solution (155 mM NH_4Cl, 9.98 mM TrisBase and pH 7.4) and incubated at 37°C for 10 min. After the lysis step, samples were spinned down at 560 g for 5 min and further washed in PBS at 560 g for 5 min. The isolated WBCs were resuspended in PBS at the concentration of 1×10^6 cells/ml. Next, the cells were stained as described below. Flow cytometric immunophenotyping was performed on WBC samples according to standard protocols with combinations of pre-titered fluorochrome-conjugated antibodies with FcR Blocking Reagent (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) at 4°C for 10 min as previously described (21, 22): CXCR4 PE (REA649), CD45 FITC (REA747), CD31 PerCP-Vio700 (REA730), CD3 FITC (REA613), CD4 PE-Vio770 (REA623), CD8 VioGreen (REA734), CD133 APC (AC133), CD34 PE (AC136), VEGFR2 PE-Vio770 (REA1116) (all from Miltenyi Biotech), and CD28 APC (28.2, BD Biosciences, Franklin Lakes, NJ, USA). In order to exclude dead cells, fixable viability dye eFluor™ 780 (eBioscience, San Deiego, CA, USA) was added to the staining mix. T_{ang} cells were defined by the expression of CD31 and CXCR4 among $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells. The analysis of the expression of CD28 was done among different subpopulations (**Figure 1**). EPCs were defined in the mononuclear

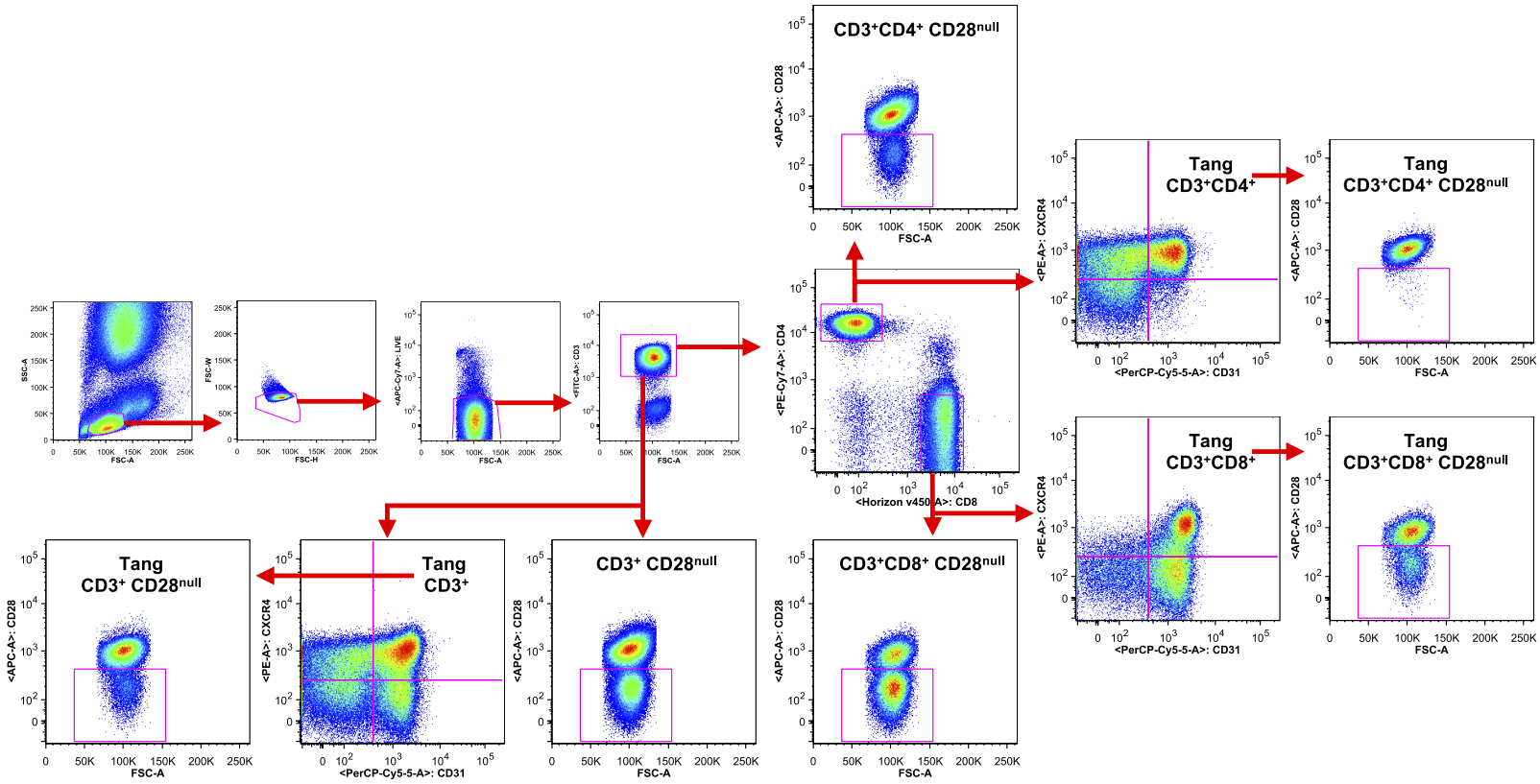

FIGURE 1 | Gating strategy used to characterize T cell subpopulations. WBCs isolated from one representative SLE patient were analyzed by flow cytometry. Representative gating strategy analysis is shown. The axis scales for fluorescence are reported as log; the axis scales for SSC, FSC are reported as linear.

cellular gate as $CD45dimCD34^+$ cells co-expressing CD133 (early EPCs $CD133^+$) or CD133/VEGFR2 (late EPCs $CD133^+VEGFR2^+$) as elsewhere described (10, 23–27). The panels with antibody and fluorochromes used for each staining are listed in **Supplemental Information**. In each T_{ang} acquisition at least 1.5×10^5 of lympho gate events were recorded and for EPC analysis at least 1×10^6 lympho-mono gate events were acquired. Quality control included regular check-up with BD™ Cytometer Setup & Tracking Beads (BD Biosciences). To automatically assess fluorescence compensation, MACS Comp Bead Kits (Miltenyi Biotec) as well as the antibodies used in the assay were utilized. In order to evaluate T_{ang} and very rare cells (EPCs) in peripheral blood we used Fluorescence Minus One (FMO) control procedure to evaluate non-specific fluorescence when defining positive events as previously described (28), since it does not contain the antibody in the detector of interest representing the best control for any given marker of interest in a multicolor staining combination. Representative FMO for the analysis of very rare EPC subpopulation is shown in **Supplemental Figure 1**. All data collection was performed on FACS ARIAII using BD FACS Diva software (all from BD Biosciences), and data analysis was performed using the FlowJo software 9.9.6 (Tree Star, Ashland, OR, USA).

Human Umbilical Vein Endothelial Cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from normal term umbilical cord vein by type II collagenase perfusion (Worthington, Lakewood, NJ, USA). HUVEC cultures were maintained in complete E-199 medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 20% heat inactivated FBS (PAA Laboratories-GE Healthcare, Toronto, Canada), 1% L-glutamine, 100 U/ml penicillin–streptomycin and 250 ng/ml Amphotericin B (all from MP Biomedicals, Santa Ana, CA, USA) at 37°C in CO_2 5%. Confluent cells were passaged with a 0.25% trypsin/EDTA (Gibco-ThermoFisher Scientific). HUVEC monolayers were incubated with sera from aPL-negative subjects at 1:2 dilution. In particular, subjects were stratified as follows: those with high percentage (>66th percentile) and the others with low percentage (<33th percentile) of T_{ang} $CD3^+$ cells.

IL-6, MMP-9, IFN-γ, ICAM-1, and ELAM-1 mRNA Expression Levels in HUVECs

The expression levels of IL-6, MMP-9, IFN-γ, inter-cellular adhesion molecule 1 (ICAM-1) and endothelial cell leukocyte adhesion molecule 1 (ELAM-1, alias E-Selectin encoded by the SELE gene) on HUVECs were evaluated by Real-Time PCR (RT-PCR) with Applied Biosystems 7500 Real-Time PCR System (ThermoFisher Scientific). HUVECs were resuspended in E-199 medium (ThermoFisher Scientific) containing 1% FBS added with 50% of serum from subjects and seeded in a 24-well plate at 5×10^3 cells/well (300 µl). Internal controls at final concentration of 50 U/ml of recombinant human IL-1β, 10 ng/ml of recombinant human TNF-α (all from R&D System) and 1 µg/ml of LPS (Sigma-Aldrich, St. Louis, MO, USA) were used as positive controls, while E-199 medium containing 1% FBS provided the negative control. After 24 h of incubation at 37°C in CO_2 5%, HUVECs were harvested and total RNA was purified using Trizol Reagent (ThermoFisher Scientific). Amplification Grade DNase I (ThermoFisher Scientific) was used to eliminate residual genomic DNA. A reverse transcription reaction was performed using SuperScript™ First-Strand Synthesis System for RT-PCR (ThermoFisher Scientific). The PCR conditions were the following: 94°C for 10 min, followed by 45 cycles of 95°C for 15 s, 60°C for 60 s and 72°C for 30 s. Quantitative RT-PCR was performed on 100 ng of cDNA using TaqMan™ Universal PCR Master Mix, no AmpErase™ UNG (ThermoFisher Scientific) by ABIPRISM 7900 HT Sequence Detection System (ThermoFisher Scientific). Quantification of mRNA expression was performed with TaqMan® Gene Expression Assay (ThermoFisher Scientific) for each target gene. Expression levels of target genes (*IL6*, *MMP9*, *IFNG*, *ICAM1* and *SELE*) were determined by the comparative Ct method normalizing the target to the endogenous gene (*GAPDH*). The following TaqMan® Gene Expression assays were used: Hs00174131_m1 (*IL6*); Hs00957562_m1 (*MMP9*); Hs00989291_m1 (*IFNG*); Hs00164932_m1 (*ICAM1*); Hs00174057_m1 (*SELE*) and Hs99999905_m1 (*GAPDH*). Relative values of target to reference ratio were expressed as fold change (RQ).

IL-6, MMP-9, IL-8, and IFN-γ Protein Expression Levels in HUVECs

The expression levels of IL-6, MMP-9, IL-8 and IFN-γ on HUVECs were evaluated by Western Blotting. HUVECs were resuspended in E-199 medium containing 1% FBS added with 50% of serum from subjects and seeded in a 24-well plate at 5×10^5 cells/well (300 µl). Internal controls at final concentration of 50 U/ml of recombinant human IL-1β, 10 ng/ml of recombinant human TNF-α (all from R&D System) and 1 µg/ml of LPS (Sigma-Aldrich) were used as positive controls, while E-199 medium containing 1% FBS provided the negative control. After 24 h of incubation at 37°C in CO_2 5%, HUVECs were harvested and lysed using RIPA lysis buffer added with Protease and Phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentration was evaluated using BCA Protein Assay Kit (ThermoFisher Scientific). Equal amounts of proteins (10 µg/lane) were migrated in NuPAGE BIS-TRIS by 4–12% SDS-polyacrylamide pre-cast gel electrophoresis in MOPS buffer 1× for 50 min at 200 V and transferred to nitrocellulose for 7 min using iBlot Transfer Stacks Nitrocellulose and iBlot® Gel Transfer Device (ThermoFisher Scientific). Membranes were blocked for 1 h at room temperature in PBS/0.1% Tween 20 (P/T) (Bio-Rad Laboratories) containing 5% non-fat milk powder (Mellin, Milan, Italy), and incubated overnight at 4°C with 1:1,000 of anti-human IL-6 (D3K2N), anti-human MMP-9 (D6O3H), anti-human IFN-γ (3F1E3) (all from Cell Signaling Technology, Danvers, MA, USA), anti-human IL-8 (6217, R&D Systems) and 1:2,000 of anti-human α-tubulin (B-5-1-2, Sigma-Aldrich). After washes, membranes were incubated in PT/5% non-fat milk powder plus anti-mouse or anti-rabbit Ig-G HRP-conjugated secondary antibodies (MP Biomedicals, Santa Ana, CA, USA) for 1 h at RT and revealed using ECL Plus Detection System (ThermoFisher Scientific). Signals

were detected using radiographic films (Kodak, Rochester, NY, USA). Image J software (LI-COR Biosciences, Lincoln, NE, USA) was used to analyze and quantify densitometry values. Protein expression levels were normalized to the housekeeping gene, α-tubulin, and expressed as relative protein levels.

HUVECs Proliferation Assay

The effect of stimulation with sera on HUVEC proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma-Aldrich) (29–31). HUVECs were resuspended in E-199 complete medium and incubated in a 96-well plate, 5×10^3 cells/well for 24 h at 37°C in CO_2 5%. Then, the complete medium was removed, and the cells were cultured in E-199 medium with 1% FBS overnight at 37°C in CO_2 5%. Afterwards, the medium was removed, and the cells were stimulated with 100 µl of E-199 medium containing 1% FBS added with 10% of serum from subjects. Each treatment was performed in triplicate. After 24 and 72 h of incubation at 37°C in CO_2 5%, 10 µl of 10 mg/ml of MTT (final concentration 0.5 mg/ml) was added to each well and incubated at 37°C in CO_2 5% for 4 h. The formed formazan crystals were dissolved in dimethyl sulfoxide (150 µl/well) (Sigma-Aldrich) and the absorbance was read at 570 nm using a microplate scanning spectrophotometer (ELISA reader, OrganonTeknika, Netherlands). The percentage of proliferating cells was evaluated as follows: 100 × (absorbance of considered sample)/(absorbance of control). The experiments were performed on sub-confluent cell cultures in order to prevent contact inhibition, which can condition the results.

Statistical Analysis

Statistics were calculated with GraphPad Prism 6 software. All data in box plot are presented as Tukey's box plot where the higher whisker represent 75th percentile plus 1.5 times interquartile range (IQR) and the lower whiskers represent 25th percentile minus 1.5 times IQR. The Shapiro–Wilk test was used to evaluate the Gaussian distribution of overall data. Statistical comparisons between the different groups of subjects were calculated with non-parametric analyses (Mann–Whitney non-parametric U-test or Kruskal–Wallis test, when appropriated) when no Gaussian distribution was found and exact p values were obtained, otherwise T-students' test was used. Correlation among variables was evaluated using the Spearman's rank correlation coefficient or Pearson's correlation coefficient according to the data's Gaussian distribution. p values <0.05 were considered significant.

RESULTS

Clinical Characteristics

Demographic data, clinical characteristics and pharmacological treatments of the study subjects are reported in **Table 1**. The autoantibody profiles of SLE patients and HCs are detailed in **Table 2**. The rate of positivity in non-criteria aPL test was very low and positivity for non-criteria aPL was never isolated.

TABLE 1 | Demographic, clinical characteristics, and pharmacological treatments of the study subjects.

	SLE	HCs	p
Number of subjects	20	10	
F, n (%)	20 (100)	10 (100)	1.00
Age, mean (SD)	33 (5)	29.8 (3.8)	0.08
Caucasian, n (%)	17 (85)	10 (100)	0.53
CV risks factors			
BMI, mean (SD)	25.1 (4.5)	22.3 (1.7)	0.07
Obesity (BMI > 30), n (%)	3 (15)	0 (0)	0.53
Smoking (ongoing), n (%)	6 (30)	3 (30)	1.00
Smoking (past), n (%)	4 (20)	0 (0)	0.27
Hypertension, n (%)	1 (5)	0 (0)	1.00
Dyslipidemia, n (%)	2 (10)	0 (0)	0.54
Subclinical atherosclerosis (IMT > 0.9) or plaques, n (%)	0 (0)	0 (0)	1.00
Clinical and serological characteristics			
Disease duration, months (SD)	109 (56)	–	
SLEDAI-2K, mean (SD)	3.4 (2.6)	–	
SLEDAI-2K, range	0–10	–	
SLICC-SDI, mean (SD)	0.3 (0.4)	–	
Cutaneous involvement, n (%)	14 (70)	–	
Mucosal involvement, n (%)	5 (25)	–	
Articular involvement, n (%)	15 (75)	–	
Serositic involvement, n (%)	7 (35)	–	
Renal involvement, n (%)	6 (30)	–	
Neurological involvement, n (%)	2 (10)	–	
Hematological involvement, n (%)	13 (65)	–	
C3 mg/dl, mean (SD)	87.8 (21.8)	–	
C4 mg/dl, mean (SD)	16.6 (10)	–	
CRP mg/dl, mean (SD)	0.56 (1.04)	–	
ESR mm, mean (SD)	11 (0.3)	–	
Ongoing treatment			
Low dose aspirin, n (%)	10 (50)	–	
Oral anti-coagulant, n (%)	2 (10)	–	
Lipid-lowering drugs, n (%)	1 (5)	–	
Anti-hypertensive drugs, n (%)*	3 (15)	–	
Anti-malarial drugs, n (%)	19 (95)	–	
Steroids, n (%)	16 (80)	–	
Steroids, daily dosage (mg), mean (SD)	5 (4.2)	–	
Steroids, cumulative dosage (g), mean (SD)	15.6 (12.2)	–	
Disease modifying antirheumatic drugs, n (%)§	15/20 (75)	–	

**angiotensin-converting enzyme inhibitors in three patients for renal involvement, combined with one beta-blockers in one case; § azathioprine, cyclosporin, methotrexate, cyclophosphamide, mycophenolate or belimumab. p values < 0.05 were considered significant.*

BMI, body max index; CRP, C-reactive protein; CV, cardiovascular; ESR, erythrocyte sedimentation rate; HCs, healthy controls; IMT, Intima Media Thickness; SD, standard deviation; SLEDAI-2K, SLE disease activity index-2000; SLICC-SDI, Systemic Lupus International Collaborating Clinics damage index; SLE, systemic lupus erythematosus.

Angiogenic T Cells and Peripheral Endothelial Progenitor Cells Are Decreased in SLE Patients

First, we characterized WBCs for the expression of T_{ang} and EPC subpopulations in our cohort. Phenotypic characterization of WBCs showed that the percentage of T_{ang} $CD3^+CD4^+$ subpopulation was reduced in SLE patients as compared to HCs (p = 0.04). Similar results were observed for $CD3^+$ (p = 0.07) and $CD3^+CD8^+$ (p = 0.12) T_{ang} cells (**Figure 2A**). SLE patients showed a significantly lower percentage of EPCs $CD133^+$ (p = 0.027) and particularly of EPCs $CD133^+VEGFR2^+$

TABLE 2 | Autoantibody profiles of patients and controls.

	SLE	HCs
ANA, n (%)	20 (100)	1 (10)
Anti-extractable nuclear antigen positivity, n (%)	9 (45)	0 (0)
Anti-double stranded DNA, n (%)	13 (65)	0 (0)
LA, n (%)	8 (40)	0 (0)
aCL IgG, n (%)	7 (35)	1 (10)
aCL IgM, n (%)	3 (15)	0 (0)
aCL IgA, n (%)	2 (10)	0 (0)
anti-β2GPI IgG, n (%)	6 (30)	0 (0)
anti-β2GPI IgM, n (%)	1 (5)	0 (0)
anti-β2GPI IgA, n (%)	1 (5)	0 (0)
anti-β2GPI D1, n (%)	5 (25)	0 (0)

aCL, anti-cardiolipin antibodies; anti-β2GPI, anti-β2 glycoprotein I antibodies; D1, domain 1; HCs, healthy controls, ANA, anti-nuclear antibodies; LA, lupus anti-coagulant; SLE, systemic lupus erythematosus.

(p = 0.012) when compared to HCs (**Figure 2B**) (**Supplemental Table 1**).

Since aPL antibodies are additional CV risk-factors, we investigated the percentage of T_{ang} and EPCs among SLE patients positive for aPL; however, our analysis showed that there was no significant difference in the percentage of circulating T_{ang} and EPC subpopulations (data not shown).

Furthermore, correlations between the percentage of T_{ang} and EPC subpopulations were not found among patients or HCs.

Angiogenic $CD28^{null}$ T Cells Are Increased in SLE Patients

Analyzing the senescent profile of T cells in relation to the expression of CD28 receptor, an increased percentage of $CD28^{null}$ cells was observed in SLE compared to HCs (p = 0.002) in $CD3^+$ peripheral blood T cells. This increase was mainly evident in T $CD3^+CD8^+$ subpopulation (p = 0.006), although was also present in the T $CD3^+CD4^+$ subset (p = 0.020) (**Figure 3A**).

Moreover, the percentage of senescent $CD28^{null}$ cells within the T_{ang} $CD3^+$ subset was increased in SLE patients (p = 0.019) when compared to HCs. In particular, among T_{ang} $CD3^+$ cells, the down regulation of CD28 expression was mainly evident among T_{ang} $CD8^+$ cells (p = 0.04) (**Figure 3B**, **Supplemental Table 2**) and among T_{ang} $CD4^+CD8^+$ cells (p = 0.01) (**Supplemental Figure 2B**).

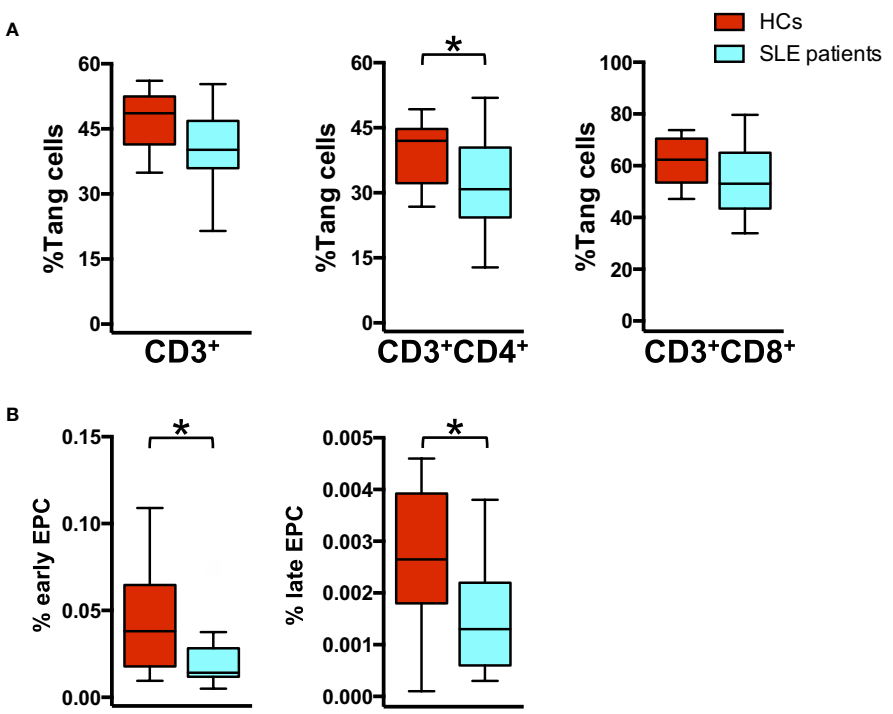

FIGURE 2 | Impaired percentage of endothelial progenitor and T_{ang} cells in SLE patients. WBCs isolated from HC and SLE patients were analyzed by flow cytometry for the identification of T cell lineage, T_{ang} and EPC subpopulation. **(A)** The percentage of T_{ang} cells for each T cell subpopulation is represented as Tukey's box plot. SLE patients show reduced percentage of T_{ang} cell subpopulations than HCs. **(B)** Differences in the early $CD133^+$ and late $CD133^+VEGFR2^+$ EPC percentage among WBCs from HCs and SLE patients are represented as Tukey's box plot. SLE patients show reduced percentage of EPC subpopulation than HCs. The y axis scale is reported as linear. Statistical analysis of the differences was performed by Mann–Whitney test. p values <0.05 were considered significant: *p < 0.05.

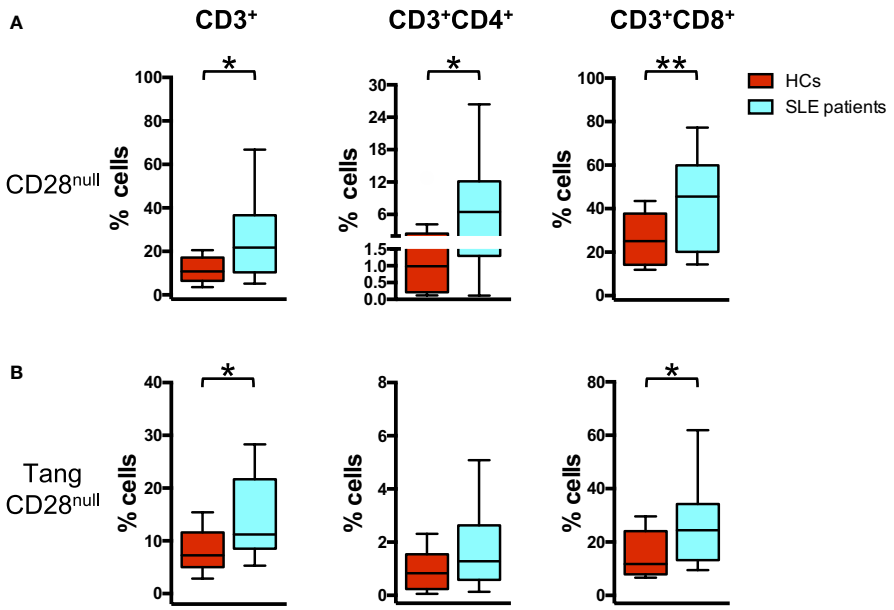

FIGURE 3 | Senescent angiogenic T cells characterize SLE patients. WBCs isolated from HC and SLE patients were analyzed for the differences in the percentage of $CD28^{null}$ cells in T_{ang} and parent T cell subpopulations using flow cytometry. **(A)** The percentages of $CD28^{null}$ cells for each T cell subpopulation are represented as Tukey's box plot. SLE patients show higher percentage of $CD28_{null}$ cell subpopulations than HCs. **(B)** The percentages of $CD28^{null}$ cells within each T_{ang} cell subpopulation are represented as Tukey's box plot. SLE patients show higher percentage of $CD28^{null}$ T_{ang} cell subpopulations than HCs. The y axis scale is reported as linear. Statistical analysis of the differences was performed by Mann–Whitney test. p values <0.05 were considered significant: *$p < 0.05$, **$p < 0.01$.

Circulating Levels of Peripheral Angiogenic T Cells and Endothelial Progenitor Cells Decrease According to the Disease Activity

Analyzing the variation of the percentage of T_{ang} and EPCs subsets in relation to clinical findings, we found that the percentages of T_{ang} $CD3^+CD4^+$ cells were inversely related to SLEDAI-2K (**Figure 4A**). The same could be seen for EPCs in relation to inflammatory markers, where the percentages of early EPCs $CD133^+$ cells were inversely associated to ESR and CRP. We also observed a positive correlation comparing the percentage of late EPCs $CD133^+VEGFR2^+$ subset with ESR (**Figure 4B**). No correlations were found between T_{ang} percentage and ESR or CRP.

It is well established that the lipid profile influences the CV risk, but is unknown if it could be related with the $CD28^{null}$ senescent status of T_{ang} cells in SLE patients. Analyzing the T_{ang} senescent status in relation to lipid profile, a direct correlation between the serum level of total cholesterol and percentage of $CD28^{null}$ T_{ang} $CD3^+$ and $CD28^{null}$ T $CD3^+CD4^+$ cells was observed. Similarly, LDL cholesterol serum levels were positively correlated with the percentage of senescent $CD28^{null}$ in T_{ang} $CD3^+$ or $CD4^+$ cells (**Figures 4C, D**) (**Supplemental Table 3**).

No correlations were found between cell percentage numbers and BMI values (data not shown).

The Percentage of Circulating T_{ang} Cells Directly Correlate With Serum Levels of IL-8

IL-8 and MMP-9 modulate endothelial homeostasis. Therefore, we analyzed if the T_{ang} subpopulation and the senescent $CD28^{null}$ subset could be associated with serum levels of these factors. As above detailed, SLE patients and HCs were stratified by the tertile of the percentage of circulating T_{ang} $CD3^+$ cells (low, $<33^{th}$ percentile; high, $>66^{th}$ percentile). Individuals with high T_{ang} $CD3^+$ cells percentage displayed higher levels of IL-8 (**Figure 5A** left panel). Accordingly, IL-8 serum levels significantly correlated with the percentage of circulating T_{ang} $CD3^+$ cells in the whole cohort (**Figure 5A**, right panel). In addition, serum levels of IL-8 directly correlated with the percentage of T_{ang} $CD3^+CD4^+$ cells while, as expected, were overall inversely related to the $CD28^{null}$ T_{ang} subpopulations ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$) (**Figure 5B**). SLE patients and HCs with high percentage of T_{ang} $CD3^+$ cells presented serum levels of IL-6, MMP-9 and IFN-γ similar to those with lower percentage of T_{ang} $CD3^+$ cells (data not shown). However, a trend towards statistical significance emerged when MMP-9 serum levels were correlated to T_{ang} $CD3^+$ cells (**Figure 5C**, left panel). Furthermore, MMP-9 levels were directly related to the percentage of the pluripotent bone marrow progenitor $CD34^+$ (**Figure 5C**, right panel) (**Supplemental Table 4**). Details of T_{ang} subsets in SLE patients and HCs stratified by the tertile of the percentage of circulating T_{ang} $CD3^+$ cells are shown in **Supplemental Table 5**.

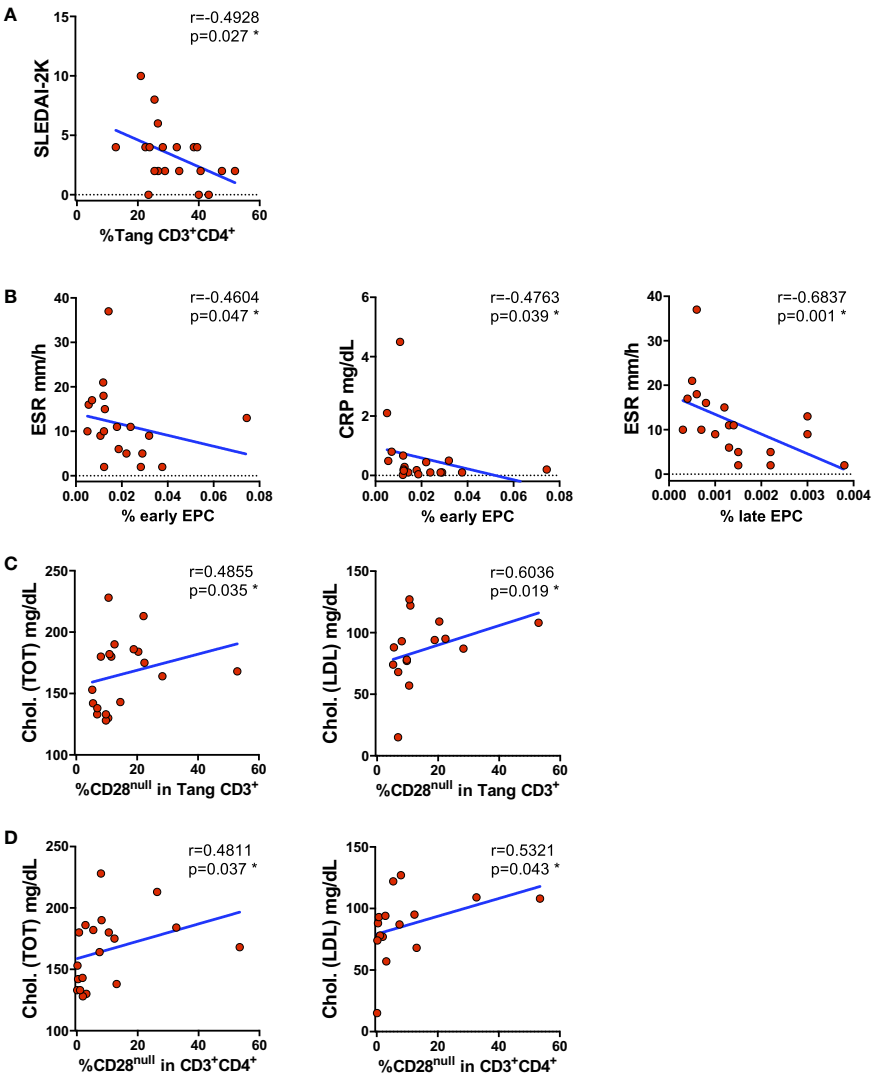

FIGURE 4 | Impaired percentage of circulating EPCs and T_{ang} is disease activity related. WBCs isolated from SLE patients were analyzed for T_{ang} and senescent $CD28^{null}$ T_{ang},; EPC subsets and the percentages of cells were correlated with clinical findings. **(A)** Correlation between the percentages of T_{ang} $CD3^+CD4^+$ cells and SLEDAI-2K is shown. The percentage of T_{ang} $CD3^+CD4^+$ cells inversely correlates with SLEDAI-2K. **(B)** Correlation between the percentages of EPCs depicted as early $CD133^+$ or late $CD133^+VEGFR2^+$ cells and systemic inflammatory markers is shown. The percentage of EPCs inversely correlates with the serum levels of systemic inflammatory markers CRP and ESR. **(C)** Correlation between the percentages of $CD28^{null}$ within T_{ang} $CD3^+$ cells and total or LDL cholesterol is shown. The percentages of the senescent$CD28^{null}$ T_{ang} cells directly correlate with cholesterol serum levels. **(D)** Correlation between the percentage of $CD28^{null}$ cells within $CD3^+CD4^+$ subpopulation and total or LDL cholesterol is shown. The percentages of senescent$CD28^{null}$ T_{ang} $CD3^+CD4^+$ cells directly correlate with cholesterol serum levels. The axis scales are reported as linear. Correlations are expressed as Spearman r values, p values <0.05 were considered significant: *p < 0.05.

HCs displayed significantly higher serum levels of IL-6, IL-8 and MMP-9 compared to SLE patients, whereas serum IFN-γ was similar between the two groups of subjects (**Supplemental Table 6**).

A Pro-Inflammatory Phenotype in HUVECs Emerges Upon Stimulation With Sera From Subjects With Low Percentage of T_{ang} Cells

To explore the interplay of T_{ang} cells with the endothelium, we analyzed the response of HUVECs to the stimulation with serum from subjects (patients and HCs) stratified according to their percentage of circulating T_{ang} $CD3^+$ cells as above described. HUVECs treated with sera from subjects with a low percentage of T_{ang} $CD3^+$ cells displayed higher mRNA as well as protein expression levels of IL-6 than cells treated with sera from subjects with higher percentage of T_{ang} $CD3^+$ cells (p = 0.02 and p = 0.04, respectively) (**Figure 6A**). Similarly, treatment with sera from subjects with a low percentage of T_{ang} $CD3^+$ cells resulted in higher mRNA expression levels of both ICAM-1 and ELAM-1 compared to HUVECs treated with sera from subjects with higher percentage of T_{ang} $CD3^+$ cells (**Figure 6B**). Although we did not detect differences in IL-8, MMP-9 and IFN-γ expression levels when HUVECs were stimulated with sera from subjects

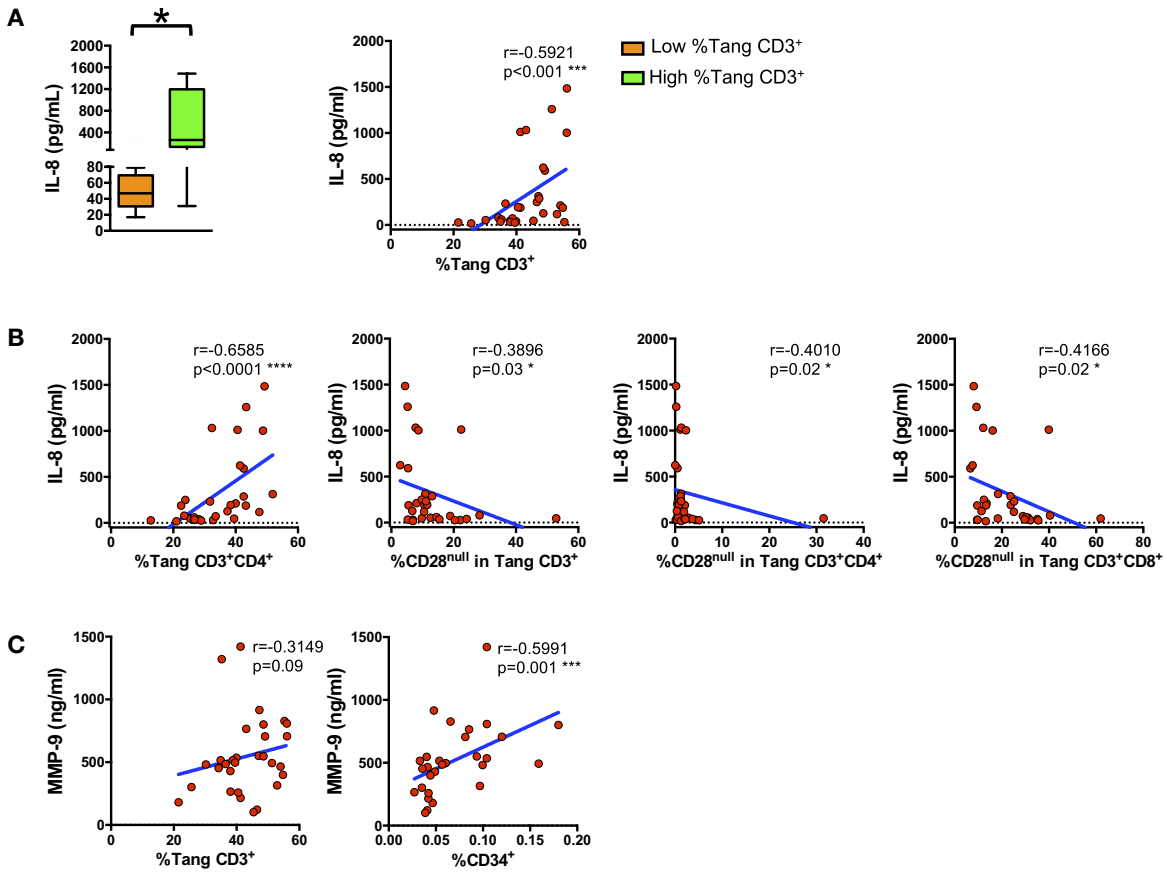

FIGURE 5 | Circulating proangiogenic factors reflect angiogenic circulating cells related compartments. WBCs isolated from subjects were analyzed for T cell lineage. T_{ang} and EPC markers and the percentages of cells were correlated with IL-8 and MMP-9 serum levels. **(A)** The differences between the serum IL-8 levels of subjects with low percentage of circulating T_{ang} $CD3^+$ cells and of subjects with high percentage of circulating T_{ang} $CD3^+$ cells are represented as Tukey's box plot (left panel). Right panel shows the correlation between the percentages of T_{ang} $CD3^+$ cells and IL-8 serum level in the whole cohort. IL-8 serum levels directly correlate with the percentages of T_{ang} $CD3^+$ cells. **(B)** The correlation between serum levels of IL-8 and the percentage of T_{ang} $CD3^+CD4^+$ and of $CD28^{null}$ cells within each T_{ang} cell subpopulation in both HCs and SLE patients are shown. IL-8 serum levels inversely correlate with the percentage of senescent $CD28^{null}$ T_{ang} cells. **(C)** The correlation between MMP-9 and the percentage of circulating $CD34^+$ or T_{ang} $CD3^+$ cells for the whole cohort are shown. MMP-9 serum levels directly correlate with the percentage of $CD34^+$ or T_{ang} cells. The axis scales are reported as linear. Statistical analysis of the differences was performed by Mann–Whitney test. Correlations are expressed as Spearman r values, and significance levels are indicated. p values <0.05 were considered significant: *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

with low or high percentage of T_{ang} $CD3^+$ cells, suggestive data emerged for MMP-9. Indeed, protein expression levels were positively related to the percentage of T_{ang} $CD3^+$ cells approaching statistical significance and were inversely correlated to the percentage of $CD28^{null}$ T_{ang} subpopulation (**Figure 6C**) (**Supplemental Table 7**).

Upon treatment with serum samples from SLE patients and HCs, HUVECs presented similar mRNA and protein expression levels of study mediators (**Supplemental Tables 8** and **9**).

