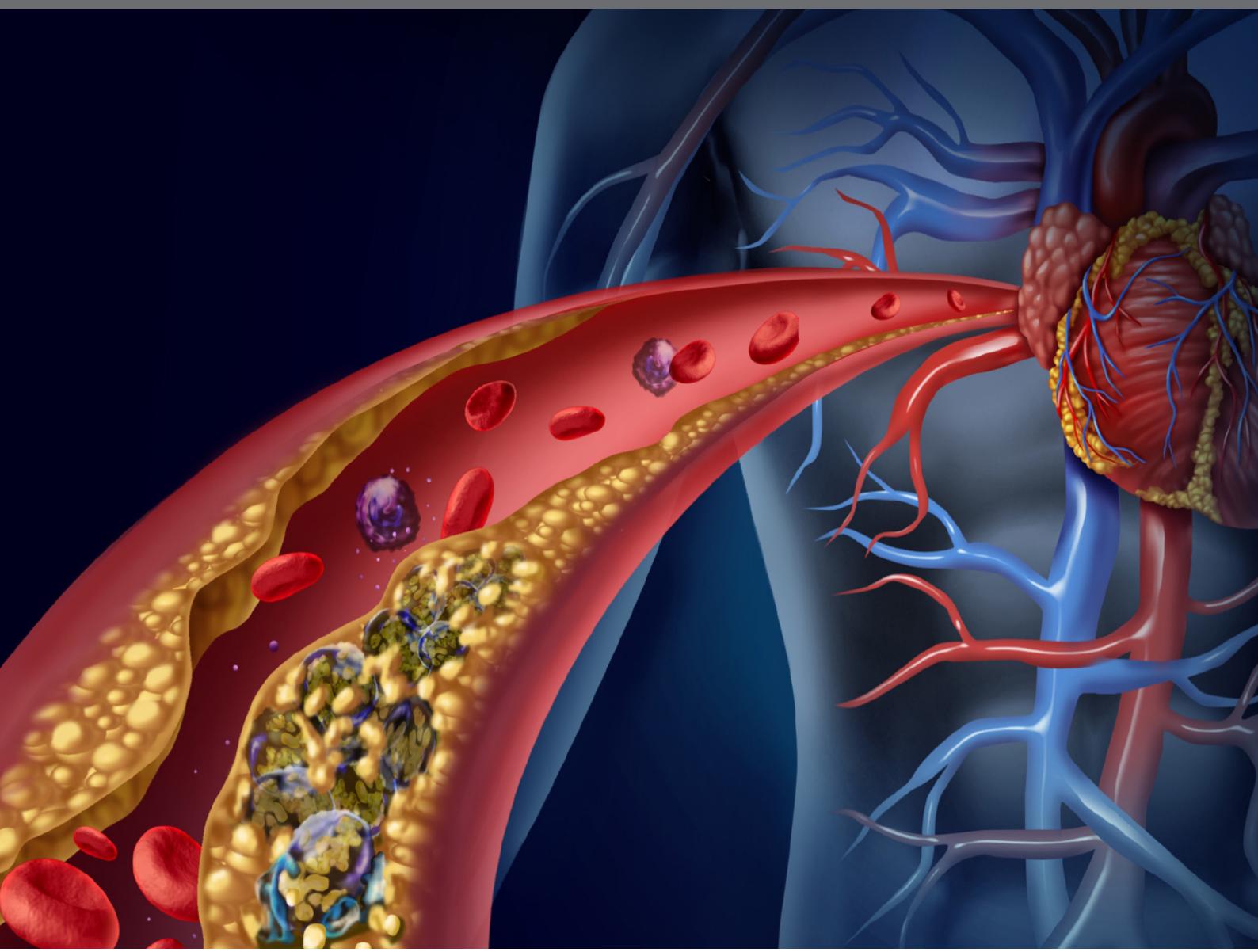
NEW TRENDS IN VASCULAR INFLAMMATION RESEARCH: FROM BIOLOGY TO THERAPY

EDITED BY: Masanori Aikawa, Ichiro Manabe and Nikolaus Marx
PUBLISHED IN: Frontiers in Cardiovascular Medicine



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frontiers

ISSN 1664-8714
ISBN 978-2-88945-637-6
DOI 10.3389/978-2-88945-637-6

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NEW TRENDS IN VASCULAR INFLAMMATION RESEARCH: FROM BIOLOGY TO THERAPY

Topic Editors:
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Recruitment of circulating leukocytes into the arterial wall leads to the development of inflamed atherosclerotic plaques. Accumulation of activated macrophages within the lesions may promote the lesion development and trigger clinical complications. Understanding mechanisms that induce and regulate this cascade of events may lead to new therapies.

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Citation: Aikawa, M., Manabe, I., Marx, N., eds. (2019). New Trends in Vascular Inflammation Research: From Biology to Therapy. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-637-6

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frontiers in Cardiovascular Medicine

EDITORIAL
published: 28 August 2019
doi: 10.3389/fcvm.2019.00102

Editorial: New Trends in Vascular Inflammation Research: From Biology to Therapy

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Keywords: inflammation, monocytes/macrophages, platelets, thrombosis, aging, vascular disease, atherosclerosis, vein graft disease

Editorial on the Research Topic

New Trends in Vascular Inflammation Research: From Biology to Therapy

The evidence from basic science and clinical studies has established the role of inflammation in atherosclerosis and other vascular diseases. Many patients on potent drugs for modifiable risks, such as cholesterol-lowering statins and PCSK9 inhibitors, still suffer from vascular complications, including acute myocardial infarction. To tackle such residual risk, new medical therapies that more specifically target mechanisms for excessive inflammation may be needed. We believe that exploring novel mechanisms for vascular inflammation is a first stride toward the development of such new medical solutions. This Research Topic features 18 articles on new trends in vascular inflammation research with a focus on disease mechanisms (Part 1) on the one hand and new therapies (Part 2) on the other hand, all authored by leaders in vascular inflammation biology.

OPEN ACCESS

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

Received: *18 June 2019*
Accepted: *15 July 2019*
Published: *28 August 2019*

Citation:
Aikawa M, Manabe I and Marx N (2019) Editorial: New Trends in Vascular Inflammation Research: From Biology to Therapy. Front. Cardiovasc. Med. 6:102. doi: 10.3389/fcvm.2019.00102

PART I: UPDATES ON THE MECHANISMS FOR CARDIOVASCULAR INFLAMMATION (12 ARTICLES)

Accumulating evidence suggests that monocytes and macrophages are heterogeneous and their subpopulations may have distinctive roles in vascular inflammation. Buscher et al. discuss monocyte heterogeneity with a focus on "patrolling" monocytes. Their article offers the emerging knowledge of the roles and kinetics of this monocyte subset as well as new technologies for identification and functional assays. Decano and Aikawa then provide the updates for macrophage biology in vascular disease. They focus on the mechanisms for activation, changes in intracellular metabolism, and current understanding of heterogeneity, and further discuss new paradigms of discovery science in vascular inflammation.

Thrombogenicity is a key feature of inflamed vessels, particularly in the diabetic milieu. Pechlivani and Ajjan discuss mechanisms for the imbalance of thrombotic and fibrinolytic factors, pathways responsible for increased thrombogenicity in diabetes, and therapeutic agents for thrombosis. This review emphasizes the importance of targeting diabetes-specific mechanisms for thrombosis.

While pro-inflammatory pathways may contribute to vascular disease, the impact of impaired protective mechanisms that support the hemostasis of non-diseased vessels also deserves similar levels of attention. An article by Yurdagul et al. discusses the role of defective efferocytosis of macrophages, a mechanism that clears apoptotic cells and promotes the resolution of inflammation,

in the formation of necrotic core and the onset of acute thrombotic events. Miyazaki and Miyazaki then review the contribution of impaired protein catabolism to atherogenesis, focusing on the ubiquitin-proteasome pathway, autophagy, and the calpine system.

Many studies have reported the role of non-coding RNAs in cancer and neurologic disorders. More recently, we have learned that various non-coding RNAs contribute to cardiovascular diseases. In vascular biology, the evidence for the role of long non-coding RNAs, as compared to microRNAs, remains scant. Haemmig et al. overview how long non-coding RNAs promote vascular inflammation and future perspectives of this area.

Implantation of a autologous vein graft to bypass an obstructive coronary or peripheral artery is a common procedure. Rates for the occlusion or narrowing of vein grafts, however, are unacceptably high. Better understanding of underlying mechanisms will help to establish new therapies that prevent vein graft failure. An article by de Vries and Quax provides a comprehensive review of inflammatory mechanisms for the vein graft lesion development.

Members of the Krüppel-like factor (KLF) family of zinc-finger containing transcription factors regulate many biological processes. Accumulating evidence has implicated KLFs in cardiovascular biology. Two comprehensive reviews focus on two different contexts. Sweet et al. overview the role of KLFs in the biology of cell types related to vascular diseases (e.g., endothelial cells, smooth muscle cells, monocytes/macrophages), and strategies for pharmacologic modulations. Manabe and Oishi then discuss the biology of KLFs in key metabolic organs such as the liver and skeletal muscles and their disorders, and provide future perspectives.

Two articles review aging from different angles. Sanada et al. discuss cell senescence and dysregulation of innate immunity that contribute to chronic low-grade vascular inflammation in the elderly. Katsuumi et al. link cellular senescence with age-related disorders, such as heart failure, atherosclerotic vascular diseases, and metabolic syndrome.

At the end of Part I on the mechanisms for vascular disease, Yamazaki and Mukouyama review the role of pericytes in vascular disease with a specific emphasis on their heterogeneity.

PART II: EMERGING EVIDENCE ON NEW THERAPIES FOR VASCULAR INFLAMMATION (6 ARTICLES)

This part covers a wide range of translational vascular medicine that spans from experimental validation of therapeutic targets to cardiovascular outcome trials. Sena et al. propose that cathepsin S is a potential therapeutic target for vascular inflammation and calcification. Peripheral artery disease is a global burden which shows an increasing prevalence and incidence worldwide. An original report by Nishimoto et al. demonstrates that activation of Toll-like receptor 9-mediated signaling by cell-free DNA released from ischemic tissues promotes macrophage activation and impairs blood flow recovery in the ischemic limb. An original report by Akita et al. demonstrates that the blockade of the IL-6 receptor suppresses atherogenesis in mice. Katsuki et al. then provides a comprehensive review of nanotechnology-based drug delivery and imaging for cardiovascular disease with a focus on inflammation. Rahman and Fisher provide a comprehensive review on the experimental and clinical evidence for the regression of atherosclerotic lesions and underlying mechanisms. They particularly focus on the role of macrophages. The last article by Aday and Ridker overviews the strong clinical evidence for the inflammatory aspects of atherosclerosis based on large cardiovascular outcome trials, including CANTOS that directly tested the effects of an anti-inflammatory therapy.

AUTHOR CONTRIBUTIONS

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

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

frontiers in Cardiovascular Medicine

REVIEW
published: 19 December 2017
doi: 10.3389/fcvm.2017.00080

Patrolling Mechanics of Non-Classical Monocytes in Vascular Inflammation

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Non-classical monocytes have emerged as the preeminent vascular housekeepers. Continuous intravascular screening is enabled by slow patrolling on the endothelium and allows a rapid response to local perturbations. Intravital imaging has been crucial to elucidate the molecular mechanisms and migratory phenotype of patrolling. In this review, we discuss technical requirements of intravital microscopy such as imaging modalities, labeling strategies, and data analysis. We further focus on patrolling kinetics and adhesion receptors in different organs and vascular beds including arteries during homeostasis and vascular inflammation and define pertinent questions in the field.