HUVECs Proliferation Positively Correlates With IL-8 Serum Levels and With Endothelial Progenitor Cells

To investigate further the protective role of T_{ang} over endothelial cells we analyzed the proliferation of HUVECs in response to serum stimulation by MTT assay. Although no differences in HUVEC proliferation upon stimulation with serum from subjects with low or high percentage of T_{ang} $CD3^+$ cells were observed [median (IQR) 57% (34–82) and 67% (62–84), respectively] at 24 h, at 72 h relevant correlation were found. Indeed, as shown in **Figure 7**, endothelial cell proliferation correlated positively with IL-8 serum levels and early EPCs $CD133^+$ subset at 72 h (**Supplemental Table 10**).

Treatment with sera from SLE patients and HCs resulted in a similar modulation of the proliferation rate of HUVECs (**Supplemental Table 11**).

DISCUSSION

The IMMENSE study provides novel insights into the evaluation of CV subclinical alterations in SLE by unveiling some potential

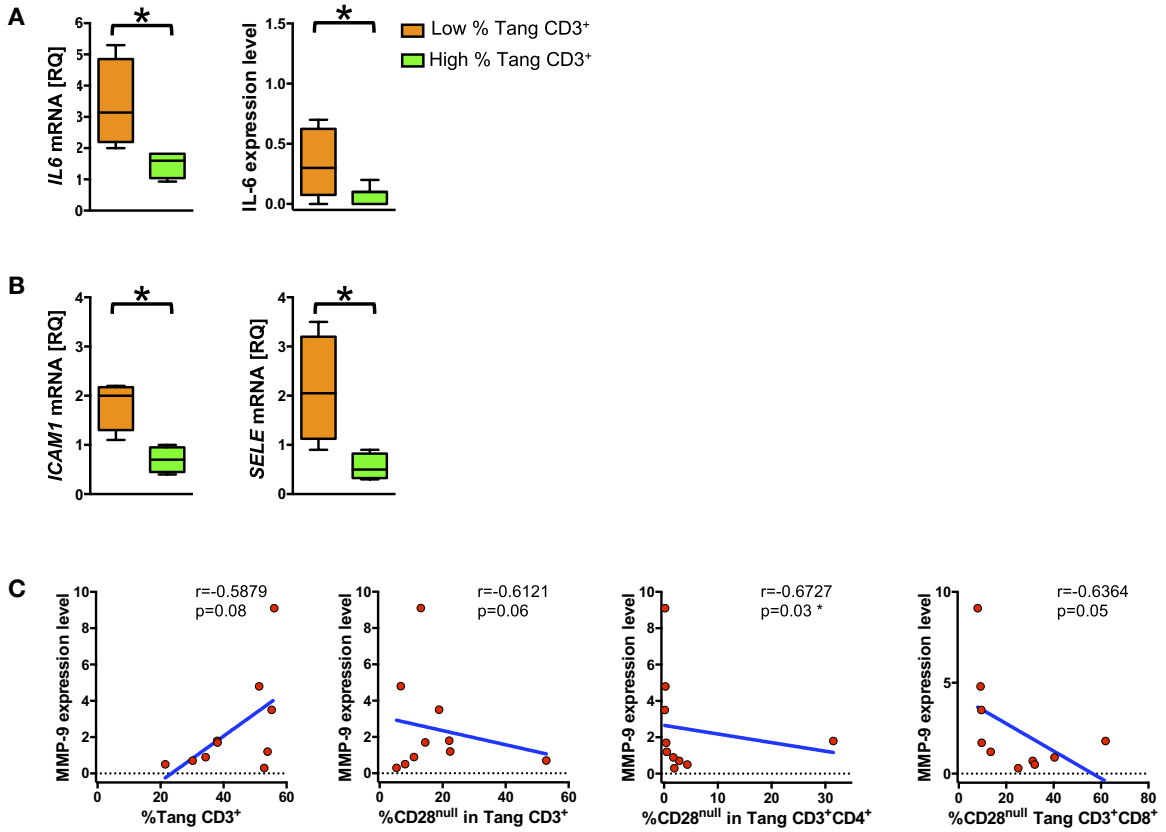

FIGURE 6 | Low percentage of circulating T_{ang} cells correlates with pro-inflammatory endothelial phenotype. HUVECs were treated for 24 h with serum from subjects (SLE patients and controls) with high or low percentage of T_{ang} $CD3^+$ and then analyzed for mRNA and protein expression level. **(A)** The differences between of mRNA and protein expression level of *IL6* from HUVECs treated with serum of subject with low percentage of circulating T_{ang} $CD3^+$ cells and HUVECs treated with serum of subjects with high percentage of circulating T_{ang} $CD3^+$ cells are shown. Data are represented as Tukey's box plot. HUVECs stimulated with serum from subjects with low percentage of T_{ang} $CD3^+$ cells show higher IL-6 expression. **(B)** The differences of ICAM1 (left panel) and *SELE* (right panel) mRNA expression between HUVECs treated with serum of subject with low percentage of circulating T_{ang} $CD3^+$ cells and HUVECs treated with serum of subject with high percentage of circulating T_{ang} $CD3^+$ cells are represented as Tukey's box plot. HUVECs stimulated with serum from subjects with low percentage of T_{ang} $CD3^+$ cells show higher adhesion molecules expression. **(C)** $HUVEC_S$ were stimulated for 24 h with serum from subjects with high or low percentage of T_{ang} $CD3^+$, and the MMP-9 expression levels were correlated with the percentage of circulating T_{ang} and $CD28^{null}$ T_{ang} cells. The expression of MMP-9 is related with the percentage of T_{ang} cells and declines according to the percentage of circulating $CD28^{null}$ T_{ang} cells. The axis scales are reported as linear. Relative Quantification (RQ) expresses fold of change of target to reference. Statistical analysis of the differences was performed by Mann–Whitney test. Correlations are expressed as Spearman r values, and significance levels are indicated. p values <0.05 were considered significant: *p < 0.05.

aspects of the complex interplay between T_{ang}, EPCs, and endothelial cells. The focus of the study was on circulating T_{ang} cells, which were recently demonstrated to be a potentially useful biomarker reflecting vascular alterations in CV and autoimmune diseases (10, 11, 32, 33).

In our highly selected young SLE patients without previous CV events and a low rate of traditional CV risk-factors, we found a mild decrease of T_{ang} cells in patients in comparison with HCs, confirming our previous observation (34). Notably, we detected for the first time that T_{ang} reduction in SLE was particularly evident among $CD4^+$ subpopulation and inversely related to disease activity as evaluated by SLEDAI-2K. Conversely, the only two other available studies reported a similar percentage of T_{ang} cells between SLE patients and HCs (11, 35), pointing out a decrease of $CD28^+$ T_{ang} cells in SLE patients with CVD. These results suggested that the CD28 expression should be used to redefine the pro-angiogenic T_{ang} cells (12). A reduced proportion of T_{ang} cells has been reported also in patients with rheumatoid arthritis (RA), especially in those who had experienced CV events (36).

Given the relevance of the immunosenescent phenotype of T_{ang} cells in relation to CV burden, we then focused on $CD28^{null}$ T_{ang} subsets, reporting an increase in $CD28^{null}$ T_{ang} among $CD3^+$, $CD4^+$ and $CD8^+$ subpopulations in SLE patients. Our results agree with the studies by Lopez and coworkers, who were the first to describe an increase of this cell subpopulation among lupus subjects (11, 37). Noteworthy, they observed that the increase of $CD28^{null}$ T_{ang} cells was most relevant among patients with CVD, independently from age, gender, disease duration, disease activity, comorbidities, and use of drugs. Interestingly, $CD28^{null}$ T_{ang} cells appeared to be related to high circulating levels of pro-inflammatory mediators (TNF-α, IFN-

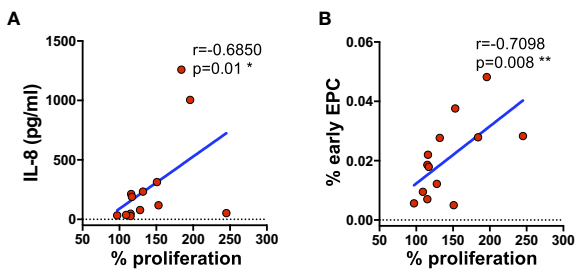

FIGURE 7 | HUVEC cell proliferation directly correlates with the serum concentration of IL-8 and the percentage of circulating subpopulation. HUVEC proliferation was evaluated by MTT after 72 h of incubation with serum from subjects (SLE patients and controls) with high and low percentage of T_{ang} $CD3^+$. Correlation between the percentage of proliferating cells and IL-8 serum levels **(A)** or the percentage of EPCs $CD133^+$ subpopulation **(B)** is shown. Correlations are expressed as Spearman r values, and significance levels are indicated. p values <0.05 were considered significant: *p < 0.05, **p < 0.01.

α) and to the positivity for anti-dsDNA and anti-Ro autoantibodies. Overall these data confirmed the evidence raised in the general population on a close relation between $CD28^{null}$ T cells percentage and CV events: circulating levels of $CD4^+CD28^{null}$ T cells were expanded in a cohort of patients with unstable angina, provided a strong and independent predictor of mortality in patients with heart failure and were found as tissue-infiltrating T cells in unstable atherosclerotic plaques (37–40). The evidence of the detrimental role of this cell subset on CV system has been confirmed even in autoimmune diseases other than SLE. Some authors found an increased number of $CD4^+CD28^{null}$ T cells as a possible distinctive feature of RA patients with high CV risk, measured by the cIMT and by the brachial artery flow mediated vasodilatation (41).

The data on the decrease of T_{ang} and the raise of $CD28^{null}$ T_{ang} cells highlighted in the IMMENSE study are particularly relevant given the highly selected composition of our study group, which included exclusively young patients without previous CV events and a low rate of conventional CV risk-factors. Indeed, our observation suggested that the deterioration of the T_{ang} compartment is an early event in disease course, preceding the development of an overt CVD. The inverse correlation of T_{ang} cells with disease activity suggests that SLE-specific mechanisms could mediate the deterioration of this T cell subset. This should not be surprising, as immunosenescence might be driven not only by aging but also by repeated antigen stimulations as happens in systemic autoimmune diseases such as SLE (42). Besides the loss of CD28, several additional processes occur, influencing the number and function of circulating immune cells, such as telomere attrition and DNA damage (43).

We could not observe any significant correlation between $CD4^+CD28^{null}$ T cells and disease specific markers, including criteria or non-criteria aPL. However, $CD28^{null}$ T_{ang} $CD3^+$, as well as $CD28^{null}$ T_{ang} $CD3^+CD4^+$ cells, were directly correlated with total and LDL cholesterol serum levels. Interestingly, this clinical correlation is hereby described for the first time in SLE patients, further reinforcing the importance of the T_{ang} loss of CD28 in relation to CV risk. In 2015, a negative correlation between total cholesterol serum levels and T_{ang} $CD3^+$ cells had been found in a cohort of healthy subjects, but not confirmed among RA patients (36). The differences between healthy subjects and patients might be ascribed to the striking prevalence of dyslipidemia in RA subjects enrolled in that study, which was as high as 36% (36).

Moreover, our study shed light on the interplay between T_{ang} and endothelial cells. Hence, T_{ang} cell percentages were found to directly correlate with serum levels of IL-8 and MMP-9, two well-characterized pro-angiogenic mediators (44). As expected, the $CD28^{null}$ counterpart of T_{ang} cells was negatively correlated with IL-8 serum levels, reinforcing the potential involvement of this inflammatory subset in mediating endothelial dysfunction (11, 36–39). *In vitro* experiments were conducted using sera from subjects stratified upon the percentage of $CD3^+$ T_{ang} cells: conclusions on the effects of $CD3^+$ T_{ang} cells on endothelial cell phenotype and proliferation could thus be derived only indirectly. The evidence that sera from subjects with a low percentage of T_{ang} cells had detrimental effects on HUVEC proliferation and phenotype, differently from sera of those with high T_{ang} cell number, is in agreement with the notion that T_{ang} cells exert a proangiogenic potential. Observed results might have been biased by additional cellular or soluble mediators: indeed, surely T_{ang} do not provide the only determinant of serum cytokine levels and several additional cell types contribute to circulating cytokines such as NK, monocytes and dendritic cells (45, 46).

In our study, we found a lower percentage of EPCs in SLE patients in comparison with HCs, in agreement with the majority of studies on lupus subjects, possibly due to increased apoptosis. Indeed, $CD34^+AnnexinV^+$ circulating cells were expanded in a cohort of SLE patients in clinical remission, compared with controls (47). Furthermore, one study reported also increased apoptosis of hematopoietic stem cells (HSCs) and decreased $CD34^+$ HSCs in the bone marrow of patients with active SLE, which could affect the $CD34^+VEGFR2^+$ EPCs (48). Decreased percentage of EPCs was described in SLE patients without any apparent clinical correlation, supporting the hypothesis of chronically decreased levels throughout the disease (49). In our patients, all with low activity and damage indexes, no correlation was found between EPCs percentage and clinical features. Recent studies in SLE patients showed also an impairment in EPCs' function with a decreased ability to produce VEGF, to migrate and to proliferate (50).

The limitations of the study consist in the low number of enrolled patients and, in particular, the low number of aPL positive patients which prevented further analysis. The low disease activity of enrolled patients might account for the unexpectedly lower levels of serum cytokines that we observed in SLE subjects compared to HCs and might possibly have impinged the accuracy of statistical analysis when evaluating the association between cell subsets and SLEDAI-2K.

However, the strength of the IMMENSE study is related to the enrollment of young female SLE patients and matched HCs, with

a low rate of modifiable CV risk-factors, in order to overcome possible confounders in the analysis of CV parameters. Furthermore, enrolled patients had a clinically inactive–serologically active SLE being the best candidates to evaluate, since disease was under control with first-line therapies, minimizing the possible interference of therapies with the number of circulating cells.

CONCLUSIONS

As a whole, the IMMENSE study supports the hypothesis that T_{ang} subpopulation potentially exerts a key role in mediating CV risk among SLE patients, confirming the heterogeneous nature of these cells (51). This work further unveils the complex interplay between T_{ang} and EPC subsets, even though we acknowledge that future studies exploiting more sophisticated experimental approaches are needed to gain mechanistic insights into the crosstalk between T_{ang} and endothelial cells.

Most importantly, we observed that the percentage of circulating pro-angiogenic T_{ang} decrements very early in disease course, with an increase in the rate of senescent inflammatory $CD28^{null}$ subset. These modulations of T_{ang} cell percentage might account for detrimental effects on the endothelium. Our preliminary and descriptive data suggest that T_{ang} might exert their effects on the endothelium *via* the pro-angiogenic mediators IL-8 and MMP-9, as documented by the following lines of evidence: i) the strong correlation between T_{ang} and these factors, ii) the inverse correlation of IL-8 with $CD28^{null}$ T_{ang} subpopulations, iii) the perturbed endothelial phenotype induced by the stimulation with sera from subjects with low number of circulating T_{ang} CD3+ cells, and iv) the correlation between endothelial cell proliferation and IL-8 serum levels.

Our observation confirms the relationship between T_{ang} subsets and the endothelium in SLE, highlighting the necessity to extend these observations longitudinally in wider cohorts of lupus patients, potentially leading to the identification of surrogate markers to early stratify SLE subjects according to the future risk of CV events.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of each participating center [approval numbers 170187 (University of Ferrara), 2793 (University of Brescia) and 2017_10_24_3 (Istituto Auxologico Italiano)]. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB, CC, and MF designed the study, selected the patients and control subjects to be recruited in the study, collected clinical data, acquired funding, and wrote the final version of the manuscript. CC collected *in vitro* data and performed the statistical analysis. ER performed experiments, analyzed the data, and contributed to the writing of the manuscript. DP, CB, and AG performed experiments and contributed to the writing of the manuscript. ES, MG, PA, AT, and FF participated in data interpretation and contributed to the writing of the manuscript. IC designed the study and contributed to the writing of the manuscript. PM contributed to the writing of the manuscript. FC and SP designed the study, performed experiments, collected flow cytometric data, coordinated the research activities, and wrote the manuscript. 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/fimmu.2020.572876/full#supplementary-material

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Mycobacterium bovis Bacille-Calmette-Guérin Infection Aggravates Atherosclerosis

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Tuberculosis has been associated with increased risk of atherosclerotic cardiovascular disease. To examine whether mycobacterial infection exacerbates atherosclerosis development in experimental conditions, we infected low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice with *Mycobacterium bovis* Bacille-Calmette-Guérin (BCG), an attenuated strain of the *Mycobacterium tuberculosis* complex. Twelve-week old male *Ldlr*$^{-/-}$ mice were infected with BCG (0.3–3.0x10^6 colony-forming units) via the intranasal route. Mice were subsequently fed a western-type diet containing 21% fat and 0.2% cholesterol for up to 16 weeks. Age-matched uninfected *Ldlr*$^{-/-}$ mice fed with an identical diet served as controls. Atherosclerotic lesions in aorta were examined using Oil Red O staining. Changes induced by BCG infection on the immunophenotyping profile of circulating T lymphocytes and monocytes were assessed using flow cytometry. BCG infection increased atherosclerotic lesions in *en face* aorta after 8 weeks (plaque ratio; 0.021±0.01 vs. 0.013±0.01; p = 0.011) and 16 weeks (plaque ratio, 0.15±0.13 vs. 0.06±0.02; p = 0.003). No significant differences in plasma cholesterol or triglyceride levels were observed between infected and uninfected mice. Compared to uninfected mice, BCG infection increased systemic CD4/CD8 T cell ratio and the proportion of Ly6C^{low} non-classical monocytes at weeks 8 and 16. Aortic plaque ratios correlated with CD4/CD8 T cell ratios (Spearman's rho = 0.498; p = 0.001) and the proportion of Ly6C^{low} non-classical monocytes (Spearman's rho = 0.629; p < 0.001) at week 16. In conclusion, BCG infection expanded the proportion of CD4$^+$ T cell and Ly6C^{low} monocytes, and aggravated atherosclerosis formation in the aortas of hyperlipidemic *Ldlr*$^{-/-}$ mice. Our results indicate that mycobacterial infection is capable of enhancing atherosclerosis development.

Keywords: mycobacterium, Bacille-Calmette-Guérin, tuberculosis, atherosclerosis, inflammation, T cells, monocytes

INTRODUCTION

It is estimated that a quarter of the world population has latent tuberculosis infection, and about 10 million people develop active tuberculosis each year globally (1, 2). Patients with a history of active tuberculosis have an increased risk of myocardial infarction, ischemic stroke, and peripheral arterial disease, suggesting that mycobacterial infection has a role in atherosclerotic cardiovascular disease (3–6). Although these studies accounted for common traditional cardiovascular risk factors, there is a possibility of residual confounding effects from measured and unmeasured characteristics that may not be fully controlled for in human population-based studies (7). Therefore, there is a need to explore the relationship between mycobacterial infection and atherogenesis in experimental animal models. Furthermore, perturbations in circulating T cell and monocyte subsets have been described in tuberculosis (8, 9). These immune cells play an important role in atherosclerosis development (10), but their correlation with atherosclerotic plaque in the setting of mycobacterial infection is not well characterized.

The pro-atherogenic effects of infectious agents were first described more than 4 decades ago, when Fabricant *et al* reported experimental induction of atherosclerosis by Marek's virus in chickens (11). Bacterial pathogens including *Chlamydia pneumoniae* (12–14), *Helicobacter pylori* (15), and periodontal organisms such as *Porphyromonas ginvivalis* (16) have been associated with increased atherosclerosis formation in hyperlipidemic animal models. Several potential mechanisms linking infection and atherosclerosis have been described, including direct pathogen invasion of vascular tissue and indirect effects via inflammatory and immune mechanisms (17–19). Previous animal studies using *Mycobacterium bovis* Bacille-Calmette-Guérin (BCG), an attenuated strain of the *Mycobacterium tuberculosis* complex, have shown different modulating effects of infection in atherosclerosis development. In hypercholesterolemic rabbits, subcutaneous *M. bovis* BCG injections enhanced atherogenesis (20). However, a recent study in *ApoE*3 Leiden.CETP* mice showed reduced atherosclerotic plaque formation after intravenous inoculation of *M. bovis* BCG (21). Notably, the latter study was confounded by a significant reduction of plasma cholesterol levels in the mice infected with *M. bovis* BCG, which likely mediated the final atherosclerosis outcome in this model (22).

To further assess the effects of mycobacteria in atherogenesis, we infected low-density lipoprotein receptor knockout ($Ldlr^{-/-}$) mice with *M. bovis* BCG via the intranasal route. We used this route of infection to mimic the natural respiratory route of acquisition of mycobacterial infections (23). We used $Ldlr^{-/-}$ mice, as *M. bovis* BCG infection was not expected to induce significant plasma cholesterol changes in this atherosclerosis model (24). We aimed at assessing whether *M. bovis* BCG infection exacerbates atherosclerosis development and induces changes on the immunophenotyping profile of circulating T cell and monocytes. We report that *M. bovis* BCG infection expanded the proportion of circulating $CD4^+$ T cell and $Ly6C^{low}$ monocytes, and aggravated atherosclerosis formation in murine aorta.

MATERIALS AND METHODS

Mice, Diet, and Study Setting

Twelve-week old male C57BL/6J $Ldlr^{-/-}$ mice were purchased from the Jackson Laboratory and housed at the Laboratory Animal Medical Services (LAMS) within the University of Cincinnati. Mice were anesthetized and inoculated with *M. bovis* BCG [0.3–3.0x10^6 colony-forming units (CFUs)] via the intranasal route. Mice were subsequently fed a western-type diet (WD) containing 21% fat and 0.2% cholesterol (Envigo TD.88137 diet) for up to 16 weeks. Age-matched uninfected $Ldlr^{-/-}$ mice fed with an identical WD served as controls. Mice were weighed every 2 weeks after initiation of WD to assess for differences in total body weight between groups. The protocols for animal experiments were conducted as per the University of Cincinnati Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) guidelines.

Atherosclerotic Lesion Assessment

Mice were euthanized at 8 and 16 weeks of WD to examine atherosclerotic lesions in aortic root sections and *en face* aorta using Oil Red O staining. Twelve frozen sections per sample were examined throughout the aortic root. Plaque ratios were determined based on plaque area per total area at the aortic root. For *en face* assessments of total aorta, the extent of plaque was measured using the plaque size per aorta area ratio using ImageJ (NIH, Bethesda, MD). Plaque composition was assessed in aortic root sections. We used CD68 antibody (Abcam ab955) at 1:200 dilution and SMC alpha actin (Abcam ab5694) at 1:100 dilution for immunofluorescence staining to assess macrophage and smooth muscle content, respectively. Images were captured using immunofluorescence microscopy (Olympus BX61) and the areas of positive fluorescence per total area of plaque ratios were estimated. Fibrosis was detected by Sirius red staining.

Circulating Lipids Assessment

Blood was collected via intra-cardiac puncture immediately after euthanasia. Plasma was separated for analysis of circulating lipids. Total plasma cholesterol and triglyceride levels were measured using enzymatic assays (Infinity^{TM} reagents). Lipoprotein distribution was assessed using fast protein liquid chromatography (FPLC).

Immunophenotyping of Circulating T Cells and Monocytes

T cell and monocyte subsets were assessed using flow cytometry. After two rounds of red blood cell lysis, FcγII/III receptors were blocked using anti-mouse CD32/CD16 for 15 min at 4°C (Leinco clone YT1.24). Cells were stained using antibodies for 30 min at 4°C at 1:100 dilution in FACS buffer (buffered salt solution with 0.5% bovine serum albumin; Leinco). The T cell panel included the following antibodies and conjugated fluorochromes: CD45.2 (clone 104)/brilliant violet 711 and CD3e (clone 145-2C11)/PerCP-Cy5.5 from BD Biosciences; CD4 (GK1.5)/FITC, CD8b (H35-17.2)/PE-Cy7, CD44 (IM7)/Brilliant Violet 421, CD25 (PC61.5)/APC, Foxp3 (clone NRRF-30)/PE from eBioscience. The monocyte panel included CD45.2 (clone 104)/brilliant violet

711, CD11b (clone M1/90)/APC-Cy7, CD11c (clone HL3)/PE-Cy7, CD115 (clone T38-320)/PE, Ly6G (clone 1A8)/PerCP-Cy5.5, and Ly6C (clone AL-21)/APC from BD Biosciences. Cells stained with the T cell panel antibodies were washed and fixed with Fix/Perm buffer and then stained for intracellular FoxP3 (eBioscience clone NRRF-30/PE). Cells stained with the monocyte panel antibodies were washed and fixed with Fix/Perm buffer and then stained for intracellular NOD2 (Novus Biologicals clone 2D9/Alexa Fluor 488). Flow cytometric data were acquired using a BD™ LSR II. Data were analyzed using FlowJo v10 software. We used Fluorescence minus one (FMO) controls to set our gates.

Mycobacteria CFU Enumeration

Right lungs and spleens were harvested for CFU enumeration. Lung and spleen tissues were homogenized in 5 ml of sterile phosphate-buffered saline (PBS) and serially diluted on 7H10 agar (262710, BD Diagnostic) supplemented with 2.5 mg/L amphotericin B (A9528, Sigma), 26 mg/L polymyxin B sulfate (P4932-5MU, Sigma), 20 mg/L trimethoprim lactate (T0667-260mg, Sigma), 50 mg/L carbenicillin disodium (C3416-1G, Sigma), and OADC enrichment (R450605, Fischer) (25). CFUs were quantified following humidified incubation at 37°C for 2 to 3 weeks.

Statistical Analyses

We used unpaired Student's t-test for group comparisons of numeric variables and flow cytometry data. To assess the correlation between immune parameters and plaque ratio, we used the Spearman's correlation test. Analyses were carried out in Stata v12 (College Station, TX); p values <0.05 were considered statistically significant. All p values were 2-tailed.

RESULTS

To confirm that inoculation with *M. bovis* BCG via the intranasal route induced a persistent mycobacterial infection in our murine model, we cultured lung and spleen homogenates of mice at time of euthanasia. Mean *M. bovis* BCG CFU in the infected group was $3.6x10^6 \pm 1.6x10^6$ CFU/g in lung and $1.1x10^6 \pm 6.5x10^5$ CFU/g in spleen at 8 weeks post challenge. The *M. bovis* BCG CFU remained detectable at $5.2x10^5 \pm 4.9x10^5$ CFU/g in lung and $1.1x10^6 \pm 9x10^5$ CFU/g in spleen by 16 weeks. Mycobacterial colonies were not detectable from the lungs or spleens of uninfected control mice. These findings confirmed that we were able to establish persistent mycobacterial infection in our experimental model.

M. bovis BCG Increases Atherosclerosis in *En Face Aorta*

M. bovis BCG infection significantly increased atherosclerotic lesions in *en face* aorta after 8 weeks (plaque ratio; 0.021 ± 0.01 vs. 0.013 ± 0.01; p = 0.011; **Figures 1A, C**) and 16 weeks of inoculation (plaque ratio, 0.15 ± 0.13 vs. 0.06 ± 0.02; p = 0.003; **Figures 1B, C**). The aortic root sections showed similar plaque involvement in infected and uninfected mice at week 8 (plaque ratio, 0.15 ± 0.06 vs. 0.13 ± 0.07; p = 0.402; **Figure 2A**) and week 16 (plaque ratio; 0.28 ± 0.05 vs. 0.28 ± 0.06; p = 0.726; **Figure 2A**). Plaque composition analysis of aortic root sections revealed decreased smooth muscle-specific alpha-actin expression (0.09 ± 0.05 vs. 0.17 ± 0.08; p = 0.002; **Figure 2B**), decreased necrotic core content (0.18 ± 0.09 vs. 0.25 ± 0.09; p = 0.033; **Figure 2B**), and increased fibrosis (0.41 ± 0.12 vs. 0.33 ± 0.09; p = 0.034; **Figure 2B**) among *M. bovis* BCG-infected mice compared to uninfected mice. Similar content of $CD68^+$ macrophages was observed in aortic root sections of *M. bovis* BCG-infected and uninfected mice (0.161 ± 0.05 vs. 0.157 ± 0.05; p = 0.779; **Figure 2C**). We were unable to detect mycobacteria in atherosclerotic lesions by acid fast bacilli staining or immunofluorescence staining using anti-mycobacterium Ag85B antibodies (**Supplementary Figure 1**). Overall, our experiments showed that *M. bovis* BCG exacerbated the extent of atherosclerosis within the aorta. However, the effect of mycobacteria in atherosclerosis was not noticeable in the aortic root, perhaps because this area of high turbulence is already prone to plaque formation in the setting of high fat diet and hyperlipidemia (26), regardless of infection.

M. bovis BCG Infection Does Not Induce Significant Changes in Body Weight or Circulating Lipids in $Ldlr^{-/-}$ Mice

Mice infected with *M. bovis* BCG displayed no differences in total body weight through the course of 16 weeks of WD, compared to uninfected control mice (baseline body weight, 24.2 ± 1.5 vs. 23.8 ± 1.5, p = 0.552; body weight at 8 weeks of WD, 34.4 ± 3.6 vs. 36 ± 5.1, p = 0.287; body weight at 16 weeks of WD, 35.2 ± 3.2 vs. 33.8 ± 2.5, p = 0.292; **Supplementary Figure 2A**). As expected in this model and diet conditions, mice were overall hyperlipidemic, but there were no significant differences in plasma cholesterol (1,160 ± 230 mg/dL vs. 1,278 ± 298 mg/dL; p = 0.359; **Supplementary Figure 2B**) or triglycerides (340 ± 125 mg/dL vs. 413 ± 154 mg/dL; p = 0.284; **Supplementary Figure 2C**) between infected vs. uninfected mice at 16 weeks. FPLC chromatograms showed a similar distribution of triglyceride and cholesterol fractions at week 16 (**Supplementary Figures 2D, E**). Similarly, no significant differences in plasma cholesterol (887 ± 444 vs. 1073 ± 353; p = 0.151; **Supplementary Figure 2B**) or triglycerides (256 ± 138 vs. 319 ± 98; p = 0.101; **Supplementary Figure 2C**) were observed between infected vs. uninfected mice at week 8. These results indicated that the aggravated atherosclerosis findings associated with *M. bovis* BCG were not related to increased amounts of circulating lipids induced by infection.

M. bovis BCG Infection Induces an Expansion of $CD4^+$ T Cells and Monocytes

Figures 3A–H summarize the flow cytometry gating strategy and key immunophenotyping findings of circulating T cells and

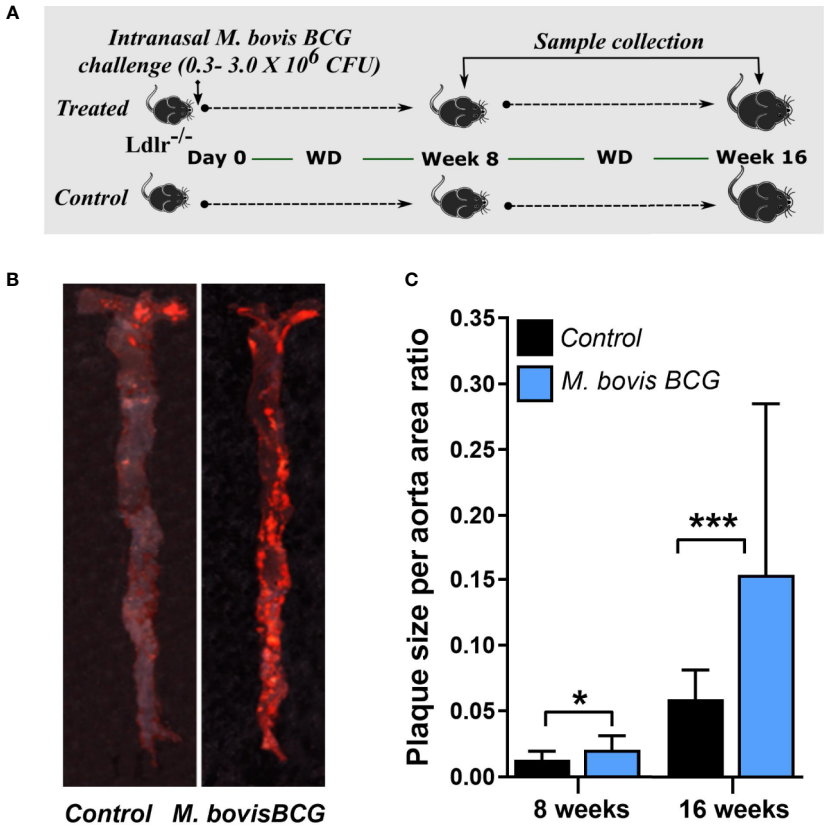

FIGURE 1 | *M. bovis* BCG increases *en face* aorta atherosclerosis by 8 and 16 weeks post-challenge. **(A)** Twelve-week old male *Ldlr*$^{-/-}$ mice were inoculated with *M. bovis* BCG (0.3–3.0x10^6 CFU) via the intranasal route. Mice were fed a western-type diet for up to 16 weeks. Age-matched uninfected *Ldlr*$^{-/-}$ mice fed with an identical diet served as controls. **(B)** Atherosclerotic lesions in *en face* aorta were examined using Oil Red O staining at weeks 8 and 16. Data are representative of the aortae of one *M. bovis* BCG-infected mouse and one control mouse at 16 weeks. **(C)** Plaque burden was quantified by the plaque size per aorta ratio in *M. bovis* BCG-infected (blue) and control mice (black). Data are mean ± SD. n = 20 mice per group pooled from 2 independent experiments. Significance was determined by Student's *t*-test. *$p < 0.05$; ***$p < 0.001$.

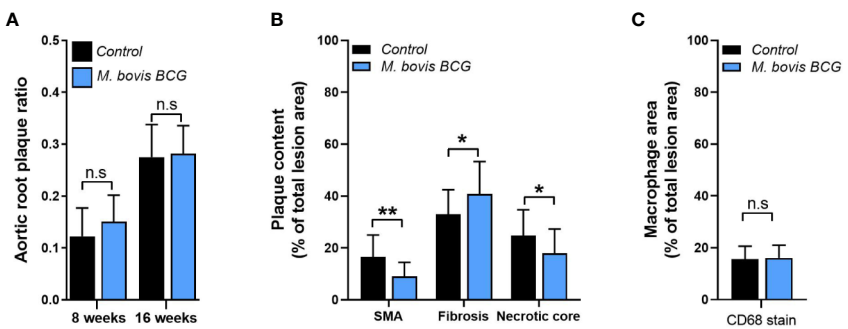

FIGURE 2 | *M. bovis* BCG does not increase extent of aortic root atherosclerosis. *Ldlr*$^{-/-}$ mice were inoculated with *M. bovis* BCG (0.3–3.0x10^6 CFU) via the intranasal route. Mice were fed a western-type diet for 8 to 16 weeks. Age-matched uninfected *Ldlr*$^{-/-}$ mice fed with an identical diet served as controls. **(A)** Plaque area per total area of aortic root ratios were quantified in *M. bovis* BCG-infected (blue) and control mice (black) at weeks 8 and 16. **(B)** Aortic root plaque composition of smooth muscle (using smooth muscle alpha actin staining; SMA), fibrosis content (using Syrius red staining), and necrotic core were quantified as percentage per total lesion area at week 16. **(C)** Macrophage content using CD68 staining was quantified as percentage per total lesion area at week 16. Data are mean ± SD. n = 20 mice per group pooled from 2 independent experiments. Significance was determined by Student's *t*-test. *$p < 0.05$; **$p < 0.01$; n.s., non-significant ($p > 0.05$).

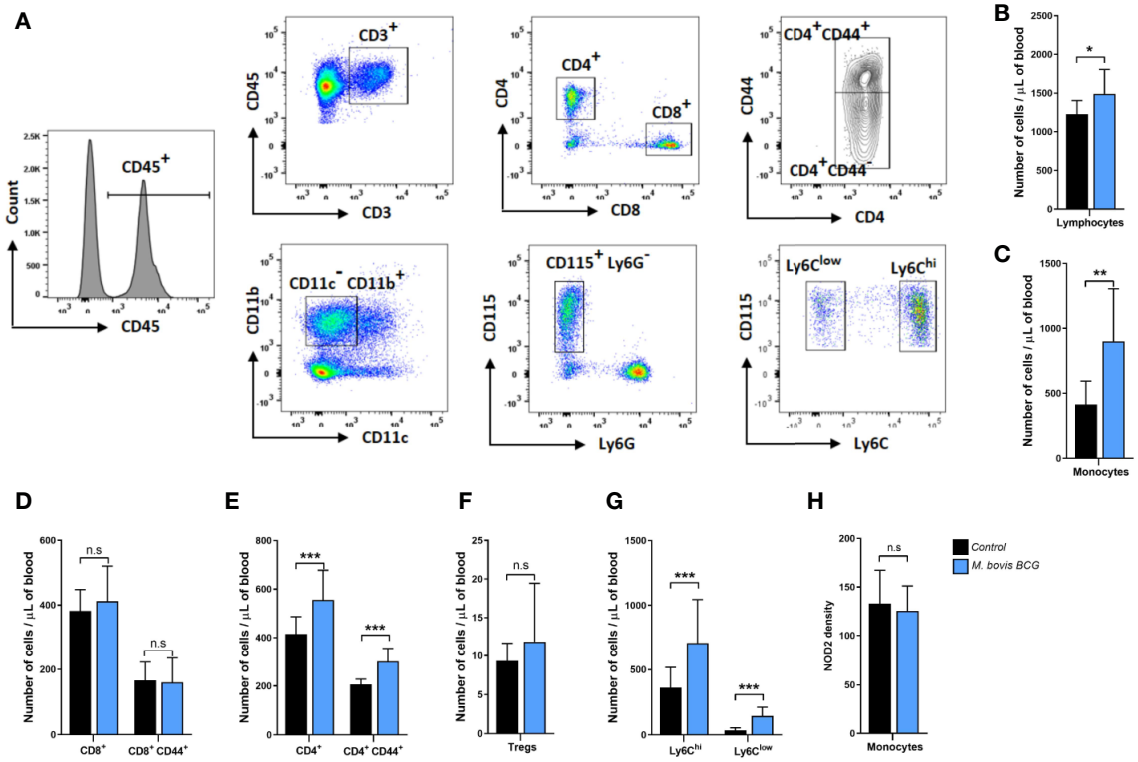

FIGURE 3 | *M. bovis* BCG induces CD4$^+$ T cell and monocyte activation by week 8. Flow cytometry analysis of blood lymphocytes and monocytes from *M. bovis* BCG-infected and control mice at 8 weeks. *Ldlr*$^{-/-}$ mice were inoculated with *M. bovis* BCG (0.3–3.0x10^6 CFU) via the intranasal route. Mice were fed a western-type diet for 8 weeks. Age-matched uninfected *Ldlr*$^{-/-}$ mice fed with an identical diet served as controls. **(A)** Representative flow cytometry plots showing the gating strategy used for identifying subsets of T cells and monocytes. T cells (upper panel) were defined as CD45$^+$ CD3$^+$ cells and seperated into CD4$^+$ and CD8$^+$ T cell subsets. Expression of CD44 was further assessed within T cell subsets. Monocytes (lower panel) were defined as CD45$^+$ CD3$^-$ CD11b$^+$ CD11c$^-$ CD115$^+$ Ly6G$^-$ cells. Monocyte subsets were defined based on Ly6C expression. **(B–H)** Number of lymphocytes **(B)**, monocytes **(C)**, T cell subsets **(D–F)**, monocyte subsets **(G)** and monocyte NOD2 MFI **(H)** from *M. bovis* BCG-infected (blue) and control mice (black). Data are mean ± SD. n = 10 mice per group. Significance was determined by Student's *t*-test. $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., non-significant ($p > 0.05$).

monocytes in *M. bovis* BCG-infected and control mice. *M. bovis* BCG infection induced an increase in circulating T lymphocytes (1,490 ± 316 cells/ul vs. 1,227 ± 177 cells/ul; $p = 0.034$; **Figure 3B**) and monocytes (901 ± 405 cells/ul vs. 414 ± 180 cells/ul; $p < 0.003$; **Figure 3C**) by 8 weeks post-infection. When we assessed T lymphocyte subsets, *M. bovis* BCG infection increased total CD4$^+$ T cell counts (556 ± 122 cells/ul vs. 416 ± 71 cells/ul; $p < 0.005$; **Figure 3E**) but not CD8$^+$ T cells (413 ± 109 cells/ul vs. 382 ± 66 cells/ul; $p = 0.468$; **Figure 3D**). There was an increased number of CD4$^+$ T cells expressing the activation marker CD44 in BCG-infected vs. uninfected mice (211 ± 102 cells/ul vs. 150 ± 61 cell/ul; $p < 0.027$; **Figure 3E**). CD4$^+$ FoxP3$^+$ T cells were similar between groups (12 ± 8 cells/ul vs. 9 ± 2 cells/ul; $p = 0.155$; **Figure 3F**). When we assessed monocyte subsets, *M. bovis* BCG infection increased the numbers of Ly6C^{high} (709 ± 335 cells/ul vs. 362 ± 155 cells/ul; $p < 0.008$; **Figure 3G**) and Ly6C^{low} (145 ± 68 cells/ul vs. 35 ± 19 cells/ul; $p < 0.001$; **Figure 3G**) monocytes at week 8. NOD2 receptor density was similar in total monocytes and monocyte subsets of BCG-infected and uninfected mice (mean fluorescence density in total monocytes, 125 ± 26 vs. 133 ± 34; $p = 0.577$; **Figure 3H**).

The CD4/CD8 T Cell Ratio and the Proportion of Ly6C^{low} Monocytes Correlate With the Extent of Aortic Plaque

M. bovis BCG infection induced an increase in the CD4/CD8 ratio (1.24 ± 0.17 vs. 0.97 ± 0.28 at week 8; $p < 0.001$; 1.47 ± 0.18 vs. 1.11 ± 0.23 at week 16; $p < 0.001$; **Figure 4A**). In addition, we observed that *M. bovis* BCG infection led to an increase in the proportion of Ly6C^{low} non-classical monocytes (19 vs. 9% at week 8; $p < 0.001$; 23 vs. 9% at week 16; $p = 0.009$; **Figures 4B–D**), compared to uninfected mice. CD4/CD8 ratio (Spearman's rho = 0.498; $p = 0.001$; **Figures 5A–B**) and the proportion of Ly6C^{low} monocytes (Spearman's rho = 0.629; $p < 0.001$; **Figures 5C–D**) correlated with aorta plaque formation at week 16. None of the other immunophenotyping parameters studied correlated with aortic plaque.

DISCUSSION

M. bovis BCG infection increased the extent of atherosclerosis formation in the aortas of WD-fed hyperlipidemic *Ldlr*$^{-/-}$ mice.

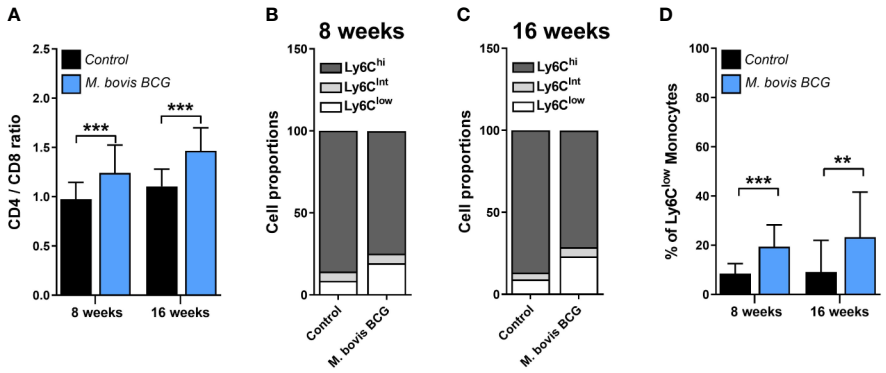

FIGURE 4 | *M. bovis* BCG increases the CD4/C8 ratio and the proportion of Ly6C^{low} monocytes. *Ldlr*$^{-/-}$ mice were inoculated with *M. bovis* BCG (0.3–3.0x10^6 CFU) via the intranasal route. Mice were fed a western-type diet for 8 to 16 weeks. Age-matched uninfected *Ldlr*$^{-/-}$ mice fed with an identical diet served as controls. **(A)** CD4/CD8 T cell ratios from *M. bovis* BCG and control mice at weeks 8 and 16. **(B, C)** Mean proportions of Ly6C^{low}, Ly6C^{intermediate} and Ly6C^{high} monocyte subsets in blood on **(B)** week 8 and **(C)** week 16. **(D)** Percentage of Ly6C^{low} monocytes in blood. Data are means ± SD for A, D. Mean for B, C. n = 10 mice per group; pooled from 2 independent experiments. Significance was determined by Student's *t*-test. $^{**}p < 0.01$; $^{***}p < 0.001$.

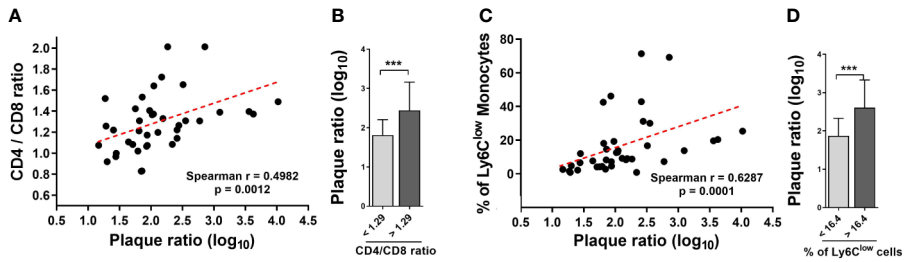

FIGURE 5 | The CD4/CD8 T cell ratio and the proportion of Ly6C^{low} monocytes correlate with the extent of aortic plaque by week 16. *Ldlr*$^{-/-}$ mice were inoculated with *M. bovis* BCG (0.3–3.0x10^6 CFU) via the intranasal route. Mice were fed a western-type diet for 16 weeks. Age-matched uninfected *Ldlr*$^{-/-}$ mice fed with an identical diet served as controls. **(A)** Spearman correlation of blood CD4/CD8 T cell ratios with aorta plaque ratio. **(B)** Aorta plaque ratio in mice grouped as below or above CD4/CD8 ratio mean. **(C)** Spearman correlation of the proportion of Ly6C^{low} monocytes and aorta plaque ratio. **(D)** Aorta plaque ratio in mice grouped as below or above mean proportion of Ly6C^{low} monocytes in blood. n = 40; data are pooled from 2 independent experiments. Significance was determined by Spearman's correlation test for **(A, C)**. Significance was determined using Student's *t*-test for **(B, D)**. $^{***}p < 0.001$.

Circulating lipid levels were not significantly increased in BCG-infected mice compared to uninfected mice, and therefore do not explain the observed differences in atherosclerosis. Compared to uninfected controls, *M. bovis* BCG-infected mice exhibited increased CD4$^+$ T cell and monocyte driven systemic immune activation. Overall, our results indicate that mycobacterial infection is capable of enhancing atherosclerosis development.

In a prior study of rabbits inoculated with two subcutaneous injections of *M. bovis* BCG and fed with cholesterol-supplemented diet, infected rabbits displayed increased atherosclerotic lesions in thoracic aorta compared to uninfected controls with similar plasma cholesterol levels (20). However, a recent study in *APOE*3-Leiden.CETP* mice showed that intravenous inoculation of *M. bovis* BCG was associated with decreased atherosclerosis formation in the aortic root after 6 weeks of infection (21). In the latter model, infection induced lower plasma cholesterol levels compared to uninfected controls, which may have affected the atherosclerosis outcome of the experiments. Our data shows that *M. bovis* BCG infection is capable of increasing atherosclerosis formation in aorta, under similar hypercholesterolemia conditions. Furthermore, we show for the first time that inoculation with *M. bovis* BCG via the respiratory route (which is the most common route of acquisition of mycobacterial infection in humans) exacerbated atherosclerosis and thus supports a pathogenic role of mycobacterial infection in plaque formation.

Population-based studies have indicated an increased risk of atherosclerotic cardiovascular disease in persons with a history of tuberculosis disease (3–6). In addition, recent studies have shown that latent tuberculosis infection is associated with higher rates of coronary artery stenosis and spontaneous acute myocardial infarction (27, 28). These data in humans suggest that the interplay between mycobacteria and cardiovascular disease can occur at different stages of mycobacterial infection. We were able

to recover viable mycobacteria from lung and spleen tissues after 16 weeks of *M. bovis* BCG inoculation, suggesting that our model may be more representative of conditions where there is mycobacterial persistence. This is true for active tuberculosis disease, but may also occur within the spectrum of subclinical tuberculosis and latent tuberculosis infection, as the "latent" state encompasses a wide range of host-pathogen interactions (29), some of which may result in residual bacterial replication and/or enhanced systemic immune activation (30–32).

We found that *M. bovis* BCG infection induced monocyte and $CD4^+$ T cell driven systemic immune activation, which is in line with results from prior studies of host immune responses to mycobacteria (20, 33). The contribution of these immune cells in atherosclerosis development has been well characterized (34), and likely provides a mechanistic link between infection and atherogenesis. Both the CD4/CD8 T cell ratio and the proportion of $Ly6C^{low}$ monocyte were associated with plaque burden in our study. However, a limitation of our study is that we did not conduct in-depth mechanistic experiments to assess the role of specific immune or mycobacterial parameters in atherogenesis. Future studies detailing specific tissue dissemination—including interactions of *M. bovis* BCG, inflammatory cells, and other stromal cells within atherosclerotic plaque—are needed. Whether targeted immune mechanisms mediate the effects of mycobacterial infection in atherosclerosis can be assessed in future studies. Of note, an increased systemic CD4/CD8 T cell ratio was recently found to be strongly and independently associated with coronary artery disease in elderly individuals (35). In human atherosclerotic plaque, there is a progressive expansion of the $CD4^+$ T cell compartment as atherosclerotic lesions evolve (36). Although a decreased systemic CD4/CD8 T cell ratio has also been associated with increased human atherosclerosis, this phenomenon has been observed primarily in persons living with HIV/AIDS (37).

M. bovis BCG infection increased the number of circulating monocytes in our model. Monocytosis has been observed in human and murine myocardial infarction (38). Furthermore, a recent study demonstrated that monocyte recruitment from the circulation into aortic plaque is required for atherosclerosis progression (39). When monocyte subsets were analyzed, *M. bovis* BCG-infected mice showed a higher proportion of non-classical $Ly6C^{low}$ circulating monocytes, which may be in response to increased endothelial injury, and could drive increased fibrosis in vascular tissue (40). Alternatively, our results may indicate a pro-atherogenic contribution of non-classical $Ly6C^{low}$ monocytes in atherogenesis. Despite $Ly6C^{low}$ monocytes being known to promote endothelial repair (41), a recent study indicated that these cells are involved in early plaque development (42). Furthermore, experimental inhibition of CCR5, a chemokine receptor preferentially involved in $Ly6C^{low}$ monocyte recruitment to atherosclerotic plaque (43), has resulted in decreased atherosclerosis formation (42, 44). Of note, triggering of the NOD2 receptor has been reported to promote conversion of $Ly6C^{high}$ into $Ly6C^{low}$ monocytes with patrolling properties (45). We did not see significant differences in NOD2 receptor expression between BCG-infected and uninfected mice; however, these results do not exclude the possibility of differential downstream NOD2 signaling. Monocytes exposed to *M. bovis* BCG develop a prolonged pro-inflammatory phenotype via epigenetic changes in histone methylation at the level of bone marrow progenitors, a phenomenon coined as trained immunity (46). In addition to central trained immunity, peripheral trained immunity of blood monocytes and tissue macrophages has also been described (47, 48). Trained monocyte-derived macrophages have an augmented production of pro-atherogenic cytokines including IL-1β, IL-6, and tumor necrosis factor-α, and are more prone to foam cell formation upon exposure to a second non-specific stimulus (49, 50). Thus, trained immunity is gaining recognition as a plausible mechanistic link between infection and atherosclerosis development (22, 51). Furthermore, classical pro-atherogenic stimuli such as oxidized-LDL also induce a trained immune phenotype in monocytes (50), suggesting that both infectious and non-infectious triggers might contribute to atherosclerosis through shared disease pathways. Future studies assessing trained immunity and epigenetic reprogramming of $Ly6C^{low}$ and $Ly6C^{high}$ monocytes and their effects in atherosclerotic plaque may provide insights into mechanisms of atherosclerosis in the setting of mycobacterial infection.

In conclusion, *M. bovis* BCG infection increased the extent of atherosclerosis development in the aortas of WD-fed hyperlipidemic $Ldlr^{-/-}$ mice. Our results indicate that mycobacterial infection is capable of enhancing atherosclerosis development, and provide experimental evidence for previously reported links between tuberculosis and atherosclerotic cardiovascular disease in humans.

DATA AVAILABILITY STATEMENT

The primary data supporting the conclusions of this article will be made available by the authors, upon reasonable request.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

MH, JQ, CF, GD, and DH contributed to the conception and design of the study. MH, JQ, SJ, SS, AM, DK, EK, and RK conducted the investigation and experiments. MH, SJ, DK, and EK performed statistical analyses. All authors interpreted data. MH wrote the first draft of the report. 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/fimmu.2020.607957/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Detection of mycobacteria in atherosclerotic lesions. Frozen sections of aortic roots isolated from *M. bovis* BCG-infected and uninfected *Ldlr*$^{-/-}$ mice were histologically stained with acid fast reagents by placing in Carbol-fuchsin solution (Sigma-Aldrich, catalog No. HT801) for 1 min followed by 2 min of counterstain with Malachite Green solution (Sigma-Aldrich, catalog No. HT802). The frozen sections were also subjected to immunofluorescence staining with the anti-*Mycobacterium tuberculosis* antibodies Ag85B (Abcam, catalog No. ab43019) with DAPI counterstain to identify the nucleus. The scale bars represent 100 µm. Note that no mycobacteria were detectable in the atherosclerotic lesions from either *M. bovis* BCG infected or uninfected mice.

SUPPLEMENTARY FIGURE 2 | *M. bovis* BCG infection does not induce significant changes in body weight or circulating lipids in *Ldlr*$^{-/-}$ mice. *Ldlr*$^{-/-}$ mice were inoculated with *M. bovis* BCG (0.3–3.0x10^6 CFU) via the intranasal route. Mice were fed a western-type diet for 8 to 16 weeks. Age-matched uninfected *Ldlr*$^{-/-}$ mice fed with an identical diet served as controls. **(A)** Body weight at baseline, 8 weeks, and 16 weeks of western-type diet in *M.bovis* BCG infected (blue) and control mice (black). **(B)** Plasma cholesterol levels in mg/dL at weeks 8 and 16. **(C)** Plasma triglyceride levels in mg/dL at weeks 8 and 16. Data are means ± SD. n = 20 mice per group; pooled from 2 independent experiments. Significance was determined by Student's t-test. n.s., non-significant ($p > 0.05$). Fast protein liquid chromatography (FPLC) profiles of total cholesterol **(D)** and tryglicerides **(E)** in *M.bovis* BCG infected and control mice at week 16.

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SYSTEMATIC REVIEW
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Immunosenescence Study of T Cells: A Systematic Review

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Background: Aging is accompanied by alterations in immune response which leads to increased susceptibility to infectious diseases, cancer, autoimmunity, and inflammatory disorders. This decline in immune function is termed as immunosenescence; however, the mechanisms are not fully elucidated. Experimental approaches of adaptive immunity, particularly for T cells, have been the main focus of immunosenescence research. This systematic review evaluates and discusses T cell markers implicated in immunosenescence.

Objective: To determine the best flow cytometry markers of circulating T cells associated with immunosenescence.

Methods: We systematically queried PubMed, MEDLINE, EBSCO, and BVS databases for original articles focused on two age groups of healthy humans: 18–44 (young adults) and >60 (older adults) years. In accordance with the Cochrane methodology, we synthesized data through qualitative descriptions and quantitative random effects meta-analysis due to extensive heterogeneity.

Results: A total of 36 studies conducted in the last 20 years were included for the qualitative analysis and four out of these studies were used to perform the meta-analysis. A significant decrease in naïve T cell subset was observed in older adults compared to young adults. Primary markers used to identify senescent cells were loss of CD28 and increased expression of CD57 and KLRG1 in terminally-differentiated memory T cell subset in older adults. Moreover, we observed an increase in proinflammatory cytokines and decrease in telomere length in old adult T cells. It was not possible to perform quantitative synthesis on cell markers, cytokines, and telomere length because of the significant variations between the groups, which is attributed to differences in protocols and unreported measurements, thus generating a high risk of bias.

Conclusions: Heterogeneity among studies in terms of data report, measurement techniques and high risk of bias were major impediments for performing a robust statistical analysis that could aid the identification of eligible flow cytometry markers of immunosenescence phenotype in T cells.

Keywords: cytokines, T cells, immunosenescence, immunosenescence markers, flow cytometry

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INTRODUCTION

The immune system presents a series of age-associated changes that affects its capacity to respond to new challenges. This cellular state, classically referred to as immunosenescence, increases susceptibility to infectious diseases, cancer, and autoimmunity (1). Immunosenescence is characterized by: (i) decreased response to new invading infectious agents, (ii) unsupported memory T cell response, (iii) increased susceptibility to autoimmune diseases, and (iv) chronic low-grade inflammation "inflammaging" (2, 3).

Most studies have focused on evaluating age-associated changes in T cell populations. These cells undergo continuous remodeling as a result of constant interaction with multiple stressors from the internal and external environments. Consequently, reorganization of the immune system is generated throughout lifetime (4). Given the need to maintain the naïve cell repertoire, T cell population is particularly sensitive, thus responding to both chronic or latent infections and new pathogens through clonal expansion and differentiation to effector subpopulation (1).

One of the major age-associated changes that occur in the immune system is thymic involution, which results in variations in number of naïve T cells; with a most notable decrease in CD8 rather than CD4 T cells, since the latter maintain their populations by homeostatic proliferation. This decrease is accompanied by a decline in T cell receptor (TCR) clonal diversity and increase in memory subpopulation, with accumulation of terminally-differentiated T cells that are either dysfunctional or exhausted (5, 6).

Several studies have demonstrated that terminally-differentiated CD8 T cells are less dependent on TCR activation, but more sensitive to innate signals (7). This convergence of characteristics of innate and adaptive immunity in T cells has been described in CD8 T cells that express CD27 and CD28 membrane receptors and exhibit senescence characteristics that includes: (i) low proliferative activity, (ii) shortening of telomeres, (iii) decreased telomerase activity, (iv) expression of senescence-associated markers (e.g., CD57 and KLRG1) and intracellular molecules (e.g., p38 and γH2AX), (v) expression of NK cell markers including inhibitors (KLRG1 and NKG2A) and activators (NKG2C and NKG2D), and (vi) secretion of large amounts of IFNγ and TNFα (7–10).

Immunosenescence is multifactorial and highly dependent on the environment, antigenic challenges, and epigenetic modifications that are particular to individual experiences (immunobiography) (2, 11). Due to the complex diversity of immune aging, it has been proposed that senescence of circulating T cells can be possibly evaluated through the expression of multiple markers such as CD27, CD28, CD57, KLRG1, CD45 isoforms (RA/RO), and production of proinflammatory molecules including IL-6 and TNFα (12). This systematic review aimed to determine the best markers measured by flow cytometry for identifying immunosenescence phenotype in human T cells.

METHODS

Study Design and Protocol Registration

Our systematic review considered the PRISMA checklist for reporting and design of systematic reviews. The study protocol was registered in PROSPERO (ID protocol: CRD42020171342) on 05/07/2020.

Search Strategy

We systematically queried PubMed, MEDLINE, EBSCO, and BVS from 1 January, 2000 to 28 October, 2020. The search strategy included only MeSH and DeCS terms and studies published in English and Spanish (**Table S1**). We also employed the advanced search filters to retrieve only experimental articles on human immune cells.

Eligibility Criteria

To reduce data variability and make accurate comparisons between articles, only published experimental studies regarding flow cytometry analysis of human T cells were evaluated. Memory subsets, senescence and exhaustion-associated markers, cytokine production, and telomere length were the immunosenescence characteristics of interest.

Two age groups were compared: young adults (18–44 years) and older adults (>60 years). The pediatric population was excluded as a result of the high variability in their humoral and cellular response (13). Individuals aged between 45 and 60 years were considered middle-aged adults, thus representing an intermediate population. Therefore, we excluded this group in order to compare the young and older adults only.

Reviews, studies using animal models, and studies using diseased persons were excluded for this systematic review. Only studies published after 2000 were selected since articles from previous years use different measuring techniques and reagents which made their comparison with recent studies difficult.

Study Selection and Data Collection Process

Duplicates were excluded and two reviewers independently screened the titles and abstracts of retrieved studies by applying the inclusion and exclusion criteria. Finally, reviewers selected the articles whose full text is to be read and evaluated for data extraction. Any disagreement over the eligibility of particular studies was resolved by consensus.

Following the final selection of 36 articles, a data-charting form was jointly developed by the team and bibliographic details of the selected articles were registered in **Figure 1**. To classify the data, four categories were defined: (1) memory subsets, (2) senescence and exhaustion-associated markers, (3) cytokines, and (4) telomere length. Each section was assigned to teams of two or more reviewers that extracted the required information in the data-charting sheets. Where possible, the mean/median, standard deviation (SD)/standard error (SE), and the number of events recorded in flow cytometry were registered. If a value was missing, the authors were contacted to provide the details. In situations of lack of response from the authors, a qualitative description of the results was done.

Different domains of results were made: (i) for memory T cell subsets, Naïve (N), Central Memory (CM), Effector Memory (EM), and Terminal Effector (TE) subsets were defined by markers such as CD27, CD28, CCR7, CD45RA, CD45RO, and CD95; (ii) CD57, KLRG1, PD1, CTLA4, TIM3, LAG3, p16, p21, and γH2Ax were defined as immunosenescence and/or exhaustion markers; (iii)

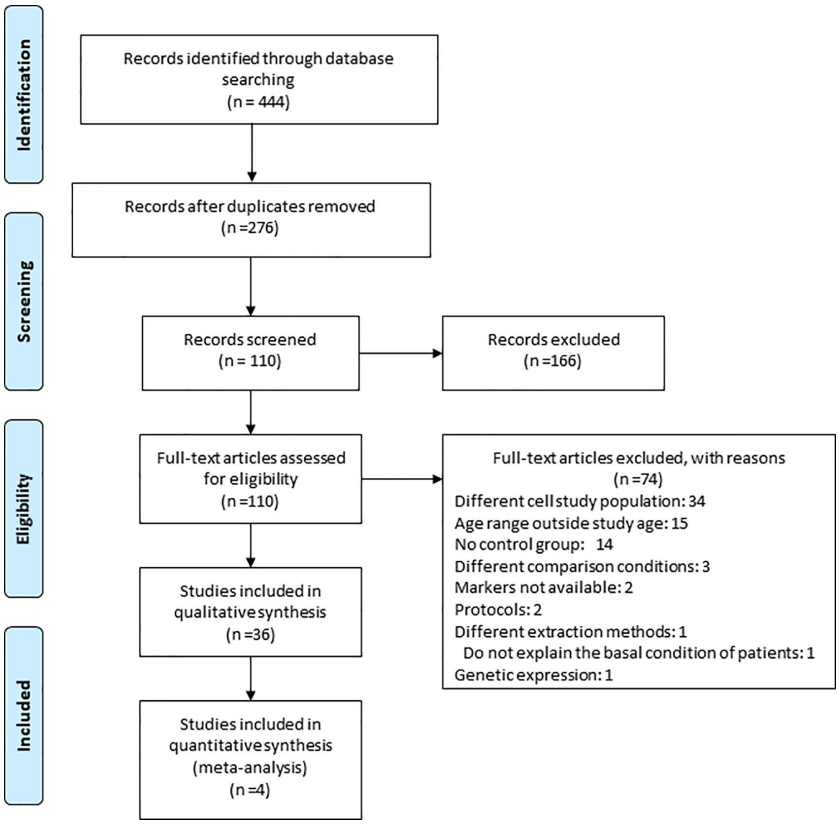

FIGURE 1 | Study flow diagram. The search strategy performed in this article led to the initial selection of 418 potential papers. 303 were retrieved from PUBMED/MEDLINE, 115 from EBSCO and 0 from BVS in the identification phase. After duplicates were removed, a total of 263 papers were identified. From these, 157 articles were excluded based on title and abstract. Further investigation of the remaining 106 full text articles led to the exclusion of 70 articles and the inclusion of 36 studies for the qualitative synthesis among which 4 studies allowed us to perform a meta-analysis.

intracellular IFNγ, TNFα, IL-2, IL-4, IL-6, IL-10, granzyme B, and perforin were analyzed. Finally, (iv) telomere length was reported as Kb or MFI by flow cytometry. Some articles reported an additional group referred to as "super-old" which was also extracted when available.

Risk of Bias Assessment

We did not find a standard assessment tool to evaluate the risk of bias and quality of basic research articles in immunology. Consequently, we designed a table with 14 questions to detect the risk of bias associated with selection, design, methodology, and results (see **Table S2**). For this review, we considered that the articles assessing CMV status without bias were the ones that performed the screening and defined the positive and negative groups. Two reviewers independently scored articles according to description quality as: completely (1), partially (0.5) or not reported (0) and 0 to 100 percent rating was given, with 100% rating for the lowest risk of bias. The analysis was carried out globally and presented in **Table S2**. The risk of bias is illustrated in **Figures 2** and **3**.

Synthesis of Results

Only four articles had the data required for a meta-analysis. This information was synthesized in forest plots. A qualitative description of the remaining papers was done and presented in a narrative form using tables and figures. Where possible, funnel plots were used to assess potential small study bias in meta-analysis containing ten or more studies and absence of statistical heterogeneity (14). Nonetheless, this was not possible due to lack of papers suitable for meta-analysis in our study.

Statistical Analysis

The data available for quantitative analysis was frequency of the memory T cell populations. We used the mean and SD of each specific group count to calculate the standardized mean (SM) and standardized mean difference between groups. The summary estimate of effect had 95% confidence interval for each comparison. Weightings were done by the inverse variance method. Effects were summarized by the random effects meta-analysis. Effect size and confidence intervals of global and subgroup analysis were plotted using forest plots. There was no sufficient data to conduct a sensitivity analysis.

We assessed heterogeneity by visual inspection of the forest plot and quantified it using the I^2 statistic, which describes the proportion of variability in the meta-analysis explained by heterogeneity of the studies rather than sampling error. Values ≥50% indicate significant heterogeneity between studies in the meta-analysis.

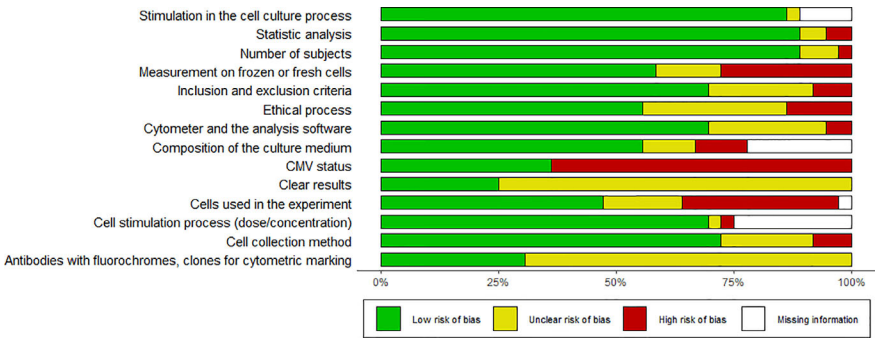

FIGURE 2 | Risk of bias summary. Shows the bias results of all papers reviewed.

RESULTS

Study Selection and Characteristics

The search strategy performed in this article led to an initial selection of 444 potential articles. 321 articles were retrieved from PUBMED/MEDLINE, 123 from EBSCO, none from BVS in the identification phase. After duplicates were removed, a total of 276 studies were identified (**Figure 1**). Out of these studies, 166 articles were excluded based on title and abstract. Further assessment of the remaining 110 full text articles led to the exclusion of 74 articles (**Table S3**) and inclusion of 36 studies for the qualitative synthesis, out of which, 4 studies were eligible for the performance of meta-analysis (15–50).

Characteristics of all included studies are shown in **Table S4**. All studies were characterized by at least two groups of interest (young and old) and experiments done on T cells obtained from peripheral blood and that these cells were used immediately for analysis or frozen for later measurement. Of the 36 studies included, 17 measured CD4 and CD8 T cells; 15 measured CD8; three measured CD4; and one measured γ/δ T cells. 15 studies measured changes in memory subsets, 21 measured markers associated with senescence and exhaustion, 15 measured cytokine expression, and five measured telomere length. 28% of the studies considered CMV serology. In these articles, measurement of intracellular cytokines was performed *via in vitro* stimulation, while analysis of markers and memory subpopulations were performed *ex vivo*.

Risk of Bias Within Studies

Heterogeneity in methodology is a risk of bias for evaluating evidence in this type of study. There was high variability among the included studies. For instance, cryopreservation can affect the expression of membrane proteins and intracellular molecules which are used for the classification of memory subsets and identification of senescence markers. Only 28% of the studies did not describe whether the measurement was done on frozen or fresh cells (15, 22, 23, 26, 28–31, 49, 50); 33% of the articles did not report the number of cells used in each experiment (15, 17, 18, 20, 22, 24–26, 28, 31, 35, 36), while only 31% clearly described the antibodies with fluorochrome and clone used (15, 21, 22, 28, 31, 39, 41, 43, 44, 46, 48). The factor with the highest risk of bias is the clarity in the presentation of the results, since only 25% of the studies reported measures of central tendency (16, 27, 33, 37, 41, 42, 47, 48, 50). Most of the studies reported only the graphics and p-value, thus making it difficult to quantitatively compare results and rate evidence. It would be useful to standardize study protocols in order to obtain reliable results that can be compared.

Synthesized Findings

Memory T Cell Subsets

As shown in **Table 1**, among the 36 eligible studies, 15 characterized T cell subsets with different combinations of defining markers. Of these studies, eight (53.3%) reported memory T cells using CD27 and CD45RA (15, 18, 20–23, 25, 28); four (26.6%) used CCR7 and CD45RA (26, 31, 32, 37); two (13.3%) used CD28 and CD95 (34, 45); and one (6.6%) used CCR7 and CD45RO (17), thus confirming the lack of homogeneity for determination of memory subpopulations of T cells. Only five studies (33.3%) reported donor CMV status and memory subsets conjointly (15, 18, 20, 26, 28). For CD8 T cells, 12 studies reported memory subset changes. Six of the studies reported memory subsets exclusively in CD8 T cells (20, 21, 25, 26, 37, 45), six reported differences in CD4 and CD8 T cells (17, 18, 22, 23, 31, 32), two studies reported memory changes exclusively in CD4 T cells (28, 34), and one study exclusively reported changes in γ/δ T cells (15). Herein, we report the subset frequency trends across studies by comparing the young and old populations (**Table 1**).

CD8 T Cells

12 studies explored CD8 T cell subsets (100%). Regarding the naïve compartment, eight (66.6%) reported a higher frequency of naïve CD8 T cells in young adults compared to older adults (20, 22, 23, 25, 31, 32, 37, 45), and just Riddel et al., evaluated CMV status, and both CMV+ and CMV- groups showed this higher frequency in young adults (20). Among the other four studies (33.3%), three did not compare naïve subsets between young and older adults (18, 21, 26), whereas one (8.3%) showed non-significant changes between the groups, and this particular study did not take CMV status into consideration, which could be altering the conclusion drawn by the researchers (17).

In this item, only three papers (22, 37, 45) were eligible for the performance of meta-analysis, which revealed that CD8 naïve T

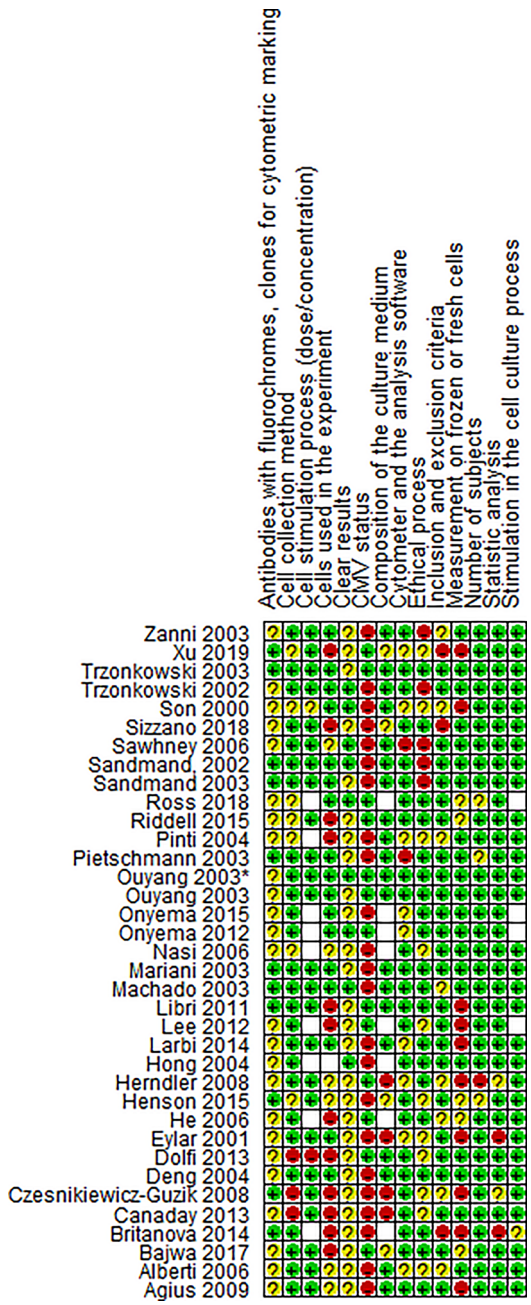

FIGURE 3 | Risk of bias summary for paper. Review authors's judgments about each risk of bias item for each included study.

cells had a higher frequency in young adults compared to older adults, with a mean difference of 33.86 (95% CI = 28.40–39.32, $p < 0.00001$). However, none of these reported CMV status, so the decrease in naive cells in older adults could also be due to CMV infection and not only age. Heterogeneity was high (I^2 = 82%). Effect analysis showed a direction toward the older adults and significant effect of age. Super-olds were not addressed for CD8 T cells due to lack of information (**Figure 4**). Of note, Britanova et al. (22) did neither reported the cryopreservation process nor statistical analysis conducted. Neither Hong et al. (37, 45) nor Zanni et al. (37, 45) fully reported the antibodies along with their clone and fluorochrome used. In this regard, given the aforementioned risks of bias, results should be interpreted with caution.

The memory compartment is divided into CM and EM. CM CD8 T cells were especially heterogeneous between studies. Of these studies, three (25%) reported no significant differences between groups (17, 20, 37): once again, just Riddel et al., considered CMV status and found that even though there was no significant difference between CMV- young and older adults, in the CMV+ older adults there was a lower frequency of this population compared to their younger counterparts (20). This same finding was reported by another article (8.3%), however in this study, CMV status was not assessed (31).Two other articles (16.6%) showed increased CM CD8 T cells in older adults compared to young adults (32, 45). The remaining six (50%) articles did not compare between groups (41.6%) (18, 21, 25, 26) and neither showed significant changes (8.3%) (23) nor interrogated this subpopulation (8.3%) (22). As stated for the naive compartment, these results are highly biased due to a lack of CMV status consideration given that only one of the seven articles that compared CM CD8 T cells, just one made the important distinction between CMV+ and CMV- adults.

On the other hand, EM CD8 T cells in three articles (26%) were elevated in older adults (23, 32, 37). In line with this, although Zanni et al. (8.3%) characterized T cells with CD28 and CD95, the bulk effector compartment was interrogated and it was found that effector CD8 T cells were also higher in the older adults (45). In contrast, Czesnikiewicz-Guzik et al. (8.3%) reported an increased EM cells in young adults (31). The remaining seven studies did not show any trend, since four (33.3%) of them did not compare between groups (18, 21, 25, 26); one (8.3%) did not show significant changes (17), and one (8.3%) did not interrogate this subpopulation (22). Interestingly, when the study included CMV status as a variable (8.3%), the CMV- older adults, unlike their CMV+ counterparts, had a statistically significant increase of this subset compared to CMV- young adults (20).

In four (33.3%) studies, TE CD8 T cells were increased in older adults (20, 23, 31, 37). This was observed in both CMV- and CMV+ groups, when this variable was included (20). Similarly, Zanni et al. (8.3%) reported a higher frequency of effector T cells in older adults (45). The remaining seven studies did not show any trend, since four (33.3%) of them did not compare between groups (18, 21, 25, 26); two (16.6%) showed no differences between groups (17, 32), and one (8.3%) did not investigate this subset (22) (**Table 1**).

CD4 T Cells

Among the eight articles reporting CD4 populations (100%), particularly for naïve T cell compartment, six (75%) studies reported significantly increased naïve subpopulation in young adults (22, 23, 28, 31, 32, 34). and just Libri et al., reported CMV status, showing that this increase was significant only for CMV+ donors (28). One (12.5%) of them reported non-significant changes (17), however as was mentioned for CD8 T cells, this

TABLE 1 | Characteristics of studies reporting memory subsets.

No.	References	Year	Lymphocyte definition	Elderly	Young	CMV status	Memory definition	Stimulation	Elderly (mean ± SD, %)				Young (mean ± SD, %)			
				n	n				N	CM	EM	E	N	CM	EM	E
1	Xu et al. (15)	2019	γ/δ	12	12	Yes	CD27 CD45RA	No	↓	NS	NS	↑	↑	NS	NS	↓
2	Sizzano et al. (17)	2018	CD4/CD8	7	7	No	CCR7 CD45RO	No	NS	NS	NS	NS	NS	NS	NS	NS
3	Bajwa et al. (18)	2017	CD4/CD8	103	48	Yes	CD27 CD45RA	No	NC	NC	NC	NC	NC	NC	NC	NC
4	Riddell et al. (20)	2015	CD8	125		Yes	CD27 CD45RA	No	↓	NS	NS	↑	↑	NS	NS	↓
5	Henson et al. (21)	2015	CD8	8	8	No	CD27 CD45RA	Yes	NC	NC	NC	NC	NC	NC	NC	NC
6	Britanova et al. (22)	2014	CD4/CD8	7	10	No	CD27 CD45RA	No	18,8 ± 12,6	NM	NM	NM	37,5 ± 8,1	NM	NM	NM
7	Larbi et al. (23)	2014	CD4/CD8	15	15	No	CD27 CD45RA	No	↓	NS	↑	↑	↑	NS	↓	↓
8	Dolfi et al. (25)	2013	CD8	5	5	No	CD27 CD45RA	No	↓	NC	NC	NC	↑	NC	NC	NC
9	Lee et al. (26)	2012	CD8	43	62	Yes	CCR7 CD45RA	No	NC	NC	NC	NC	NC	NC	NC	NC
10	Libri et al. (28)	2011	CD4	± 67	± 40	Yes	CD27 CD45RA	No	↓	↑	↑	↑	↑	↓	↓	↓
11	Czesnikiewicz-Guzik et al. (31)	2008	CD4/CD8	26	31	No	CCR7 CD45RA	No	↓	↓	↓	↑	↑	↑	↑	↓
12	Nasi et al. (32)	2006	CD4/CD8	10	12	No	CCR7 CD45RA	No	↓	↑	↑	NS	↑	↓	↓	NS
13	Alberti et al. (34)	2006	CD4	20	12	No	CD28 CD95	No	35,6 +/- 2,3	45,9 +/- 2,8	11,8+/- 1,3		55 +/- 3,6	34,8 +/- 2,8	8+/- 2,4	
14	Hong et al. (37)	2004	CD8	17	17	No	CCR7 CD45RA	No	9.5 +/- 2.6	NS	51.3 +/- 3.4	32.3 +/- 3.7	46.4 +/- 4.1	NS	28.2 +/- 2.8	18.0 +/- 2.5
15	Zanni et al. (45)	2003	CD8	10	10	No	CD28 CD95	No	3.6 +/- 1.4	42.3 +/- 6	54 +/- 6		40.6 +/- 5	36.6 +/- 5	16.8 +/- 9	

*1. Information on Vδ1, mean ± SD not reported, Y were CMV- and O were CMV+ (**Figure 1B**) 2. The significant difference in the percentages of CD4 and CD8 subsets was between young and semi supercentenarians (**Figure 1**) 3. The distributions of CMV-specific CD4 and CD8 T cells among the memory compartments (**Figure S3A**) 4. Results from multiple linear regression fitting age and CMV response as covariates for CD8+ T cell subset composition (1B/**Table S1**) 5. Senescent characteristics in the memory subsets (**Figure 1**) 6. Values in CD8 (**Table 1**) 7. Impact of aging on T cell phenotype and function, values in CD8 (2A and 2 B) 8. Senescent and inhibitory characteristics in the memory subsets (**Figure S1**) 9. Association of CMV infection with the frequency of CD8+ T cell subsets in young and elderly people. (**Figure S1**) 10. Frequencies of each population within total CD4+ T cells are represented by grouping via age and CMV status, changes in N, EM and E subsets were CMV dependent (**Figure 1C**) 11. Values in CD8 T cells (**Figures 3B, D**) 12. The old group is centenarian (**Figure 2**) 13. Stimuli with PMA/ionomycin did not change CD95 and CD28 expression. Stimulation was carried out to analyze modifications in the intracellular production of cytokines (**Table 1**) 14. (**Figure 1B**) 15. Stimuli with PMA/ionomycin did not change CD95 and CD28 expression. Stimulation was carried out to analyze modifications in the intracellular production of cytokines (**Table 1**). The figures and tables correspond to the original article cited. N, naive; CM, central memory; EM, effector memory; E, effector; ↑, increased; ↓, reduced; NS, no changes or no significant changes between the groups; NM, not measured; NC, not compared between the groups; NR, not reported.*

lack of change could be explained by the low number of subjects and neglecting the CMV status of the participants, while the other (12.5%) did not compare between groups (18).