Keywords: monocytes, patrolling, arteriosclerosis, arteries, microcirculation, venules

OPEN ACCESS

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

Received: *07 September 2017*
Accepted: *23 November 2017*
Published: *19 December 2017*

Citation:
Buscher K, Marcovecchio P, Hedrick CC and Ley K (2017) Patrolling Mechanics of Non-Classical Monocytes in Vascular Inflammation. Front. Cardiovasc. Med. 4:80. doi: 10.3389/fcvm.2017.00080

INTRODUCTION

Monocytes have been implicated in many inflammatory diseases (1, 2). They are composed of at least two murine monocyte populations with distinct functional and molecular properties (3). Ly6C$^+$ CCR2$^+$ CX3CR1$^-$ classical monocytes are abundant in the blood and in several non-inflamed organs (spleen, lung, liver, and brain), and readily extravasate to many inflammatory sites (3). In contrast, Ly6C$^-$ CCR2$^-$ CX3CR1$^+$ non-classical monocytes predominantly remain in the vascular system (3) and engage in long-term migration along the endothelium with or against the flow, a process termed patrolling (4). Transcriptomic and functional comparison suggests that CD14$^-$CD16$^+$ monocytes are the human counterpart to patrolling monocytes (=non-classical monocytes; both terms are interchangeable) in mice (5–7). Non-classical monocytes can derive from classical monocytes and have a lifespan of several days in humans (8) and mice (9, 10). Activated endothelial cells attract patrolling monocytes for scavenging and neutrophil-mediated necrosis (11). Similarly, endothelium of the pulmonary circulation of tumor-bearing mice attracts patrollers that subsequently orchestrate an antitumor response by recruiting NK cells (12). Viruses or nucleic acids induce a TLR7-mediated response in patrollers that results in the production of TNF-α, IL-1β, and CCL3 (5). Non-classical monocytes often exert anti-inflammatory and prohomeostatic effects (5, 11, 13). However, they can also have pro-inflammatory functions depending on the disease-specific context (13–15). Further insights into patrolling mechanisms will be critical to understand and therapeutically target the leukocyte response in cardiovascular disease (16).

In vitro culture of endothelial cell layers has been instrumental in understanding monocyte behavior (17). However, the full repertoire of adhesion molecules and signaling cues underlying effective patrolling still remains obscure. As a result, patrolling cannot be studied *in vitro* using purified ligands as immobilized substrates. Moreover, data suggest that the molecular and migratory phenotype differs between vessel compartments [arteries vs. venules (18)] and tissues [ear dermis

venules (19) vs. kidney cortex circulation (11) vs. mesenteric venules (19, 20)]. Therefore, intravital microscopy in anesthetized mice is paramount to the study of patrolling monocytes. It provides an *in situ* characterization of migration patterns, endothelial interactions, and the local orchestration of a dynamic leukocyte response. This review elaborates on the imaging technology, labeling strategies, migration phenotypes, and molecular requirements of patrolling monocytes throughout the circulation in healthy and inflammatory conditions.

LABELING MODALITIES

Although CX3CR1-GFP and Nr4a1-GFP mice are widely used to image patrollers, there are currently no reporter mouse strains with highly specific endogenous markers. Alternative approaches to imaging patrolling monocytes *in vivo* are feasible but require a number of experimental considerations.

Specificity (true negative rate) and sensitivity (true positive rate) determine the value of any labeling strategy. Many reporter mice lack sensitivity, i.e., many non-targeted cells are also labeled. The CX3CR1-GFP mouse is widely used for studies of the mononuclear phagocyte system. The CX3CR1 locus had been replaced with an eGFP construct (knock-in), resulting in cytosolic GFP fluorescence (21). In heterozygotes (CX3CR1-GFP$^{+/-}$), several myeloid lineages are GFP$^+$ including monocytes, dendritic cells, tissue-resident macrophages, brain microglia, and subsets of NK and T cells (21). Monoallelic expression of CX3CR1 seems sufficient for adequate chemokine receptor function, although this has not been tested rigorously, and alterations have been reported (22). Homozygous GFP expression (CX3CR1-GFP$^{+/+}$) results in a CX3CR1 knockout. Comparing CX3CR1-GFP$^{-/+}$ with CX3CR1-GFP$^{+/+}$ in littermates is useful for understanding the functional role of CX3CR1 in monocytes (23). Due to the long half-life of unmodified eGFP, the eGFP signal does not correlate well with the endogenous CX3CR1 expression (24). Non-classical and classical monocytes show a high and intermediate GFP expression, respectively (**Figure 1**). With high sensitivity photomultiplier tubes in modern microscopes, both monocyte subsets are detectable. Thorough controls are required to ensure the sensitivity of the GFP signal, such as complementary flow cytometry data (e.g., to show that recorded CX3CR1-GFP$^+$ cells in a specific disease model are indeed patrolling monocytes and not classical monocytes or other leukocyte lineages).

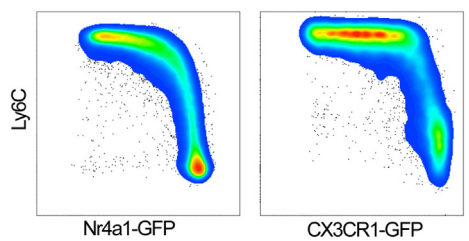


FIGURE 1 | GFP expression in classical (Ly6C^{high}) and non-classical monocytes (Ly6C^{low}) of Nr4a1-GFP (left panel) and CX3CR1-GFP$^{+/-}$ mice (right panel). Gated on single live CD45$^+$, CD115$^+$ CD11b$^+$ monocytes.

To overcome the issue of CX3CR1-GFP expression in other blood leukocytes, these mice were crossed with IL2RG$^{-/-}$ Rag2$^{-/-}$ knockout strains (19). Here, monocytes remain the only GFP$^+$ population in the blood (19). However, the global gamma chain deficiency and lack of T cells in Rag2$^{-/-}$ mice severely alters systemic immunity (25), limiting the applicability of this mouse model.

Another common reporter for non-classical monocytes is the transgenic Nr4a1-GFP (Nur77-GFP) mouse. This mouse was originally generated for the study of TCR activation (26). A GFP-Cre fusion protein was inserted at the start codon of Nr4a1, and fluorescence is induced by antigen stimulation. It was later discovered that Nr4a1 is mandatory for the development of non-classical monocytes in the bone marrow (27). Nr4a1-GFP reporter mice show a strong GFP signal in non-classical monocytes, whereas classical monocytes are low (27). The GFP intensity of the two monocyte subsets is about one magnitude further apart than in CX3CR1-GFP mice (**Figure 1**). This suggests that GFP^{high} and GFP^{low} discrimination in Nr4a1-GFP mice may be superior to CX3CR1-GFP mice for intravital microscopy.

In vivo labeling by antibodies or dyes (28) is an alternative or complementary approach to visualize patrolling monocytes. It is critical that azide is removed from commercially available products, which can be done by using microdialysis or spin columns. Some companies provide no azide (NA)/low endotoxin (LE) antibodies for *in vivo* applications. Depending on the abundance of the target, 1–5 μg suffice to image monocytes. Caveats include adverse effects of antibody binding, such as function blocking, receptor dimerization, internalization, or presentation of the Fc portion to Fc receptors on monocytes, endothelial, or other cells. All these can lead to unwanted activation (or inhibition) of downstream effects. The use of F_{ab} fragments circumvents the latter problem, but does not address internalization or function-blocking issues (29). As an example, the anti-CD11b antibody clone M1/70 is commonly used to tag myeloid cells *in vivo* (11), but its function-blocking effect may alter patrolling kinetics. This was shown in untreated mesentery venules (20) and in TLR7 agonist R848-treated venules of the kidney cortex (11). If two or more fluorophores are simultaneously used, color-switching experiments are required to exclude a label-dependent bias.

Injection of fluorescently labeled anti-mouse GR1 (bi-specific for Ly6C and Ly6G) antibodies in wild-type (11) or CX3CR1-GFP$^{+/-}$ mice (20) helps to discriminate classical monocytes and neutrophils from patrollers. Similarly, anti-mouse Ly-6C (clone HK1.4) can distinguish between classical (Ly6C$^+$) and non-classical (Ly-6C$^-$) monocytes in CX3CR1-GFP mice. GFP$^-$ Ly-6C$^+$ populations in the blood include neutrophils and some T cell subsets, whereas the GFP$^+$ Ly6C$^-$ subset unambiguously corresponds to patrolling monocytes in the blood. Labeling of CD115 (CSF-1R, clone AFS98), although highly specific for monocytes, is not recommended for intravital imaging, as it affects M-CSF signaling (30). To ensure that the imaged cells are located in the vessel lumen, a blood tracer (e.g., 70–200 kDa fluorophore-coupled dextran) must be coinjected. A gap in the tracer signal verifies the intraluminal position of the cell. In addition, fluorescently labeled anti-mouse CD31 (PECAM-1, clone

390) can be used to mark the endothelium. However, this antibody also labels neutrophils and platelets (31).

Together, neither genetic nor antibody labeling approaches alone achieve high specificity and sensitivity. It is therefore highly recommended to verify the validity of the labeling strategy using multiple methods.

IMAGING PLATFORMS AND TISSUE SITES

Most vascular sites feature a unique molecular environment with tissue-specific patterns of intravascular leukocyte adhesion (31, 32). Therefore, the site of imaging should be carefully chosen according to the biological question. Due to the proximity of the vessels to the tissue surface, many tissues and organs are accessible for intravital upright confocal microscopy, which enables a penetration depth of about 100 µm. This includes the ear dermis, the mesentery, the cremaster muscle, the femoral and popliteal vasculature, the spleen, and the liver. While patrolling also occurs in the microcirculation of the kidney cortex, glomeruli as main functional units cannot be assessed in their entirety using confocal imaging. For these denser and highly scattering tissues, multiphoton microscopy is the preferred imaging modality (33). Patrolling, in contrast to rolling, describes a slow motion. Therefore, acquisition speeds of 1–0.5 frames/s are sufficient to describe the kinetics of patrolling. Tiled acquisition is possible. Modern 20–25× water immersion objectives with a NA around 1.0 offer reasonably high spatial resolution and a large field of view.

A challenge to intravital imaging is the intrinsic movement of tissues due to muscle twitching, peristalsis, and cardiac and respiratory cycles, which can strongly bias kinetic readouts. Tracheal intubation helps to reduce respiration-related motions. A respirator can be used with a pause at the plateau after inspiration or expiration. The muscular tone controlled by the autonomic nervous system can be suppressed by muscle relaxants. Restraining devices can be helpful in stabilizing the target tissue, yet require proper controls to rule out artifacts that may be introduced by the immobilization apparatus. For example, the widely used stabilization device with a suction chamber applies a vacuum (34) that can trigger trauma-induced neutrophil accumulation. Similarly, physical restrainers of vessels (35) directly impact on the adventitia and physiological flow conditions, and indirectly on endothelial cell biology (36, 37).

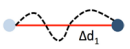
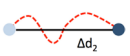
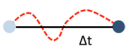
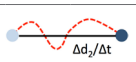
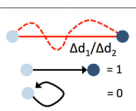
We recently developed an intravital live cell triggered imaging system for stable 2D and 3D two-photon imaging of large arteries that does not require physical restraint (33). This technology enables high-resolution video acquisition of leukocyte cell migration in the intravascular and intramural compartment of healthy and diseased arteries. The system has been optimized for the Leica platform, but can be mounted on any multiphoton microscope with external trigger control. It requires a trigger-box, non-invasive pulse oximetry, and a custom-made Arduino circuit with a Matlab-based software module to coordinate the pulse signal and frame acquisition. The system is versatile and can be used to study intra- and extravascular leukocyte behavior in many diseases, including atherosclerosis, renal artery stenosis, and vasculitis of large arteries.