Analysis of two papers (22, 34) which were eligible for performance of meta-analysis revealed that CD4 naïve T cells also had a higher frequency in young adults compared to older adults, with a mean difference of 18.65 (95% CI = 14.91–22.39, $p < 0.00001$). Unfortunately, neither of these articles measured CMV status of the participants, which leads to a significant bias of the mentioned effect. Heterogeneity was low ($I^2 = 18\%$). Effect analysis showed a direction toward the older adults and significant effect of age. When super-olds were addressed, heterogeneity increased ($I^2 = 74\%$) and the effect was not significant, presumably because of the SD in Britanova et al. (22) (**Figure 5**). Of note, Alberti et al. (34) neither reported the antibodies along with their clone and fluorochrome nor the cytometer and software analysis. In this regard, these results should be interpreted with caution.

As with CD8 T cells, the memory compartment is divided into CM and EM. In four (50%) articles, CM CD4 T cells showed an increased proportion in the aged group (23, 28, 32, 34), whereas this subset showed no differences between groups in two (25%) articles (17, 31). The remaining two (25%) studies neither compared between groups (18) nor interrogated this subpopulation (22). Interestingly, Libri et al. reported increased CM subset only in old CMV- donors (28).

Considering EM CD4 T cells, three (37.5%) articles reported higher frequencies in the older group (23, 28, 32). As in Zanni et al. in CD8 T cells, Alberti et al. (12.5%) reported the bulk effector compartment with CD28 and CD95 in CD4 T cells and found an increase in old adult (34, 45). The remaining four

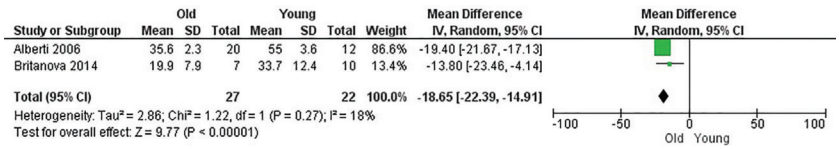

FIGURE 4 | Influence of age on naive CD8+ T cell frequency. Forest plot for the different outcomes regarding cell frequency between old and young groups. The forest plot displays the SMD (squares) and 95% confidence interval of the individual studies. The diamond in each plot indicates the overall estimate and 95% confidence interval.

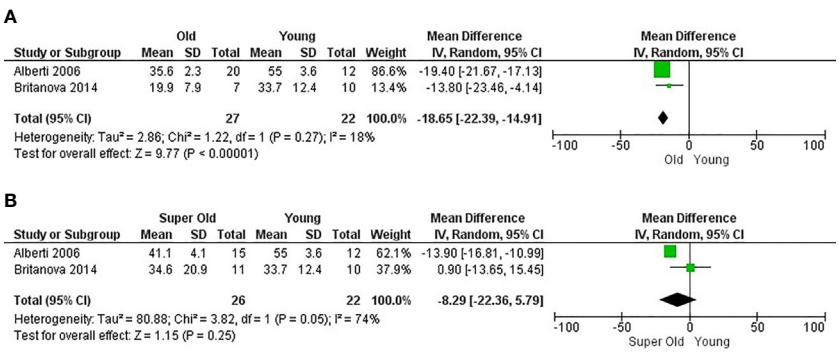

FIGURE 5 | Influence of age on naive CD4+ T cell frequency. Forest plot for the different outcomes regarding cell frequency. **(A)** Changes in the frequency of CD4+ naive T cells between old and young groups and **(B)** Changes in the frequency of CD4+ naive T cells between super old and young groups.

studies (50%) did not show any trend, since two (25%) of them showed non-significant changes (17, 31); one (12.5%) neither compared between groups (18) nor investigated this subset (12.5%) (22).

TE CD4 T cells were increased in older adults in two (25%) articles (23, 28). Similarly, Alberti et al. (12.5%) reported a higher frequency of effector T cells in older adults (34). The remaining five studies (62.5%) did not allow us to establish any trend, since three (37.5%) of them showed non-significant changes (17, 31, 32); one (12.5%) neither compared between groups (18) nor interrogated this subset (12.5%) (22) (**Figure 6**).

Of note, Libri et al. reported increased frequencies of EM and TE T cells in CMV-infected donors in both age groups as well as in old CMV+ compared to young CMV+ donors (28).

γδ T Cells

Among γδ T cells, those from young CMV+ adults had similar percentages of memory subsets with those of old CMV+ adults. In all three subsets of γδ T cells CMV- young adults had a higher frequency of naive cells than CMV+ adults. Concomitantly, an increase of TE cells was observed in the CMV+ young adult group (15) (**Table 1**).

Immunosenescence and Exhaustion Markers

21 out of 36 articles evaluated markers associated with immunosenescence and exhaustion. Of these studies, nine (42.8%) measured both CD4 and CD8 T cells (16, 24, 31–33, 36, 40, 41, 48), nine (42.8%) measured CD8 T cells (19, 21, 25, 27, 30, 35, 42, 44, 47), two (9.5%) measured CD4 T cells (28, 29), and

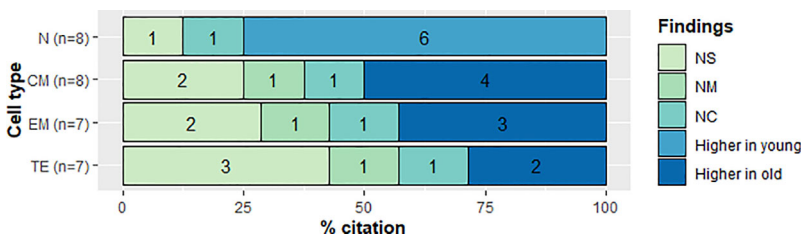

FIGURE 6 | Qualitative analysis of frequency in CD4+ T cell subsets. CD4+ T cell subset and citation percent across studies comparing young and old populations. N, naive; CM, Central Memory; EM, Effector Memory; TE, Terminally Effector; NS, No Significant change; NM, Non measured; NC, not compared.

one (4.7%) measured γδ T cells (15). Expression of these markers in different subsets of CD4 or CD8 T cells was determined in eight studies (38%) (15, 19, 21, 25, 28, 30, 33, 35, 36); 11 (52.3%) studies measured the total population of T cells (CD4 or CD8) (16, 24, 29, 31, 32, 40–42, 44, 47, 48), and the remaining two (9.5%) measured the total population and subsets of T cells (25, 27). Eight (38%) considered serology for CMV (15, 16, 27, 28, 35, 40, 42, 44) and six (28.5%) performed *in vitro* stimulation to measure expression of the markers (21, 24, 33, 40, 42, 44) (**Table 2**).

The main markers evaluated were CD57 (47.6%), CD28 (42.8%), KLRG1 (23.8%), CD95 (19%), PD-1 (19%), CTLA-4 (9.5%), p16 (9.5%), p21 (9.5%), γH2AX (9.5%), CD85j (9.5%), and CD11a (9.5%) (15, 16, 19, 21, 24, 25, 27, 28, 30, 31, 33, 35, 36, 40, 42, 44, 47, 48). The following molecules were evaluated in one article at a time: CD127, CD31, CD244, CD245, Bcl-2, p38, ICOS, LAG-3, TIM-3, 2B4, T-bet, Eomes, IL-7Rα, Bcl-2, CLA, CCR4, CD62L, CD126, CD26, CD95L, HLA-DR, CD69, and P-glycoprotein 1 (15, 16, 19, 21, 24, 25, 28–33, 40, 41).

In γδ T cells, the CD57 expression significantly increased in aged subjects CMV+ in Vδ1+ and Vδ1−Vδ2− subsets, but no significant differences in young CMV- and CMV+ in any subsets (15). CD8 T cells expressing CD57 were more frequent in older adults (19, 21, 25, 27, 31, 35, 44). The expression of this marker on various memory subsets was increased in TE population in both age categories. However, this happened to a lesser extent in young adults (21). In CD4 T cells, upregulation of CD57 was highly correlated with age (31). Likewise, the expression of CD57 was higher in TE and EM subsets than N and CM subsets in CMV seropositive subjects (28).

The percentage of CD8 and γδ T cells (Vδ1+ and Vδ1−Vδ2−) expressing KLRG1 was increased in older adults (15, 21, 25, 27, 42). Likewise, CD95 expression was increased in CD8 T cells from older adults compared to young adults (33, 36, 48). CD95 expression was highest in TE when compared to the other memory subsets in CD8 T cells (19). The frequency of CD95 expressing CD4 T cells was higher in older adults than in young adults (33, 36, 48).

On the other hand, CD28 expression decreased in CD8 T cells from older adults compared to young adults and was correlated with CMV+ status (16, 19, 27, 31, 35, 40, 44, 47). Loss of CD28 expression is more frequent in CD8 than CD4 T cells (31). Libri et al. reported low CD28 expression in EM and TE CD4 T cells compared to naïve and CM T cells (28). In general, CD4 T cells are more resistant to age-associated phenotypic changes than CD8 T cells (31).

Among the studies selected for this review, only four measured the expression of PD-1 (19, 21, 24, 25). Two reported upregulation of PD-1 in older adults compared to young adults (21, 25). In addition, PD-1 expression was upregulated in CM and EM subsets, showing that PD-1 is augmented in old and differentiated populations (19, 21, 24, 25). Interestingly, Canaday et al. reported lower expression of PD-1, higher expression of CTLA-4, and lower expression of Tim-3 in older adults (24). Dolfi et al. also reported an increased expression of LAG-3 in T cells from older adults compared to young adults (25). Due to heterogeneity among study design, these reports are not conclusive.

Proteins p16, p21, and γH2AX were measured in few articles (19, 21, 27); however, all articles reported an increased expression of these molecules in older adults. Considering the lack of studies on these molecules, it was not possible to assume their utility for identifying senescent T cells, even when these molecules have been considered as distinctive of replicative senescence in other cell types.

Cytokines

Of the 36 eligible studies, 15 reported measurement of different cytokines and serine proteases such as IFNγ, TNFα, IL-2, IL-4, IL-6, IL-10, granzyme, and perforin. Of these studies, 12 measured at least one of the above mentioned cytokines in CD8 T cells; seven in CD4 T cells, and one in γδ T cells, considering the total populations or subsets by memory, markers or antigen specificity (**Table 3**).

For CD8 T cells, ten studies measured IFNγ (20, 21, 23, 25, 38, 42, 43, 45, 48, 49), seven measured TNFα (20, 21, 23, 25, 45, 46, 49), three measured IL-2 (20, 25, 45), three measured IL-4 (43, 45, 48), two measured IL-10 (44, 45), two measured granzyme B (21, 25), one measured perforin (21), and one measured IL-6 (45). In the case of IFNγ, 70% of the studies reported a higher percentage of IFNγ-producing CD8 T cells in older adults than young adults; 20% reported non-significant statistical differences (21, 23, 25, 43, 45, 48, 49), and 10% reported a decrease (42). In the latter, it should be noted that the measurement was performed in a subset which included the KLRG1 marker (42). For TNFα, IL-2, IL-4, IL-10, and IL-6, 85.7% (21, 23, 25, 45, 46, 49), 100% (20, 25, 45), 100% (43, 45, 48), 100% (44, 45), and 100% (45) of the studies found higher percentage of CD8 T cells producing these cytokine, respectively. Two studies evaluated CD8 T cells expressing two or more cytokines simultaneously (IL-2, TNFα, and IFNγ), thus finding a higher percentage of CD8+ IL-2+ TNFα+ (21) and CD8+ IFNγ+ TNFα+ (45) subpopulations in older adults. In addition, 100% of the granzyme B (21, 25) and perforin (21) studies showed a lower proportion of CD8 T cells producing these cytotoxic molecules. In the overall analysis, the percentages of cytokines producing CD8 T cells were higher, while the percentage of serine protease was lower in older adults.

Regarding CD4 T cells, there are major heterogeneous results. For IFNγ, four out of the six studies reported a non-significant difference between the age groups (29, 38, 43, 49), the remaining reported a lower level in older adults (34, 48). Three studies measured TNFα (34, 46, 49), with 66% of them (46, 49) showing an increase in these cytokines in the elderly, as opposed to one article (33%) that reported a decrease in older adults (34). Three studies measured IL-4 (34, 43, 46), with 66% (43, 46) showing an increase in older adults, while the remaining percentage reported non-significant differences between the groups (34).

In addition, four studies evaluated the relationship between cytokine production, memory phenotype, and age (20, 21, 34, 45). Henson et al. (21) reported that naïve and EM CD8 T cells from older adults appear more polyfunctional compared to those

TABLE 2 | Characteristics of studies reporting immunosenescence markers.

No.	References	Year	Lymphocyte definition	Elderly	Young	CMV status	Populations	Stimulation	Markers in elderly									
				n	n				CD28	CD57	KLRG1	CD95	PD1	CTLA4	TIM3	p16	p21	Others
1	Xu et al. (15)	2019	γ/δ	12	12	Yes	Subsets	No	NM	↑	↑	NM	NM	NM	NM	NM	NM	↑CD85j, ↑CD244+, NC : γH2AX
2	Ross et al. (16)	2018	CD4/CD8	10	9	Yes	Total	No	↓	NM	NM	NM	NM	NM	NM	NM	NM	↓CD31
3	Onyema et al. (19)	2015	CD8	11	11	No	Subsets	No	↓	↑	NM	NC	NC	NM	NM	NC	↑	↑CD245, NC Bcl-2
4	Henson et al. (21)	2015	CD8	8	8	No	Subsets	Yes	NM	↑	↑	NM	↑	NM	NM	NM	NM	↑ p38, ↑γH2AX
5	Canaday et al.	2013	CD4/CD8	24	24	No	Total	Yes	NM	NM	NM	NM	↓	↑	↓	NM	NM	↓ ICOS in CD4 *ex vivo*
6	Dolfi et al. (25)	2013	CD8	± 38	± 37	No	Total/ subsets	No	NM	↑	↑	NM	↑	NM	NM	NM	NM	↑LAG-3 ↑2B4, ↑T-bet, ↑Eomes
7	Onyema O et al. (27)	2012	CD8	11	11	Yes	Total/ subsets	No	↓	↑	↑	NM	NM	NM	NM	↑	↑	
8	Libri et al. (28)	2011	CD4	25		Yes	Subsets	No	NC	NC	NM	NM	NM	NM	NM	NM	NM	IL-7Rα, Bcl-2
9	Agius et al. (29)	2009	CD4	± 9	± 11	No	Total	No	NM	NM	NM	NM	NM	NM	NM	NM	NM	NS: CLA, CD11a, CCR4
10	Herndler-Brandstetter et al. (30)	2008	CD8	NR	NR	1.1.1 Yes	Subsets	No	NM	NS	NM	NM	NM	NM	NM	NM	NM	NS : CD62L, CD11a, CD126,
11	Czesnikiewicz-Guzik et al. (31)	2008	CD4/CD8	41	68	No	Total	No	↓	↑	NM	NM	NM	NM	NM	NM	NM	↑ CD85j, ↓ CD26
12	Nasi et al. (32)	2006	CD4/CD8	7	7	No	Total	No	NM	NM	NM	NM	NM	NM	NM	NM	NM	NS : CD127
13	Sawhney et al. (33)	2006	CD4/CD8	25	20	No	Subsets	Yes	NM	NM	NM	↑	NM	NM	NM	NM	NM	CD4: ↑CD95L; CD8: NS CD95L
14	He et al. (35)	2006	CD8	15	22	Yes	Subsets	No	↓	↑	NM	NM	NM	NM	NM	NM	NM	
15	Pinti et al. (36)	2004	CD4/CD8	14	13	No	Subsets	No	NM	NM	NM	↑	NM	NM	NM	NM	NM	
16	Ouyang et al. (40)	2003	CD4/CD8	5	5	Yes	Total	Yes	↓	NM	NM	NM	NM	NM	NM	NM	NM	NS: HLA-DR, CD69, CD45RO/RA
17	Machado et al. (41)	2003	CD4/CD8	± 10	± 20	No	Total	No	NM	NM	NM	NM	NM	NM	NM	NM	NM	↓ P-glycoprotein 1
18	Ouyang et al. (42)	2003	CD8	70	11	Yes	Total	Yes	NM	NM	↑	NM	NM	NM	NM	NM	NM	
19	Trzonkowski et al. (44)	2003	CD8	91	63	Yes	Total	Yes	↓	↑	NM	NM	NM	NM	NM	NM	NM	
20	Trzonkowski et al. (47)	2002	CD8	65	31	No	Total	No	↓	NM	NM	NM	NM	↓	NM	NM	NM	
21	Sandmand et al. (48)	2002	CD4/CD8	15	26	No	Total	No	NM	NM	NM	↑	NM	NM	NM	NM	NM	

*1. Information of Vδ1mean ± SD not reported (**Figures 1I**, **3B**), 2. (**Figure 2A**). 3. Expression of the different markers among subpopulations of CD28/CD57, (**Figures 2**, **4**). 4. The T cell subsets from old individuals express a greater array of senescent markers relative to young individuals (**Figure 1**), 5. Dates shown in CD8 (**Figure 1D**), 6. Dates in Total CD8 T cell (**Figures 1A**, **2A**, **3B**), 7. Only in $CD28^{-}$ CD57+ cells a significantly higher p16 expression was found in the elderly compared to the Young subjects (**Figure 2B**, 3 y 4), 8. (**Figure 3A**), 9. (**Figure S1**), 10. CD8+CD45RO+CD25+ from elderly vs CD8+CD45RA+CD28+ from young (**Figure 1B**), 11. Dates in total CD8 T cells (**Figures 2A**, **5A**), 12. (**Figure 5**. and text) 13. (**Figures 2**, **3**, **4**), 14. CD57 increased and CD28 decreased in tetramer negative CD8 T cells, NS in tetramer-positive cells (**Figures 2B, C**, **3B, C**), 15. No increase observed at single-cell level on CD45RA negative T lymphocytes (**Figure 4**), 16. CMV peptide-specific CD8 (**Table 1**), 17. (**Figures 1**, **2**), 18. (**Figure 3B**), 19. Comparison between young responders and old (**Figure 1A**), 20. (**Table 1**), 21. There was no difference between the two elderly groups (Centenarians >100 years, n=25), (**Table 1**). The figures and tables correspond to the original article cited. ↑, increased in older; ↓, reduced in older; NS, no changes or not significant changes between the groups; NM, not measured; NC, not compared between the groups; NR, not reported.*

TABLE 3 | Characteristics of studies reporting cytokine.

No.	References	Year	Lymphocyte definition	Elderly	Young	1.1.2 CMV status	Populations	Stimuli	Cytokines							
				n	n				IFNγ	TNFα	IL-2	IL-4	IL-10	Granzyme B	Perforin	Others
1	Xu et al. (15)	2019	γ/δ	12	12	1.1.3 Yes	Vδ1/N vs E	1.1.4 PMA+ionomycin	↑	↑	↓	NM	NM	NM	NM	↑MIP-1α
2	Riddell et al. (20)	2015	CD8	8	8	1.1.5 Yes	Total/CD27 CD45RA	1.1.6 Anti-CD3 beads+IL-2	NS	NS	↑ CD8	NM	NM	NM	NM	
				7	7		CMV specific CD8/CD27 CD45RA	Peptide+IL-2	NS	NS	NS	NM	NM	NM	NM	
3	Henson et al. (21)	2015	CD8	8	8	1.1.7 No	CD27 CD45RA	1.1.8 Anti-CD3 beads	↑	↑	NM	NM	NM	↓	↓	
4	Larbi et al. (23)	2014	CD8	15	15	1.1.9 No	CD8	1.1.10 PMA+ionomycin	↑	↑	NM	NM	NM	NM	NM	
5	Dolfi et al. (25)	2013	CD8	44	54	1.1.11 No	1.1.12 Total/ virus specific CD8	1.1.13 Peptide or SEF	↑	↑	↑	NM	NM	↓	NM	↓MIP-1β
6	Agius et al. (29)	2009	CD4	± 8	± 8	1.1.14 No	Total	1.1.15 Peptide	NS	NM	NM	NM	NM	NM	NM	
7	Alberti et al. (34)	2006	CD4	20	12	1.1.16 No	CD95 CD28	1.1.17 PMA+ionomycin	↓	↓	NM	NS	NM	NM	NM	
8	Deng et al. (38)	2004	CD4/CD8	11	5	1.1.18 No	CD4/CD8	1.1.19 Peptide	NS	NM	NM	NM	NM	NM	NM	
9	Ouyang et al. (42)	2003	CD8	70	11	1.1.20 Yes	CD8+KLRG1 + CD8 +KLRG1−	1.1.21 PMA+ionomycin	↓KLRG1 +	NM	NM	NM	NM	NM	NM	
10	Pietschmann et al. (43)	2003	CD4/CD8	79	75	1.1.22 No	CD4/CD8	1.1.23 PMA+ionomycin	↑CD8	NM	NM	↑	NM	NM	NM	
11	Trzonkowski et al. (44)	2003	CD8	91	63	1.1.24 Yes	CD8	1.1.25 Peptide then PMA +ionomycin	NM	NM	NM	NM	↑	NM	NM	
12	Zanni et al. (45)	2003	CD8	10	10	1.1.26 No	CD95 CD28	1.1.27 PMA+ionomycin	↑	↑	↑	↑	↑	NM	NM	↑ IL-6
13	Sandmand et al. (46)	2003	CD4/CD8	14	25	1.1.28 No	CD4/CD8	1.1.29 PMA+ionomycin	NM	↑	NM	NM	NM	NM	NM	
14	Sandmand et al. (48)	2002	CD4/CD8	14	24	No	CD4	PMA+ionomycin	↓	NM	NM	↑	NM	NM	NM	
							CD8		↑	NM	NM	↑	NM	NM	NM	
15	Eylar et al. (49)	2001	CD4/CD8	40	48	1.1.30 No	CD4/CD8	1.1.31 Antibody anti-CD3+PMA	↑ CD8	↑	NM	NM	NM	NM	NM	

*1. Information of Vδ1mean ± SD not reported; NS in Vδ2+ ((**Figures 2A, D**), 2. Measurement of double and triple positive populations for IFN/Y, TNF-a and IL-2, finding an increase in the elderly in the percentage of total CD8+ producers of IL-2 and TNF-a (CD8+ IL-2+ TNF-a+), as well as an increase in CD8 + CMV + being these triple positive for IL-2, TNF-a and IFN-y (**Figures 5B, D**, **6B, D**), 3. ↑ IFN-y in CD27+CD45RA+, CD27–CD45RA– and CD27–CD45RA+; ↑ TNF-a in CD27+CD45RA+ and CD27– CD45RA+; ↓ Granzyme B in CD27+ CD45RA–, CD27– CD45RA– and CD27– CD45RA+; ↓Perforin in CD27+CD45RA+, CD27–CD45RA– and CD27–CD45RA+ (**Figure 3C**), 4. The capacity of CD4 T cells to produce cytokines no change between the groups. Data of a representative case (**Figure 2D**), 5. Stimuli with CMV showed similar results as in influenza stimuli. The ability to produce multiple cytokines simultaneously was decreased in the elderly compared with young (**Figures 5**, **S2B, S2C**). 6. Values in blood, IFN γ-producing CD4 in elderly in blisters is significantly reduced compared to young population (**Figure 2E**) 7. ↓IFN-y: CD95–CD28+ and CD95+ CD28+; ↓ TNF-a: CD95+ CD28+; Nonagenarians (n=15): ↓ IFN-y in CD4+ CD95–CD28+ and CD4+ CD95+ CD28+; ↑ IL-4 in CD4+ CD95+ CD28+ (**Figures 3**, **4**, **5**). 8. Lower mean fluorescent intensity of IFN-y in T cells compared with that in young subjects (**Figures 2B, D**). 9. (**Figure 4**), 10. ↑ IFNγwas just for old women; NS in IL-4- producing CD4 in women (**Figures 1**, **2**), 11. (**Figure 3**), 12. ↑IL-4: CD95+ CD28-; ↑IL-6: CD95+ CD28+ and CD95+ CD28-; ↑IL-10: CD95+ CD28– (**Figures 4**, **5**). 13. Centenarians (>100-year-olds, n = 21): Increase in TNF-a-producing cells among CD8+ cells, but not CD4+ cells (**Figure 2**), 14. Centenarians (>100-year-olds, n = 25): Increase in TNF-a and IL-4-producing cells among CD8+ cells and increased only in IL-4 for CD4 compared to young (**Figure 1**), 15. (**Table 1**). The figures and tables correspond to the original article cited. ↑ is increased in older. ↓, reduced in older; NS, no changes or no significant changes between the groups; NM, not measured; PMA, phorbol myristate acetate; SEF, Staphylococcus enterotoxin F.*

from young adults, while Riddell et al. (20) reported no significant difference between the groups. Moreover, Alberti et al. and Zanni et al. reported increases in both Th1 and Th2 cytokine-producing CD4 and CD8 in non-naïve T cells in older adults (34, 45).

Finally, only one study evaluated the aforementioned cytokines in γδ T cells, finding that older adults have a higher percentage of IFNγ and TNFα-producing γδ T cells and decreasing numbers of IL-2 producing cells (15).

Telomere Length

Five studies measured telomere length: three (60%) on CD8 T cells (20, 30, 39); one (20%) on CD4 and CD8 T cells (50), and one (20%) on γδ T cells (15). Three (60%) measured telomere length in total population of T cells (30, 39, 50), while the remaining two (40%) measured telomere length in memory T cell populations (15, 20) (**Table 4**). Xu et al. showed a decrease in telomere length in EM cells compared to naïve and CM cells; however, in Vδ1+ and Vδ1− Vδ2− subsets of γδ T cells, they also found that CD57+ cells had a significant shortening in telomere length in all γδ T cell types when compared to CD57−. No comparison was done between the age groups (15).

The studies that measured telomere length in CD8 T cells (20, 30, 39, 50) showed that the different memory subsets and whole CD8 T cells had significant reduction of telomere length in older adults compared to young adults. Riddell et al. stratified young and older adults based on their CMV status and found that young CMV+ adults had shorter telomeres in all memory subsets compared to their CMV- counterparts. In older adults, the difference between CMV+ and CMV- was not significant. In older adults, the difference between CMV+ and CMV- was not significant (20). Son et al. showed a reduction of telomere length in CD4 T cells as a function of increasing age (50). CD4 T cells from both young and older adults showed longer telomeres than CD8 T cells (50). Telomere shortening may be a useful marker for immunosenescence in T cells, but other mechanisms are also involved in restricting their proliferative capacity (20).

DISCUSSION

The generation and maintenance of antigen-specific memory T cells is crucial for long-term immune protection and effective vaccination. Four memory subsets have been canonically described: naïve, central memory (CM), effector memory (EM), and terminally-differentiated effector cells (TE) which can be characterized by presence of surface markers including CCR7, CD45RA/RO, CD27, CD28, CD62L, and CD95, among others (51, 52). Despite the fact that total numbers of T cells remain relatively constant with aging, significant changes have been observed in the composition of memory subpopulations (i.e., naïve vs. memory cells) (53).

All included studies that compared the distribution of memory populations between young and older adults found a considerable reduction of the naïve subset in older adults, both in CD4 (17, 22, 23, 28, 31, 32, 34) as seen in CD8 (17, 22, 23, 25, 31, 32, 37, 45), although the magnitude of the latter was much greater (31, 32). This finding is consistent with literature reviews in the field and the low percentage of naïve cells could partially explain the increased susceptibility in older adults to new infections and development of malignant pathologies (54, 55). As shown in **Figures 4** and **5**, a meta-analysis was performed with four studies and significant results were only found when comparing the naïve subset from young and older adults. However, the risk of bias was high in two of these studies (**Figure 3**); therefore, these results should be evaluated with caution.

One of the factors that most contributed to this heterogeneity is the definition of age groups. Populations designated as "young adults" were aged 18 to 44 years and populations of "older adults" were aged 60 to 107 years. Similarly, the creation of subgroups within the older adults to assess whether the observed differences increased linearly with age is also done arbitrarily and adds to this high heterogeneity, thus negatively affecting integrative analysis in the field of immunosenescence (**Table 5**). The highest variability was observed in the "older" group, whose cutoff point was 70 (22, 32), 80 (17–19, 21, 23, 34),

TABLE 4 | Characteristics of studies reporting telomere length.

No	References	Year	Lymphocyte definition	Elderly	Young	1.1.32 CMV Status	Memory definition	Elderly (mean ± SD)				Young (mean ± SD, %)			
				n	n			N	CM	EM	E	N	CM	EM	E
1	Xu et al. (15)	2019	γ/δ	9		Yes	CD27 CD45RA/ CD57+	NC	NC	NC	NC	NC	NC	NC	NC
2	Riddell et al. (20)	2015	CD8	27	38	Yes	CD27 CD45RA	5,7 ± 1,92	4,6 ± 1,42	4,0 ± 1,26	4,1 ± 1,32	10,1 ± 2,82	8,2 ± 2,78	7,0 ± 2,57	7,7 ± 2,73
3	Herndler-Brandstetter et al. (30)	2008	CD8	NR	NR	Yes	CD45RARO/ CD28 CD25	5.1 ± 0.4				7.1 ± 0.1			
4	Mariani et al. (39)	2003	CD8	10	18	No	Total	↓				↑			
5	Son et al. (50)	2000	CD4/CD8	30	22	No	Total	CD4+: 6.7 ± 2 CD8+:5.5 ± 1.7				CD4+: 8.7 ± 1.7 CD8+: 6.9 ± 1.8			

*1. Vδ1 decrease of telomere length from Naïve to EM (**Figure 3H**), 2. (**Figure 3B**), 3. CD45RO+CD25− from elderly vs CD45RA+CD28+ from young (**Figure 3**), 4. Young vs 80 years old (**Figure 3B**), 5. Third group: 80–94 years (n = 19) CD4: 6.2 ± 1.9; CD8: 5.1 ± 1.9 (**Table 1**). The figures and tables correspond to the original article cited. NS, no changes or no significant changes between the groups; NM, not measured; NC, not compared between the groups.*

TABLE 5 | Summary: suggestions for diminishing heterogeneity in the study of immunosenescence.

- To Perform screening of CMV status since it is an associated confounder with aging.
- To Establish Consensus among experts to define age groups to be able to compare studies.
- To Be more descriptive in the research protocol detailing the stimuli, the number of cells, culture media, antibodies, fresh or frozen cells.
- To Report measurements and statistical values for all experiments (not just the p-value).
- To Show and report both positive and negative results.

90 years (28, 31, 45) or adults ≥65 years of age (15, 20, 25, 26, 37). In the "super-olds" classification, adults aged 70 to 100 years were included (18, 22, 34) and the least variable classification was that of "centenarians", which included adults aged 98 to 107 years (17, 32).

The inclusion of age groups with ranges not greater than 10 years in research protocols would greatly facilitate the comparison between studies carried out by different groups within the field, thus making findings more significant. Another alternative that could improve the quality of immunosenescence research is the addition of longitudinal studies that correlate the percentages of memory populations with aging. Using this method, studies where non-statistical significance was found in comparisons between groups could find negative correlations between the percentage of CD4 and CD8 naïve cells with age (17, 20, 28), as well as positive correlations between CD4 memory cells (17, 28) and CD8 TE (17, 20) with age. In this way, a first approach to evaluate the overall effect of aging on different cellular subpopulations could be done.

However, these studies also have their limitations and should only be complementary to subgroup comparisons, since centennial populations may behave like young or middle-aged adults and differ from older adults. For instance, Britanova et al. (22) showed a decrease in naïve cells with aging in the correlation analysis, but long-lived adults had a higher percentage of naïve T cells than older adults, thus suggesting the existence of homeostatic mechanisms or healthy aging in long-lived adults.

Achieving a reduction in variability could aid a better understanding of the redistribution of memory populations since, unlike the naïve subpopulation, their behavior showed contradictory results in the studies included, which made it impossible to assess the global effect of aging on these populations. One of the factors that could affect the modulation of these populations is the presence of chronic infections such as CMV (56). This particular viral infection is known to impact and shape immune cell differentiation and exhaustion, acting in many cases as a confounder of age itself in immunosenescence studies (15). As stated before, only a third of all included articles in this systematic review reported donor CMV status as a screening method. Here, we sought to discuss the main results in light of their CMV status.

Multiple linear regression analysis performed by Libri et al. allow them to discriminate the effect of CMV seropositivity and age itself on CD4 T cell memory subset frequency and found that age and CMV serostatus both contribute to the decrease in naive T cells during ageing but the increase in CM, EM and TE T cells in old individuals is apparently the result of age itself for CM subset and primarily result of CMV infection for EM and TE subsets (28).

Riddel et al., using linear regression as well, showed a different distribution of memory subsets of CD8 T cells with age depending on the CMV status of the individuals. Although the reduction of the naive compartment and increase of the TE with age was a shared feature, the CM and EM subsets had different behaviors in the CMV+ and the CMV- groups, which could contribute to the understanding of the immunomodulatory effect of CMV infection (20). For instance, CMV+ older adults had not only a reduction of the naive compartment, but of the CM subset as well, suggesting that chronic infection has an effect on these two subsets, which are particularly important to respond to new infections and maintain long-lasting memory of vaccine-induced response, respectively. On the other hand, CMV- older adult cells seem to not differentiate all the way to TE cells, but some of them seem to be able to stay with an EM phenotype, since there was also an increase in older adults compared to young adults. This could provide CMV- older adults with a better suited response to reinfection, as well as diminishing the inflammatory environment produced by TE cells, indicating that CMV infection leads to "inflammaging" through TE induction (20, 26). Of note, Bajwa et al. only reported results in memory subsets following CMV-specific T cell expansion in seropositive individuals (18).

On the other hand, analysis of memory subsets within γδ T cells showed significant changes in the naive and TE compartments. Due to a lack of a seronegative CMV older adult group, an age-dependent change is not possible to infer. A decrease in the frequency of naive cells in all three subsets of γδ T cells between CMV+ and CMV- young adults shows the immunomodulatory impact of CMV infection on memory generation and maintenance. Concomitantly, an increase of TE cells was observed in CMV+ young adults, suggesting that CMV infection induces γδ T cells to differentiate. As for the CMV+ older adult, no significant changes were reported by Xu et al. in comparison to CMV+ young adults, showing that this change of proportions of the different memory subsets is dependent on CMV serostatus and not age (15).

Similarly, some authors (19, 21, 25) reported important functional changes in both naïve and memory populations, thus suggesting greater differentiation in cells from older adults. For this reason, the use of a third marker associated with differentiation and/or functionality such as CD57 or CD28 could complement memory assessment, thereby improving flow cytometry panels for study of immunosenescence and exhaustion (19, 27).

In literature, different markers have been reported to evaluate immunosenescence and exhaustion in T cells. These markers are expressed when cells start to activate and differentiate into effector populations and are therefore increased in people who have experienced a higher number of antigenic encounters throughout their life. One of these markers is CD28, a co-

stimulatory molecule required for T cell activation that diminishes its expression under repeated antigen stimulation, making the cells less prone to correctly activate (19, 31). Furthermore, CD57 has been described as a marker of terminal differentiation, thus increasing in T cells from old people. Onyema et al. (19) used these two markers to classify CD8 T cells and found that, in young donors, 80% of the cells were CD28+CD57−, indicating a less differentiated and exhausted CD8 population. In old donors, this population was diminished, while an immunosenescent CD57+ phenotype was more prevalent.

Donors used in experiments carried out by He et al. (35) were all CMV+ and results observed as increased CD57 and decreased CD28 expressions in the older group were obtained exclusively in CD8 T cells A2-NLV tetramer negative, as opposed to reports from Ouyang et al. (40) that also showed decreased CD8 CD28+ T cells from seropositive old people but in tetramer-specific cells. Change of CD57 expression in γδ T cells was only statistically significant in older adults compared to CMV- young adults. Since there was no difference between CMV+ and CMV- young adults, or CMV+ young and older adults, age and CMV infection could have a synergic effect causing the upregulation of CD57 (15).

On the other hand, Pera et al. found a significant increase in $CD28^-$ CD4 T cells in CMV^+ individuals compared to CMV^- individuals. Similarly, the frequencies of $CD28^-$ CD8 T cells were generally higher in CMV^+ individuals. In contrast, the effect of age on $CD28^-$ CD4 and CD8 T cells is small. They concluded that the diminished expression of CD28 in T cells strongly suggests that these observations reflect CMV-associated immunomodulation rather than normal immunosenescence (57).

KLRG1 is also expressed in differentiated cells and mediates inhibitory effects (7). The expression of this molecule was found to increase in T cells from older adults (15, 21, 25, 27, 42), suggesting that this marker could possibly reflect an immunosenescent state. CD95 is a multifunctional receptor that is upregulated in response to activation and, depending on signals from the environment, can induce cell death (33). An increase in this receptor was found in CD8 T cells from older adults compared to young adults (33, 36, 48). On these cells, expression of CD95 could regulate apoptotic signaling, thereby making senescent cells resistant to apoptosis. However, it is important to clarify that due to the heterogeneity of the studies, a definitive conclusion cannot be made.

Of note, It seems that Ouyang et al. (42) divided their study in CMV donor status known and CMV donor status not known. Results shown regarding increased KLRG1 in older people and reduced IFN-γ in CD8 KLRG1+ T cells in our analysis were taken from the former because experiments carried out in the CMV donor status known group included increased KLRG1 expression in CD8 CMV-specific T cells in the old group.

The proteins PD-1, CTLA-4, and LAG-3 are inhibitory receptors that can be expressed after persistent antigenic stimuli, leading to functional exhaustion of T cells characterized by proliferative failure and loss of cytokine production (24). The p16 and p21 proteins belong to the cyclin-dependent kinases family which regulates cell cycle progression. Their expression can increase as a consequence of genotoxic stress and inhibiting cell proliferation as a preventive measure for malignant transformation (58). Similarly, γH2AX protein is activated in response to DNA damage. Although few studies were found to measure these molecules, they report an increased expression of these proteins in older group, in accordance to what is reported in literature as key characteristics of cellular senescence (21).

It should be noted that although some articles did report CMV status, their results did not fit our study design, for example, Onyema et al. reported differences in each age group when CMV status was considered for CD57, CD28, P16 and p21 expression, but these conclusions were drawn on CD3 gate only, before the subsequent gating process (27). Libri et al, reported low CCR7, CD28 and IL-7Ra and high CD57 expression on naturally CMV-expanded CD4 EM and TE T cells when compared to N and CM CD4 T cells (28).

These markers are surface or intracellular proteins whose expression may be affected due to methodological factors such as cryopreservation of cells and/or *in vitro* stimulation (59). Therefore, it is advisable to standardize the methodological designs, since they constitute a factor with a high risk of bias within our review, making it impossible to draw conclusions.