KINETIC ANALYSIS OF PATROLLING

Blood-borne leukocytes interact with the endothelium in an orchestrated manner to leave the blood stream and exert their function in the surrounding tissue (31, 38). Members of the selectin and integrin families as well as cytokine receptors are sequentially engaging, resulting in capture, rolling, arrest, and extravasation. Although this process, referred to as the leukocyte adhesion cascade, differs qualitatively and quantitatively among different vascular beds and environmental signals, common key patterns have emerged (31, 38). As the leukocyte adhesion cascade consists of distinct migration steps, kinetic analysis of patrolling cells aids to delineate underlying molecular processes. These are summarized in **Table 1**.

Path length and displacement describe the total (circuitous) path length and the direct distance (beeline), respectively. Confinement ratio is defined as the ratio of path length and displacement. A value of 1 signifies a straight path, and a value close to 0 a meandering/circular motion. Importantly, these parameters can change as a function of the length of the video recording, which therefore needs to be standardized and noted in the method sections. A recording time of 30 min has been found to be sufficient in most circumstances. Under inflammatory conditions, some patrollers show longer durations of interactions, which may necessitate longer recordings. Velocity is calculated as distance traveled over time and expressed as µm/min. While the velocity in a patrolling population in healthy vessels is mostly homogenous, disease conditions can provoke irregular patterns, e.g., in atherosclerotic arteries (18), that can be plotted as velocity over time. The metric "dwell time" has been used to describe short static phases, particularly in glomerular capillaries (39). Since patrolling can occur with or against the blood flow, the flow bias is an insightful parameter.

TABLE 1 | Microkinetic parameters to describe patrolling.

	Start End	Unit	Description
Displacement		ΔXY_{end}-XY_{start} in µm	Beeline traveled within a time period (e.g., 1 min)
Path length		Path length in µm	Strongly affected by motion artifacts. False measurements also affect the confinement ratio
Duration		Total patrolling time in seconds	Depends on recording time. A minimum recording time of about 30 min is required in venules, and about 20 min in arteries
Velocity		Path length per time in µm/min	Minimum time ideally >10 min. Jerky patrolling can be plotted as velocity over time
Confinement ratio, straightness		Displacement/path length. No unit	Straightness of the path. 1 = straight path. 0 = start and end position overlap

By aligning all start points of all tracks, the dominant patrolling direction can be plotted (e.g., as tracks or rose plot). Numbers of active patrollers per vessel segment need to be normalized to vessel surface area visible in the intravital recording to account for out-of-focus segments or disease-related vessel perturbations (such as atherosclerotic plaque). To determine these parameters, several manual and automatic tracking tools are available in Fiji (ImageJ) (40) or Imaris (Bitplane).

Motion artifacts of intravital recordings can significantly affect kinetic measurements. Non-linear and linear transformations (translation, rigid body, affine, or scaled rotation) can be corrected during post-processing. If the automated tracking algorithm (e.g., using the centroid of the cell) works to more precision than the image resolution, an artificial sub-pixel back-and-forth motion will occur, resulting in a systematic overestimation of the path length and underestimation of the confinement ratio. Noise filters that remove sub-pixel movements smaller than the image resolution can remedy this issue.

The lack of highly specific reporter models and the difficulty of precisely distinguishing patrolling from other steps of the adhesion cascade poses challenges for data analysis. Criteria for the identification of patrollers include stable patrolling for 60 s or longer. In arteries, a 90-s threshold is recommended to safely discriminate motion artifacts and slow rolling from active patrolling. Patrolling velocity in microvessels and arteries is about 12 and 36 μm/min, respectively (11, 18, 20). To discriminate rolling from patrolling, a velocity threshold of 2 standard deviations (SDs) below the mean rolling velocity should be applied. Since the selectin requirements of non-classical monocyte rolling have not been studied, a clear definition of monocyte rolling before patrolling is not yet available.

Blood flow imposes directional shear forces on intravascular leukocytes. In the dermal, mesenteric, and kidney microcirculation, intravascular patrolling occurs mostly independent of the blood flow (migration regardless of flow direction). However, in arteries, a strong downstream flow bias has been detected (preferential migration with flow direction; **Figure 2**). The velocity and meandering migration paths are currently the only kinetic parameters that identify patrolling monocytes throughout the circulation.

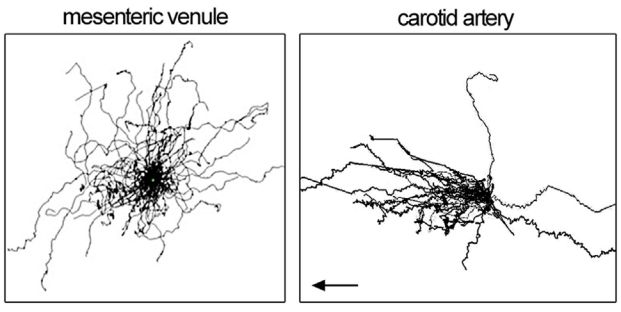


FIGURE 2 | Patrolling tracks with centered start coordinates as spider pot. Each line represents one patrolling monocyte. Data show patrolling in mesenteric venules (left) and carotid artery (right panel). Left panel is adopted from Carlin et al. (11) and right panel from Quintar et al. (18). Flow direction in the left panel is not available.

TISSUE-SPECIFIC MOLECULAR REQUIREMENTS

Molecular requirements of patrolling are both site- and stimulus-specific with regard to adhesion receptors. Table S1 in Supplementary Material highlights the main findings currently available in the literature.

In homeostatic conditions, arterioles, capillaries, and postcapillary venules are populated by patrollers (11, 19, 20, 41). Several investigations in mice highlighted the critical role of integrins. Blockade of the leukocyte integrin LFA-1 ($\alpha_L\beta_2$) results in immediate detachment of patrolling monocytes in all healthy tissues studied (19). In most cases, this leads to an increase of blood-borne non-classical monocytes as measured by flow cytometry, suggesting that around one-third of the marginal pool of non-classical monocytes is constantly engaged in vascular patrolling (19). Integrin α_L knockout mice also showed abolished patrolling although this global knockout does not allow unambiguous conclusions. Patrolling is reduced by around 50% in *ICAM-1*$^{-/-}$ mice, and additional knockout of ICAM-2 completely eliminates patrolling (11). *ICAM-2*$^{-/-}$ alone does not affect patrolling, suggesting that ICAM-1 is the major endothelial ligand for LFA-1 in patrolling monocytes, and ICAM-2 is a redundant binding partner (11). CD11b (integrin α_M, Mac-1) inhibition does not reduce the numbers of patrollers in steady state venules of the ear dermis (19) and glomerular capillaries (41) but decreases dwell time and path length in the latter (41). Endothelial CCN1/CYR61 as a potential CD11b ligand is required for effective patrolling in mesenteric venules (20). Under homeostatic conditions, the chemokine receptor CX3CR1 is irrelevant in most vessels but not in uninflamed glomerular capillaries of the kidney (41). Treatment with pertussis toxin, a potent inhibitor of $G_{\alpha i}$ signaling required for integrin activation, does not affect patrolling in steady state mesenteric venules (11). Intravital microscopy of the ear dermis after adoptive transfer of human monocytes (5) and flow chamber experiments on human umbilical vein endothelium (HUVEC) (17) confirmed the role of integrin LFA-1 in human $CD14^{dim}CD16^{+}$ monocyte crawling on uninflamed tissue. Furthermore, blocking of CX_3CL1 or VEGFR2 intensified patrolling via unknown mechanisms *in vitro* (17). These data show that LFA-1 integrin is mandatory for patrolling in all conditions, whereas the role of Mac-1, ICAM-1/2, and the CX3CR1 chemokine receptor varies.

Capillaries of the kidney glomerulus are a key target of renal inflammation and injury (42). Interestingly, many patrolling mechanisms seem to be different here. Using multiphoton intravital microscopy, about five non-classical monocytes were detected per hour in one glomerulus with an average dwell time of about 15–20 min (39, 41). Deficiency of CX3CR1 (using CX3CR1-GFP$^{+/+}$ mice) or the combined blockade of β_2 (CD18) and α_4 (CD49d) integrins reduced the number of patrollers in uninflamed conditions (41). CD18 or α_4 integrin inhibition alone does not have an effect (41). During the anti-glomerular basement membrane (GBM) antibody response (these antibodies trigger glomerulonephritis as in Goodpasture syndrome), primary adhesion of patrollers requires LFA-1, and the dwell time is reduced after CD11b blockade (41). During anti-GBM inflammation,

CX3CR1 knockout mice showed changes in patroller recruitment and dwell times in a time-dependent manner (41). These data emphasize that adhesion requirements differ depending on the environmental and spatial context.

There are phenotypic similarities between monocyte patrolling and neutrophil "crawling." The latter describes a slow, integrin Mac-1 (CD11b)-dependent meandering motion along the endothelium (43). P-selectin glycoprotein ligand 1 (PSGL-1) engagement, platelet interactions, and LFA-1-mediated arrest are mandatory for effective neutrophil crawling (43, 44). While both monocyte patrolling and neutrophil crawling include upstream and perpendicular motion and require endothelial ICAM-1 (43), only neutrophil crawling is known to necessitate an endothelial chemotactic gradient (45) and eventually results in site-directed extravasation (43). Moreover, in contrast to patrolling, crawling is evident only on activated endothelium. Of note, $CXCR6^+$ NKT cells also show patrolling along liver sinusoids (46). Thus, neutrophil crawling and monocyte patrolling are two separate entities. Molecular pathways may be similar, but this remains to be investigated.

TOLL-LIKE RECEPTORS (TLRs) AND PATROLLING

Patrolling has been studied in several mouse models of inflammation. TLRs act as pattern recognition receptors that monitor damage- and pathogen-associated molecular pattern molecules in the blood stream (47, 48). Direct application of TLR agonists on the vessel ("painting") mounts a local response in a time-dependent manner (11, 18, 20), whereas systemic use is not suitable for imaging due to pan-endothelial activation (49). The painting results suggest that perivascular tissue-intrinsic mechanisms suffice to intensify patrolling. TLRs are highly expressed on non-classical monocytes (5, 11). The impact of direct TLR stimulation of monocytes has not been investigated using intravital microscopy.

Painting of the mesenteric vasculature with agonists for TLR2 (Pam3CSK), TLR3 [Poly(I:C)], TLR4 (LPS), or TLR5 (flagellin) induce an time-dependent increase in patrolling (50). An early increase after 30–60 min is seen after TLR2 and TLR9 activation, whereas TLR3 and TLR4 promote a late accumulation (around 3 h) (50). TLR2 and TLR9 are the strongest inducers in these experimental settings, leading to about 9- to 10-fold more patrolling monocytes after 3 h (50). An increase of patrolling upon TLR7-activation by R848 (Resiquimod) has been shown in the dermis (ear) (11, 19), the mesentery (19, 20), the kidney (11), and the carotid artery (18). Notably, R848 attracts patrollers to both the arterial (18) and venular (11) endothelium, suggesting a conserved endothelial response. In all tissues and across all TLR stimulants except for TLR9 agonists (50), patrolling becomes more intense and meticulous after stimulation, as evidenced by longer dwell times (reduced velocity), longer tracks, and lower confinement ratios. In contrast to homeostatic conditions, blockade of $G_{\alpha i}$ signaling by pertussis toxin, Mac-1 (CD11b) by antibody inhibition or CX3CR1-deficiency impede the upregulation of patrolling after TLR7 stimulation (11) (Table S1 in Supplementary Material). It thus seems that vascular activation by most TLRs suffices to intensify local surveillance by patrolling monocytes.

Patrolling monocytes can initiate a local neutrophil response *via* a TLR7-dependent paracrine secretion of pro-inflammatory cytokines, such as IL-1β, KC, TNF, CCL3, or IL-6 (11). Activated platelets are required to effectively ramp up patrolling and signal subsequent neutrophil recruitment in mesenteric vessels (20, 51–53). Interestingly, TLR3 and TLR4 agonists lead to early (30–60 min) neutrophil accumulation that is followed by patrolling monocytes, indicating a TLR-specific temporal response of leukocyte recruitment to the activated endothelium (50).

It remains unclear how "painting" of TLR agonists works. Several biological components could play a role. Pericytes can actively support abluminal leukocyte behavior in the subendothelial space (54, 55). Moreover, laminins as active constituents of the basement membrane affect the endothelial phenotype (56) and leukocyte extravasation (57). An active supply of adhesion receptors to the endothelial surface from the lateral border recycling compartment is another example how the endothelium can actively shape interaction with blood-borne leukocytes (58). The study of these functional units might shed light on the microenvironmental signals required for effective patrolling.

PATROLLING KINETICS IN VENULES AND ARTERIES

In addition to venules (11, 19, 20), arterioles (4), and capillaries (11, 41), it was recently demonstrated in mice that the healthy arterial endothelium of large arteries is also monitored by patrolling monocytes (18). Hence, patrolling seems to be a universal surveillance mechanism throughout the circulation. However, molecular and biophysical conditions differ in microvessels and macrovessels and between venous and arterial endothelium (59). The vascular wall shear stress is low in venules and high in small precapillary arterioles (37). Due to their large circumference, large arteries have an intermediate shear stress profile (60, 61), which impacts on the functional phenotype (62). Moreover, different gene expression patterns between endothelial cells of the venular and arterial tree determine many differences in the molecular landscape involved in the leukocyte adhesion cascade (59). The details of monocyte patrolling in arteries, veins, arterioles, venules, and capillaries remain to be explored.

In homeostatic conditions, patrolling occurs at a velocity of 9 μm/min in venules of the kidney cortex (11), 17 μm/min in the dermal microcirculation (19), and about 36 μm/min in carotid arteries (18) (**Table 2**). Only in arteries, a clear downstream bias of patrolling was found (in direction of the flow; **Figure 2**). The dermal and mesenteric circulation as well as *in vitro* patrolling on HUVEC cells (17) showed hairpins (straight tracks with one sharp turn), loops, waves (meandering), and mixed forms (19). In arteries, predominantly the wave pattern was observed, whereas others only rarely occurred (18). The confinement ratio was determined at 0.6, 0.5, and 0.2 for kidney cortex venules, mesenteric venules, and the carotid artery, respectively. It is possible that the arterial confinement ratio is somewhat underestimated due to uncompensated motion artifacts (overestimation of the total path length). These observations point

TABLE 2 | Microkinetic analyses of patrolling monocytes in different vascular beds.

		Carotid artery (18)		Dermal venules (19)	Kidney cortex (11)		Lung (12)	Mesenteric venules (20)
		Basal	R848	Basal	Basal	R848	Basal	Basal
Velocity	μm/min	36	19	17	≈9	≈7.5	10.2 ± 0.3	≈9
Duration	s	284	343	14	≈540	≈1,300	nd	≈1,200
Length	μm	134	124	249	≈80	≈150	nd	≈200
Confinement ratio		0.22	0.10	0.63	≈0.6	≈0.3	nd	≈0.55
Displacement	μm	31	12	162	≈28	≈41	nd	nd

nd, not determined.

to an active role of shear forces and the position in the vascular tree on the agility of patrollers. Similarly, other leukocytes show cell type-specific reactions to shear forces. T cells preferentially migrate against the flow over short distances (63–65), whereas neutrophils show a downstream flow bias (64, 66). Kinetic measurements of patrolling monocytes in different vascular networks are summarized in **Table 2**.

Besides flow conditions, differing repertoires of endothelial adhesion receptors between venules and arteries could also account for differences in observed kinetics. This is supported by the finding of a differential requirement for the integrins LFA-1 and VLA-4 ($\alpha_4\beta_1$) in arteries. VLA-4 blockade in R848-treated arteries alone is not effective (18). Blocking LFA-1 reduces patrollers by 50%, and sequential blockade of VLA-4 leads to a further 25% reduction (18). In contrast, LFA-1 blockade alone in R848-treated dermal or kidney cortex venules suffices to abolish patrolling completely (11, 19). A similar observation was made in uninflamed glomulerula of the kidney (41). In all tissues studied, stimulation with TLR agonists leads to a significant decrease of the confinement ratio, pointing to a higher dwell time (Table S1 in Supplementary Material). These data emphasize that large arteries are unique entities with regard to monocyte patrolling.

Analysis of integrin requirements in arteries compared to venules suggest site-specific mechanisms (18). However, monocyte heterogeneity (67) within the non-classical subset with subset specific vascular tropism could also contribute to this phenomenon. This possibility has not been sufficiently studied so far. The intermediate subset in humans ($CD14^+CD16^+$) has not yet been described in mice. The function of the MHC-II^+ subset of non-classical monocytes remains unclear. New multiplexed single-cell technologies will help to classify human and mouse monocyte patrollers with high resolution.

PATROLLING IN ATHEROSCLEROTIC ARTERIES

Monocyte recruitment to the neointima is a disease-defining process in atherogenesis (2, 16, 68). It has been shown that monocyte rolling on explanted atherosclerotic endothelium is mostly P-selectin dependent (69), and adhesion is driven by VCAM-1 and its ligand integrin $\alpha_4\beta_1$ (70, 71). Endothelial ICAM-1 and VCAM-1 expression is upregulated at lesion sites (72, 73). Genetic depletion or blockade of VCAM-1 leads to reduced plaque buildup (74). However, the concept of classical and non-classical monocyte subsets and their distinct functions was unknown at the time of these studies. While classical monocytes adhere early to plaque-prone endothelium, extravasate, and contribute to the lesional macrophage population ($F4/80^+$ $Ly6C^-$ $I\text{-}A^{b+}$ phenotype) (75–77), far less is known about non-classical monocytes.

Western diet is known to trigger monocytosis and is thought to mainly affect the classical monocyte population (75, 77). Advances in intravital imaging have allowed to quantify the intravascular accumulation of non-classical monocytes in murine carotid arteries (18, 33). Wild-type C57Bl/6J mice fed western diet for 4–6 weeks and $apoE^{-/-}$ mice fed western diet showed an 8- and 22-fold increase in the number of patrolling monocytes on the arterial endothelium, respectively (Video S1 in Supplementary Material) (18). Thus, it is reasonable to hypothesize that a concurrent relocation of non-classical monocytes to atherogenic endothelia throughout the body results in a pseudo-reduction of these cells in the blood. This leads to a systematic bias in the analysis of blood-borne monocytes.

How does patrolling take place in large arteries? Intravital imaging showed that monocytes can directly interact with the endothelium from free flow (18, 35), possibly with the help of platelets (78). Kinetic analyses suggest that patrolling is preceded by arrest (18), suggesting an integrin-dependent adhesion step before patrolling. While most non-classical monocytes showed patrolling behavior (40%) in plaque-prone arteries, some were also arrested (15%), rolling (20%), or showed mixed phenotypes (18). The latter includes cells with alternating patterns of patrolling and fast rolling (>60 μm/s). Patrolling velocity (33 vs. 21 μm/s) and confinement ratio (0.2 vs. 0.05) in plaque vicinity is significantly decreased compared to plaque distant sites (18), indicating that local endothelial cues can trigger meticulous patrolling. Mean duration of patrolling was observed between 4 and 7 min in atherosclerotic conditions (18). However, many cells engaged only in short phases of patrolling with subsequent fast rolling, followed again by slow patrolling (18). This observation points to intermittent engagement of selectin receptors that enable fast leukocyte rolling (38). A viable candidate for capturing and rolling is PSGL-1 (ligand for endothelial P-selectin and E-selectin). PSGL-1 is expressed in non-classical and classical monocytes (79). Of note, differential adhesion receptor requirements have been found in short- and long-term patrolling of human $CD14^{dim}CD16^+$ human monocytes *in vitro* (17). Detailed insights on heterogeneity and spatial arrangements of the endothelial receptor landscape affecting monocyte patrolling and rolling are currently unavailable.

What is the fate and function of plaque-patrolling monocytes? Most patrollers in plaque-prone arteries detach eventually and are carried away in the circulation. While patrollers can arrest and extravasate under certain inflammatory conditions have been described (19, 80), the extent and relevance of this pathway during atherogenesis is still debated. Further studies are required to establish the identity, the migratory route, and the phenotype of these cells in atherosclerosis.

Recent data suggest an endothelial protective effect of patrolling monocytes in early atherogenesis (18). Nr4a1$^{-/-}$ *Apoe*$^{-/-}$ mice on western diet develop aggravated atherosclerosis (81, 82), and it has been proposed that Nr4a1-deficiency causes hyper-inflammatory lesional macrophages (82). However, an additional explanation could be that patrolling monocytes confer early endothelial protection during hyperlipidemia. In this line, patroller-deficient Nr4a1$^{-/-}$ mice show increased endothelial damage compared to wild-type controls as assessed by electron microscopy (18). Pleiotropic effects of Nr4a1-deficiency impede unambiguous conclusions.

To overcome these problems, a non-classical monocyte-specific knockout mouse was recently developed by excising the E2 superenhancer region upstream of the Nr4a1 promoter (83). In this mouse model, macrophages retain normal levels of activation during inflammatory conditions. The non-classical monocyte population is completely ablated. *E2*$^{-/-}$*Ldlr*$^{-/-}$ (LDL receptor knockout to trigger atherosclerosis) bone marrow chimeric mice on high cholesterol diet developed increased plaques along the aortic root (84). As shown by intravital microscopy, even in the non-plaque-prone femoral vasculature, patrolling activity was elevated during Western diet feeding beginning within 1 day. This required CD36, one of the receptors for oxidized low-density lipoprotein (OxLDL). Western diet feeding or OxLDL binding to CD36 induced F-actin formation, in part through adapter protein DAP12 and a member of the Src family kinase (84). These data hint at a chronic diet-induced inflammatory phenotype of non-classical monocytes that leads to increased endothelial recruitment even at a distance to plaque development.

CONCLUSION

Vascular housekeeping by patrolling monocytes is a crucial process required for endothelial homeostasis. Many disorders entailing vascular inflammation might trigger increased patrolling activity including chronic kidney disease (85, 86), tumors (87), HIV infection (88), myocardial infarction (89), atherosclerosis (18), and medium and large-vessel vasculitis (90). Targeting monocyte patrolling may be thus be a useful therapeutic approach. Recently, it was shown that non-classical monocytes in lung allografts are involved in acute graft rejection by mediating neutrophil recruitment (91). Adverse effects of non-classical monocyte have also been found in dendritic cell remodeling after spine injury (92). To exploit the effects of patrolling in clinical settings, ways to selectively increase or abolish the non-classical monocyte population need to be explored. Alternatively, or in addition, strategies to selectively manipulate patrolling behavior may become available.

There is a high interest in understanding the molecular foundation of patrolling in different parts of the vasculature. Many studies have contributed to a deeper understanding of the biological impact in health and disease. Yet, compared to the vast body of work on neutrophil and lymphocyte recruitment, a multitude of questions remains to be addressed. High-resolution intravital microscopy will be a key technology in this endeavor.

AUTHOR CONTRIBUTIONS

KB researched the published papers and wrote a draft of the review. KL revised the review and wrote parts. PM discussed data and wrote a section. CH reviewed the review.

FUNDING

This work was funded by a grant from the National Institutes of Health, R01 HL115232 (KL), and a grant from the Deutsche Forschungsgemeinschaft, BU-3247 (KB).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fcvm.2017.00080/full#supplementary-material.

Video S1 | ILTIS intravital microscopy of the unrestrained carotid artery in CX3CR1-GFP/ApoE-KO mice at a heart rate of about 300/min. Collagen is blue (second harmonics), and the blood red (Dextran). Blood flow right to left.