Moreover, these markers seem to be accompanied by dysregulated cytotoxic ability and reduced telomere length. This decrease is among the hallmarks of aging (60). In the few studies that included this measurement, it was found that there was a decrease in telomere length in the TE subset compared to the other memory populations, as well as in the older adults relative to the young adults. This decrease in telomere length is a consequence of replicative senescence due to repeated antigen exposure in TE cells and due to the accumulation of DNA damage throughout life in older adults. Telomere shortening has been associated with an increased risk of mortality (20). Of particular note, conclusions drawn from Herndler-Brandstetter et al. on telomere length could reflect clear-cut age derived immune changes as experiments were conducted exclusively on seronegative individuals (30). Despite being a characteristic marker of cellular aging in T cells, not many studies perform this measurement when evaluating immunosenescence. This can be explained by the difficulty of the technique, high cost, and requirement of highly qualified personnel (20).

In addition, these functional markers have been associated with a high production of proinflammatory cytokines in older adults (61); however, the results of our review showed a tendency toward an increase in the percentage of both pro- (IFNγ, TNFα, IL-2, IL-6) and anti-inflammatory (IL-4 and IL-10) cytokine-producing T cells and decrease in expression of serine proteases. In CD4 T cells, the percentages of TNFα and IL-4 producing cells in older adults were both high, thus, a predominance toward any of the helper profiles (Th1 or Th2) could not be determined. As for γδ T cells, only one study evaluated this population, in which a proinflammatory cytokine production increase was

observed in older adults in the Vδ1 subpopulation. However, since it is a single study, it does not provide sufficient evidence for changes in cytokine production in these cells with aging from which conclusions can be made.

Even though the population included in this review were people reported as healthy and of a certain age range, other variables that present a valid comparison to identify whether intracellular cytokine measurement by flow cytometry can be used as marker for immunosenescence can be observed. Among these variables are the cytokines, culture stimuli, cell population, and techniques employed.

In literature, there is a list of both anti- and proinflammatory cytokines that have been associated with aging. In the case of proinflammatory cytokines, measurement is commonly done in CD8 T cells and the main cytokines measured are IL-6, TNFα, IL-2, IL-1B, IL-8, IL-18, and IFNγ (62). However, the studies reviewed in this study only account for four out of seven cytokines, with IFNγ cytokine being the most frequently measured, leaving aside cytokines such as IL-6, which has been classically reported to be increased in serum of old individuals (63). On the other hand, in the case of anti-inflammatory cytokines, IL1-Rα, IL-4, IL-10, and TGFβ are reportedly measured in both CD4 and CD8 T cells (62); nevertheless, we found that only five studies measured IL-4 or IL-10, while the remaining studies measured none. Finally, although the assessment of serine proteases is related with the cytotoxic profile of CD8 (64), only two studies measured perforin and granzyme B. Production of this cytolytic molecule seems to be modulated mainly by age, given that CMV+ older adults secreted lower levels of this cytokine than both CMV- and CMV+ young adults. However, a definitive conclusion cannot be drawn since there was no a CMV- older adult group (15). This shows that there is still no consensus on cytokines that should be measured for the assessment of aging.

The stimulus for induction of cytokine production is of great importance since it has been reported that variations in culture conditions, as well as the type and time of stimulation can influence the production of cytokines (65, 66). For instance, the three studies that evaluated polyfunctionality showed different results and, when looking at the culture conditions, it is done differently in each of them. In the case of Dolfi et al., specific stimulation with viral peptides was performed, while in the case of Riddell et al. and Henson et al., non-specific stimulation, anti-CD3 pearls and IL-2, and PMA/ionomycin were respectively performed (20, 21, 25).

Another important element for getting comparable and reproducible results is the cell population in which the measurement is made. Two options were mainly found in this review: measurement in total populations of T cells (CD4/CD8/γδ) and specific antigen populations. As an example, Riddell et al. and Dolfi et al. evaluated the response of CD8 T cells to a specific stimulus with CMV peptides (20, 25). In the case of Riddell et al., the response of specific CMV T cells was directly measured by tetramers, finding an increase in the population that co-produced IL-2/TNFα/IFNγ and IL-2/IFNγ to a greater extent in the older adults (20). On the other hand, Dolfi et al. reported non-significant differences between young and old individuals despite performing the same stimulus with CMV peptide. The latter could be explained by the measurement being made on total CD8 instead of antigen-specific CD8 T cells (25).

The technique for measurement of cytokines considered in this study was flow cytometry, which is performed by measuring intracellular cytokines, inhibiting vesicular transport or at the level of a cell culture supernatant using a cytometric bead array. However, cytokine measurement can be performed using other techniques such as ELISA or ELISPOT which can detect molecules in the plasma of an individual, supernatant of a culture, or directly in reactive cells. Evidence has shown that these techniques are complementary rather than comparable, since they do not have a direct correlation with their results (67). Therefore, there is no standardized method of cytokine measurement, such that studies based exclusively on one of these techniques do not represent the outlook of cytokines as a marker of immunosenescence.

Our systematic review has some limitations. Age groups across studies included a fair degree of variability. Similarly, 75% of the studies did not report values in the results of the experiments (i.e., number of cells used, mean or median, SD, among other descriptives) and, despite contacting the authors, we were unable to access majority of the data. Consequently, only one meta-analysis, in one of our result domains, namely, the memory T cell subsets, could be done throughout our analysis. This limitation suggests that the heterogeneity between the age ranges of the study groups and variation between the protocols does not allow robust conclusions.

To reduce the variability of the data and make accurate comparisons between the studies, only studies with flow cytometry analysis were taken into account, excluding several studies, especially those reporting cytokines or cytoplasmic proteins that are usually measured by other experimental techniques. The non-distinction between fresh and frozen cells when carrying out or reporting the experiments may also influence the presence, absence or quantity of certain membrane proteins, as well as cytokine production and telomerase activity. Finally, most of the studies in basic sciences report the experiments in which their alternative hypothesis is verified, and rarely negative data is reported. We consider that this can generate a publication bias and we observe this phenomenon even in the absence of a feasible funnel plot construction.

CONCLUSION

In conclusion, it was impossible to perform statistical analysis between the age groups for immunosenescence markers, cytokines, and telomere length due to the fact that data obtained from the included studies had high heterogeneity attributable to the different protocols employed. Therefore, we could not determine the best immunosenescence markers. However, based on the articles reviewed, we propose some essential items in the study of immunosenescence (**Table 6**).

TABLE 6 | Recommendations for the study of immunosenescence in T cells by flow cytometry.

- To evaluate memory, use a combination of markers that favor identifying at least four basic memory subsets such as CD45RA or CD45R0 along with CCR7 or CD62 and a third one to access memory in-depth, such as CD95 or CD57.
- Evaluate cytokine production measure: (a) Pro-inflammatory cytokines: one or more of the following IFN-y, TNF-a, or IL-2. (b) Anti-inflammatory cytokines: one or more of the following: IL-10 or IL-4.
- We consider that the decrease in CD28 expression and the increase in the expression of CD57 and KLRG1 are the ones that best describe an immunosenescent state.
- Telomere length is the feature that best describes T cells senescence.
- Although p16, p21, and H2AX are hallmarks of aging, not many studies use these markers to evaluate immunosenescence.

We considered that achieving methodological consensus in immunosenescence research is an aspect that requires special attention (**Table 5**).

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.

AUTHOR CONTRIBUTIONS

IR: Coordination, design, search, selection, review of papers, and writing of the manuscript. NR, ML, LM, MM, JO, OR, and EV: Design, search, selection, review of papers, and writing of the manuscript. EH: Design, review, and correction of the manuscript. CP: Coordination, design, writing, and review of the manuscript. 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/fimmu.2020.604591/full#supplementary-material

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REVIEW
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Purinergic Signaling in Controlling Macrophage and T Cell Functions During Atherosclerosis Development

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Atherosclerosis is a hardening and narrowing of arteries causing a reduction of blood flow. It is a leading cause of death in industrialized countries as it causes heart attacks, strokes, and peripheral vascular disease. Pathogenesis of the atherosclerotic lesion (atheroma) relies on the accumulation of cholesterol-containing low-density lipoproteins (LDL) and on changes of artery endothelium that becomes adhesive for monocytes and lymphocytes. Immunomediated inflammatory response stimulated by lipoprotein oxidation, cytokine secretion and release of pro-inflammatory mediators, worsens the pathological context by amplifying tissue damage to the arterial lining and increasing flow-limiting stenosis. Formation of thrombi upon rupture of the endothelium and the fibrous cup may also occur, triggering thrombosis often threatening the patient's life. Purinergic signaling, i.e., cell responses induced by stimulation of P2 and P1 membrane receptors for the extracellular nucleotides (ATP, ADP, UTP, and UDP) and nucleosides (adenosine), has been implicated in modulating the immunological response in atherosclerotic cardiovascular disease. In this review we will describe advancements in the understanding of purinergic modulation of the two main immune cells involved in atherogenesis, i.e., monocytes/macrophages and T lymphocytes, highlighting modulation of pro- and anti-atherosclerotic mediated responses of purinergic signaling in these cells and providing new insights to point out their potential clinical significance.

Keywords: T lymphocytes, extracellular ATP and adenosine, CD39 and CD73, P1 and P2 receptors, atherosclerosis, macrophage, necrotic core, oxLDL

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Abbreviations: AC, adenylate cyclase; ADO, adenosine; ADP, adenosine diphosphate; ATP, adenosine diphosphate; CKD, chronic kidney disease; DAMPs, damage-associated molecular patterns; DC, dendritic cells; GM-CFS, granulocyte-macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; IFN, interferon; IL, interleukin; LDL, low-density lipoproteins; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein-1; MMP9, Matrix Metallopeptidase 9; NLRP3, NACHT, LRR and PYD domains-containing protein 3; NO, nitric oxide; NTPDase, ectonucleoside triphosphate diphosphohydrolase; oxLDL, oxidized low-density lipoprotein; PAMPs, pathogen-associated molecular pattern; ROI, reactive oxygen intermediates; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; SMC, smooth muscle cells; TGF-β, transforming growth factor β; TLR, toll-like receptors; TNF-α, tumor necrosis factor α; UDP, uridine diphosphate; UTP, uridine triphosphate; VCAM-1, vascular cell adhesion protein 1.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of the arteries, characterized by the development of characteristic lesions named atheromatous plaques (1, 2). It represents the most diffuse pathological state of peripheral and coronary artery disease, as well as of cerebrovascular disorders (3). Factors participating in the atherosclerotic process have been identified, among them: genetic predisposition, hyperlipidemia, metabolic dysregulation (obesity, diabetes), hypertension and smoking (4). A role for microorganisms has also been hypothesized, at least for the initial stages of atherosclerosis (**Figure 1**, topic 1) (5–8). The first steps of atherogenesis are characterized by endothelium activation and changes in lipid permeability. Expression of VCAM-1, ICAM-1, P-Selectin and different cytokine receptors allows endothelial adhesion of immune cells (monocytes, lymphocytes, neutrophils) (9). Permeation of cholesterol-containing low-density lipoproteins (LDL) in the inner lining of the artery wall and their oxidation (oxLDL) by reactive oxygen species (ROS) favor leukocyte activation and amplification of the pro-inflammatory background (**Figure 1**, topic 2) (1). Upon expression of scavenger receptors, engulfment of oxLDL and migration to the intima, circulating monocytes become macrophages (foam cells) that dying in the plaque release engulfed lipids (**Figure 1**, topics 3, 4). Necrotic immune cells, debris, extracellular lipids and cholesterol crystals are not cleared efficiently and accumulate within the plaque-forming the so-called "necrotic core" (**Figure 1**, topics 5, 8–9) (2).

Macrophages are central in atherosclerosis as they participate in all stages of atheroma formation (10–12). Circulating monocytes are captured by the activated endothelium and undergo differentiation into macrophages and changing their phenotype according to stages of the atherosclerotic process. They perform different tasks ranging from perception of danger signals, engulfment of lipids and dead cells, secretion of inflammatory (ROS, activating cytokines) but also pro-resolving molecules (12). Atheromas are mainly populated by pro-inflammatory M1 macrophages but also by DC able to perform antigen presentation to T lymphocytes (13, 14). Interestingly, while M1 macrophages promote atherogenesis, M2 are atheroprotective (15). Macrophages are activated by the Th1 cytokine IFN-γ to produce ROI and NO (**Figure 1**). IFN-γ is fundamental for the pathogenesis of atherosclerosis and endowed with the ability to cause atheroma even in the absence of immune cells (16, 17). Adaptive immunity takes part in the pathogenesis of atherosclerosis (18, 19). Although monocytes migrating through the intima are more abundant than T lymphocytes, these latter cells are crucial for the formation of the lesion as they produce activation signals for macrophages thus amplifying their contribution to atheroma formation (20). Differentiation of naïve $CD4^+$ lymphocytes to effector and memory T cell subsets take place during atherogenesis (21). Antigen presentation by lesional macrophages and DC enables T cells to recognize antigens promoting the pro-inflammatory response underlying atherosclerosis (**Figure 1**, topic 10). Among them: LDL, oxLDL, beta 2 microglobulins, HSP60, and apo B-100 (22, 23). T cell polarization into Th1 and Th17 populations induce production of TNF-α, IL-17a and IFN-γ pro-inflammatory cytokines (16, 24, 25); however, Treg anti-inflammatory IL-10 and TGF-β cytokines have also been detected in the atheromatous lesions (23). Therefore, T cells secrete pro- and anti-inflammatory cytokines that direct evolution and stability of the plaque (19, 26).

Fibroblasts proliferate and secrete collagen, proteoglycans and elastin that accumulate in the intima (**Figure 1**, topic 11) (27). Immune cells promote not only atheroma formation but also its evolution with complications, damage, and sometimes disruption (23, 28). Thrombotic complications may also occur as a consequence of endothelial damage, rupture of the fibrous cap and exposure of prothrombotic material which triggers platelet activation and lead to blood coagulation. Plaque fracture is a very dangerous event threatening patients' life. It is highly dependent on the plaque composition as it is more frequent in atheromas rich in macrophages and poor of fibroblasts and consequently in collagen fibers (29). However, plaque destabilization and rupture is still an unpredictable event and strategies to stabilize the lesion represent a challenging problem.

In the present review, we will illustrate the importance of purinergic signaling in modulating pro- and anti-atherogenic responses, particularly in T cells and macrophages. We will also highlight the potential of purinergic receptor agonists and antagonists for new therapeutic strategies to treat atherosclerosis.

PATHOGENESIS OF ATHEROSCLEROSIS

Although abnormal lipid accumulation in the artery wall during atheroma formation is considered the main hallmark of the disease (30), there is still debate on triggering factors and stressors taking part in the initial stages of the disease. While there is a consensus on the participation of innate and adaptive immunity in chronic inflammation underlying atherogenesis, less is known on signals activating immune cells. Danger signals, i.e., pathogen-associated molecular patterns (PAMPs) derived from viruses and bacteria, as well as danger-associated molecular patterns (DAMPs), which are intracellular or endogenous molecules, have been linked to atherosclerosis (31). Among PAMPs, bacterial lipopolysaccharide (LPS), cytomegalovirus (CMV) and human immunodeficiency virus (HIV); while among DAMPs indicated to take part in atherogenesis: minimally modified LDL, oxidized LDL, oxidized phospholipids, advanced glycation end-products, high-mobility group box 1 and heat shock proteins. Interestingly, fatty acids can induce sterile vascular inflammation (**Figure 1**, topic 1) (32).

Cytokines play a fundamental role in atherosclerosis and associated comorbidities (e.g. psoriasis, SLE, CKD) (33–37). Pro- and anti-atherogenic cytokines have been shown. To the first group belong molecules with pro-inflammatory activities, such as interferons (IFNs) (α, β, γ), interleukin-(IL)1β, IL-6, IL-17a, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor (TNF)-α while among anti-atherogenic cytokines, transforming growth factor (TGF)-β, IL-10, and IL-35. The preeminent effect of pro-atherogenic cytokines ranges from

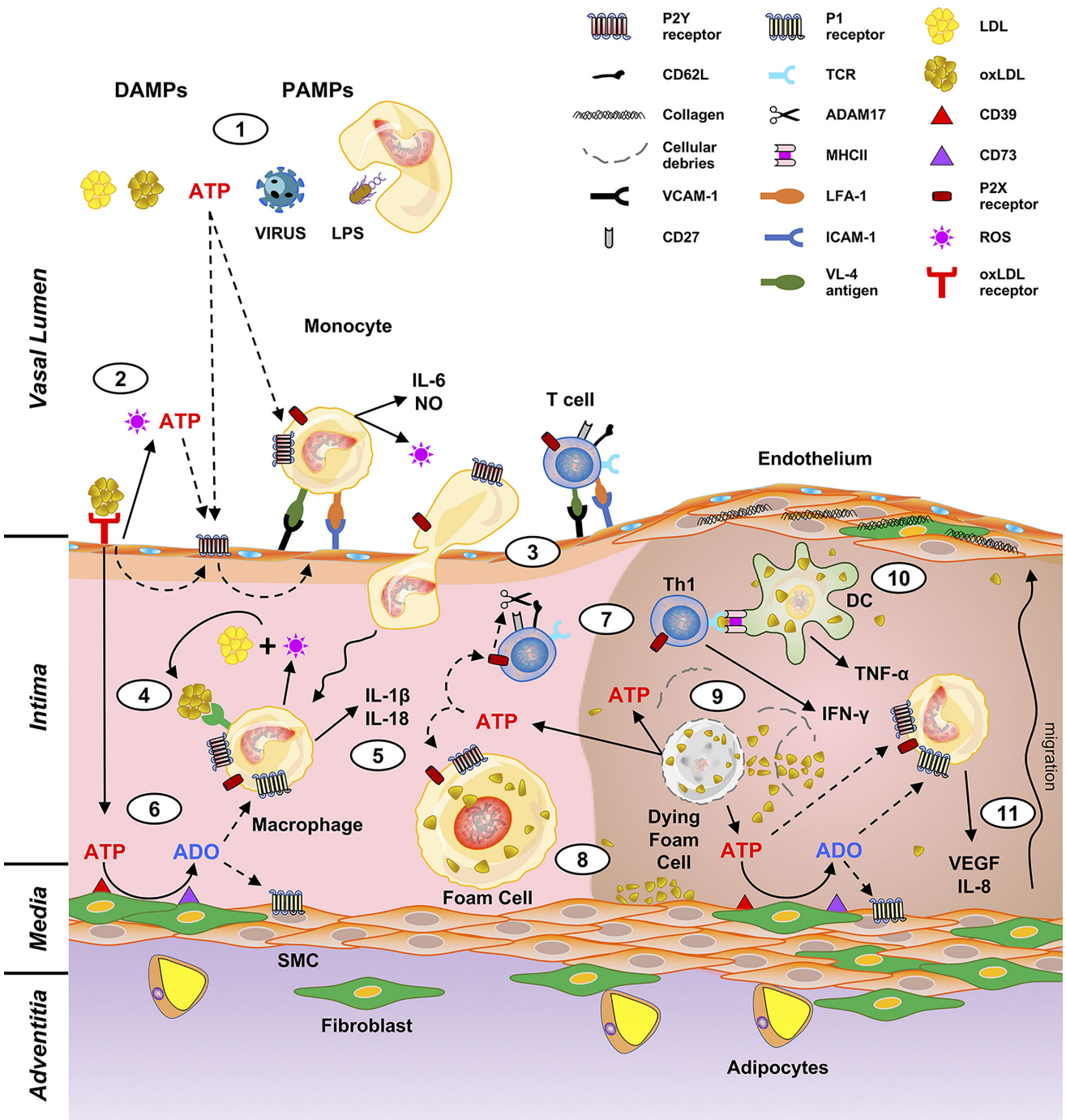

FIGURE 1 | Putative role of purinergic signaling in driving macrophages and T cell activation in atheroma development. DAMPs (LDL, oxLDL, extracellular ATP) and PAMPs (viruses, microbes, LPS) trigger the production of cytokines and oxygen species by monocytes (1). This induces release of ATP from the endothelial cells and expression of the leukocyte adhesion molecules (vascular cell adhesion molecule 1, VCAM-1; intercellular adhesion molecule 1, ICAM-1) (2) thus prompting adhesion and extravasation of monocytes and lymphocytes (3). Macrophage derived ROS oxidate LDL to oxLDL (4) and stimulate IL-1β and IL-18 production (5). Extracellular ATP is also converted to ADO by CD39 and CD73, which are expressed by the intima cells (6). ADO exerts a down-modulation of the immune response therefore has a protective effect. On the contrary, ATP acts as a proinflammatory molecule inducing the cleavage of CD62L by ADAM17 and T cell polarization to a Th1 phenotype (7). Upon engulfment of oxLDL, macrophages become foam cells (8). The atheroma "necrotic core" (right part of the figure) forms by accumulation of dying foam cells, lipids, cholesterol crystals and immune cells (9). Pro-inflammatory IFN-γ and TNF-α are released upon antigen presentation to T lymphocytes by DC (10), and in turn promote IL-8 and VEGF secretion, with consequent fibroblasts and SMC migration and proliferation (11).

induction of the synthesis of other cytokines, amplifying the pro-inflammatory activities of immune cells, to upregulation of endothelial adhesion molecules, thus favoring attachment and diapedesis of monocytes and lymphocytes (38).

PURINERGIC RECEPTORS

Nucleotides and nucleosides are not just accumulated and used within the cell but they are also secreted and synthesized extracellularly where they serve as intercellular messengers. ATP, ADP, UTP, UDP and adenosine, just to cite some, present at high concentrations within the cell where they exert multiple roles, bind extracellularly to evolutionary conserved P2 (activated by nucleotides) and P1 (activated by adenosine) plasma membrane receptors (**Figure 2**). Signal transduction of these receptors modulates cell and tissue pathways involved in tissue metabolism, gastrointestinal and hepatic function, circulation, nervous tissue response and immune defense (39–41). Interestingly, dysregulation of the purinergic signaling network has been implicated in the pathogenesis of allergic and neurological diseases, tissue fibrosis and cancer (42–46). Extracellular nucleotides participate in normal circulation physiology, but also in the onset of pathologic states that develop into the blood vessels, such as in blood hypercoagulability, thrombosis, atherosclerosis (40, 47, 48).

P2 Receptors

P2X Receptors

They are grouped into two subfamilies, namely: P2X and P2Y (49) (**Figure 2**). P2X receptors comprise seven subtypes (P2X1-P2X7). They are highly conserved, trimeric, ATP-gated ion channels, selective for monovalent and divalent cations Na^+, K^+, Ca^{2+}, Mg^{2+}. Upon binding of the ligand, some of them desensitize (50, 51). Interestingly, the existence of lipid rafts and the level of cell membrane cholesterol can modulate the sensitivity of P2X receptors to ATP (52, 53). For the many responses they mediate in the circulatory apparatus, P2X receptors have been chosen as therapeutic targets for the cardiovascular system (54, 55).

The seventh subtype is an exception between P2X for its permeability transition and for not desensitizing in the presence of ATP (56). Its potential interest in atherogenesis is very high. Involvement of P2X7 in energy metabolism has been shown in mice; accordingly, deletion of the receptor induces lipid accumulation, fat mass distribution increase and gain of weight

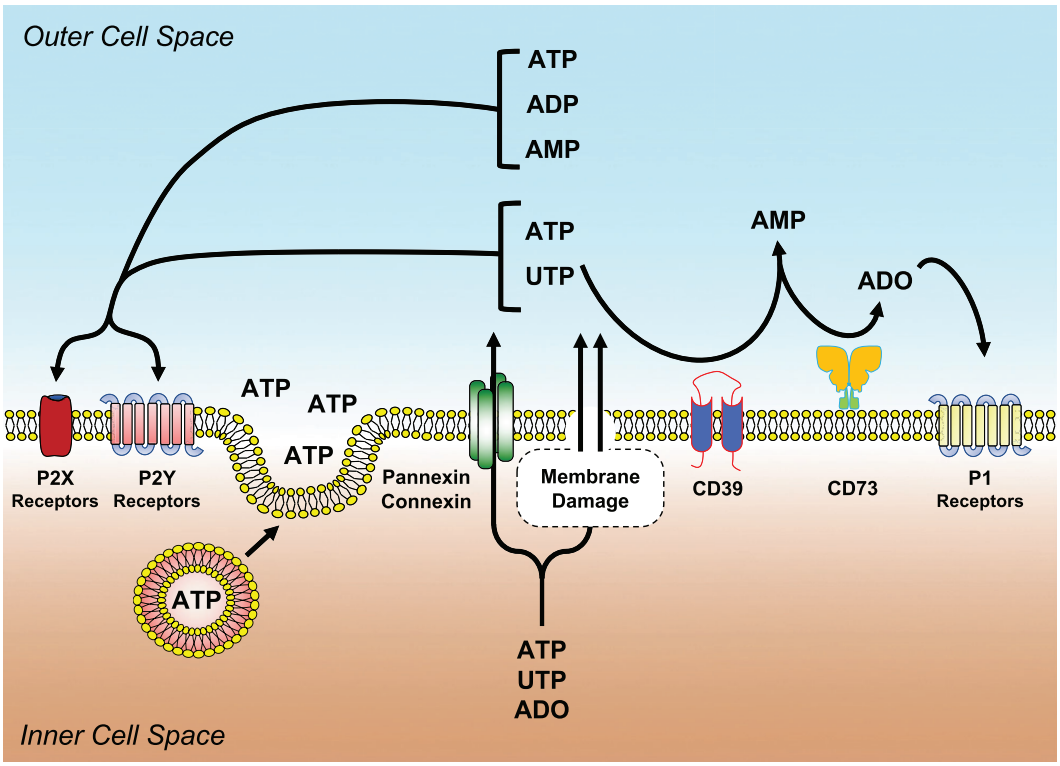

FIGURE 2 | Main molecular components of the purinergic signaling network. Nucleotides (ATP, ADP, UTP, UDP, etc.) and nucleosides (ADO) can be released or transported extracellularly as a consequence of shear stress membrane damage, hypoxia, apoptosis, necrosis and infections. Once liberated, they bind and activate purinergic P2 (P2Y and P2X) or P1 (A_1, A_{2A}, A_{2B}, A_3) receptors. ADO is generated from the enzymatic conversion of ATP/ADP to AMP by the ectonucleoside triphosphate diphosphohydrolase CD39 and with the hydrolysis of AMP to ADO by the ecto-5′-nucleotidase CD73. ADO activates P1 receptors.

(57). The receptor is also endowed with the ability to induce transcription and secretion of inflammatory cytokines such as IL-1β, IL-18 (**Figure 1**, topic 5) and IL-6 which are central in atherosclerosis (58–60). Hence, Toll-like receptors (TLR) and P2 purinergic receptors induce activation of inflammasomes (61). Their activation by extracellular ATP causes IL-1β and IL-18 release (62). Interestingly, engulfment of lipids by macrophages increases the sensitivity of TLR to their ligands and activates NLRP3 (also known as NOD-, LRR- and pyrin domain-containing 3, NALP3) inflammasome (63, 64). NLRP3 is activated by two signals: the first being microorganisms or inflammatory cytokines endowed of the ability to activate transcription factor NF-κB, upregulate NLRP3 proteins and expression of the inactive form of the cytokines; while the second step is mediated by different stimuli among which extracellular ATP through activation of the P2X7 receptor (65). An important confirmation on the importance of P2X7 in atherogenesis comes from the animal model, where the absence of this subtype inhibits inflammasome activation and improves atherosclerosis (66).

P2Y Receptors

P2Y receptors include eight subtypes named: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (67). They have a membrane topology with seven-transmembrane domains and couple intracellularly to G_q/G_{11} or $G_{i/0}$ proteins (67) (**Figure 2**). They differ in agonist specificity. $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ subtypes are preferentially activated by ADP (68), whereas $P2Y_6$ by UDP. $P2Y_2$ is activated by UTP or ATP, while $P2Y_4$ and $P2Y_{11}$ are selective for UTP and ATP, respectively (69). $P2Y_{14}$ is activated by UDP-glucose (69). P2Y receptors modulate several physiological responses.

Endotheliocytes release ATP in response to blood flow changes, hypoxia, or damaging agents (40, 70, 71). Moreover, ATP and other nucleotides are released from all dying cells and act as DAMPs activating and recruiting immune cells (58) (**Figures 1** and **2**). Interestingly, oxLDL favor nucleotide release from endothelial cells (72). Triggering of the $P2Y_2$ receptor by ATP secreted by endothelial cells upon stimulation with oxLDL induces expression of receptors for advanced glycation end-products and adhesion molecules (73). Furthermore, the release of ROS and ATP/UDP from endothelial cells upon exposure to oxidized low-density lipoprotein (oxLDL), induce autocrine $P2Y_1$-mediated upregulation of ICAM-1 and VCAM-1 with subsequent stimulation of leukocyte adhesion (74) (**Figure 1**, topic 2).

As an example, platelet aggregation is dependent on adenosine ADP/$P2Y_{12}$-mediated amplification of thrombin effects. During platelet aggregation, the ADP receptor $P2Y_{12}$ plays a pivotal procoagulant role as shown by the benefits gained by its inhibition with the receptor blocker Clopidogrel in patients with cardiovascular disease such as in acute coronary syndrome, recent stroke and arterial disease (75, 76). In abdominal aortic aneurysm, a condition characterized by dilatation of the abdominal aorta which involves antigen-driven T cells in the site of inflammation (77), Clopidogrel reduces the content of cytotoxic $CD8^+$ T cells in the aortic wall and has an ameliorative role in the disease (78). Indeed, P2Y receptors would be central in inducing endothelium activation and atherogenic modifications at least in the double negative knockout ($P2Y_1^{-/-}$/$ApoE^{-/-}$) mice. In these animals, the $P2Y_1$ subtype contributes to TNF-α-induced ICAM-1 and VCAM-1 exposure with consequent leucocyte recruitment in inflamed femoral arteries (79). Moreover, reduction of the aortic sinus lesions associated to a decrease in macrophages infiltration and to a diminished VCAM-1 expression in endothelial cells of $P2Y1^{-/-}ApoE^{-/-}$ compared to $ApoE^{-/-}$ mice suggests that atherosclerotic lesions are due to endothelial or smooth muscle cells expression of $P2Y_1$ receptors (80). Interestingly, bacterial lipopolysaccharide (LPS) upregulates lectin-like oxLDL receptor in endothelial cells (81), which in turn induces a $P2Y_1$- and $P2Y_1$-mediated upregulation of ICAM-1 and VCAM-. This prompts leucocyte adhesion to endothelial cells (74). Similarly, the $P2Y_2$ subtype promotes atherosclerosis in mice by inducing the expression of the same adhesion molecules. Matrix metalloproteinase-2 proteolytic activity was reduced in atheroma of $P2Y_2^{-/-}$ $ApoE^{-/-}$ mice (82). Another potentially very interesting for the pathogenesis of atherosclerosis is the $P2Y_6$ subtype. Hence, $P2Y_6$ is upregulated during vascular inflammation induced by TNF-α or LPS stimulation in mice, and its inhibition or ablation reduces the vascular inflammatory response (83). These findings suggest that P2Y receptors could be targeted for therapeutic purposes in atherosclerosis.

P1 Receptors

P1 receptors are activated by adenosine (ADO) that can be transported outside the cell by specific membrane transporters or generated extracellularly by ATP and ADP hydrolysis (see next paragraph) (**Figure 1**). ADO concentration in the extracellular fluids ranges from 100 to 500 nM in homeostatic conditions while it augments to low micromolar for the presence of inflammation or during hypoxia and ischemia (84, 85).

ADO or ADORA receptors consist of four subtypes: A_1 (ADORA1), A_{2A} (ADORA2A), A_{2B} (ADORA2B) and A_3 (ADORA3). They are seven-transmembrane G-protein-coupled receptors that associate with G-proteins. Depending on receptor subtype, ADO activates (A_{2A}, A_{2B}) or inhibits (A_1, A_3) adenylate cyclase (AC) (86). ADORA receptors also differ in ligand affinity, being A_1, A_{2A} and A_3 subtypes activated by low (10–50 nM) ADO concentrations while on the contrary, A_{2B} needs around 1 mM ADO for activation (87).

P1 receptors induce multiple responses (42, 85, 88). Extracellular ADO is very important to dampen acute inflammation thus preventing tissue injury. ADO-mediated immunosuppressive mechanisms are mainly based on inhibition of pro-inflammatory cytokine secretion, production of suppressive cytokines and induction of regulatory immune cells. Endothelial cells use adenosinergic signaling to regulate the leakiness through the endothelial monolayer of the brain capillaries, for the passive exchange of solutes and proteins (89, 90); however, the use of P1 agonists, particularly of the A_{2A} subtype has to be carefully evaluated for the side effects deriving from the T cell migration through the blood-brain barrier (91). Interestingly, A_{2A} receptor signaling has also been indicated as a target for limiting aneurysm formation (92); A_3 antagonism

reduces hypercholesterolemia in ApoE$^{-/-}$ mice (93). Therefore, ADO and its receptors represent promising pharmacological targets to treat atherosclerosis.

Ectonucleotidases

Extracellular nucleotide concentration in homeostatic conditions is low or close to zero. This is due to the hydrolyzing activity of different plasma membrane ectonucleotidases transforming ATP to ADP and then to ADO (**Figure 2**). Besides avoiding of accumulation of nucleotides in the extracellular *milieu*, these enzymes degrade P2 receptor agonists (i.e., nucleotides) lowering their concentration thus reducing the efficiency of stimulation. Conversely, their activity augments the amount of ADO thus increasing the probability of activating P1 receptors (42). Shifting from P2 to P1 activation has quite often the consequence of changing purinergic-mediated responses from pro- to anti-inflammatory, thus preserving tissue integrity (94).

Different ectonucleotidase families have been described: ectonucleotide pyrophosphatase/phosphodiesterase (NPP), alkaline phosphatases, ectonucleoside triphosphate diphosphohydrolases (NTPDases, among which CD39 or NTPDase1) and ecto-5'-nucleotidase (CD73). CD39 catalyzes the conversion of ATP or ADP to AMP, while CD73 hydrolyzes AMP to ADO (95–97) (**Figure 2**). Ectonucleotidases play a central role in immune regulation, thus preventing the development of conditions favoring autoimmune diseases (94). Moreover, the generation of ADO by ectonucleotidases reduces tissue damage and ameliorates tissue physiology in hypoxia-related disease states (98, 99). CD39 has been associated with resistance to thrombus formation in injured mice arteries (100) while in CD73$^{-/-}$ mice, absence of the enzyme does not directly affect thrombosis, but indirectly lowers it by increasing CD39 expression, particularly on monocytes (101, 102). CD39 likely exerts multiple and sometimes apparently contrasting effects in atherosclerosis. The absence of this gene in hyperlipidemic mice decreases atheroma formation and it was hypothesized that this effect resulted from multiple contributions, i.e.: decreased platelet activation, increased plasma HDL concentration and augmented cholesterol efflux (103). Expression of CD39 is crucial in neointimal formation after vascular injury in mice as its absence impairs the migration of vascular smooth muscle cells (104).

MODULATION OF MACROPHAGES BY PURINERGIC SIGNALING DURING ATHEROGENESIS

P2 Mediated Effects

Macrophages and DC express both P2X and P2Y receptor subtypes that are involved in modulating responses ranging from cytokine secretion, giant cell formation, production of oxygen radicals and antigen presentation.

Immunohistochemistry demonstrates that $P2Y_6$ is upregulated in the atherosclerotic aortic segment of ApoE$^{-/-}$ mice after 4-week of cholesterol-enriched diet, with the accumulation of $P2Y_6$ expressing macrophages into the plaque. Interestingly, Suramin or PPADS treatments were able to reduce the plaque size, without modification of the number of macrophages and smooth muscle cells (105). $P2Y_6$ receptor mRNA increases in aortic portions with atherosclerosis, while expression of the mRNA for other P2Y subtypes (P2Y1, $P2Y_2$, $P2Y_4$) remain unchanged (105). However, the participation of the $P2Y_6$ receptor to atherosclerosis in mice seems to be dependent on the experimental model used. A reduction in atherosclerotic plaque formation in the aortic arch was observed in high fat-fed LDLR knockout mice lacking the $P2Y_6$ receptor in bone marrow-derived cells, but not in other mouse models (106). P2X7 is highly expressed in immune cells, particularly in macrophages where it is involved in IL-1β and IL-18 processing and release (59, 107–110). Macrophages are the main source of IL-1β, which is responsible for inflammation linked to atherosclerosis. It can thus be hypothesized that stimulation of P2X7 by extracellular ATP released within the atheroma induces the release of this pro-inflammatory cytokine (111–113). The efficacy of the A740003, a P2X7 specific antagonist, in decreasing vessel inflammation further supports its role in atherosclerosis and gives a new chance for the local pharmacological targeting of atherosclerosis (113).

IFN-γ is also a central mediator in atherosclerosis (114). IFN-γ potentiates IL-1β release from primary human monocyte-derived DC. Indeed, IFN-γ also upregulates expression of the P2X7 subtype, which in turn prompts IL-1β secretion (115, 116). IL-18 and its functional receptor have been detected in human endothelial cells, SMC and macrophages, and are implicated in atherogenesis (117). Since P2X7 expressed by human macrophages is also involved in ATP stimulated IL-18 release it again represents a suitable candidate for pharmacological targeting of atherosclerosis (108).

The centrality of NLRP3 inflammasome in atherosclerosis has also been well ascertained (118). Different approaches have been successfully attempted to inhibit the protein complex both *in vitro* and *in vivo*. This latter has shown a positive effect on experimentally induced atherosclerosis (119–121). Extracellular ATP is among stimuli that potently activate NLRP3, therefore, it is very promising for therapeutic purposes the observation that deficiency of a single purinergic receptor, namely the P2X7 subtype, is sufficient to block NLRP3 inflammasome and ameliorate the clinical picture of atherosclerosis in mice (122).

MODULATION OF T LYMPHOCYTES BY PURINERGIC SIGNALING DURING ATHEROGENESIS

P2 Mediated Effects

P2Y and P2X receptor activation lead the inflammatory processes of the vessels favoring interactions between leukocytes, platelets and vessel wall. The $P2Y_{12}$ subtype has attracted interest for its pro-thrombotic and pro-inflammatory

role both in Apolipoprotein E-deficient mice and in humans (123). Contribution of the ADP receptor in modulating atherogenesis in the mouse model would be at least in part due to the induction of platelet α-granule release that would increase recruitment of inflammatory cells (124).

During atheroma formation, platelets induce a phenotype change and INF-γ secretion in human $CD4^+$ T lymphocytes; but administration of the $P2Y_{12}$ receptor blocker Prasugrel to human volunteers completely inhibits platelet-mediated pro-inflammatory changes induced in Th cells. Therefore, anticoagulant therapy with Prasugrel may provide therapeutic benefits both from direct platelet inhibition and also by downregulating the immune response (125). Clopidogrel, another $P2Y_{12}$ inhibitor decreases expression of the purinergic receptor by leukocytes, ameliorates atheroma conditions and stabilizes aortic sinus plaques increasing the number of atheroprotective regulatory $CD4^+CD25^+$ T (Treg) cells in $ApoE^{-/-}$ mice (126, 127). Although atherosclerosis is characterized by migration of different immune cells through the vessel wall, at least in the mouse model, lymphocytes are already present within the normal/noninflamed aorta before the onset of atheroma; while macrophages and DC that perform T cell antigen presentation are recruited into the artery wall. This migration is partially dependent on L-selectin (CD62L) both in normal and atherosclerosis-prone $ApoE^{-/-}$ mouse aorta (128). Shedding of CD62L occurs during lymphocyte activation and rolling; interestingly, activation of the P2X7 receptor triggers the shedding of CD62L in leukocytes (**Figure 1**, topic 7) (129, 130).

P1 Mediated Effects

It is long known that ADO has anti-inflammatory properties (58). Curiously, the potent anti-inflammatory drug methotrexate is responsible for ADO release that activating A_2 receptors expressed by immune cells, reduces their presence in the inflamed tissue (131). Since ADO acts as a down-modulator of the immune response, it exerts atheroprotective functions by reducing the secretion of pro-inflammatory cytokines, thus lowering immune-mediated tissue damage (58). The role of $CD8^+$ T lymphocytes in atherosclerosis has been the object of intense debate. However, a recent report has shed light on this issue and on the involvement of CD39 ectonucleotidase in conferring a regulatory and atheroprotective phenotype to $CD8^+$ cells. This is associated with a reduction in cytokine production through increased CD39 expression in both mouse and human atherosclerotic lesions (132).