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

The reviewer SM and handling Editor declared their shared affiliation.

frontiers in Cardiovascular Medicine

REVIEW
published: 03 August 2018
doi: 10.3389/fcvm.2018.00097

Dynamic Macrophages: Understanding Mechanisms of Activation as Guide to Therapy for Atherosclerotic Vascular Disease

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An emerging theory is that macrophages are heterogenous; an attribute that allows them to change behavior and execute specific functions in disease processes. This review aims to describe the current understanding on factors that govern their phenotypic changes, and provide insights for intervention beyond managing classical risk factors. Evidence suggests that metabolic reprogramming of macrophages triggers either a pro-inflammatory, anti-inflammatory or pro-resolving behavior. Dynamic changes in bioenergetics, metabolome or influence from bioactive lipids may promote resolution or aggravation of inflammation. Direct cell-to-cell interactions with other immune cells can also influence macrophage activation. Both paracrine signaling and intercellular molecular interactions either co-stimulate or co-inhibit activation of macrophages as well as their paired immune cell collaborator. More pathways of activation can even be uncovered by inspecting macrophages in the single cell level, since differential expression in key gene regulators can be screened in higher resolution compared to conventional averaged gene expression readouts. All these emerging macrophage activation mechanisms may be further explored and consolidated by using approaches in network biology. Integrating these insights can unravel novel and safer drug targets through better understanding of the pro-inflammatory activation circuitry.

Keywords: macrophages, cardiovascular diseases, drug development, inflammation, cell metabolism

OPEN ACCESS

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

Received: *27 December 2017*
Accepted: *02 July 2018*
Published: *03 August 2018*

Citation:
Decano JL and Aikawa M (2018) Dynamic Macrophages: Understanding Mechanisms of Activation as Guide to Therapy for Atherosclerotic Vascular Disease. Front. Cardiovasc. Med. 5:97. doi: 10.3389/fcvm.2018.00097

INTRODUCTION

In the last few decades, accumulating evidence has supported modulation of inflammatory signals, and regulation of immune cell to cell interactions in atherosclerosis are key therapeutic strategies for atherothrombotic disease (1–3) The recent Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) trial, involving over 10,000 patients, show conclusive proof that reduction of inflammation, specifically targeting the interleukin-1β (IL-1β) pathway activation, independent of LDL cholesterol lowering, can significantly lower coronary artery disease (CAD) morbidity and mortality. Antagonizing the IL-1β signaling resulted in marked reduction of plasma high-sensitivity C-reactive protein (hs-CRP) levels among patients with elevated hs-CRP levels and history of myocardial infarction, which eventually led

to decreased major adverse cardiac/CV events (MACE and MACE+) (4) hs-CRP is a predictive marker of the severity of atherosclerosis and extent of future cardiovascular events (5, 6). Success of anti-inflammatory drug trials are reliant on rigorous basic science research, integrating a plurality of approaches. These include analyses of pathologic specimens, tightly controlled *in vitro* experiments and extensive use of pre-clinical small animal models to gather basic mechanistic information about the disease. In the context of chronic inflammation in cardiovascular disease, basic science research in macrophage biology has undoubtedly been the guiding compass for pursuing this anti-inflammation focus of atherosclerosis therapy.

MACROPHAGE ACTIVATION IN VASCULAR INFLAMMATORY DISEASE

Both acute and chronic forms of vascular inflammation are typified by the multitude of vasculitides and atherothrombotic pathologies. CAD, peripheral artery disease, vein graft failure, and arterio-venous fistula failure have seen various macrophage subtypes playing crucial roles. They either drive disease progression or cessation, or promote vessel repair and healing (7). Understanding the various phenotypes that allow macrophages to be categorized into subclasses with stereotyped behavior and function is crucial. This helps design strategies to precisely modulate immune signaling in vascular inflammation (1–3, 8). By limiting cellular subpopulations promoting plaque development, intimal cell proliferation, and tissue damage may be mitigated. This may also spare the subpopulation deemed beneficial for achieving disease control and resolution to allow return to homeostasis. In addition, understanding the profound adaptability, and plasticity of macrophages is key to knowing how to trigger phenotypic and functional changes within these cells and how far they can be reprogrammed.

What we have learned from the past is that both *in vitro* modeling of human and mouse primary macrophages complemented by experiments on small animal models of vascular disease have been important in elucidating mechanisms of macrophage activation and their role in the progression of the atherothrombotic lesions in CAD (9, 10). It is known that majority of the release of matrix metalloproteinases, MMPs, in human atherosclerotic plaques may derive from macrophages and foam cells, and to a lesser extent from smooth muscle cells (SMCs) and endothelial cells (ECs) (11). Excessive activation of proteases in the lesion lead to increased degradation of fibrillar collagen. This determines plaque integrity, leading to friable and unstable lesions. This may also lead to adverse remodeling prompting rupture and embolism of plaque debris, often seen in plaques (12, 13). Our preclinical studies used genetically-altered mouse strains to demonstrate that MMP-collagenases, major macrophage products, indeed promote the paucity of plaque collagen (14, 15). In vein grafts, MMP-2 and MMP-9 may play important roles in degrading the basement membrane which leads to enhanced infiltration of pro-inflammatory monocyte and macrophage populations (16, 17). These unstable plaque features are also most prominent among CAD patients with elevated low density lipoprotein (LDL) cholesterol levels (18), elevated lipoprotein (a) [Lp(a)] (19) and other metabolic derangements, reiterating a close interplay between inflammation and dysregulation of lipid handling (and other metabolic syndromes). LDL modifications, LDL cholesterol efflux and reverse cholesterol efflux all contribute to how cholesterol crystals instigate initial stages of atherosclerosis.

The process begins with recruiting monocytes from the circulating blood, followed by several processes including their differentiation into macrophages, foam cell formation and activation of the NOD-like receptor-pyrin domain (PYD)-containing-3 (NLRP3) inflammasome complex. Cholesterol crystals may cause phagolysosomal damage in macrophages priming them to activate NLRP3. Activation of NF-κB induces macrophages to produce pro-IL-1β and a pro-form of NLRP3. Upon activation of NLRP3, activated caspase-1 cleaves the pro-IL-1β releasing IL-1β, which amplifies the cascade of inflammatory signals (20, 21) including IL-6, tumor necrosis alpha (TNF-α) and pro-thrombotic initiators such as tissue factor (coagulation factor III). Clinical relevance for this is reflected in patients with typically high serum LDL cholesterol levels having increased incidence and severity of CAD and subsequent MACE sequelae. This paved way for the extensive use of statins in aggressively lowering elevated LDL cholesterol levels. Eventually, aggressive lipid lowering therapy indeed improved survival rates, as well as MACE/MACE+ outcomes as shown in clinical studies (22–25). However, certain patient populations did not benefit much due to having a baseline coronary atheroma predominantly dictating their prognosis (MACE) (26, 27). This leads to the burgeoning field of statin related research focused on elucidating mechanisms of statins that mitigate inflammation in cardiovascular disease, independent of their cholesterol lowering action.

For recalcitrant cases, LDL cholesterol levels can be further lowered by increasing availability of hepatic clearance via LDL receptors. Here, reduction of proprotein convertase subtilisin/kexin type 9 (PCSK9) activity and circulating levels have been quite effective, as seen in the success of drugs like evolucumab (Repatha) (28) and alirocumab (Praluent) (21, 29). Whether these inhibiting antibodies can reduce macrophage activation in coronary lesions remains to be proven. Interestingly, with successful reduction of LDL cholesterol levels in the at-risk population, physicians have inevitably selected for and identified a subpopulation of patients with considerable CAD morbidity despite sufficiently lowered LDL cholesterol levels. Since then, multiple points of evidence recognize inflammation, beyond hyperlipidemia, as a key regulatory hub by which CAD risk factors, co-morbid metabolic disease, and cardiovascular adverse events intersect (5, 30).

MECHANISMS FOR MACROPHAGE ACTIVATION

Traditional Thoughts on Macrophage Inflammatory Pathways in CAD

For years, many thought of resting macrophages as being activated in disease states into polarized subclasses that are

diametrically opposed. Either they are "classically-activated" or pro-inflammatory, designated as "M1" and "alternatively-activated" or non/anti-inflammatory/pro-resolving "M2." However, with much utilization of techniques allowing for multiple parametric assessments like single cell assays (FACS, etc), -OMICs profiling, and networks medicine approach, a more recent multi-dimensional model has emerged (31–35). Unsurprisingly, many authors have shied away from the M1 and M2 designations. Our recent study used single cell gene expression analysis to reveal that interferon gamma (INFγ)-induced "classically-activated" human primary macrophages remain largely heterogeneous, which is not consistent with a traditional "polarization" theory (36).

Acknowledging that macrophage subclasses fall into a spectrum of activation that is beyond bi-directional is the currently accepted paradigm. However, it may be still helpful, from a drug development and therapeutic research stand point, to think of macrophages in CVD to be either pro-inflammatory state(s) or any state(s) that is otherwise, most likely either immunosuppressive or pro-resolving (2, 37), (**Figure 1A**) because cause-effect relations are clear in these systems. So-called M1 macrophages are the ones that generate pro-inflammatory cytokines like TNF-α, inducible nitric oxide synthase (iNOS), and IL-6 (38). INFγ or lipopolysaccharide (LPS) is used to promote the pro-inflammatory phenotype *in vitro* (32) by triggering toll-like receptor (TLR)4 signaling associated pathways NF-κB, Notch, and INFγ/STAT-1. The NF-κB and NLRP3 inflammasome pathways are enhanced in pro-inflammatory macrophages. As stated above, experiments in small animal models reveal microscopic cholesterol crystals that activate the NLRP3 inflammasome resulting in the secretion of interleukin 1 family of cytokines (20). In another pathway, Delta-like 4 ligand (Dll4)-triggered Notch signaling can activate the NF-κB pathway to induce pro-inflammatory mediators such as iNOS, IL-1β, and CCL2/MCP-1, while Dll4 suppression reduce expression of pro-inflammatory factors of so-called M1 macrophages (8, 39–41). More recently, we demonstrated that the interplay between ADP-rbosylation enzymes PARP9 and PARP14 regulates the balance of pro- vs. anti-inflammatory macrophages (36).

The loosely characterized anti-inflammatory or alternatively activated type of macrophages (traditionally called M2-like cells) is a general designation to refer to different subclasses that generate molecules that either suppress activity of the M1-like cells, initiate efferocytosis or promote resolution of inflammation (31) (**Figure 1A**). In mouse models, unlike in humans, these M2-like cells are identified with markers including arginase 1, mannose receptor C type 1, Ym1 and Fizz1 (38, 42). Although these markers do not identify M2-like macrophages in humans, information gleaned from using these murine M2-like cells may yield genes and proteins that are present and crucial in human macrophages for controlling inflammation. To produce M2-like phenotype *in vitro*, resting or activated macrophages need to be stimulated with any of the following chemokines: IL-4, IL-13, and IL-10, (42, 43) in contrast to the LPS or IFNγ stimulated M1-like cells. To avoid confusion, recent guidelines for macrophage subclass nomenclature propose to call these *in vitro* stimulated cells M(LPS), M(IFNγ), M(IL-4) or M(IL-10) (32).