CONCLUSIONS

Atherosclerosis is a leading cause of death in developed countries and it has been the target of multidisciplinary therapeutic approaches to reduce the relevant burden of life loss and health spending. Data coming from extensive epidemiological, clinical and experimental studies show that lifestyle habits are crucial to prevent atherosclerosis. Several strategies have been tested to treat the disease, among them: cholesterol-lowering agents, blood pressure reducing drugs, anti-inflammatory agents (corticosteroids, monoclonal antibodies to cytokines) and anti-P-selectin antibodies (133). Indeed, no definitive answers on the efficacy of these clinical approaches have been obtained. Therefore, novel therapeutic solutions are highly required (2, 33, 133).

ADO, for example, behaves as a down-modulator of immune cell activation as shown in many *in vitro* studies as well as in animal models and clinical trials. Besides anti-inflammatory properties and inhibition of cholesterol accumulation into the vessels, ADO also shows anti-thrombotic effects, thus having an atheroprotective potential sufficient to prompt clinical trials particularly involving the A2A receptor (134–137).

Macrophages and lymphocytes are central in the evolution of atherosclerosis for their ability to produce signals feeding the underlying pro-inflammatory background of the disease (19, 26, 38).

IL-1β has a pivotal role in atherosclerosis, and purinergic signaling is the main triggering way for its release. Interestingly, Losartan, an angiotensin II receptor blocker used to treat hypertension, inhibits LPS/ATP-induced IL-1β secretion by suppressing NLRP3 inflammasome (119). The NALP3/P2X7 tandem has a well-ascertained role in inflammation. An important result obtained in the animal model consists in the observation that the absence of the P2X7 subtype impairs lesional inflammasome activity and ameliorates the disease, pointing to the centrality of this receptor as a trigger of NLRP3 induced inflammation (122). Due to the importance of NLRP3 in atherosclerosis, different ways have been proposed for its inactivation (120, 121, 138). Interestingly, the P2X7 antagonist A740003 also shows an effect in decreasing IL-1β secretion and MMP9 activity in ex-vivo cultures of atheromatic cells, independently from NLRP3 (113). Therefore, further studies are needed to shed light on the activation of this latter P2X7 dependent proinflammatory pathway.

The $P2Y_2$ receptor subtype has also been endowed with pro-inflammatory properties in the $ApoE^{-/-}$ mouse model, and pro-thrombotic capacities in human coronary artery endothelial cells. It would therefore be worthy to pharmacologically target this receptor in the attempt of reducing inflammation and thrombosis in atherosclerosis (139). Involvement of the $P2Y_6$ receptor in the inflammatory background underlying atherosclerosis has been shown both in mice and humans (105, 140). This subtype is expressed in murine atherosclerotic plaques and is involved in NO production and IL-6 secretion in murine macrophages (105). The P2Y6 receptor subtype plays a role in immune cell activation and recruitment to the arterial wall, most likely by inducing MCP-1 and CCR2 overexpression, accompanied by modulation of the CCL2-mediated signaling (106, 141, 142). Accordingly, leukocyte migration and lesion size induced by the $P2Y_6$ agonist UDP are decreased in $P2Y_6R^{-/-}$ mice. Accordingly, mice deficient in both $P2Y_6$ and low-density lipoprotein, LDL,

receptor show lower atherosclerotic lesion sizes and lipid accumulation in the aorta. Recent studies on $P2Y_6$ pro-inflammatory effects had shed light on vascular inflammation in the presence of bacterial LPS. The $P2Y_6$ receptor antagonist MRS 2578 shows a positive effect in down modulating a nuclear factor κB reporter and expression of pro-inflammatory genes in human microvascular endothelial cells *in vitro* (83). Moreover, inflammation and uptake of cholesterol by macrophages are lower in atheroma of $P2Y_6^{-/-}$ mice, candidating the subtype as a therapeutic target for atherosclerosis (66, 106, 140, 143).

$P2Y_{12}$ receptor represents a further very promising molecule for the treatment of the disease as its inhibitor Ticagrelor reduces cardiovascular events in patients with acute coronary syndrome and decreases inflammatory endothelial activation and vascular dysfunction in $ApoE^{-/-}$ mice (144, 145). Moreover the efficacy of Prasugrel, another $P2Y_{12}$ receptor blocker, in abolishing pro-thrombotic and pro-inflammatory responses of platelets and $CD4^+$ T cells in humans, may also provide an indirect positive effect on the inflammatory response underlying the genesis of atheroma and also in cardiovascular diseases involving T cells (125). Concerning $CD8^+$ T lymphocytes, although their identification in the atherosclerotic lesions has already been reported a few decades ago, however, both atheroprotective and pro-atherogenic roles have been proposed, depending on the animal or research model used (146). Different $CD8^+$ subpopulations would have a particular role in atherosclerosis. Two putatively protecting phenotypes have been identified and would be MHC class I-restricted $CD8^+$ lymphocytes and regulatory CD8+CD25+ T cells (146); moreover, a role has been attributed to CD39 ectonucleotidase in reducing IFN-γ and TNF-α production by $CD8^+$ in atherosclerotic lesions in mice (132). Another important point is that inhibition of the A_{2A} receptor reduces the formation of foam cells, making this receptor putatively interesting to inhibit lipid accumulation within the intima (134, 135). Another issue to be further explored is the involvement of TLR receptors in atherosclerosis, being TLR9 a first candidate for future studies (138, 147). It would also be worthy to check whether the expression of TLR is modulated by nucleotides during atherosclerosis. Experiments performed in hypercholesterolemic mice showed that oxidized phospholipids are proatherogenic; therefore, it would be interesting to check whether extracellular ATP may amplify this response (148).

The attenuation of the inflammatory background of atherosclerosis would be a desirable first step to treat the disease; rapidly expanding knowledge on the effects mediated by extracellular nucleotides and nucleosides on immune and non-immune cells participating in atherosclerosis will hopefully give a new chance of introducing new therapeutic compounds to treat inflammation and therefore atherosclerosis (93, 149). Another challenge consists of finding new ways for the *in situ* delivery of anti-atherosclerotic drugs, to block atheroma progression and possibly revert it. Nano- and micro-particles could likely be a new and possibly efficient way to administer drugs directly to the atherosclerotic lesions (150).

AUTHOR CONTRIBUTIONS

DF, FC, and AL conceived the review and wrote the manuscript. FC prepared the figures. DM and CC checked and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Terminally Differentiated CD4$^+$ T Cells Promote Myocardial Inflammaging

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The cardiovascular and immune systems undergo profound and intertwined alterations with aging. Recent studies have reported that an accumulation of memory and terminally differentiated T cells in elderly subjects can fuel myocardial aging and boost the progression of heart diseases. Nevertheless, it remains unclear whether the immunological senescence profile is sufficient to cause age-related cardiac deterioration or merely acts as an amplifier of previous tissue-intrinsic damage. Herein, we sought to decompose the causality in this cardio-immune crosstalk by studying young mice harboring a senescent-like expanded CD4$^+$ T cell compartment. Thus, immunodeficient NSG-DR1 mice expressing HLA-DRB1*01:01 were transplanted with human CD4$^+$ T cells purified from matching donors that rapidly engrafted and expanded in the recipients without causing xenograft reactions. In the donor subjects, the CD4$^+$ T cell compartment was primarily composed of naïve cells defined as CCR7$^+$CD45RO$^-$. However, when transplanted into young lymphocyte-deficient mice, CD4$^+$ T cells underwent homeostatic expansion, upregulated expression of PD-1 receptor and strongly shifted towards effector/memory (CCR7$^-$ CD45RO$^+$) and terminally-differentiated phenotypes (CCR7$^-$CD45RO$^-$), as typically seen in elderly. Differentiated CD4$^+$ T cells also infiltrated the myocardium of recipient mice at comparable levels to what is observed during physiological aging. In addition, young mice harboring an expanded CD4$^+$ T cell compartment showed increased numbers of infiltrating monocytes, macrophages and dendritic cells in the heart. Bulk mRNA sequencing analyses further confirmed that expanding T-cells promote myocardial inflammaging, marked by a distinct age-related transcriptomic signature. Altogether, these data indicate that exaggerated CD4$^+$ T-cell expansion and differentiation, a hallmark of the aging immune system, is sufficient to promote myocardial alterations compatible with inflammaging in juvenile healthy mice.

Keywords: CD4+ T-cells, myocardial aging, inflammaging, NSG animals, immunosenescence, lymphocytes

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INTRODUCTION

Immunosenescence is a conserved phenomenon among a wide range of animals, including birds, reptiles and mammals, which strongly correlates with morbidity in elderly humans (1, 2). Senescence particularly manifests in adaptive immune functions, as evidenced by a reduction in naïve T cell production, an accumulation of effector and terminally differentiated T cells and a decline in antibody response following antigen stimulation in aged individuals (3, 4). In parallel to these alterations, chronic, sterile, low-grade inflammation also develops alongside aging, a process termed inflammaging, which contributes to the pathogenesis of a multitude of age-related diseases (5). T cell dysfunction during aging stands at the crossroad between immunosenescence and inflammaging. Effector memory T cells (T_{EM}) ($CD44^+CD62L^-$ in mice; $CD45RO^+CCR7^-$ in humans) may develop a senescent phenotype characterized by the expression of checkpoint inhibitory receptors and defective TCR-mediated proliferation, but increased secretion of proinflammatory cytokines (6). These paradoxical conditions result in immunodeficiency and inflammation, dampening anti-infectious responses while fostering tissue damage and autoimmunity (7). Mechanistically, T_{EM} dominates the aged immune system compartment due to a couple of factors, including thymus involution and long-life exposure to pathogenic stimuli. In particular, immune surveillance against persistent viruses, mostly cytomegalovirus (CMV), causes an inflation of the effector/memory T cell compartment and an early onset of the immune aging phenotype in humans (3, 8).

In light of the profound effects of immune aging in human health, recent studies sought to discover biomarkers for physiological and accelerated aging. Early data from the OCTO-immune longitudinal study revealed that octogenarians with a more advanced immunosenescence profile, characterized by a high frequency of CD8 over CD4 lymphocytes and poor proliferative responses to mitogen stimuli, also presented higher mortality (9). The follow-up NONA-immune longitudinal study further confirmed the positive association between a high immune risk profile and higher mortality in nonagenarians. In addition, higher levels of IL-6 were associated with poorer cognitive function and predicted future cognitive decline in the elderly (10). Most recently, longitudinal high-dimensional immune profiling of young and elderly humans generated an immune aging (IMM-AGE) score, which could better indicate immunological aging, regardless of chronological factors. At the cellular level, interindividual shifts in $CD4^+$ and $CD8^+$ T cell subsets accounted for the immune trajectories observed in aging. By selecting a set of transcripts and surface markers that represent such immune aging trajectories, the authors showed that the IMM-Age score could better predict cardiovascular disease and overall mortality than epigenetic clock markers (2).

Compelling clinical and preclinical data have now demonstrated that exaggerated immunosenescence profiles are associated with several aging-related diseases in humans. In particular, T cell dysfunction during aging has also been associated with rheumatoid arthritis, systemic lupus erythematosus, metabolic disorders, and more specifically, cardiovascular diseases. The senescent $CD4^+$ T cell compartment has been implicated in myocardial inflammation and has been shown to promote age-related tissue dysfunction. Aged T cells from heart-draining lymph nodes show preferential heart homing and a proinflammatory response in young transferred mice (11). In humans, T cell senescence due to CMV infection predicts cardiovascular mortality in the elderly population (12). Moreover, Moro-Garcia et al. found that CMV seropositivity and its ensuing T cell memory inflation correlated with more advanced heart failure progression in middle-aged patients (12, 13). In addition, CMV-independent maladaptive accumulation of $CD28^-$ T cells positively correlates with cardiovascular death, whereas memory $CD8^+CD28^+$ T cell numbers are associated with overall improved survival (14).

Taken together, these lines of evidence suggest that an accumulation of a senescent T cell compartment may contribute to age-related myocardial decline and predispose toward cardiovascular diseases. However, it remains unclear whether aged T cells would simply fuel cardiac-intrinsic age-related damage or act as primary triggers of myocardial aging even in the absence of other preexisting conditions. To mechanistically decompose this question, we generated a xenograft transplantation model in which young humanized mice were treated to harbor a senescent-like expanding human $CD4^+$ T cell compartment. Our data uncover a role for terminally differentiated T cells in promoting cardiac inflammatory shifts that match those observed in aged hearts.

MATERIALS AND METHODS

Study Approval

For mouse studies, all *in vivo* procedures were approved by the local authorities (*Regierung von Unterfranken*) and conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

The human samples were obtained from another study conducted at the university clinic of Würzburg that had previously recruited 46 patients with ST-elevation and non-ST-elevation infarction (STEMI/NSTEMI), 48 healthy control patients who had undergone elective coronary angiography without any sign of coronary artery disease, and 15 healthy volunteers (KAMI-study). Individuals with active tumors, hematological diseases, skeletal muscle diseases, autoimmune diseases, immunosuppressive treatment, or infection were excluded from the study. All parts of the study conform to the Declaration of Helsinki and have been approved by the respective ethics committee of the University of Würzburg. All patients signed an informed consent form.

Animals

Adult (2–3 months) and aged (12–16 months) C57/BL6J mice were purchased from Charles River, whereas 2–3-month-old NOD-$Prkdc^{Scid}$ $Il2rg^{tm1Wjl}$ H2-$Ab1^{tm1Doi}$ HLA-DRA*0101 and HLA-DRB1*0101 (NSG-DR1) were commercially available from

Jackson Laboratory though a dedicated-breeding project (stock # 030331). Due to the combined *Prkdc*^{Scid} and *Il2rg*^{tm1Wjl} mutations, these animals harbor no mature T or B cells, lack functional NK cells, and are deficient in cytokine signaling, making xeno-engraftment possible without signs of rejection (15). Moreover, these animals express a transgenic construct containing the mouse MHC Class II *H2-Ea* and *H2-Eb1* genes engineered to encode proteins in which the alpha1 domain (amino acids 1-85) and the beta1 domain (amino acids 1-96) were replaced by the corresponding amino acids of the human MHC Class II protein encoded by HLA-DRA1*0101 and HLA-DRB1*0101. This mutation allows human MHC-II molecules to be functionally expressed on murine antigen presenting cells, which could then prime engrafted human CD4$^+$ T cells.

The animals were housed under specific pathogen-free (SPF) conditions with a 12-h light/12-h dark cycle and standard diet provided ad libitum and were acclimatized for at least 7 days post shipment. Due to their immunodeficiency status, the NSG-DR1 mice were maintained in dedicated ventilated cabinets, and all experimental procedures were performed inside a laminar flow hood under sterile conditions.

Human peripheral blood samples were obtained from healthy subjects recruited into the aforementioned clinical study in our hospital (Department of Internal Medicine of the University Hospital of Würzburg) in a period ranging between 2018 and 2020. Blood samples were collected in CPT vacutainers coated with sodium heparin and Ficoll^{TM} Hypaque^{TM} (BD, Franklin Lakes, USA), and peripheral blood mononuclear cells (PBMCs) were obtained after gradient centrifugation (1500 *g*, 20 min, 23°C), frozen in CTL-Cryo^{TM} freezing media (CTL, Boon, Germany) and then stored in liquid nitrogen (-180°C) until further usage. To monitor the effects of aging on the peripheral blood T cell compartment, we performed eight color flow cytometry measurements on all healthy subjects who had been recruited to the aforementioned study (described below). In total, we analyzed 13 samples from young subjects (20–45 yo), 18 samples from middle-aged subjects (45–65 yo) and 26 samples from elderly subjects (65–85 yo). A description of these subjects is presented in **Supplemental Table I**.

HLA genotyping was performed by the Institute for Clinical Transfusion Medicine and Haemotherapy from the University Hospital of Würzburg as previously described (16), and only CD4$^+$ T cells from matching healthy donors (i.e., DRB1*01:01) were selected for adoptive transfer experiments. Out of 57 healthy subjects screened, we found seven matching samples suitable for transplantation into NSG-DR1 mice. Three of these subjects had undergone elective coronary angiography evaluations with excluded myocardial infarction, and the remaining subjects were healthy volunteers. A description of the healthy donors enrolled in the T cell transfer experiments is presented in **Supplemental Table II**.

Adoptive Cell Transfer

Human untouched CD4$^+$ T cells were purified from PBMC samples by magnetic cell sorting using the CD4$^+$ T cell isolation kit, Human (Miltenyi Biotec, Bergisch Gladbach, Germany). Finally, human CD4$^+$ T cells were resuspended in sterile PBS (Biochrom, Berlin, Germany) at a concentration of 1x10^7 cells per ml and adoptively transferred into NSG-DR1 mice (2x10^6 cells i.p. – referred to as day 0). Baseline characterization of the donor cells and purity checks were routinely performed by flow cytometry. As a control group, age-matched naïve NSG-DR1 littermates were investigated.

Endpoint Analysis

At 6 weeks post human CD4$^+$ T cell transfer, NSG-DR1 mice were sacrificed by cervical dislocation, then whole-body perfusion with PBS + 50 U/ml heparin (Ratiopharm, Ulm, Germany) was conducted and the organs were extracted for *ex vivo* analysis. FACS analysis of freshly isolated cells (heart and spleen) was performed. The samples intended for RNA analysis were stored in RNAlater (Qiagen, Hilden, Germany) for 24 h and then stored at -80°C. Samples intended for histological analysis were embedded in Tissue-TeK optimum cutting temperature medium (Sakura Finetek, Alphen ann den Rijn, The Netherlands) and then stored at -80°C. Heart DNA extraction was performed from heart slices by trimming the Tissue-TeK content and performing tissue digestion and DNA purification using a GeneJET genomic DNA purification kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's instructions.

In Vitro Stimulation Assay

1 million PBMC (pretransfer), NSG-DR1 recipients' splenocytes or digested heart tissues were resuspended in complete RPMI media containing 10% FCS, 1% L-Glutamine, 1% Sodium pyruvate, 1% non-essential amino acids, 1%Pen/Strep, 1 μM 2-ME (Gibco – Grovemont Cir, USA) and seeded in U-bottom 96-well plate. Cells were stimulated for 3 h with a T-cell stimulation cocktail containing Phorbol 12-Myristat 13-Acetat (81 ηM) und Ionomycin (1.34 μM) supplemented with protein transport inhibitors Brefeldin A (10.6 μM) and Monensin (2μM) (eBioscience – San Diego, USA). Following incubation time, cells were washed and used in intracellular flow cytometry analysis.

Flow Cytometry

Immunophenotyping of spleen and digested heart samples obtained from control and CD4$^+$ T cell transfer mice and human PBMCs was performed. The heart samples were enzymatically digested in type II collagenase (1,000 IU/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA) for 30 min at 37°C and then ground against a 70-μm mesh (Miltenyi Biotec, Bergisch Gladbach, Germany) in 0.5% BSS/BSA. The lymphoid organs were ground against a 30-μm cell strainer, and the splenocyte preparations underwent erythrocyte lysis. All samples were stained with zombie aqua fixable viability dye (BioLegend, San Diego, USA) for 15 min at room temperature and protected from light. Afterwards, samples were washed and resuspended in FACS buffer, and surface staining was performed in the presence of FC-blocking antibody (anti-CD16/CD32, clone 2.4G2, BD Pharmingen) for host NSG-DR1 cell panels. The following antibodies conjugated with different fluorophores were used:

Human: anti-CD45RO (clone UCHL1), anti-CD4 (OKT4), anti-CCR7 (clone G043H7), anti-CD25 (clone M-A251)anti-CD127 (clone A010D5) obtained from BioLegend (San Diego, USA), and anti- PD-1 (clone J105), anti-FoxP3 (clone 236A/E7), anti-TNF (clone Mab11), anti-IFN-γ (clone 45.B3), anti-IL-17a (clone eBio64DEC17) and anti-IL-13 (clone 85BRD) obtained from eBioscience (San Diego, USA). Mouse: anti-CD45 (clone 30-F11), anti-CD11b (clone M1/70), anti-Ly6G (clone 1A8), anti-CD11c (clone N418), anti-CD62L (MEL-14), anti-CD3e (145-2C11), anti-CD44 (IM7), and anti-CD4 (RM4-5) conjugated with different fluorophores were obtained from BioLegend (San Diego, USA). Flow cytometry measurements were performed on an Attune-NxT (Thermo Scientific, Darmstadt, Germany). Data analysis was performed using FlowJo software (FlowJo LLC Ashland, OR, USA). Compensation for spectral overlap was conducted based on single staining controls, and flow cytometry gates were set based on unstained controls and fluorescence minus one controls.

Gene Expression Analysis

RNA was extracted from mouse myocardial samples (apical region) using the tissue RNA isolation kit (RNeasy mini – Qiagen). The RNA concentration and quality were assessed in a spectrophotometer, and 200 ng was used for cDNA synthesis (iScript – Bio-Rad). TaqMan probes for quantitative PCR were used to measure the expression of genes related to cardiac stress, aging, inflammation and fibrosis. The probes used in this manuscript were *Gapdh* (Mm033002249_g1), *Hsp1a1* (Mm01159846_s1), *Myh6* (Mm00440359_m1), *Myh7* (Mm01319006_g1), *Tnf* (Mm99999068_m1), *Il1b* (Mm00434228_m1), *Mmp9* (Mm00442991_m1), *Mmp2* (Mm00439498_m1), *Col1a1* (Mm00801666_g1), *Col3a1* (Mm01254476_m1), and *Tgfb3* (Mm00436960_m1). Target mRNA levels were normalized to *Gapdh* expression levels.

RNA-Seq and Bioinformatics

RNA extracted from mouse myocardial samples (apical region) with or without human T-cell transfer were used for bulk RNA sequencing. DNA libraries suitable for sequencing were prepared from 220 ng from one control sample and from 400 ng of total RNA from all other samples with oligo-dT capture beads for poly-A-mRNA enrichment using the TruSeq Stranded mRNA Library Preparation Kit (Illumina). Sequencing was performed on the NextSeq-500 platform (Illumina) in paired-end mode with 2x75 nt read length. Sequencing data are available at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE163413. Sequencing reads were adapter trimmed and mapped to the mouse (GRCm38.p6) and human genome (GRCh38.p13) with STAR followed by computational deconvolution of mouse and human reads using the XenofilteR tool (17). Gene level based read counts were generated with featureCounts using the RefSeq annotation. The count output was utilized to identify differentially expressed genes using DESeq2. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology analyses were performed using the web server g:Profiler (18) for all upregulated transcripts (for mouse transcripts: log2 fold change >0.5, FDR<0.05). The upregulated transcripts were also analyzed against the myocardial single cell atlas derived from *Tabula muris senis* consortium (19). Briefly, the gene counts and metadata from heart cells of the *Tabula muris senis* were downloaded from: figshare.com/articles/dataset/Tabula_Muris_Senis_Data_Objects/12654728?file=23872838, and the data was further analyzed using Seurat package (version 3.1). Cells from 3- and 18-months old animals were subset as representatives of young and aged mice. In the new subset Seurat Object, clustering analysis and UMAP (Uniform Manifold Approximation and Projection) dimensionality reduction were performed using the top 3,000 variable genes and the first 20 principal components. The average expression of NSG-DR1 T cell-transferred upregulated transcripts were assigned a signature score within each cell in the Object using the "AddModuleScore" function from Seurat. The combined score of all cells from young mice was compared to cells from aged mice. Gene set enrichment analysis (GSEA) was performed as previously described (20). Briefly, normalized expression of differentially expressed genes (FDR<0.05) from all samples were organized in a tab-separated file and probed against gene sets available in the GSEA platform (https://www.gsea-msigdb.org/gsea/index.jsp). Expression profiles were compared amongst the canonical pathways from curated gene sets (https://www.gsea-msigdb.org/gsea/msigdb/collection_details.jsp#C2). Data was expressed as normalized enrichment score plus FDR with an expression heatmap depicting representative genes for each gene set. After XenofilteR deconvolution, upregulated human transcripts from T cell transferred NSG-DR1 myocardial RNA samples were selected using the XenofilteR tool (17). A list of upregulated genes that showed no counts in NSG control myocardial was used for GO and KEGG analysis in g:Profiler web-server tool.

Methylated DNA Quantification

The percentage of 5-methylcytosine (5-mC) was determined in gDNA samples obtained from mouse myocardial tissue using the MethylFlash Global DNA Methylation ELISA Easy Kit (Epigentek). Briefly, 100 ng of gDNA samples and a standard curve (ranging from 0.1 to 5% 5-mC) were adsorbed to a 96-well assay plate for 1 h at 37°C. Wells were then washed three times with washing buffer and incubated with 50 µl of 5-mC detection complex solution, consisting of a mixture of 5-mC monoclonal antibody, signal indicator and enhancer solution for 50 min at room temperature. Following incubation, the wells were washed five times with washing buffer, and 100 µl of developing solution was added. The reaction was allowed to develop for 5 min, and then 100 µl of stop solution was added. The absorbance was read in a spectrophotometer (OD450 nm). The percentage of 5-mC in myocardial samples was estimated by interpolation of unknown values to a polynomial curve.

Histology

Heart cryosections (14 µm) from NSG-DR1 mice following 6 weeks of $CD4^+$ T cell transfer were fixed in 4% (vol/vol) formaldehyde solution in PBS and blocked with carbo-free

solution (Vector Lab Inc. Burlingame, CA, USA) for 30 min and then stained with wheat germ agglutinin (WGA) FITC to stain the cardiomyocyte cell surface, followed by DAPI for nuclear counterstaining. For picrosirius red staining (PSR, Morphisto, Frankfurt am Main, Germany), heart cryosections were stained as previously described (21). For Sudan black staining, cryosections were fixed as described before and washed with 50% and 70% alcohol solution respectively. Samples were then stained for 8 min with SenTraGor^{TM} reagent (Arriani pharmaceuticals Attica, Greece) according to manufacturer's instruction. Slides were washed twice in 50% alcohol solution and three times in PBS before proceeding to antibody staining. First, sections were blocked for mouse antibody staining with M.O.M blocking kit (Vector Lab Inc. Burlingame, CA, USA) and then stained with anti-biotin antibody clone Hyb-8 (Linaris Dossenheim, Germany) for 30 min. Following primary staining, samples were washed 3 times in PBS and stained with goat anti-mouse antibody conjugated to A555 and Phalloidin A647 for 30 min. DAPI was used at the last 5 min to stain nuclei. Samples were washed three times with PBS and mounted with Mowiol media. The fluorescence images were acquired using an epifluorescence microscope (model DFC 9000GT; Leica) coupled to a high-resolution camera (sCMOS monochrome fluorescence camera; Leica) and processed using ImageJ (NIH) software.

Statistics

Graphs presented in the study were designed to display the group mean values (bars), the SEM and the distributions of each individual value plotted. Graphs and statistical analyses were performed using GraphPad Prism software (version 7.0d, GraphPad software). A P value of less than 0.05 was considered statistically significant. Differences between groups containing normally distributed data were tested using an unpaired, two-tailed t test for two groups or two-way ANOVA followed by multiple t tests for data with more than two dependent variables. The Mann-Whitney U test was used to compare two groups that in which data did not fit a normal distribution. The data not-fitting a normal distribution are presented as box plots showing the median and the 25^{th}–75^{th} percentiles, as well as each individual point.

RESULTS

Myocardial- and Immunosenescence Phenotypes Develop Synchronously

In humans, naïve T cells express the chemokine receptor CCR7 and lack the expression of activation markers such as CD45RO, while effector/memory T cells present the opposite phenotype (22). As shown in **Figure 1A** and in Supplemental **Figure 1**, the frequency of naïve $CD4^+$ T cells was reduced in the elderly population (65–85 years old) compared to a younger adult population (20-40 years old) (**Figure 1B**). Similarly, effector memory and terminally differentiated $CD4^+$ T cells ($CCR7^-$ $CD45RO^{+/-}$) were increased in the aged group versus adults (**Figure 1C**). To translate these findings to mice, we quantified naïve ($CD62L^+CD44^-$) and effector ($CD62L^-CD44^+$) T cells in the spleen of young (2–3 months) and aged mice (12–16 months) (23) (**Figure 1D**). Our findings confirmed that shifts in naïve and effector T cell populations occur similarly between mice and humans (**Figures 1E, F**, **Supplemental Figure 2**). These age-related changes in peripheral immune cell composition have been shown to intertwine with cardiac inflammation and functional decline (11). In accordance with previous findings, we herein observed that aged hearts show a higher ratio of β:α-myosin heavy chain expression (products of the *Myh7* and *Myh6* genes respectively, **Figure 1G**), as typically seen in several pathological conditions (11, 24). In addition, cytosine methylation levels, a canonical molecular clock marker (25), were increased in the aged heart (**Figure 1H**). Moreover, Sudan black staining revealed a myocardial accumulation of lipofuscin granules in 12–16 months old hearts, especially distributed within the interstitial space (**Figure 1I**), suggesting the presence of senescent cells (26). Altogether, these data reinforce the synchronicity between immune and cardiac aging.

Human $CD4^+$ T Cells Shift Toward a Senescent-Like Phenotype When Transferred Into NSG-DR1 Mice

A $CD4^+$ T cell compartment comprising inflated effector- and terminally-differentiated cells, as typically seen in aging, has been implicated in a multitude of diseases. However, it remains unclear whether these findings simply reflect the association or a causative role of immune cells in tissue senescence (27). To further dissect the contribution of a senescence-like $CD4^+$ T cell compartment in a young organism context, we developed a xenograft model in which human $CD4^+$ T cells from DRB1*01:01 subjects were engrafted into humanized immunodeficient NSG-DR1 mice expressing a matching human HLA (detailed in methods section). After a period of 6 weeks of T-cell expansion, the heart and spleens of recipients were harvested for downstream analysis (**Figure 2A**). Successful engraftment was demonstrated by the higher spleen to body weight ratio in transferred mice, together with the detection of human $CD4^+$ T cells in the recipients' spleens (**Figures 2B, C**). No alterations in animal body weight were observed over a 6-week period, indicating a lack of xenograft reaction (**Supplemental Figure 3A**).

The phenotype of human $CD4^+$ T cells was analyzed by flow cytometry before adoptive transfer and after engraftment conditions as previously described (28). After gating on live single cell $CD4^+$ events, the major T helper cell subsets were defined as naïve ($CD45RO^-CCR7^+$), effector-memory ($CD45RO^+$ $CCR7^-$), T central memory ($CD45RO^+CCR7^+$), and terminally differentiated ($CD45RO^-CCR7^-$) (**Figure 2D**). Moreover, exhausted T-cells were defined based on the surface expression of PD-1 (**Figure 2E**) (29), whereas regulatory $CD4^+$ T cells were defined $CD4^+FoxP3^+$ (**Supplemental Figure 3C**) (30). As shown in **Figures 2D–G** and **Supplemental Figure 3B**, upon adoptive transfer into NSG-DR1 mice, $CD4^+$ T cell exhibited a stark shift toward effector-memory/terminally-differentiated and exhausted phenotypes in parallel with a significant decline in the

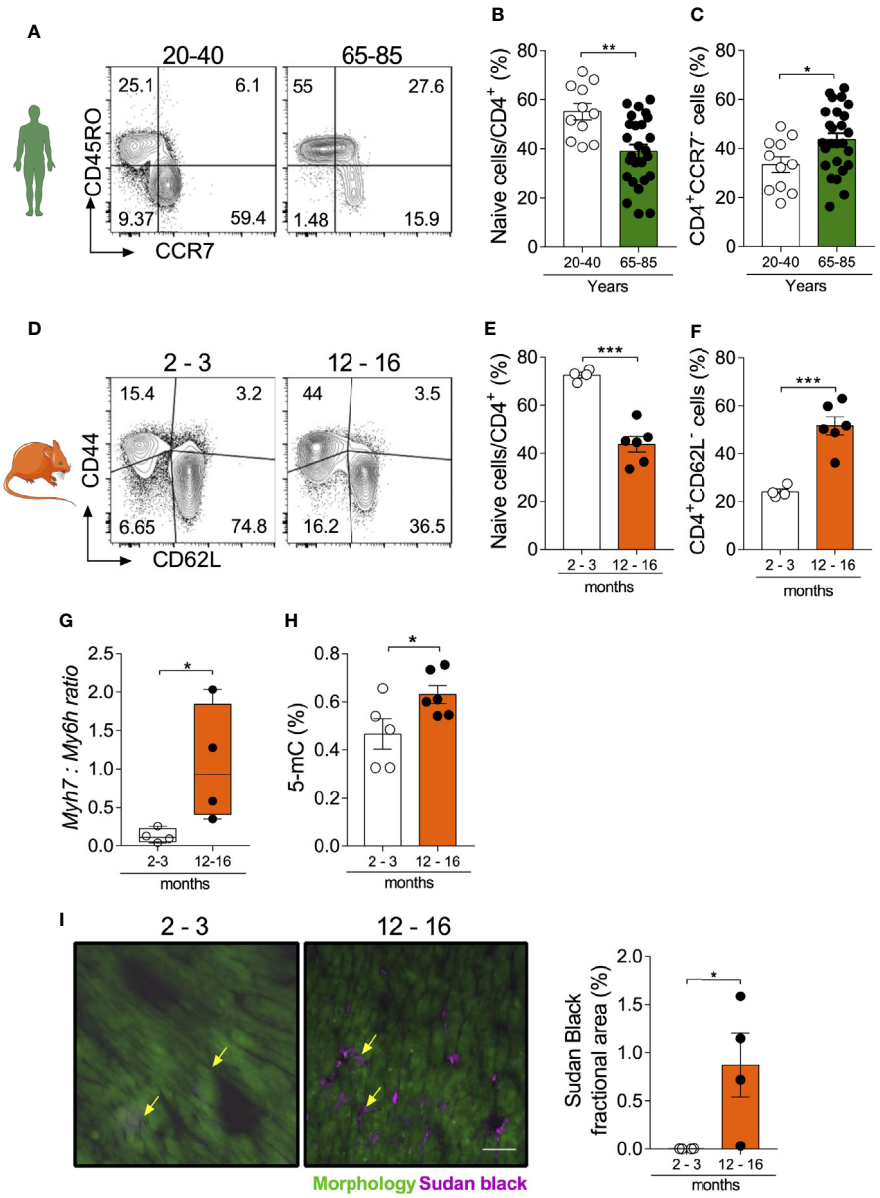

FIGURE 1 | Myocardial and immunosenescence phenotypes develop synchronously. **(A)** FACS strategy depicts the frequency of human naïve $CD4^+$ T cells ($CD45RO^-CCR7^+$) and the effector memory population ($CD45RO^+CCR7^-$). Aging is accompanied by a reduction in naïve $CD4^+$ T cells **(B)**, while effector memory/terminally differentiated cells increase **(C)**. **(D)** FACS strategy shows the distribution of naïve ($CD44^-CD62L^+$) and effector memory ($CD44^+CD62L^-$) $CD4^+$ T cells in the spleen of young (2–3 months) and aged (12–16 months) mice. The frequency of naïve $CD4^+$ T cells is reduced in aging **(E)**, while effector memory and terminally differentiated cells increase **(F)**. In immunocompetent WT animals, aging is associated with and higher *myh7:myh6* expression ratio **(G)** and increased levels of heart DNA methylation **(H)**. **(I)** Immunofluorescence of lipofuscin granules and its quantification in heart tissue from young and aged mice. Yellow arrows depict lipofuscin stained areas in both groups. Scale-bar: 100 μm. The bar graphs display the group mean values (bar), the SEM and the distribution of each individual value. Statistical analysis in **(B–I)**: Two-tailed unpaired *t* test, $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

naïve and regulatory compartments. This effect was not dependent on $CD4^+$ T cell donor's age (**Supplemental Table II**). This data confirm that we have successfully generated an experimental model able to recapitulate the hallmarks of T-cell senescence in young mice, offering a unique opportunity to decompose the causality of cardio-immune aging processes.

Human $CD4^+$ T Cell Engraftment in NSG-DR1 Mice Favors Cardiac Leukocyte Accumulation

After having observed that transferred human $CD4^+$ T cells rapidly acquire an effector/terminally differentiated phenotype in NSG-DR1 mice, we sought to investigate their myocardial

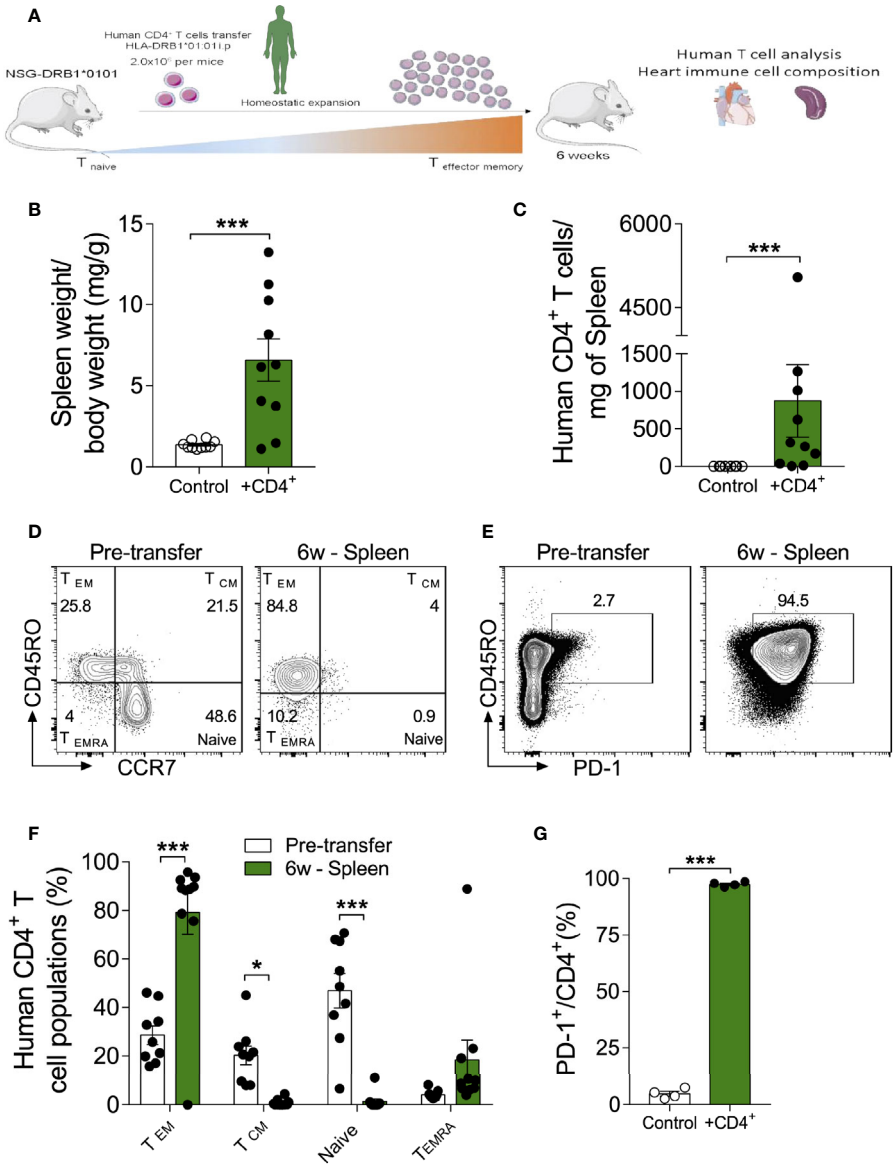

FIGURE 2 | The xenograft transplantation model renders young mice harboring a senescent-like T-cell compartment. **(A)** Experimental design: Immunodeficient NSG-DR1 mice were adoptively transferred with $CD4^+$ T cells purified from matching human donors, which readily undergo homeostatic expansion without causing xenograft reactions. Naïve NSG-DR1 mice were used as controls. Six weeks after transfer, heart and spleen tissue were collected for analysis. **(B)** Spleen weight to body weight ratio and number of $CD4^+$ T cells per milligram of spleen **(C)** depict the T cell engraftment of NSG-DR1 mice after 6 weeks. **(D)** Flow cytometry plots illustrate the frequency of naïve ($CCR7^+CD45RO^-$) and effector memory ($CCR7^-CD45RO^+$) $CD4^+$ T cells before transfer and in NSG-DR1 spleen 6 weeks afterwards. **(E)** FACS plots also illustrate the distribution PD-1^+ T cells before and after transfer in the spleen. Control group: Naïve NSG-DR1 mice. $CD4^+$ group: NSG-DR1 mice transplanted with human $CD4^+$ T cells. **(F)** Bar graph shows the shift from naïve toward an effector memory phenotype following 6 weeks of engraftment in the spleen. **(G)** Increased frequency of human PD-1^+ T cells in the spleen of NSG-DR1 mice. The bar graphs display the group mean values (bar), the SEM and the distribution of each individual value. Statistical analysis in **(B, C, G)**: two-tailed paired *t* test, $^{***}P < 0.001$, in **(F)**: two-way ANOVA followed by multiple *t* tests, $^{***}P < 0.001$ and $^{*}P < 0.05$.

distribution and possible effects on the composition of other cardiac resident leukocytes. First, we observed that most transferred human $CD4^+$ T cells found in cardiac tissue presented effector memory ($CD45RO^+CCR7^-$) terminally differentiated ($CD450^-CCR7^-$) and exhausted (PD-1^+) phenotypes (**Figures 3A–C**). No differences were detected in the Treg distribution in heart tissue when compared to pretransfer frequencies (**Supplemental Figure 4A**). Moreover, cardiac infiltrating $CD4^+$ T cells showed increased production of TNF and IL-13 following *in vitro* stimulation, compared to pre-

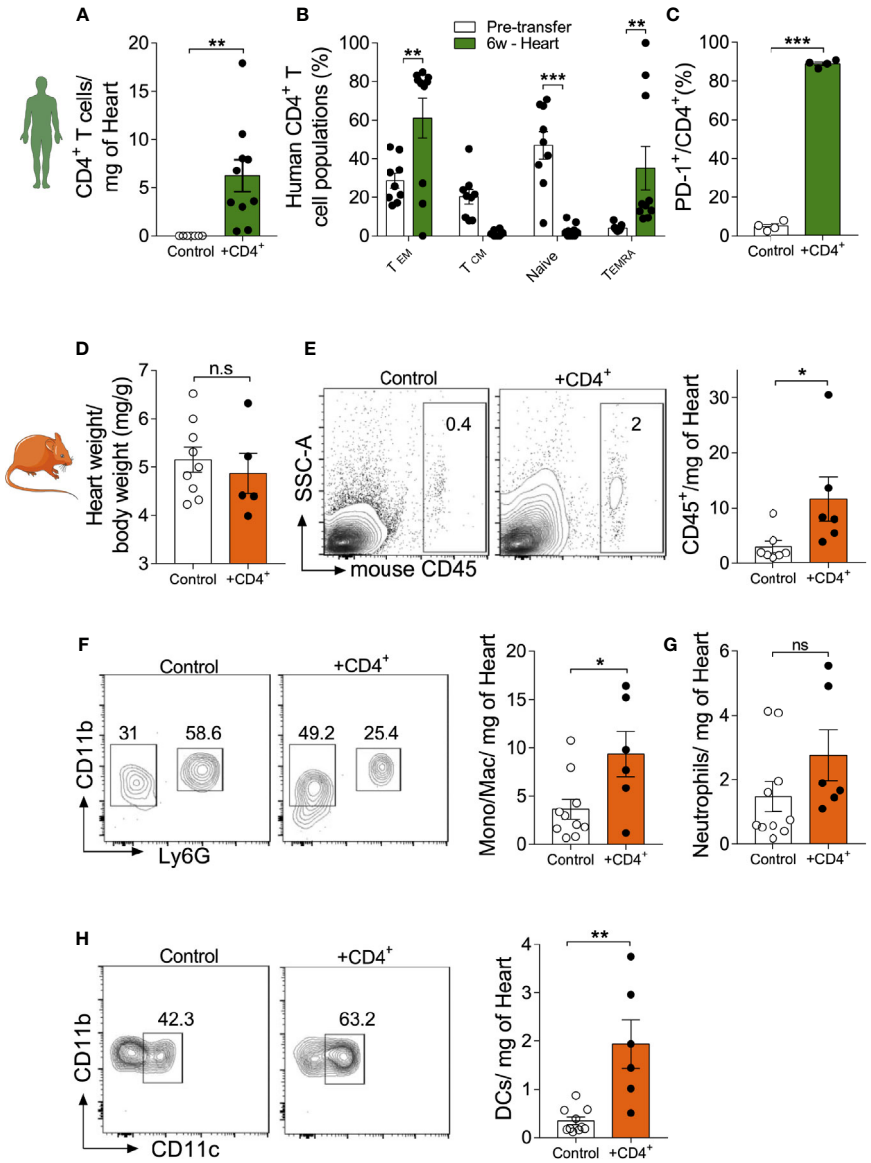

FIGURE 3 | Young mice harboring a senescent-like CD4$^+$ T-cell compartment show signs of myocardial inflammaging. Myocardial infiltration of transferred T-cells and its impact on baseline tissue inflammation. **(A)** Human CD4$^+$ T cells were detected in the myocardium of NSG-DR1 mice 6 weeks after engraftment. **(B)** CD4$^+$ T cells found in the myocardium mainly presented a terminally differentiated phenotype, classified as CCR7$^-$CR45RO$^-$, while naïve and central memory phenotypes were largely reduced after transfer. **(C)** Increased frequency of heart PD-1$^+$ T cells versus pretransfer values. **(D)** Heart weight to body weight was measured in control and CD4$^+$ T cell-transferred NSG-DR1 mice. **(E)** FACS plots depict the frequency of heart mouse leukocytes (live CD45$^+$) in control and adoptively transferred mice. The bar graph shows the number of heart leukocytes normalized per tissue weight. **(F)** Gating strategy for cardiac monocytes/macrophages (CD11b$^+$Ly6G$^-$) and neutrophils (CD11b$^+$Ly6G$^+$). The bar graph illustrates the number of monocytes/macrophages and neutrophils **(G)** in control and CD4$^+$ T cell-transferred mice per milligram of heart. **(H)** Cardiac dendritic cells were gated as Ly6G$^-$CD11b$^+$/CD11b$^+$CD11c$^+$ events. Control group: Naïve NSG-DR1 mice. CD4$^+$ group: NSG-DR1 mice transplanted with human CD4$^+$ T cells. The bar graphs display the group mean values, the SEM and the distribution of each individual value. Green bars represent parameters measured on human CD4$^+$ T cells, and orange bars represent mouse endogenous leukocyte parameters. Statistical analysis in **(B)**: two-way ANOVA followed by multiple *t* tests, ***$P < 0.001$ and **$P < 0.01$, in all other panels: two-tailed unpaired *t* test, **$P < 0.01$ and non-significant (n.s) $P>0.05$. *$P < 0.05$.

transfer values and to splenic cells (**Supplemental Figure 4B-D**). The myocardial accumulation of transferred CD4$^+$ T cells did not result in changes from heart to body weight ratios (**Figure 3D**), but promoted a mild infiltration of other murine inflammatory cells (**Figure 3E**), including monocytes/macrophages (Ly6G$^-$CD11b$^+$, **Figure 3F**) and dendritic cells (CD11b$^+$ CD11c$^+$ Ly6G$^-$, **Figure 3H**), but not neutrophils (CD11b$^+$Ly6G$^+$, **Figure 3G**).

Expanded CD4$^+$ T Cells Promote Myocardial Inflammation and Stress Response

To further assess the impact of a senescent CD4$^+$ T cell compartment on myocardial inflammation and the stress response, we analyzed the expression of genes that reflect features of cardiomyocyte physiology/stress response, inflammation/immunity and extracellular matrix remodeling/fibrosis (31–35). We observed a significant increase in beta myosin heavy chain 7 expression (*Myh7*) in heart tissue from T cell-transferred NSG-DR1 mice, paralleled by a reduction in alpha myosin heavy chain (*Myh6*) expression, similar to physiological aging (**Figure 4A**). In addition, T cell-transferred NSG-DR1 mice displayed an increased myocardial *Il6* expression, a proinflammatory cytokine associated with left ventricular dysfunction and a heart failure predictor in humans (32) (**Figure 4B**). Moreover, the expression levels of *Mmp9*, which can be produced by cardiomyocytes, neutrophils and monocytes under inflammatory conditions, was also found to be increased in T cell-transferred hearts (**Figure 4C**). Alongside with increased cardiac leukocyte numbers, these findings suggest that a senescent-like CD4$^+$ T cell compartment is sufficient to promote mild myocardial inflammation compatible with the concept of inflammaging. No differences between groups were observed concerning the myocardial expression levels of *Hsp1a1*, *Il1b*, *Tnf, Tgfb3, Col1a1*, and *Col3a1*. Other canonical markers of cellular senescence (e.g., CpG methylation and accumulation of lipofuscin granules) and myocardial aging (e.g., hypertrophy and fibrosis) were not affected by the adoptive T-cell transfer within the short period herein analyzed (**Supplemental Figure 5**).

Transcriptome Analysis Reveal an Inflammaging Phenotype in the Heart of CD4$^+$ T Cell Transferred NSG Mice

To gain mechanistic insight into the effects of terminally differentiated T cells on the heart inflammaging status, we performed bulk RNA sequencing of myocardial tissues from T cell transferred and control NSG-DR1 mice (**Figure 5A**). The major advantage of this approach is that the sequenced reads can be then aligned against the mouse and human reference

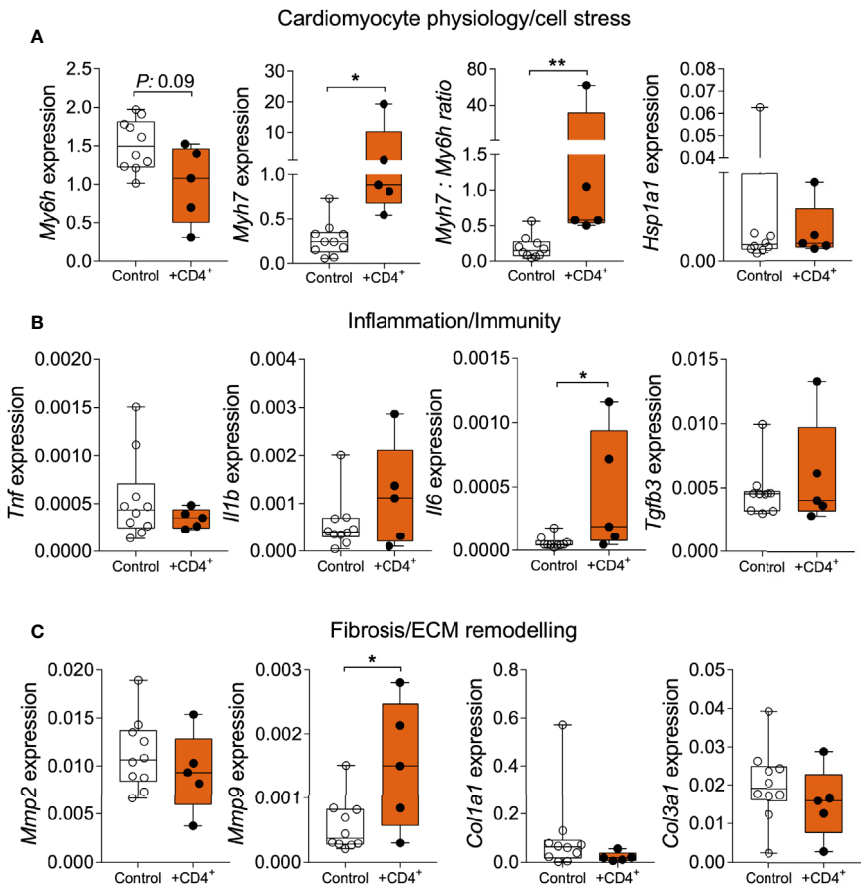

FIGURE 4 | Senescent-like CD4$^+$ T cells promote alterations in myocardial gene expression compatible with physiological aging. Relative myocardial mRNA levels of transcripts related to cardiomyocyte stress **(A)**, inflammation **(B)**, and extracellular remodeling **(C)**. Control group: Naïve NSG-DR1 mice. CD4$^+$ group: NSG-DR1 mice transplanted with human CD4$^+$ T cells. The bar graphs display the group mean values, the SEM and the distribution of each individual value. Statistical tests: Mann-Whitney. $^{**}P < 0.01$ and $^{*}P < 0.05$.

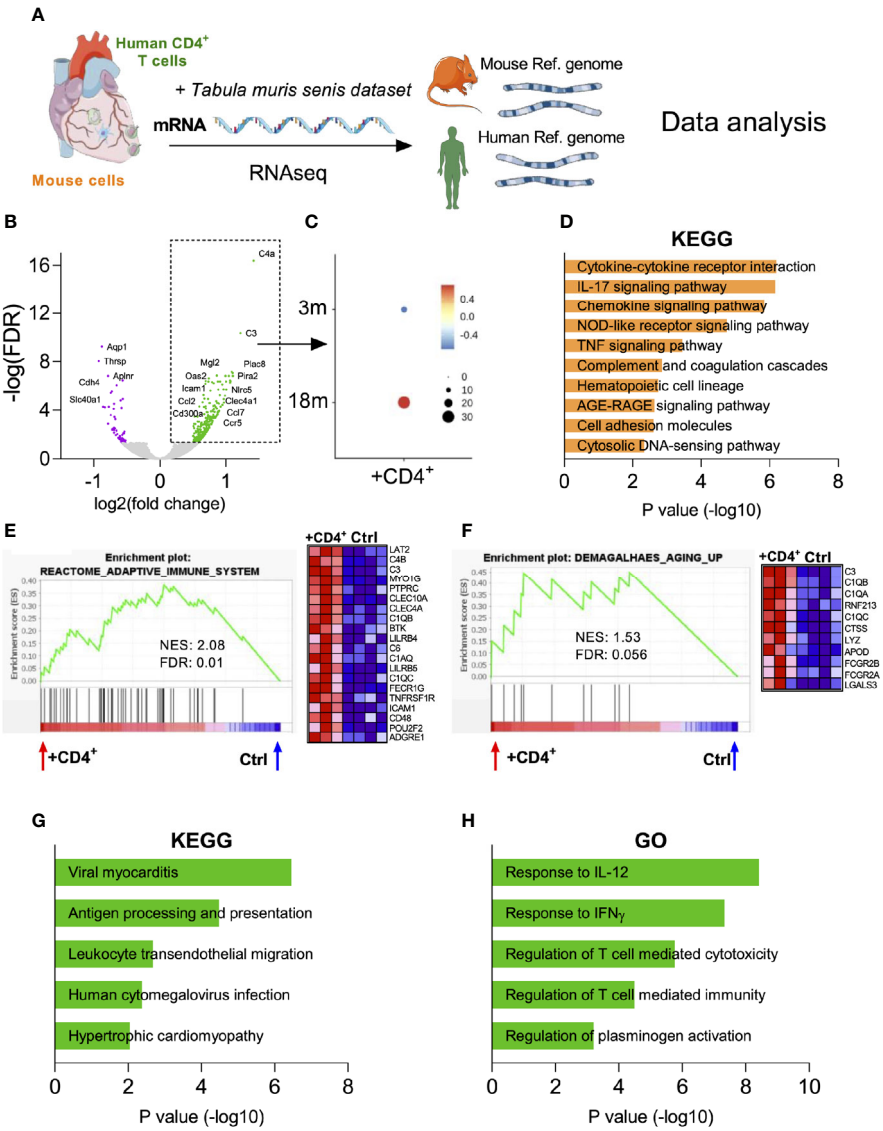

FIGURE 5 | Transcriptome analysis reveal an inflammaging phenotype in the heart of CD4$^+$ T cell transferred NSG mice. **(A)** Myocardial mRNA extracted from control and T cell transferred NSG-DR1 mice was employed in RNA-seq analysis. Transcripts were aligned against mouse genome and human genes were selected using the XenofilteR tool and further integrated with other publicly available datasets. **(B)** Volcano plots comparing the gene expression levels in myocardial tissue of T cell transferred NSG-DR1 mice versus control. The upregulated and downregulated genes (± 0.5–1.5 log2 fold, FDR<0.05) are highlighted in green and purple respectively. **(C)** The myocardial upregulated gene set identified in **(B)** was then probed in other myocardial aging datasets publicly available [*Tabula muris senis* (19)]. A score of the average expression levels (colored scale) and fraction of cells expressing the signature (circle sizes) was analyzed at single-cell level on myocardial samples obtained from 3- and 18-months old mice (3M and 18M respectively). **(D)** KEGG analysis of upregulated mouse transcripts from T cell transferred NSG-DR1 hearts. The bars represent the adjusted *P* value (Fisher's exact test). **(E, F)** GSEA analysis of "reactome adaptive immune system" and "Demagalhaes aging up" (36) in the myocardium of control and T cell transferred NSG-DR1 mice. Normalized enrichment score (NES) and FDR are depicted in the graphs. The heatmaps illustrate the genes differently expressed at each gene set. **(G, H)** KEGG and gene ontology (GO) analysis of upregulated human transcripts found in the myocardium of T cell transferred NSG-DR1 mice. The bars represent the adjusted *P* value (Fisher's exact test). Data from control (n: 4) and CD4$^+$ T cell transferred (n: 3) were acquired from two independent experiments.

genomes, meaning that it is possible to discriminate what transcripts were expressed by the murine myocardial cells or by the transferred human cells. As depicted in **Figure 5B**, hearts from T-cell-transferred mice upregulated a unique set of murine genes involved in complement pathway (*C4a, C3*), immune cell trafficking (*Icam1, Ccl2, Ccl7, Ccr5*), and immune inhibitory response (*CD300a, Clec4a1*). To investigate whether this transcription signature could be also upregulated in the heart during physiological aging, we compared it against cardiac single-cell transcriptomic data available from the *Tabula muris senis* consortium (19). The *Tabula muris senis* is a bold initiative that provides an unprecedented and comprehensive analyses of

age-related molecular signatures in mice. It includes bulk and single-cell-sequencing RNA atlases covering 23 tissues and organs throughout the mouse lifespan, offering a unique resource to be compared with our own sequencing findings. The analysis revealed that the myocardial gene set found to be upregulated in young NSG mice harboring an expanding T-cell compartment resembles the signature observed in aging hearts (**Figure 5C**).

Unsupervised enrichment pathway analysis and Gene set enrichment analysis (GSEA) further revealed a T-cell-triggered upregulation of pathways related to myocardial inflammation (e.g., Cytokine-cytokine receptor interaction, IL-17 and TNF signaling pathways) and canonical aging pathways such as advanced glycation end products signaling (AGE/RAGE) (**Figures 5D, E**). Furthermore, the myocardial gene expression signature found in T-cell-transferred NSG-DR1 mice overlapped with an conserved age-related gene set described in several tissues of senescent mice, rats and humans (**Figure 5F**) (36). Lastly, we deconvoluted the mouse and the human transcripts found in T-cell-transferred NSG-DR1 hearts using the XenofilteR tool (17). Enrichment pathway analysis indicated that human T cells infiltrating the myocardium acquired a pro-inflammatory phenotype, with a gene signature coinciding to viral myocarditis and cytomegalovirus infection processes (**Figure 5G**). Moreover, the transferred human T cells were enriched in pathways associated to canonical Th1 responses (IL-12 and IFN-γ), cytotoxicity and plasminogen activation (**Figure 5H**). The pro-inflammatory phenotype and sustained survival of expanded human T cells is also in line with TNF secretory profile observed in T-cell stimulation functional assays (**Supplemental Figure 4 C-D**) (37, 38).

DISCUSSION

Aging is not a disease per se; it is an unavoidable biological process. However, its pace and intensity can greatly vary between subjects, hence differentially impacting individual susceptibility to a plethora of age-related diseases. Compelling evidence has demonstrated that an overt T cell senescence profile can be associated with several age-related conditions, especially cardiovascular diseases (11–14, 39–43). However, whether the immune system fuels local inflammation due to underlying age deterioration or triggers tissue age-related changes is not completely understood. To address this question, we have developed a xenograft model in which human $CD4^+$ T cells rapidly shift towards an aged-like terminally-differentiated phenotype in young NSG-DR1 hosts. The major advantage of this model is that it recapitulates several hallmarks of T cell senescence in otherwise young and healthy mice, offering therefore a unique opportunity to decompose the mechanisms of aging (44, 45).

Herein, we observed that transferred $CD4^+$ T cells expanded systemically, mildly infiltrated young healthy hearts and promoted myocardial alterations that recapitulates some of the shifts typically seen in the physiological aging process, such as the recruitment of inflammatory immune cells and upregulation of pro-inflammatory genes. Most strikingly, our bulk RNA sequencing analysis identified a distinct myocardial transcriptomic signature in young NSG-DR1 mice harboring a senescent-like T-cell compartment, which matched other conserved age-related signatures reported in previous studies (19, 36). Altogether, these findings strongly suggest a causal relationship between immunosenescece and myocardial aging.

The accumulation of senescence-associated T cells (SA-T) has already been implicated in tissue dysfunction and higher morbidity in humans (2). In addition to its acute role in autoimmune diseases and allograft rejection (46), SA-T cells were also found in chronic low-grade inflammation and tissue dysfunction that developed during physiological aging. SA-T cells have been found in the visceral adipose tissue of aged mice among macrophages and B cells. Despite presenting defective TCR-mediated proliferation, SA-T cell stimulation has been reported to result in abundant production of TNF, IL-6, and osteopontin (47). Similarly, tertiary lymphoid structures enriched in $PD\text{-}1^+$ $CD4^+$ T cells are observed in chronic kidney disease developed in aged mice and in humans. Although the contribution of $PD\text{-}1^+$ $CD4^+$ T cells is not clear, global deletion of $CD4^+$ T cells resulted in a dampened inflammatory response and improved kidney function (48). Infiltrating T cells expressing IFN-γ are found in aged brains in close proximity to neural stem cells and local IFN-γ signaling has been shown to impair neural stem cell proliferation, providing a possible cause for its decline during aging (49).

A recent elegant study by Desdín-Micó et al. indicated that the premature induction of T-cell senescence in young mice drives systemic inflammaging and multi-organ morbidity, being that sufficient to recapitulate some major features of aging process (50). In a previous study we had reported that the heart-draining lymph nodes of aged mice also showed a great enrichment for Th1-polarized effector $CD4^+$ T cells, and that genetic models of T helper cell ablation attenuated the myocardial inflammaging (11). Most recently, Kallikourdis's team has shown that T-cell costimulation blockade blunts the age-related myocardial functional decline (51), providing further functional evidence for a causal link between T-cell development and myocardial aging. In humans, an exaggerated accumulation of memory T cells has been widely associated to increased cardiovascular risk, heart failure progression and overall mortality (8, 12, 14). More recently, Alpert and coauthors developed a refined human immunological age score (IMM-AGE) based on longitudinal high-dimensional flow cytometry, proteomics, and transcriptomic assessments which has been shown to predict cardiovascular events and overall mortality (2).

Humanized NSG mouse strains have been used to allow a meaningful assessment of human T cell functionality in the absence of a graft versus host reaction, especially in the context of infectious disease and cancer research (52–55). On top of the standard NSG genetic makeup, the mouse strain used in the current study was further engineered to express a hybrid murine: human MHC-II (HLA-DRB1*01:01) that enables cognate antigen interactions between mouse antigen presenting cells

and human $CD4^+$ T cells in an *in vivo* context (56). Thus, the transferred human T-cells can be fully stimulated in this experimental setting. It has been previously reported that the adoptive transfer of $CD4^+$ T cells to immunodeficient mice lacking T, B, and NK cells (15) results in rapid proliferation of donor cells marked by robust differentiation towards a memory phenotype (57, 58). Notwithstanding, none of these studies have employed this humanized adoptive cell transfer model to investigate mechanisms of aging. Despite the advantages this model; namely, a decomposition of immune vs tissue aging mechanisms in a single mouse in a translational experimental setting, this model has some important limitations. Most notably, the time-points chosen for the end-point analyses in the present study (6 weeks) might have been too short to reproduce some of the long-term low-grade shifts occurring throughout the several months of a mouse lifespan. Therefore, we could not observe structural and functional myocardial alterations, despite the clear shifts in gene expression profile. Still, the observation that terminally-differentiated T cell compartment can promote a cardiac inflammaging molecular signature in young mice without preexisting cardiac conditions raises important mechanistic insights that help dissecting some causality of myocardial aging process.

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. Sequencing data are available at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE163413.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of the University of Würzburg. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Regierung von Unterfranken.

AUTHOR CONTRIBUTIONS

MDG, NH, DA, MA, MS, TH performed experiments and analysed the data; MH, NH, UH recruited the human subjects and handled the human samples; MH, MDG, IS, UH, SF and GR designed the study and interpreted data; MDG and GR drafted the manuscript 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/fimmu.2021.584538/full#supplementary-material

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MINI REVIEW
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The Dual Role of Platelets in the Cardiovascular Risk of Chronic Inflammation

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Patients with chronic inflammatory diseases often exhibit cardiovascular risk. This risk is associated with the systemic inflammation that persists in these patients, causing a sustained endothelial activation. Different mechanisms have been considered responsible for this systemic inflammation, among which activated platelets have been regarded as a major player. However, in recent years, the role of platelets has become controversial. Not only can this subcellular component release pro- and anti-inflammatory mediators, but it can also bind to different subsets of circulating lymphocytes, monocytes and neutrophils modulating their function in either direction. How platelets exert this dual role is not yet fully understood.

Keywords: platelet, chronic inflammation, cardiovascular risk, leukocytes, systemic inflammation

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INTRODUCTION

Systemic inflammation (SI) has been described as a consequence of increased levels of the circulating pro-inflammatory mediators that activate endothelial cells (EC). Endothelial activation is part of a normal immune system defense, but a prolonged inflammatory stimulus induces a sustained endothelial activation/dysfunction that is often associated with atherogenesis and cardiovascular events. The role of SI on cardiovascular (CV) risk has been explored in autoimmune diseases. Systemic lupus erythematosus (SLE)-like mouse models display endothelial dysfunction and cardiac hypertrophy, mediated through IL-6 and IL-1α (1). In these patients, it has been shown that B lymphocyte stimulator induced apoptosis of endothelial progenitors cells and EC (2). In addition, it has been suggested that autoantibodies play a role in endothelial dysfunction, possibly by modulating the adhesion of neutrophils (3, 4). In a model of arthritis, endothelial dysfunction was only observed in rats with a persisting imbalance between NOS and COX-2 pathways, higher plasma levels of IL-1β and tumor necrosis factor-α (TNF-α) (5). The presence of diabetes mellitus type 2 in patients with metabolic syndrome impairs the endothelial function (6). In a model of SI, the levels of endothelin-1 and endocan are related to endothelial dysfunction (7). However, there are some circumstances in which the relationship between SI and endothelial dysfunction is less clear. In Systemic Inflammatory Response syndrome, the levels of dysfunctional EC were associated with mortality and organ dysfunction independently of inflammatory markers (8).

The link between inflammation and endothelial dysfunction has been confirmed by the inhibition of molecules related to SI. Anti-TNFα antibodies reduces sE-selectin and sVCAM expression (9) and decreases endothelium-dependent relaxation (10, 11). EC treated with etanercept revert the apoptosis induced by TNF-α (12). JAK inhibitors improve endothelium dependent vasorelaxation, endothelial cell differentiation and lipoprotein profiles, while decreasing pro-inflammatory cytokines in SLE-like syndrome (13). Glucocorticoids decrease IL-1β and TNF-α

levels, improving the function of endothelium in rheumatoid arthritis (RA) (14). Patients with CV risk factors had increased levels of IL-1β and its gene expression signature and blocking IL-1β with canakinumab was observed to prevent recurrent cardiovascular disease (CVD) (15). Agents with nucleoside triphosphate hydrolase activity decrease platelet-leukocyte-endothelium interaction, the transcription of pro-inflammatory cytokines, microvascular platelet-neutrophil aggregate sequestration, activation marker expression on platelets (PLTs) and neutrophils contained in these aggregates, leukocyte extravasation, and organ damage (16). Furthermore, dihydroartemisin inhibits the occludin downregulation induced by TNF-α, improving the permeability of EC (17) and the inhibition of cannabinoid receptors reduces leukocyte-adhesion and improves microvascular blood flow (18). The pre-treatment of primary cultured human umbilical vein endothelial cells (HUVECs) with sevoflurane reduces ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) IκBα, and NF-κB activation, and blocks the adhesion of leukocytes (19).

Products with anti-inflammatory properties can improve endothelial function. Lactobacillus plantarum 299v supplements decreased inflammatory markers (20), while the active form of vitamin D diminishes IL-6 secretion and increases the angiogenic capacity of myeloid angiogenic cells via CXCL10 down-regulation (21).

FEATURES OF ENDOTHELIAL DYSFUNCTION

The inflammatory phase that leads to endothelial dysfunction is initiated by TNF-α and subsequently amplified by IL-1, IL-6 and downstream mediators. Endothelial dysfunction refers to the failure by ECs to perform their physiological functions, often due to a maladaptive response to pathological stimuli. The phenotypic features of endothelial dysfunction include the upregulated expression of endothelial leukocyte adhesion molecules (ELAMs) [E-selectin, ICAM-1 and VCAM-1]. On leukocytes, activated ECs induce the affinity of counter-receptors for ELAMs and secrete and display chemokines on the luminal surface. Endothelial dysfunction also includes a compromised barrier function, the secretion of microvesicles, an increased vascular smooth muscle tone, and the increased production of vasoconstrictor substances, the reduced resistance to thrombosis via platelet aggregation and oxidative stress upregulation (22–24). It was described that high levels of IL-8 and TNF-α up-regulate CX3CR1 expression on platelet-monocyte aggregates, increasing adhesion to activated endothelium (25). In mouse models, increased IL-17 was associated with reactive oxygen species formation, circulating inflammatory leukocytes and endothelial dysfunction (26), while higher levels of resistin, TNF-α, IL-1β, and MMP-9 expression were associated with the levels of inflammatory infiltrates in artery walls (27).

Beyond the activation of the well-known signaling cascades, the stimulation of EC induces gene expression via microRNAs (miRNA) and epigenetic modifications. The overexpression of miR100 in ECs attenuates leukocyte-endothelial interaction, represses the mammalian target of rapamycin complex 1 signaling, stimulating endothelial autophagy, and attenuates NF-κB signaling. Local miR100 expression is inversely correlated with an inflammatory cell content (28). miR181b inhibits downstream and upstream NF-κB signaling in response to activation (29), while the NF-κB target genes (VCAM-1, ICAM-1, E-selectin, and tissue factor) (30) and miR223 are associated with HUVEC dysfunction (31). Additionally, IFN-α, through miR155, promotes an endothelial dysfunction signature in HUVECs characterized by transcription suppression and the mRNA instability of eNOS and by the upregulation of MCP-1 and VCAM-1 and enhanced neutrophil adhesion (32).

Endothelial microparticles (MPs), shed as a result of the activation of EC are considered a source of important information on the status of ECs and vascular function (33, 34). Circulating levels of endothelial MPs reflect a balance between cell stimulation, proliferation, apoptosis, and cell death (35) and are increased by inflammatory stimuli, mediated by the activation of NF-κB and associated with oxidative stress intensity (36, 37). Endothelial MPs are increased in autoimmune diseases (38) and serve as markers for vascular dysfunction and their effects depend on their cargo and on the surface molecules. Recently, levels of endothelial MPs have been associated with disease activity in SLE patients and CV risk (39).

MPs from RA patients had higher expression of TNF-α on the surface compared to healthy donors (HD), increasing apoptosis and autophagy levels on EC and correlating with clinical RA activity (40).

PLATELETS AND SYSTEMIC INFLAMMATION

PLTs have come to be recognized as active players in SI. After activation, PLTs participate in the vasculature inflammation and damage, atherogenesis and thrombosis (41–45). Wide ranges of stimulus are able to activate PLTs. The strong PLT activation was achieved with the ligation of the agonist thrombin, collagen and ADP to the PLTs receptors: protease-activated receptor 1, GPVI and P2Y1 or P2Y12 respectively (46–48). Other non-classical pathways are able to activate PLTs due to the expression of Toll-like receptors (TLR), TNF-α receptor, IL-1β receptors and C-type lectin-like receptors (49–53). Some autoantibodies presents in autoimmune disease patients such as anti-citrullinated protein, anti-β2 glycoprotein I and anti-D4GDI have also the ability to induce PLT activation through FCγRIIa (54–56).

However, PLTs also participate in the resolution of inflammation as anti-inflammatory elements. How PLTs sense the signal to exert pro- or anti-inflammatory functions is not yet fully known. However, it is known that PLTs exert their functions by releasing soluble factors and interacting with cells. The dual role of PLTs in inflammation (57, 58) may be the result of differences in the PLT packing of molecules, activated-dependent release by different stimuli, the kinetics of

TABLE 1 | Evidences of dual role of expressed/secreted platelet factors.

Factor	Pro-inflammatory	Anti-inflammatory
PF4	• Monocytes: ↑ phagocytosis (59) ↑ respiratory burst (59) ↑ pro-inflammatory cytokines (59) • Neutrophils: ↑ NETs formation (60) • Endothelial cells: ↑ Leukocyte recruitment (60)	• T lymphocytes: ↓proliferation (61) ↓Th17 differentiation (62) ↓granzyme B (61)
IL-1	↑ endothelial activation (63, 64) ↑ EC damage (63) ↑ neutrophil adhesion (63)	
P-selectin	• Monocytes: ↑ pro-inflammatory cytokines (65) • Neutrophils: ↑ NETs formation (66)	• *In vitro*: ↓neutrophil adhesion to activated EC (67)
sCD40L	Plasmatic levels correlated CV risk and pro-inflammatory cytokines (68, 69) ↑ B cell isotype switching (70)	• Monocytes: ↓TNF-α (71) ↑IL-10 (71) Correlation with IDO, Treg (68)
TGF-β		• T lymphocytes: ↓T cell function and proliferation (72, 73) ↓Th1, Th17 response (72) ↓IFN-γ production (72, 73) ↓granzyme B and perforin (73) ↑Treg differentiation (72)
HMGB1	• Monocytes: ↑ migration and accumulation in tissues (74, 75) ↓apoptosis (74) • Neutrophils: ↑ NETs formation (76, 77)	

release and the *de novo* synthesis of soluble factors and their binding to certain molecules on the surface of leukocytes.

Pro-inflammatory and Anti-inflammatory Soluble Factors Released by PLTs

Some of the released factors of PLTs are synthesized *de novo*, whereas others are stored and are secreted from granules as pro-thrombotic, immunoregulatory molecules and growth factors immediately after activation. Molecules from dense granule components contribute to hemostasis and coagulation. Molecules from α-granules contain multiple cytokines, mitogens, pro- and anti-inflammatory factors and other bioactive molecules that are essential regulators in the complex microenvironment (**Table 1**).

Platelet Factor 4 (PF4, also called CXCL4) is the most abundant protein secreted by activated PLTs and is deposited on endothelium. Higher levels of circulating PF4 have been observed in patients with chronic inflammation (78–80). PF4 increases phagocytosis, respiratory burst, survival and the secretion of inflammatory cytokines in monocytes (59). PF4 blocking reduces the inflammation of vasculature and CV events by reducing leukocyte recruitment and the generation of neutrophil extracellular traps (NETs) by neutrophils (60, 81, 82).

PF4 also acts as an anti-inflammatory factor on T lymphocytes (83), limiting Th17 differentiation by suppressing RORγ expression (62). A lack of PF4 induces the rejection of cardiac transplantation by increasing levels of IL-17 and T cell mediated inflammation. We has observed that PF4 decreases T lymphocyte proliferation and granzyme B expression in CD8+ T lymphocytes (61), explaining how higher levels of PF4 in a malignant context can limit T lymphocyte stimulation (61).

Stimulated PLTs are able to secrete and store IL-1β (63). PLT levels were closely associated with plasmatic IL-1β levels (15). This cytokine activates HUVECs, inducing neutrophil adhesion and endothelium damage (63). The co-culture of PLTs from SLE with HUVECs increased EC damage and inflammatory marker expression in an IL-1β dependent manner (64). In experimental models of inflammation, IL-1α secreted by activated PLTs also played a crucial role in SI (84–86).

The soluble P-selectin secreted from α-granules is also implicated in inflammatory responses. P-selectin from activated PLTs induces the release of 3-10 folds of inflammatory cytokines by monocytes (65) and also promotes NETs formation (66). Elevated levels of circulating soluble P-selectin may contribute to early vascular disease by promoting the adhesion of leukocytes to the endothelium (87). However, soluble P-selectin can prevent *in vitro* adhesion of neutrophils to activated endothelium (67).

Circulating soluble CD40L (sCD40L) is secreted mainly by activated PLTs. In human immunodeficiency virus (HIV) patients, sCD40L levels correlated with pro-inflammatory cytokines (68). Moreover, PLTs support B cell isotype switching through CD40L-CD40 binding (70). In patients with an increased CV risk plasmatic sCD40L was increased and correlated with disease activity and with pro-inflammatory cytokines (69). The addition of thrombin-activated PLTs to TLR-stimulated monocytes has been seen to reduce TNF-α and IL-6 secretion and induce IL-10 production, and were abolished by blocking sCD40L (71). In HIV, sCD40L levels were correlated with IDO enzymatic activity and Treg frequency, in addition to induced Treg expansion and differentiation (68).

TGF-β is a potent anti-inflammatory factor, produced mainly by PLTs, which suppresses T lymphocytes function and is involved in Treg differentiation. The culture of CD4+ T lymphocytes with PLTs enhances Th1 and Th17 cytokine production but the TGF-β secreted by PLTs activates Treg suppressing Th1 and Th17 response (72). PLTs inhibit CD4+ and CD8+IFN-γ production, proliferation and granzyme B and perforin expression in a TGF-β dependent manner (73).

PLTs also release the damage-associated molecular pattern molecule high-mobility group box 1 (HMGB1) contributing to thrombosis process (87) promoting monocytes migration, suppressing monocyte apoptosis via TLR4-ligation and the monocyte accumulation at the site of vascular thrombosis (74, 75) and promote NETs formation (76, 77).

TABLE 2 | Effects of PLT binding to other cells.

	Ligands of interaction	Effect on bound cells
Endothelial cells	GPIIB/IIIa- ICAM1 CD40L-CD40	Adhesion (88) Expression E-selectin VCAM-1 (89) Secretion IL-8 MCP-1 (89)
Neutrophils	GPIβα-Mac-1 P-selectin-PSGL-1	Arrest to endothelium (90–92) NETs formation (66, 93)
Monocytes	P-selectin-PSGL-1 GPIβ-CD11b CD147-CD147 CD40L-CD40	Secretion MCP-1, IL-8, TNF-α, MMP9 (94, 95) Expression TF (96) Differentiation M1 macrophages (97) Secretion IL-10 (71, 98, 99) Reduction TNF-α, IL-1β (71, 99)
Lymphocytes	P-selectin-PSGL-1 P-selectin-ALCAM	• *(SLE) B cells*: Upregulation CD86, BAFF (100) Secretion IL-10 (100) • *(Psoriasis) CD4+:* Secretion IL-17 (101) • *Healthy donors:* Less proliferation (102–105) Less pro-inflammatory cytokines (102–105)

Binding of PLTs to Leukocytes and Endothelial Cells

The interaction of PLTs with leukocytes and EC contributes to SI by favoring the arrest of leukocytes on endothelium, the production of inflammatory cytokines and NETs formation. Under certain circumstances, the binding of PLTs to leukocytes decreases the inflammatory response, participating in the resolution of thrombo-inflammation (**Table 2**).

Under inflammatory stress, PLTs have a firm adhesion to endothelium via GPIIB/IIIa-ICAM-1 in a fibrinogen dependent manner (88). CD40L expressed by PLTs induces the expression of E-selectin, VCAM-1 and ICAM-1 on endothelium and the secretion of IL-8 and MCP-1 (89), increasing the recruitment of leukocytes.

Although the P-selectin-PSGL-1 (P-selectin glycoprotein 1) axis is essential to the binding of PLTs to leukocytes, other pathways are involved in this process: P-selectin-ALCAM, GPIβα-Mac1, CD40L-CD40, P-selectin-CD15, JAM-C-Mac1, TREM1L-TREM1, CD36-trombospondin-CD36, and CD147 pathway (94, 106, 107).