IL-4 polarization of macrophages involve the Krüppel-like factor-4 (KLF-4) pathway and the IL-4/STAT-6 pathway. Paucity of KLF-4 in macrophages produce elevated expression of pro-inflammatory genes iNOS,and TNFα (44). MCP-1-induced protein (MCPIP) generated by KLF4 inhibits M1 activation. Likewise, it also promotes an M2-like phenotype. Furthermore, IL-4 triggered STAT6 induction promotes KLF4 expression, which mediates M2 activation through MCPIP activity as stated above (45). Peroxisome proliferator-activated receptors (PPARs) are also considered to be promoters of an anti-inflammatory phenotype (46). IL-4 and STAT-6 mediate transcription of several metabolism-related genes and regulators including PPARγ (47). Furthermore, absence of PPARγ in macrophages fail to induce oxidative metabolism and are also unable to exhibit M2-like phenotype (48). Independent from IL-4, IL-10 has recently been identified to promote mitochondrial fitness during pro-inflammatory activation of macrophages by mitigating the mitochondrial damage caused by reactive oxygen species (ROS) after iNOS activation, essentially paving the way for control, and resolution of inflammation (43).

Position along the macrophage phenotype/activation spectrum may simply be a function of the balance or imbalance of pro-inflammatory vs. anti-inflammatory factors present in every macrophage. Macrophages modeled *in vitro* are inherently heterogenous (**Figure 1B**). This means that although most of every single macrophage may be sitting at or near the extremes of the M1/M2 spectrum, some will remain near or at the median of the spectrum. The state of activation for groups of macrophages accumulating in local areas of the chronically inflamed tissue may be a function of the combined effect of the activation states of each macrophage single cell state. It is tempting to simplify that attenuating any and all M1-like macrophage populations will attenuate inflammatory burden. Disease state can therefore be reverted back to homeostasis. However, reducing pro-inflammatory activation without balancing the pro-resolving macrophage population and/or leaving a "patrolling" subpopulation might foreseeably lead to a more vulnerable immune system (8). That is why aggressive anti-inflammatory therapy targeting only macrophage accumulation and activation may increase incidence of severe infections. Calibrating the overall balance may lead to the development of efficient and safe therapies with minimal risk for unfavorable immunologic consequences. For example, inhibiting a specific signaling mechanism such as the Dll4-Notch pathway may suppress damaging macrophage products while promoting protective factors (39–41, 49).

Mechanisms Through Metabolic Reprogramming

Glycolytic Energy Preference of the Activated Macrophage

Evidence suggests that metabolic pathways are vital regulators of macrophage activation (50). As early as 1963, researchers recognized how metabolism affects monocyte and macrophage

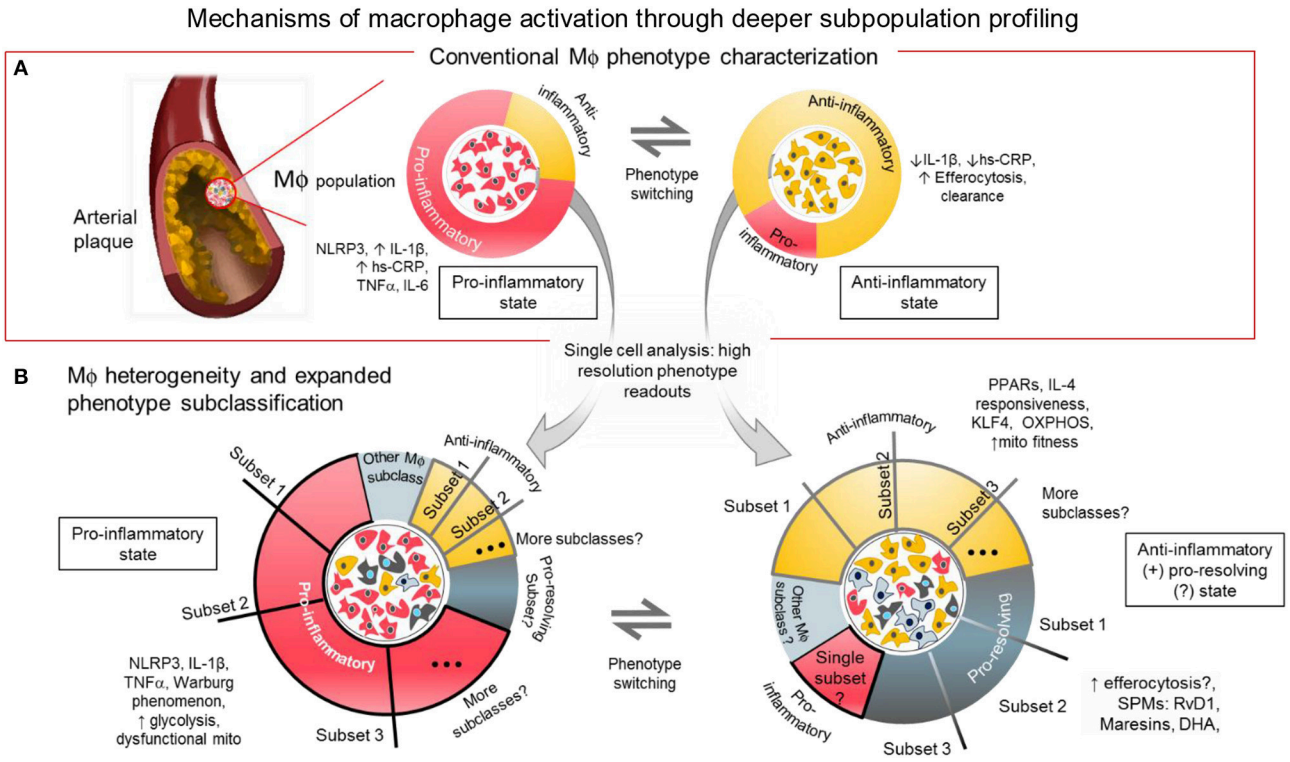

FIGURE 1 | Mechanisms of macrophage activation through deeper subpopulation profiling. **(A)** Conventional characterization of macrophages. Macrophages and foam cells in atherosclerotic lesions comprise a mixture of phenotypes and different activation states. The balance of proinflammatory, and anti-inflammatory/pro-resolving states may determine the fate of the plaque whether it will be stable or prone to rupture. The balance of inflammatory signaling molecules, metabolic states and presence of SPMs, among others, may influence the level of macrophage activation in distinct local regions within the atherosclerotic plaque. **(B)** Macrophage heterogeneity and expanded subclassification. The balance of factors within the macrophages may depend on the number and size of subpopulations with distinct functional phenotypes as revealed by single cell analysis. One or more subset populations within a specifically activated macrophage population and a disproportional balance of any one subset may drive the outcome of disease progression. Identifying the driver population(s) may be the key to identifying regulators of the disease.

physiology. Based on experimental findings, alveolar macrophages, and circulating monocytes tend to have better physiologic function when energy is being fueled by aerobic respiration. Moreover, the macrophages were noted as capable of aerobic respiration in greater magnitudes than neutrophils and monocytes, and that inhibiting part of oxidative phosphorylation (OXPHOS) had a depressive impact on phagocytic activity (51). Later, G.C Hard showed that peritoneal macrophages from immune mice (activated) challenged by injecting a virulent strain of *C. ovis* intraperitoneally, had higher production of lactic acid compared to peritoneal macrophages from non-immune mice (resting macrophages). It was not clearly distinguished whether the peritoneal macrophages from the non-immune mice were truly differentiated and not an admixture of macrophages and patrolling monocytes in the peritoneal cavity. Still, his findings are among the first to show enhanced glycolysis through increases in lactate production along with decreased O_2 uptake in immune activated primary macrophages (52). After more than a decade, experiments with thioglycolate-elicited mouse peritoneal macrophages show surprisingly high amounts of hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase implying further glycolytic induction in activated macrophages. In contrast, resting macrophages from saline injected mice showed less than 10% activity of 6-phosphofructokinase, complementing the prior observation that glycolytic rate is increased dramatically during phagocytosis or increased secretory activity (53). These early studies into macrophage metabolism research have broaden an entire field for mining biological mechanisms that allow us to appreciate immune activation as a reflection of metabolic state. Pivotal insights on macrophage metabolic reprograming came when it was reported that pro-inflammatory type polarization of macrophages with either LPS or INFγ could switch bioenergetic preferences of macrophages from OXPHOS to the glycolytic route (54, 55), a phenomena many observed to be reminiscent of the Warburg phenomenon happening in cancer cells (56–58). Other evidence supporting this, demonstrates that stimulation of TLRs by LPS increase *PFKFB3* (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3) expression, resulting in an increase of the key glycolytic allosteric regulator fructose 2,6-bisphosphate and a glycolytic flux (59).

Role of Metabolites in Classical Activation of Macrophages

Afterwards, work done in the lab of Luke O'Neill showed how metabolite key players in the glycolytic pathway, tri-carboxylic acid cycle (TCA) and OXPHOS play important roles in determining inflammatory activation states of immune cells like macrophages (56). They confirm that LPS causes macrophages to switch their core metabolism from OXPHOS to glycolysis. Inhibiting glycolysis could also suppress LPS mediated IL-1β secretion, albeit not TNFα, in mouse macrophages. While LPS does decrease expression of mitochondrial genes, there is a concomitant increase on levels of the TCA intermediate succinate. Their findings show that succinate: (1) stabilizes HIF-1α which support IL-1β release; (2) increases succinylation of several proteins; and (3) reduces desuccinylation of Sirt5 (60), a known epigenetic regulator of metabolism and inflammation. This Warburg-like phenomenon in macrophages implicate reprogramming at the epigenetic level through changing levels of acetyl-CoA potentially affecting increased acetylation and decreased deacetylations of histone proteins (61) Pro-inflammatory macrophages also have increased glucose transporter 1 (GLUT1) expression and availability to drive glucose uptake. This causes both hyper inflammatory proteome and transcriptome seen in RAW264.7 cells overexpressing GLUT1, leading to elevated secretion of inflammatory mediators, increase in reactive oxygen species (ROS) production and oxidative stress intracellularly (62) LPS-stimulated TLR4 increases mammalian target of rapamycin (mTOR) signaling, which induces the expression of lactate dehydrogenase and hypoxia induced genes (63, 64). Both events shunt acetyl-CoA away from TCA consumption. Moreover, this activation also leads up to elevated levels of an isoform of *PFKFB3* favoring glycolysis (63–65).

Metabolic Changes in Anti-inflammatory Type of Macrophages

On the other hand, anti-inflammatory conditioning of macrophages have an opposite effect of promoting OXPHOS while reducing preference for glycolysis (66). IL-4 for instance promotes OXPHOS resulting in elevated oxygen consumption rate (OCR) in RAW264.7 cells. Extracellular acidification rate (ECAR), a measure of lactate production (anaerobic glycolysis) is also lower compared to pro-inflammatory LPS stimulated RAW264.7 cells (67). But as a caveat, LPS and/or IFNγ-polarized macrophages may not repolarize into an anti-inflammatory phenotype (IL-4 re-stimulation) if there is too much mitochondrial dysfunction brought out by LPS-induced hyper-glycolysis and nitric oxide production (68). This results in inhibition of OXPHOS in the mitochondria (66). This also prevents plasticity of LPS+IFNγ conditioned macrophages to convert into an anti-inflammatory phenotype (66) underlying the importance of OXPHOS in the resolution of inflammation or the direction of macrophage activation. In this case of proinflammatory macrophages resistant to phenotype switching, IL-10 may be the key signaling molecule that may aid IL-4 and accomplish the phenotype switch.

Arguably considered an anti-inflammatory chemokine, IL-10 could increase clearance of dysfunctional mitochondria through mitophagy. IL-10 activates STAT3 signaling which in turn activates DDIT4 transcription factor leading to inhibition of the NLRP3 inflammasome activated mTORC1 pathway, thereby releasing the restraint against autophagy. DDIT4 itself promotes clearance of ROS-damaged mitochondria by mitophagy while IL-10 maintains membrane gradient potential of mitochondria of primary macrophage promoting organelle integrity and fitness during macrophage activation. This allows for rapid resolution of inflammation (43). All these changes in bioenergetics during macrophage polarization may be accompanied by a changing metabolome and specifically, a shift in the lipidome as well.