The interaction of PLTs with neutrophils through GPIβα-Mac-1 and P-selectin-PSGL-1 is crucial for the development of thrombo-inflammation and vascular damage by arresting neutrophils to endothelium and the induction of NETs formation (66, 90–93). The neutrophil-platelet aggregates can also be seen in tissues in acute coronary syndrome and the skin of psoriatic patients (108, 109). Stimulated TLR4 PLTs, through the binding with neutrophils, induced robust neutrophil activation and formation of NETs (93). Induced NETs formation by PLTs was abolished blocking their binding with neutrophils (110). The increase of neutrophil-platelet aggregates in the circulation of autoimmune disease patients correlates with neutrophil activation (111) and vascular abnormalities (112) which was abolished with intravenous immunoglobulins plus prednisolone treatment (112). However, even all the current evidences supports that interaction of PLTs with neutrophils are involved in inflammatory process and vasculature damage, this interaction could have also anti-inflammatory consequences depending on the neutrophil status. The addition of PLTs to previously stimulated TLR-neutrophils downregulates degranulation markers expression and the secretion of elastase (113).

Although monocyte-PLT aggregates are increased in CVD (114), the interaction of PLTs with monocytes have pro- or anti-inflammatory consequences depending on the experimental assay and the activation status of monocytes. As pro-inflammatory consequence, thrombin-activated PLTs through P-selectin-PSGL-1 binding induces the expression of MCP-1 and IL-8 in resting monocytes (95). P-selectin expressed on the surface of PLTs induced a rapid tissue factor expression by monocytes (96). The binding of PLTs to monocytes through GPIβ-CD11b induces a M1 macrophage phenotype that produce TNF-α (97). Via CD147 axis, RA patients had more circulating intermediate monocytes-PLTs aggregates, increasing the TNF-α and MMP-9 secretion (94). Autoimmune patients with a higher CV risk have more monocyte-PLT aggregates and its associated with the activated state of monocytes (115). As anti-inflammatory consequences, it has been observed that the binding of PLTs to activated monocytes induces IL-10 production and decreases TNF-α and IL-1β production, and were abolished by the blocking of P-selectin-PSGL-1, CD40L-CD40 axis and Ca_2+ chelator (71, 98, 99).

Although the binding of PLTs to lymphocytes has preferably anti-inflammatory consequences, it was demonstrated a contribution in inflammatory process. High levels of lymphocytes-PLTs aggregates were observed in SLE and psoriasis. In SLE, lymphocytes-PLTs aggregates had an up-regulation of CD86, B cell activation factor receptor and IL-10 production and correlated positively with plasmatic levels of IgG, IgA, IL-10, sCD40L and renal manifestation, and correlated negatively with IgM levels (100). In psoriasis, the IL-17+ CD4+ had higher levels of bound PLTs and anti-TNF-α drugs normalize the numbers (101), while in HIV there are more lymphocytes-PLTs aggregates and are associated to D-dimer levels, increasing the CV risk (116).

We observed that CD4+ T lymphocytes-PLT aggregates had a reduced proliferation and production of pro-inflammatory cytokines. A less severe phenotype and a decreased CV risk was observed in RA patients with higher levels of circulating CD4+T lymphocytes-PLTs aggregates (102). The addition of PLTs to lymphocytes from RA synovial fluids decreased their proliferation and the secretion of IFN-γ, IL-17, and increased IL-10 production (103). PLTs MPs cultured with Tregs prevented the differentiation into IL-17– and IFN-γ-producing cells in a P-selectin dependent manner (104). The co-cultures of CD4+ CD25- T cells with PLTs induced Tregs and this effect was abolished by the blocking of glycoprotein A repetitions predominant (105).

In HIV, the aberrant function of CD8+ T lymphocytes was abolished when these cells were co-cultured with PLTs from HD, implying that direct contact with PLTs and TGF-β secretion contributed to this functional improvement (117). In later stages of experimental autoimmune encephalomyelitis (EAE), there was an increase CD4+ T lymphocytes-PLTs aggregates through the interaction of P-selectin-ALCAM, down-regulating their activation, proliferation and the production of IFN-γ, crucial for the spontaneous resolution of EAE. The blocking of CD4+-PLT aggregates exacerbate EAE (118).

Platelet to Lymphocyte Ratio in Systemic Inflammation

The platelet to lymphocyte ratio (PLR) has emerged as a reliable marker of SI. Although elevated counts of PLTs and low counts of lymphocytes *per se* has been associated with worse prognosis of CVD, increase CV mortality and morbidity and SI (119, 120), PLR predicts better the outcomes of CVD. The role of PLR as an independent marker in CVD and SI has been extensively reviewed (121, 122). In patients with chronic inflammatory diseases, PLR is elevated and it correlates with markers of SI (122–125).

However, there are confounding factors that alter PLR. Sex and ethnic origin also modulates PLR (126) as well as drugs that affect blood cell maturation in the bone marrow (127, 128). Other confounding factors of PLR may be the technical limitations of PLR measurements, such as EDTA dependent agglutination (129, 130).

CONCLUSIONS

PLTs have been considered to play a pro-inflammatory role in SI. However, their binding to leukocytes and EC and the secretion of immunomodulatory molecules during activation also have anti-inflammatory consequences. Different effects were observed with platelets from healthy donors or chronic inflammatory patients. In addition, the binding to each subpopulation of leukocytes has distinctive consequences. Further research is necessary to reveal how platelets exert their dual function.

AUTHOR CONTRIBUTIONS

CZ and EC: literature review, manuscript preparation. SV: oversight, editing, and planning. All authors contributed to the article and approved the submitted version.

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The Role of Inflammation and Immune Activation on Circulating Endothelial Progenitor Cells in Chronic HIV Infection

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Endothelial inflammation and damage are the main drivers of cardiovascular risk/disease. Endothelial repair is mediated in part by recruitment of bone marrow endothelial progenitor/endothelial colony forming cells (EPC/ECFC). People with HIV (PWH) have increased cardiovascular risk and the impact of infection in endothelial repair is not well defined. The low frequencies and challenges to *in vitro* isolation and differentiation of EPC/ECFC from PBMCs had made it difficult to study their role in this context. We hypothesized that HIV driven inflammation induces phenotypic changes that reflects the impact of infection. To test this hypothesis, we evaluated expression of markers of trafficking, endothelial differentiation, and angiogenesis, and study their association with biomarkers of inflammation in a cohort of PWH. In addition, we investigated the relationship of circulating endothelial progenitors and angiogenic T cells, a T cell subset with angiogenic function. Using a flow cytometry approach, we identified two subsets of circulating progenitors LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ in PWH. We found that the phenotype but not frequencies were associated with biomarkers of inflammation. In addition, the percentage of LIN4$^-$CD45^{dim}CD34$^+$ was associated with serum levels of lipids. This data may provide a new tool to better address the impact of HIV infection in endothelial inflammation and repair.

Keywords: endothelial progenitor cells, HIV infection, endothelial inflammation, T cell activation, endothelial repair

INTRODUCTION

In people with HIV (PWH), immune activation and inflammation is associated with cardiovascular risk independently of the traditional risk factors or duration of antiretroviral treatment and CD4 counts (1–12). PWH has twice the risk of cardiovascular disease compared to the general population and the standard cardiovascular risk prediction scores underestimate cardiovascular risk in these patients (1, 13).

Endothelial inflammation and injury play a critical role in the pathogenesis of cardiovascular risk/disease. The homeostasis, maintenance and repair of endothelial cells is mediated in part by bone marrow-derived endothelial cell progenitors also called endothelial colony forming cells (EPC/ECFC) (14–16). The low frequency, absence of specific markers, and the overlap with hematopoietic progenitor markers creates challenges for study this population in circulation (14, 17–20). In *in vitro* cultures of PBMC, two subsets of progenitor cells have been isolated, the "putative endothelial progenitor cells" called EPC/ECFC that can undergo clonal expansion, differentiate into endothelial cells, and promote neo-vasculogenesis (14, 21). The other subset of progenitor cells is the "angiogenic or colony forming unit endothelial cells" (CFU-EC). These cells have hematopoietic origin, and while they cannot differentiate into endothelial cells, they promote vasculogenesis by the secretion of critical growth factors (17, 22). In addition, recent evidence had shown that a subset of T cells can promote angiogenesis by secreting growth factors that drives proliferation and differentiation of endothelial progenitor cells. Because of these properties these T cells are called angiogenic T cells (T_{angs}) (22, 23).

EPC/ECFCs have been studied as potential biomarker of disease and their levels in circulation have been associated with cardiovascular risk and events (24, 25). In contrast in PWH, studies of the frequencies of circulating EPC/ECFC have shown contradictory results and their role in HIV associated cardiovascular risk is not well defined (26–32). The challenges to isolate and culture *in vitro* endothelial progenitor cells from PWH suggest that in this setting some functional properties of these cells are compromised (29). Accordantly, one study reported that factors present in the serum from PWH alters the functional properties of EPC/ECFC isolated from cord blood of healthy volunteers. These observations propose that systemic inflammation in the setting of HIV infection can influence the functionality with no changes in their frequency (33).

In this study, we hypothesize that systemic inflammation and immune activation induces changes in their phenotype that reflects the impact of HIV infection. To address this question, we developed a high parameter flow cytometry approach to study the relationships between the phenotype of endothelial progenitors and serum levels of biomarkers of inflammation, and angiogenic T cells.

TABLE 1 | Patient Characteristics.

	Patients (n = 36)
Age, yr, median (IQR)	52 (45-57)
Gender n (%)	
male	31 (86.1)
female	5 (13.9)
Race/Ethnicity n (%)	
White	24 (66.7)
African American	2 (5.6)
Hispanic	8 (22.2)
Other	2 (5.6)
Clinical Characteristics	
Smoker, n (%)	12 (33.3)
Diabetes, n (%)	5 (13.8)
Hypertension diagnosis, n (%)	11 (30.6)
Lipid-lowering therapy (%)	12 (33.3)
Statins n (%)	11 (30.6)
Aspirin n (%)	10 (27.8)
BMI Kg/m^2, median (IQR)	27.03 (24.46, 32.62)
SBP mmHg, median (IQR)	130 (121, 140.5)
DBP mmHg, median (IQR)	79 (74, 83)
FRS 10 yr %, median (IQR)	11.2 (9, 18.4)
HIV infection, median years, (IQR)	14 (10, 20)
T cell counts, median (IQR)	
CD4 counts (cells/μL)	662 (503, 856)
CD8 counts (cells/μL)	557.5 (436.5-913.8)
CD4 nadir (cells/μL)	331.5 (91.25, 404.5)
CD4/CD8 ratio	1.03 (0.734, 1.58)
ART, n (%)	
Tenofovir	24 (64)
Abacavir	11 (30.6)
NNRTI	11 (30.6)
PI	13 (36.1)
INSTI	17 (47.2)
Clinical Laboratory, median (IQR)	
Total Cholesterol mg/dL	184.5 (145,.5, 216.3)
LDL mg/dL	108 (79, 129,3)
HDL mg/dL	46 (34, 64.75)
Triglycerides mg/dL	123.5 (85.75, 177.5)
Biomarkers, median (IQR)	
IL-8 (pg/mL)	3.482 (2.718, 4.843)
hsIL-6 (pg/mL)	1.806 (1.28, 2.527)
IL-6R (ng/mL)	38.46 (31.69, 46.410)
TNFα (pg/mL)	2.491 (1.896, 3.129)
TNFRI (ng/mL)	2.784 (2.297, 3.1740)
hsCRP mg/mL	1.223 (0.6645, 2.566)
sCD163 (mg/L)	0.1769 (0.1414, 0.265)
sCD14 (mg/L)	1.705 (1.561, 1.88)
D-dimer mg/L	0.3512 (0.2362, 0.4547)
TFPI (ng/mL)	31.2 (28.7, 37.13)
sICAM1 (μg/mL)	332.2 (259.6, 407.7)
sVCAM1 (μg/mL)	407.7 (360.1, 552.1)

SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure, ART, antiretrovirals, FRS, Framingham Risk Score; DM, Diabetes Mellitus, BMI, Body mass index, LDL, Low-Density Lipoprotein cholesterol; HDL, High-Density Lipoprotein cholesterol; IL-8, interleukin 8; IL-6, interleukin 6; IL-6R, interleukin 6 Receptor; TNF, Tumor Necrosis Factor; TNFRI, Tumor Necrosis Factor Receptor I, hsCRP, high sensitivity C-Reactive Protein; sCD163, soluble CD163; sCD14, soluble CD14; TFPI ,Tissue Factor Pathway Inhibitor; sICAM1, soluble intercellular adhesion molecule1; sVMAC1, soluble vascular adhesion molecule1.

MATERIAL AND METHODS

Participants

Participants (PWH, n= 36) were recruited at the HIV Clinic in Minneapolis, Minnesota (Hennepin Healthcare) under an institutional review board approved protocol (**Table 1**). Biomarkers and clinical chemistry are described in **Supplementary Material and Methods**. Patients and healthy controls were consented and studied in NIAID/CCMD intramural program IRB approved HIV clinical research studies (**Supplementary Table S1**). Healthy volunteers were obtained through the NIH Blood Bank and were recruited at the MedStar University Hospital (n = 6). Georgetown University. All participants signed informed consent.

Flow Cytometry

Frozen PBMCs from PWH and healthy controls were thawed and rested overnight. Cells were stained with LIVE/DEAD (Invitrogen, CA). Cells were incubated with 1μg/mL human IgG (Sigma, MO) to block Fc receptors followed by a cocktail of

mAbs (**Supplementary Materials and Methods**, **Supplementary Table S2**). Samples were acquired with a BD Symphony and analyzed using Flowjo software.

Statistical Analysis

Associations between circulating progenitor cell subsets and serum levels of biomarkers (**Supplementary Table S3–S5**) were performed using Pearson Correlation Coefficient and two sample t-test. Because of the multiple comparison p-value <0.01 was considered significant.

RESULTS

Two Circulating Subsets LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ of Progenitor Cells Are Detected in PBMCs From PWH

The EPC/ECFC have been defined by the expression of CD34$^+$KDR$^+$ (CD309), however, these markers are expressed by other cells (14, 20, 34). From *in vitro* cultures of PBMCs, two progenitors have been identified, the EPC/ECFC have been defined as CD45 negative whereas, the angiogenic or colony forming unit endothelial cells (CFU-EC), which are hematopoietic origin and express CD45 (14, 15, 18, 19, 22).

To overcome the challenges to isolation and culture EPC/ECFC cells from PBMCs from PWH and determine the impact of infection, we developed a flow cytometric panel that allow us their detection in frozen PBMCs, and to study their phenotype. Because of the low frequencies, the overlap of surface makers with different cell types in blood that can lead to false positive events we used a cocktail of mAbs (LIN4) to exclude all the lineage cells from hematopoietic origin (**Supplementary Material and Methods Table S2** and **Supplementary Figure S1**). Using this approach, we identified two subsets of cells expressing CD34$^+$ similar to that described in the cultures of PBMCs (14, 15, 18, 19, 22). One population was CD45$^-$ (LIN4$^-$CD45$^-$CD34$^+$) and one expressed CD45$^+$ gate (expressing CD45^{dim}) and through the manuscript we call this population as LIN4$^-$CD45^{dim}CD34$^+$ (**Supplementary Figure S1**).

Having validated this flow cytometry panel in frozen PBMCs, we next analyzed the frequency of circulating progenitor in a cohort of 36 PWH with a median time from the diagnosis of HIV infection 14 (10, 20) years (**Table 1**). Participants had a median age 52 (45-57) years, 86% were male sex at birth, 67% were white, 33% smokers and median BMI was 27 kg/m2 and a moderate Framingham Risk Score (FRS) median 11.2. All PWH were receiving cART for more than a 1 year with HIV-RNA levels <200 copies/mL and have a median CD4 T cell count of 662 cells/μL (**Table 1**). The clinical and laboratory characteristics, biomarkers of inflammation, coagulation and vascular inflammation are described in **Table 1**.

The proportion of LIN4$^-$ cells (expressed as the frequency of total live cells) in PBMCs from PWH was % 0.140 (IQR: 0.065 to 0.240), as depicted in **Figure 1B** (**Figure 1A** gating strategy). Lower frequencies of the LIN4$^-$CD45$^-$ compared to the LIN4$^-$CD45$^+$ subset were observed (**Figure 1C**).

The frequency of the LIN4$^-$CD45$^-$CD34$^+$ progenitor cells were lower than LIN4$^-$CD45^{dim}CD34$^+$ subset, $p< 0.0001$ (**Figure 1D**). In addition, stable frequencies of these subsets in PBMCs were measured in two time points of an interval of six months apart (**Supplementary Figure S2**).

These results showed that two circulating CD45$^-$CD34$^+$ and CD45^{dim}CD34$^+$ progenitor cells can be detected during chronic HIV infection, and steady frequencies were observed during approximately 6 months interval of stable virologic control.

Circulating LIN4$^-$CD45^{dim}CD34$^+$ Progenitor Cells Are Associated With Levels of Lipids During HIV Infection

To evaluate the impact of HIV infection on LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ subsets, we next studied the association between the proportion of circulating cell progenitors and biomarkers of inflammation and coagulation, lipids profile, and cardiovascular risk (**Supplementary Tables S3, S4**). No associations were observed between the frequency of LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ subsets and the plasma levels of biomarkers of inflammation (**Supplementary Table S3**).

In contrast, the frequency of circulating LIN4$^-$CD45^{dim}CD34$^+$ subset showed a statistically significant although weak associations with the levels of total cholesterol (R= 0.533, p= 0.001), LDL cholesterol (R= 0.647, p<0.001), and triglycerides (R= 0.466, p= 0.004), **Supplementary Table S4**. To better understand these relationships, we performed a multivariate analysis using DM (diabetes), total cholesterol, LDL cholesterol and triglycerides as variables. We found that the frequency of LIN4$^-$CD45^{dim}CD34$^+$ was associated with LDL cholesterol, R^2 = 0.44, $p< 0.01$ (estimated coefficient 0.00277, 95% CI 0.00085, 0.00469) and triglycerides, R^2 = 0.29, $p< 0.01$ (estimated coefficient 0.00065, 95% CI 0.00023, 0.00107).

These data suggest that the levels of biomarkers of systemic and endothelial inflammation may not influence the frequencies of cell progenitors in circulation in this study group of PWH. In addition, the results highlight a potential link between serum lipids levels and the frequencies of LIN4$^-$CD45^{dim}CD34$^+$ but not LIN4$^-$CD45$^-$CD34$^+$ progenitor cells.

Expression of Endothelial Markers by Circulating Endothelial Progenitors Are Associated With Biomarkers of Inflammation

One limitation to study the *in vivo* functional properties of endothelial progenitors in PWH is the challenge to *in vitro* culture and differentiate them from PBMCs (30, 33). In addition, the *in vitro* culture may select clones that can proliferate and differentiate in endothelial progenitor cells and may not represent the impact of the infection in the overall population of cell progenitors. To overcome these challenges, we hypothesized that the inflammatory environment associated with HIV infection is reflected in their phenotype.

We developed a multidimensional flow cytometry approach to study the relationships between the expression levels of

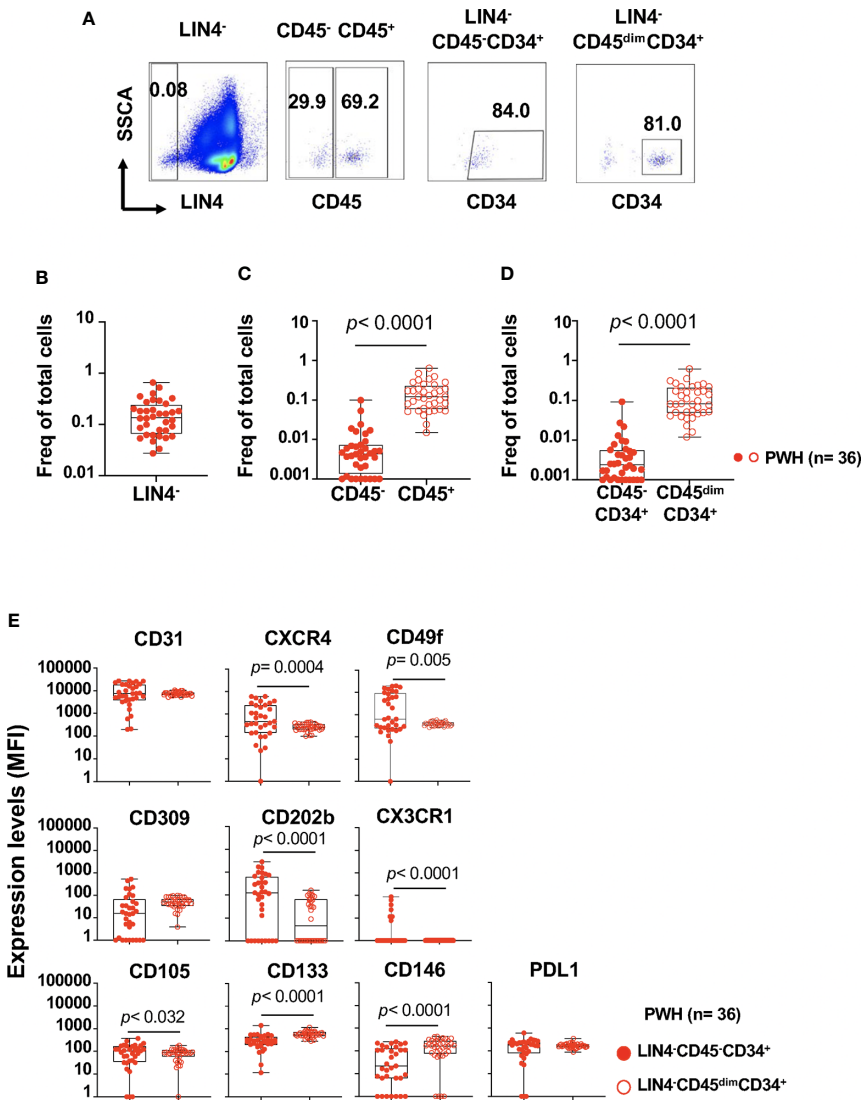

FIGURE 1 | Detection and phenotype of two circulating cell progenitors LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ in PBMCs from PWH. PBMCs from PWH (n= 36) were thawed and rested overnight. PBMCs were stained with LIVE/DEAD followed by a cocktail of mAbs: LIN4, CD45, CD3, CD8, CD34, CD31,CD105, CD49f, CD133, CD146, PDL-1, CD14, CD309 and CD202b (**Supplementary Table S2**). Full minus one (FMO) was used control. **(A)** Representative dot plots of the gating strategy. Frequency of: **(B)** LIN4$^-$, **(C)** LIN4$^-$CD45$^-$ and LIN4$^-$CD45$^+$, **(D)** LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ cells are represented as the frequency of total live cells. **(E)** Expression of the markers CD31, CXCR4, CD49f, CD309, CD202b, CX3CR1, CD105, CD133, CD146 and PD-L1 in the LIN4$^-$CD45$^-$CD34$^+$ (closed red circle) and LIN4$^-$CD45^{dim}CD34$^+$ (open red circle) cells are shown as median fluorescence intensity (MFI). Whiskers represent median and IQR. Comparison between subsets was performed using non-parametric Wilcoxon test. *P* value < 0.05 was considered significant.

markers associated with progenitor cells (CD34, CD133, CD49f), endothelial cell markers (CD31, CD105, CD309, CD202b, CD146, PD-L1), and homing receptors (CXCR4, CX3CR1); and serum levels of biomarkers of inflammation (**Supplementary Table S2** and **Figure 1E**).

We found that both LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ expressed the homing receptors CD31, CXCR4 and CD49f (the laminin receptor) involved in trafficking and homing of progenitor cells. In addition, LIN4$^-$CD45$^-$CD34$^+$ showed significant higher expression levels of CXCR4, CD49f, CD202b, CD105 and CX3CR1 than the LIN4$^-$CD45^{dim}CD34$^+$ subset (**Figure 1E**). In contrast, LIN4$^-$CD45^{dim}CD34$^+$ subset expressed higher levels of the CD133 and CD146 surface markers. Similar expression levels between the subsets were observed for CD31, CD309 and PD-L1 (**Figure 1E**). The analysis of the frequency of cell subsets expressing these markers showed similar results with the exception of CD202b expression. The frequency of CD202b$^+$ LIN4$^-$CD45^{dim}CD34$^+$ cells expression was higher than LIN4$^-$CD45$^-$CD34$^+$ (**Supplementary Figure S3B**).

We next determine the potential influence of systemic inflammation in the phenotype of the progenitors (**Supplementary Table S5**). We found a positive association, although weak, between the serum levels of CRP and the expression of endothelial markers CD309 (R= 0.582, p= 0.002) and PD-L1 (R= 0.507, p= 0.004) in LIN4$^-$CD45$^-$CD34$^+$ subset. In addition, the serum levels of IL-6 were associated with expression of CD105 (R= 0.465, p= 0.009).

In contrast in the LIN4$^-$CD45^{dim}CD34$^+$ subset, the expression of PD-L1 was positively associated with the serum levels of IL-6 (R= 0.459, p= 0.005), TNFRI (R= 0.454, p= 0.005), **Supplementary Table S5**. No associations were observed with endothelial inflammation biomarkers including sICAM, and sVCAM (data not shown).

Altogether these data suggest that in the context of HIV infection, biomarkers of inflammation have a potential effect on the expression of markers associated with endothelial differentiation in both LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ subsets.

We next evaluated whether HIV viral replication and its associated inflammatory environment has an effect in the phenotype of these subsets (**Supplementary Table S1** and **Figure 2**). PWH with viral loads > 50 copies/ml showed significant lower frequencies of LIN4$^-$, LIN4$^-$CD45$^-$ and LIN4$^-$CD45$^-$CD34$^+$ compared to those with suppressed viral replication by cART and healthy volunteers (**Figure 2A**). In contrast, no changes were observed on the LIN4$^-$CD45$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ (**Figure 2A**). The phenotype of the subset LIN4$^-$CD45$^-$CD34$^+$ showed similar levels of expression except for the expression of CD117 (**Figure 2B** and **Supplementary Figure S4A**). In contrast, reduced expression levels of CXCR4, CD49f and CD117 was noted in the LIN4$^-$CD45^{dim}CD34$^+$ subset (**Figure 2C** and **Supplementary Figure S4B**).

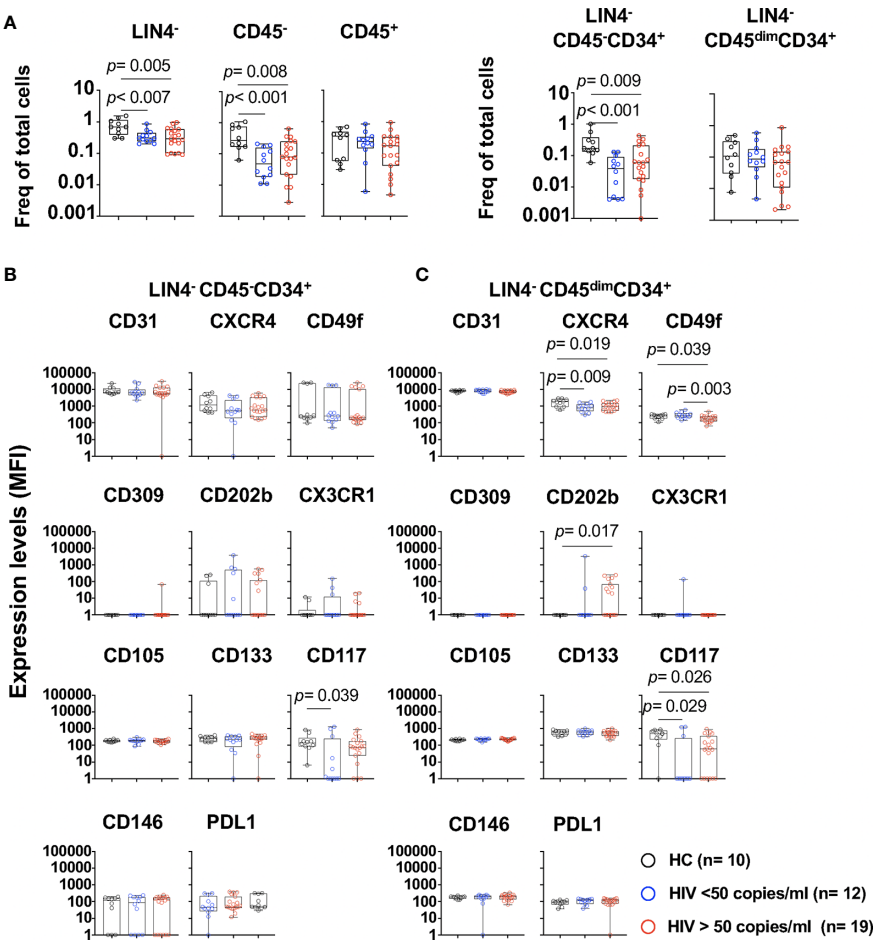

FIGURE 2 | Phenotype of circulating cell progenitors LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ in PWH. PBMCs from healthy control (HC, n=10), PWH with viral loads < 50 copies/ml (n= 12), and > 50 copies/ml (n= 19) were thawed and rested overnight. PBMCs were stained with LIVE/DEAD followed by a cocktail of mAbs: LIN4, CD45, CD34, CD31, CXCR4, CD105, CD49f, CD133, CD146, PDL-1, CD309, CD117 and CD202b (**Supplementary Table S2**). **(A)** Percentage of LIN4$^-$, LIN4$^-$CD45$^-$ and LIN4$^-$CD45$^+$(Left panel), and percentage of LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ (Right panel). Percentages are represented as frequency of total live cells. Expression of the markers in: **(B)** LIN4$^-$CD45$^-$CD34$^+$, and **(C)** LIN4$^-$CD45^{dim}CD34$^+$ cells. Expression of the markers as shown as median fluorescence intensity (MFI). Whiskers represent median and IQR. Comparison between groups was performed using non-parametric Mann-Whitney test. P value < 0.05 was considered significant.

The frequencies of LIN4$^-$CD45$^-$CD34$^+$ or the LIN4$^-$CD45^{dim}CD34$^+$ subset were not associated with the viral load, CD4 and CD8 T cell counts or with the expression of HLADR$^+$CD38$^+$ in T cells. Moreover, weak associations, although significant were observed with the phenotype of the cell subsets. CD4 T cell counts showed association with expression of CD146 expression (R= 0.498, p= 0.005) in the LIN4$^-$CD45$^-$CD34$^+$ subset. In addition, the percentage of CD4$^+$HLADR$^+$CD38$^+$ was associated with expression of CD117 in this subset (R= 0.652, p< 0.001). In the LIN4$^-$CD45^{dim}CD34$^+$, viral load showed a trend of a negative correlation with the expression levels of CD49f (R= -0.420, p= 0.022), and the frequency of CD4 and CD8 HLADR$^+$CD38$^+$ was negatively associated with the expression of CD31 (R= -0.465, p=0.014 and R=- 0.584, p= 0.001 respectively). These results suggest that HIV and its associated immune activation may have an impact in the phenotype of these circulating cell subsets.

CX3CR1$^+$ Angiogenic T Cells and Circulating Endothelial Progenitors in PWH

Angiogenic T cells (T_{angs}) can promote vascular repair by secretion endothelial growth factors and are defined by expression of CXCR4$^+$CD31$^+$ (23). We next evaluated the potential relationship between angiogenic T cells and circulating progenitors in PWH (**Table 1**).

The study participants had a frequency of CD4 T_{angs} of 16.5% (IQR: 13.3 to 23.65), and CD8 T_{angs} 43.9% (IQR: 36.2 to 54.08), [**Figure 3B** (**Figure 3A** gating strategy)]. In addition, the frequency of CD4 and CD8 T_{angs} showed a trend of a negative correlation with the Framingham Risk Score (FRS) but did not rich statistical significance (R= -0.346, p= 0.045; and R= -0.347, p= 0.044 respectively, **Supplementary Table S6**).

CD4 T_{ang} cells were negatively associated with SBP (R= -0.510, p< 0.001) with a trend of association with DBP (R= -0.407, p= 0.014), and D-dimer levels (R= -0.407, p= 0.015). In contrast, CD8 T_{ang} cells showed a trend of negative association with SBP and DBP (R= -0.377, p= 0.023, R= -0.391, p= 0.018 respectively) but did not rich statistically significance (**Supplementary Table S6**).

CD4 and CD8 T_{angs} cells showed no correlation with the frequencies of circulating progenitors LIN4$^-$CD45$^-$CD34$^+$ and LIN4$^-$CD45^{dim}CD34$^+$ subsets (data not shown).

We next evaluated the expression of CX3CR1 in T_{ang} cells that has been reported as a marker of pro-inflammatory T cells and can mediate endothelial inflammation in the context of HIV infection (35). A higher proportion of CX3CR1$^+$CD8 T_{ang} cells compared with their CD4 counterpart was observed in PWH (**Figures 3A, C**). In addition, we found that CX3CR1$^+$ CD8 T_{ang} cells showed a trend of a positive correlation with circulating LIN4$^-$CD45$^-$CD34$^+$ but did not rich statistical significance (R= 0.327, p= 0.052). These results suggest that in the setting of HIV infection, CD8 T_{ang} cells express CX3CR1 and have the potential to promote endothelial inflammation.

DISCUSSION

Endothelial inflammation is the underlying mechanism of cardiovascular risk/disease. Endothelial repair is mediated in part by recruitment of bone marrow derived progenitor cells, and the impact of HIV infection in this process is not well defined (30, 33, 36).

Several limitations to study the role of endothelial progenitors during HIV infection includes their low frequencies in circulation, the lack of specific markers and difficulties of *in vitro* culture from PBMCs. In an attempt to overcome these challenges and to better defined the *in vivo* impact of HIV infection, we investigated their phenotype and the relationships with biomarkers of inflammation.

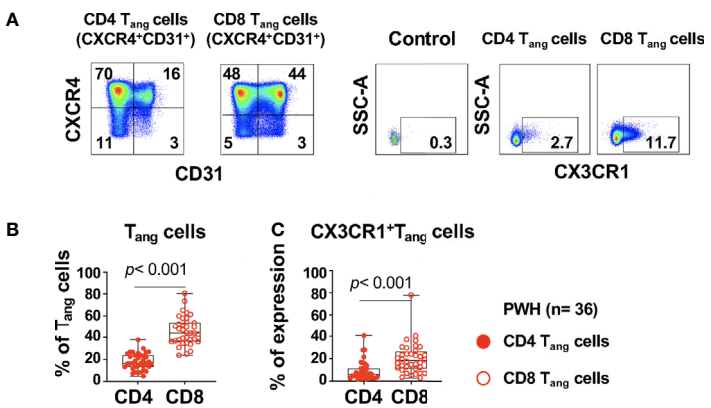

FIGURE 3 | Expression of CX3CR1$^+$CD4 and CD8 T_{ang} cells in PWH. PBMCs from PWH (n= 36) were thawed and rested overnight. PBMCS were stained with LIVE/DEAD followed by a cocktail of mAbs described in Table Flow Cytometry Panel (**Supplementary Table S2**). CD4 T cells were gated as CD3$^+$CD8$^-$ and CD8 T cells were gated as CD3$^+$CD8$^+$. **(A)** CD4 and CD8 angiogenic T cells (T_{ang}) were identified based on surface expression of CXCR4$^+$CD31$^+$. Surface expression of CX3CR1 were analyzed in CD4 and CD8 T_{ang} cells and full minus one (FMO) was used control. **(B)** Percentage CD4 and CD8 T_{ang} cells. **(C)** Expression of CX3CR1 in CD4 and CD8 T_{ang} cells expressed as frequency of the parent population. Comparison between CD4 and CD8 T_{ang} cells was performed using nonparametric Wilcoxon test. P value < 0.05 was considered significant.

We developed a high dimensional flow cytometry assay with a stringent exclusion of potential hematopoietic lineage contaminants to determine the frequencies and phenotype of circulating progenitor cells. One advantage of this approach is the distinction of two subsets of circulating progenitors ($LIN4^-CD45^-CD34^+$ and $LIN4^-CD45^{dim}CD34^+$) that are mostly not made in the previous reported studies of endothelial progenitors (36, 37). Our findings are in agreement with the observations from Echeveria et al. They reported circulating angiogenic cells defined as $CD31^+CXCR4^+CD34^+CD45^{low}$ cells, and a heterogenous population $CD31^+CXCR4^+CD34^+CD45^-$ containing circulating endothelial cells and endothelial progenitors in PWH (38). This study and our highlight the heterogeneity of circulating cell subsets associated with angiogenesis and vascular repair mechanisms.

Similar to previous reports, we found no associations between the frequencies of circulating progenitors and inflammatory biomarkers (30, 32). These inflammatory markers reflect endothelial damage and may not be involved in the mobilization and recruitment of cell progenitors (39). In addition, our study participants had a moderate FRS and although statistically significant, weak correlations were observed with the biomarkers studied. Therefore, future studies should evaluate cohorts with distinct cardiovascular risk to better define these relationships. We also evaluated the impact of viremia, and although we found reduced frequencies in circulation, no association between the viral load and the frequencies of these subsets were observed.

We observed an association between pro-atherogenic lipids including LDL and triglycerides, and the frequency of the $LIN4^-CD45^{dim}CD34^+$ subset. This subset contains the "angiogenic" myeloid progenitors ($CD45^{dim}$). Elevated lipids levels has been reported to be involved in the mobilization of hematopoietic stem and progenitor cells (40–42). Studies had shown that excess of circulating lipids drive endothelial inflammation, and particularly, LDL cholesterol is a modulator of recruitment of hematopoietic progenitors to the atherosclerotic plaques. These myeloid progenitors can differentiate into monocytes and macrophages and thereby contribute to the pathology of the vascular disease (41, 42). In addition, other studies shown that HDL cholesterol is a determinant of the number of endothelial progenitors in circulation (43).

In contrast to the frequencies, we observed that expression of markers of endothelial differentiation (CD309 and PD-L1) in the $LIN4^-CD45^-CD34^+$ were associated with biomarkers of systemic inflammation including IL-6, CRP, whether these correlations reflect an ongoing differentiation process and altered functional property needs to be determined. Accordantly, a report showed that factors present in the serum of PWH but not in uninfected individuals alter the functional properties of normal donor cord blood derived endothelial progenitor cells (33). Particularly, they found that the plasma levels of CRP in untreated HIV infected patients was negatively correlated with functional properties of endothelial progenitors. The study did not report isolation of EPC/ECFC from PWH (33).

In addition, other factors can influence the functionality of these cells including the cART. The HIV protease inhibitor Ritonavir have been shown to have cytotoxic effects *in vitro* in human endothelial cells (44). More importantly, in a longitudinal study it was evaluated the impact of the commonly used protease inhibitors and non-nucleoside reverse transcriptase inhibitors on circulating endothelial cells, EPC/ECFC and angiogenic cells (38). In this study, the authors reported a greater recovery of circulating endothelial cells and EPC/ECFC in the group treated with the protease inhibitor darunavir suggesting an active endothelial repair process in the setting of cART (38).

In the present study, we also evaluated the potential relationship of endothelial cell progenitors, and subset of T cells with angiogenic properties (23). The study showed a trend in the relationship between $LIN4^-CD45^-CD34^+$ and angiogenic CD8 T_{ang} cells expressing CX3CR1. CD8 T cells expressing CX3CR1 have been associated with the recruitment of pro-inflammatory T cells and endothelial inflammation in the setting of HIV infection (35, 45). The role of CX3CR1 in angiogenic T cell function is not well defined, in animal models, CX3CR1 expression is associated with enhanced atherosclerosis and renal impairment suggesting a pro-inflammatory potential of these cells (46). In addition, we found an association between CD4 T_{ang} cells and blood pressure. Reports had indicated that T cells are involved in regulating blood pressure and in the context of HIV infection, the T cell immune activation may alter these mechanisms (47, 48).

Altogether this study suggests a complex interplay between endothelial inflammation and repair and a potential role of T cell immune activation in the setting of HIV infection. The evaluation of the phenotype of endothelial progenitor cells provides a new tool to a better assessment of these interactions and their contribution in cardiovascular risk in PWH.

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

Individuals with HIV were recruited at the HIV Clinic in Minneapolis, Minnesota (Hennepin Healthcare) under an institutional review board approved protocol (#13-3657). Healthy volunteers' samples were obtained at the MedStar University Hospital under an institutional review board approved protocol (CR00000926). Participants from NIAID/CCMD intramural program IRB approved (91-I-0140). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MC designed the study. ZZ, JC, and TL performed the experiments. MC, ZZ, and TL analyzed and interpreted the

data and wrote the manuscript. JK, PK, CH, IS, and JB were involved in recruitment of participants of the study and wrote the manuscript. JQ performed statistical analysis. 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/fimmu.2021.663412/full#supplementary-material

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