Lipidome Changes in Proinflammatory Macrophages and the Pro-Resolving Factors

E. Dennis' group did important work on macrophage lipidomics having contributed to the LIPID MAPS consortium to develop quantitative methods for evaluating the composition, biosynthesis, and function of all macrophage lipids. In one of their findings, they identified endotoxin Kdo_2-Lipid A (KLA, a defined form of LPS) of *E.coli* activates macrophages via TLR4 similar to "regular" LPS. Prostaglandin D2 (PGD2) is the predominant eicosanoid produced after KLA—proinflammatory stimulation of RAW264.7 cells (> 120 ng/10^6 cells), with almost nil 11-HETE production in contrast. PGE2 and PGD2 increase in a dose dependent manner with both LPS and KLA stimulation. PGE2 and PGD2 increase in a more potent but similar fashion to TNFα chemokine after LPS or KLA stimulation (69). Using RAW264.7 cells, their laboratory again assessed mouse macrophage lipidome changes with KLA treatment vs. drug perturbations like statins which are clinically relevant as statins inhibit cholesterol biosynthesis pathway and are able to reduce further inflammation in CAD of patients with high LDL profile (70). KLA tended to increase almost all categories of sphingolipid analyzed, cholesterol esters, and some glycrophospholipids. Statins like mevastatin (or compactin), a parent compound of pravastatin promote lipid changes intracellularly in these macrophages. Expectedly, statins blocked KLA induced increases in desmosterol and other components of the sterol biosynthetic pathway yet had no effect on actual intracellular cholesterol levels *per se* (71).

Still, there is more to uncover regarding metabolome and lipidome changes during the phenotypic differentiation by activated macrophages. With large metabolomic/lipidomic datasets, a systems approach to analysis may be the key to uncover comprehensive understanding of the dynamics of lipid and non-lipid metabolite pathways in the macrophage. This initial characterization in the mouse system is a first step to finding desirable therapeutic targets (71). However, there is still need to further elevate these studies to clinical relevance by using human samples. To this end, Tabas et al. further advanced the utility of mass-spectrometry lipidomic profiling (6) for atherosclerosis therapeutics by demonstrating specialized pro-resolving lipid mediators (SPMs) in atherosclerotic plaques.

SPMs are generally essential fatty acids-derived autacoids (2) They found that bioactive lipid derivative resolvin D1 (RvD1) levels were low relative to pro-inflammatory lipid leukotriene B_4 (LTB_4) in vulnerable plaques of the human carotid artery. This was further confirmed in hyperlipidemic mouse models by showing that administration of RvD1 increases plaque stability, lowers oxidative stress and necrosis, and thickens fibrous caps. Their findings support a–omics assisted mechanistic rationale for SPM therapy in CAD to mitigate plaque vulnerability (37).

Another set of SPMs, the maresins which are studied extensively by the Serhan group, are macrophage derived and are produced via 14-lipoxygenation of docosahexaenoic acid (DHA) that is either converted by enzymes into mediators with two-OH groups or into autacoids that are peptide-lipid conjugates, called maresin conjugates. These SPMs promote the uptake and clearance of apoptotic cells by macrophages. Maresins also regulate portions of tissue repair (2) therefore, resolution of the inflammatory damage. SPMs can potentially influence switching of macrophage function.

Macrophage Crosstalk With Other Immune Cells

Not only are macrophages known for their plasticity, these cells have the ability to influence and be influenced by other immune cells like T cells that results in a similar macrophage phenotype switching as above. The reciprocity of T cells and macrophages through either paracrine signaling or molecular interactions may dictate the direction of inflammation. It may either drive well into a vicious cycle of unmitigated chronic pro-inflammatory atherosclerotic events or toward inflammation resolution (72). Several reports have already shown that these macrophages communicate with other immune cells via specified protein pairs on their cell surfaces. Pairs can be co-stimulatory or co-inhibitory, whereby molecular interaction between these pairs triggers downstream biomolecular cascades that may promote or limit macrophage activation in atherosclerosis (73). Oncology researchers took advantage of this T cell-macrophage crosstalk, in order to combat cancer cells (74). Immunologists have coined the term immune checkpoints to identify these pairs of proteins that interact to either promote a pro-inflammatory activation or an anti-inflammatory one (75).

One of these pairs of co-stimulatory proteins are CD40 and CD40L (ligand) which are expressed in macrophages among other cell types found in atherosclerotic vessels (76). Both CD40 and CD40L are expressed highly in atherosclerotic lesions (77) and plasma which may predict patients with features of high-risk atherosclerotic lesions corroborated with MRI (78). Abrogating CD40L activity effectively reduces release of pro-inflammatory factors together with reducing activation of macrophages by activated T cells *in vivo* using mouse models of atherosclerosis. Deletion of either CD40 or CD40L has atheroprotective effects by mitigating macrophage activation (73). CD80/B7-1 and CD86/B7-2 are a pair of recognized M1-like markers for macrophages are also expressed in dendritic cells (DCs) in atherosclerotic plaques. They co-stimulate and bind to CD28 on T cells, B cells, and other macrophages (79). CD80 and CD86 double deficiency in hyperlipidemic LDL receptor-deficient ($LDLr^{-/-}$) mice results in lesser atherosclerotic burden (80) Other co-stimulatory immune checkpoint proteins found in human atherosclerotic lesions and in pre-clinical models that tend to skew macrophages and DCs to the pro-inflammatory end of the spectrum are: OX40-OX40L, CD137-CD137L, and CD30-CD30L (81) Immune checkpoint and co-inhibitory proteins PD-1 and PD-L1/2 inhibit T cell immune response resulting in a beneficial atheroprotective effect. Stimulation of PD-L1 expression *in vitro* could attenuate the stimulatory ability on allogeneic T cell proliferation and its cytokine production, including IFNγ (82). Such effect, however, is detrimental to immune clearance of tumor cells in cancer, hence the success of the anti-PD-1 immunotherapy oncologic drug pembrolizumab (83). CD27-CD70 is a another possible co-inhibitory pair since CD27 paucity on mice models show markedly increased atherosclerotic burden. In addition, CD27 is demonstrated to be essential in maintain a healthy pool of regulatory T cells (Tregs), preventing increased apoptosis of Tregs (84). CD70 promotes macrophage function and viability, and is important for effective efferocytosis and extrusion of oxLDL. CD70 deficiency results in more advanced atheroma (85). These are just a few of the immune checkpoints that affect macrophage behavior. Other proteins include, though not limited to, CTLA-4, ICOS-ICOSL, GITR-GITRL, and TIM (81). Other potential macrophage checkpoints are CD47 and signal regulatory protein alpha, SIRPα. In cancer, activation of SIRPα by CD47 on macrophages suppress both phagocytosis and respiratory burst (74). Therefore, as expected in mouse atherosclerotic models, blockade of CD47 exerts anti-atherosclerotic effects, halting lesion progression and preventing plaque rupture and restores phagocytosis/efferocytosis (86). A more recently studied immune regulator is the V-domain containing Ig Suppressor of T cell Activation (VISTA, aka PD-1H, DD1α; gene name DIES1). VISTA is both a receptor and a ligand with immunosuppressive effects on IFNγ, and TNFα with T cells and macrophages (74). In fact, majority of VISTA+ macrophages have the anti-inflammatory M2-like phenotype (87). Other proteins that can potentially act as immune checkpoints by control macrophage behavior via T cell and other immune cell 2-way interaction include: T cell immunoglobulin and ITIM domain (TIGIT) and indoleamine-2,3-dioxygenase (IDO). More studies are, however, required to clarify their roles in the macrophage inflammatory phenotype spectrum (74).

A summary comparison of these biomolecular signatures of atherosclerosis, as evidenced by findings in studies of mouse vs. human macrophages is enumerated in **Table 1**.

Macrophage Heterogeneity in Atherosclerosis

Advances in single cell analysis provided insights of how heterogenous human primary macrophages are. Single cell qPCR reveals how heterogenous resting macrophages, M0 or M(-), are. Yet after a pro-inflammatory polarization by IFNγ activation, M(IFNγ) macrophages even have a

TABLE 1 | Comparison of the effects of some biomolecular markers of macrophage activation as seen in mouse *in vitro* & pre-clinical models of atherosclerosis vs. human atherosclerosis (including *in vitro* studies).

Biomolecular markers of macrophage activation	Effect on pro-inflammatory activation of macrophages (+/- mouse models of atherosclerosis)	References	Effect on pro-inflammatory activation of macrophages/expression in plaques of clinical atherosclerosis	References
NLRP3 inflammasome Cholesterol crystals	↑	(20)	↑	(88)
IL-1β	↑	(20, 89)	↑	(4)
IL-6, TNF-α	↑	(38)	↑	(90, 91)
TLR2/TLR4	↑	(92, 93)	↑	(94)
iNOS	↑	(95)	↑	(96, 97)
INFγ/STAT1 signaling	↑	(36)	↑	(98)
CCL2/MCP1	↑	(38)	↑	(99, 100)
IL-4, IL-13 signaling	↓	(42)	?	(101)
IL-10 signaling	↓	(43)	↓	(102)
Dll4/Notch1 signaling	↑	(38, 41, 103)	↑	(39)
Ym1	↓	(104)	*No correlation*	(105)
Fizz1	↓	(104)	↑ ?	(106) (107)
PPAR-α/-γ	↓	(46)	↓ (predicted for PPAR-α)?	(108)
CD40 –CD40L	↑	(109)	↑	(76)
CD80-CD86	↑	(80)	↑ (*in vitro*, DC only)	(110)
OX40-OX40L	↑	(103)		(111)
CD137-CD137L	↑	(112)	↑ (monocytes)	(113)
CD30-CD30L	↑ (CD30L only in LPS-RAW264.7 cells)	(114)	↑ (CD30)	(81, 115)
PD1-PD-L1/2	↓ by PD1 (Chen)/ ↑ by PD-L1/2 (Gotsman)	(116) (117)	↓ (myeloid DC)	(82)
CD27-CD70	↓ (CD70)	(84)	*?*	No definitive consensus
Hyperglycolysis	↑	(56)	↑	(118)
GLUT1	↑	(119)	↑ (*in vitro* only)	(120)
OXPHOS	↓	(56)	*?*	*No evidence*
Citrate	↑	(56)	*?*	*No evidence*
Succinate	↑	(60)	*?*	*No evidence*
Itaconate	↑	(56)	*?*	*No evidence*
Prostaglandin D2	↑	(69)	↑	(121)
Prostaglandin E2	↑	(69)	↑	(122)
Resolvins	↓	(1, 37, 123)	↓	(124)
Maresins	↓	(123)	↓	(125)

DC, dendritic cells; OXPHOS, oxidative phosphorylation pathway.

further heterogenous response showing subpopulations that are more responsive to pro-inflammatory signals, even as some populations remain resistant and reticent, maintaining an unstimulated phenotype (35, 36). As cited earlier, identifying these macrophages and contrasting them vs. the highly responsive ones may help filter out by enrichment of key regulatory genes. These genes may promote phenotype switching thereby unraveling them to be desirable therapeutic targets. These targets or pathways may be otherwise hidden when examining an average transcriptomic readout from "bulk" macrophage populations processed from conventional qPCR or mRNAseq assays instead of the high resolution readouts of single cell analysis.

FUTURE PERSPECTIVES: NEW PARADIGMS OF DISCOVERY SCIENCE AND DRUG DEVELOPMENT IN VASCULAR INFLAMMATION

With the multitude of mechanistic perspectives that make up the macrophage behavior in atherosclerosis, there are many aspects to consider when designing effective therapies with the highest potential of leaping from the laboratory bench to clinical translation. Consolidating these various angles intelligently, in order to arrive at a viable drug target in the fastest possible way, is an attractive goal for the medical science community.

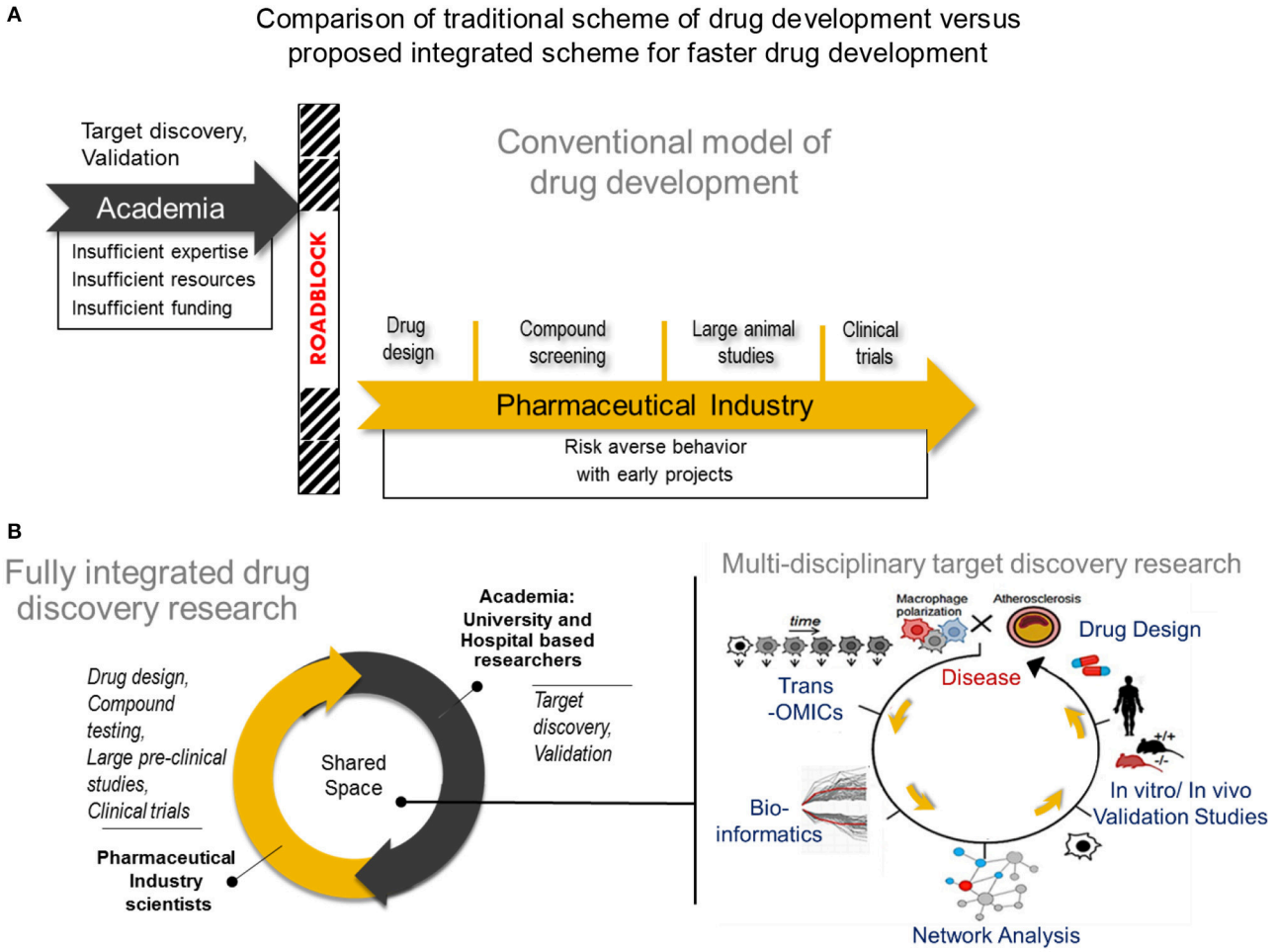

FIGURE 2 | Comparison of tradional drug development vs. proposed integrated drug discovery research **(A)** The conventional model of drug development Target discovery and validation studies are often done by academic researchers who usually have insufficient expertise and resources to conduct such studies resulting years of development. After characterizing a potential drug target, many academic investigators struggle to translate their finding into the pharmaceutical space due to a natural disconnect between academia and industry. In rare instances, academic investigators may manage to cross the roadblock and transfer their breakthroughs to industry, followed by a lengthy process of drug design, compound testing and animal studies before the drug will be considered for human studies. **(B)** A new paradigm for drug development Left) A fully integrated drug discovery research in our laboratory involves close collaboration between academic and pharmaceutical industry scientists. Right) Use of multi-omics approach to disease characterization with systems approach analysis for faster target discovery and prioritization and drug design. A right panel was reproduced from Iwata et al. (36). Trans-OMICs: genomics, transcriptomics, proteomics, epigenomics, metabolomics, lipidomics, etc.

However, due to limited resources, insufficient funding, and lack of expertise, many academic investigators may fail to develop and deliver their target discovery beyond the laboratory space (126, 127). Despite the evident strengths of pharmaceutical industry in drug development, its recent tendency to avoid investing in early, high-risk projects appears to have enhanced this gap in translation of academic target discovery into the clinic (128, 129). These roadblocks often pre-maturely remove the possibility of an otherwise promising target from being transferred to pharmaceutical development and eventually becoming a novel, even first-in-class, drug. (**Figure 2A**). Arriving at the most cost effective strategy for pharmaceutical translation requires a balancing act of carefully prioritizing the targets discovered with very rational and discreet management of resources for target(s) validation while still within the hands of the academic researcher (130).

Close collaboration between academia and industry will foster faster exchange of expertise and resources that could lead to mutually beneficial research outputs (130, 131). In such a model established in our laboratory (**Figure 2B**, left panel), drug development is facilitated when effectively combining the exploratory nature of innovative academic research and the extensive expertise in drug design and the rigor and resource intensive validation from pharmaceutical industry. Much advancement in computational biology and networks medicine, as well as steadily declining costs and time required for—omics experiments and single cell experiments also help to speed the transition of academic target discovery into

drug development. It is easier now to consolidate various biological mechanisms that define macrophage activation, and use an intergrated approach to arrive at viable targets in a more comprehensive and unbiased manner. A typical workflow of target discovery research in our laboratory is demonstrated in **Figure 2B (right panel).** We expect that paired with an ever-expanding knowledge base and expertise in performing these–omics experiments, as well as use of machine learning approaches, a more solid understanding of pathways that drive macrophage activation will emerge. Novel pathways of activation may be uncovered when we actively use network science to incorporate comprehensive readouts from–omics and single cell experiments. With all these innovative large-scale approaches in biological research and data analyses, pairing with synergistic efforts from academic and industry scientists and cell and computational biologists, more effective and better defined drugs may arrive to the market sooner. Better understanding of biological mechanisms for macrophage activation and heterogeneity through big data, particularly of clinical samples, and integrated analysis, may also lead to safer drugs that target specific subsets of populations as opposed to a more generalized approach.

AUTHOR CONTRIBUTIONS

JD and MA both contributed to the concept and key contents of this manuscript. JD drafted the manuscript and MA critically reviewed and edited it.

FUNDING

This work was supported in part by research grants from the National Heart, Lung, and Blood Institute (R01HL107550 and R01HL126901 to MA) and Kowa Company, Ltd., (Nagoya, Japan to MA).

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

frontiers in Cardiovascular Medicine

REVIEW
published: 19 January 2018
doi: 10.3389/fcvm.2018.00001

Thrombosis and Vascular Inflammation in Diabetes: Mechanisms and Potential Therapeutic Targets

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Cardiovascular disease remains the main cause of morbidity and mortality in patients with diabetes. The risk of vascular ischemia is increased in this population and outcome following an event is inferior compared to individuals with normal glucose metabolism. The reasons for the adverse vascular profile in diabetes are related to a combination of more extensive atherosclerotic disease coupled with an enhanced thrombotic environment. Long-term measures to halt the accelerated atherosclerotic process in diabetes have only partially addressed vascular pathology, while long-term antithrombotic management remains largely similar to individuals without diabetes. We address in this review the pathophysiological mechanisms responsible for atherosclerosis with special emphasis on diabetes-related pathways. We also cover the enhanced thrombotic milieu, characterized by increased platelet activation, raised activity of procoagulant proteins together with compromised function of the fibrinolytic system. Potential new therapeutic targets to reduce the risk of atherothrombosis in diabetes are explored, including alternative use of existing therapies. Special emphasis is placed on diabetes-specific therapeutic targets that have the potential to reduce vascular risk while keeping an acceptable clinical side effect profile. It is now generally acknowledged that diabetes is not a single clinical entity but a continuum of various stages of the condition with each having a different vascular risk. Therefore, we propose that future therapies aiming to reduce vascular risk in diabetes require a stratified approach with each group having a "stage-specific" vascular management strategy. This "individualized care" in diabetes may prove to be essential to improve vascular outcome in this high risk population.

Keywords: atherosclerosis, diabetes mellitus, thrombosis, endothelial dysfunction, vascular inflammation

OPEN ACCESS

Edited by:
Nikolaus Marx,
Uniklinik RWTH Aachen, Germany

Reviewed by:
Andreas Schäfer,
Hannover Medical School, Germany
Christoph Eugen Hagemeyer,
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *31 October 2017*
Accepted: *03 January 2018*
Published: *19 January 2018*

Citation:
Pechlivani N and Ajjan RA (2018) Thrombosis and Vascular Inflammation in Diabetes: Mechanisms and Potential Therapeutic Targets. Front. Cardiovasc. Med. 5:1. doi: 10.3389/fcvm.2018.00001

INTRODUCTION

The prevalence of diabetes continues to increase and is reaching epidemic proportions.[1] The vast majority of these patients have type 2 diabetes characterized early in the disease process by insulin resistance (IR) followed by pancreatic β-cell failure leading to elevated blood glucose and emergence of diabetes (1–3). A minority have type 1 diabetes, secondary to immune destruction of the β-cells.

[1]http://www.who.int.

However, these individuals can also develop IR leading to double diabetes, which has features of both type 1 and type 2 diabetes (4), and this group appears to have an enhanced vascular risk.

Despite advances in therapy, cardiovascular disease (CVD) remains the main cause of morbidity and mortality in diabetes (5, 6). Similarly to CVD in individuals without diabetes, an increased inflammatory response and an enhanced thrombotic milieu represent the two main pathological pathways underpinning atherothrombosis in diabetes. However, the adverse vascular process is accelerated in diabetes, secondary to a combination of IR and elevated blood glucose levels. Understanding the exact mechanisms involved in disease initiation and progression is crucial in order to develop effective vascular preventative and therapeutic treatment strategies (7–9).

This review summarizes the pathophysiological mechanisms responsible for increased atherothrombotic risk in diabetes, addresses current treatment modalities, and explores additional pathways that can be targeted to reduce vascular events in this high-risk population.

Pathophysiology

Both extensive vascular pathology and an enhanced thrombotic environment contribute to premature vascular occlusive events and poor clinical outcome in patients with diabetes.

A normal endothelial cell (EC) function is the key to maintain vascular health. EC dysfunction is one of the earliest abnormalities in the atherothrombotic process, which also contributes to the latter stages of the disease. Another central mechanism in vascular pathology is systemic inflammation, which promotes vascular damage. EC dysfunction and an inflammatory milieu are both associated with a prothrombotic and hypofibrinolytic environment, facilitating vascular occlusion and leading to myocardial infarction, stroke, and occlusive peripheral vascular disease, common complications in patients with diabetes.

EC Dysfunction

The endothelium plays an important role in maintaining vascular homeostasis by regulating vasodilation and vasoconstriction, thrombosis and fibrinolysis, platelet activation, platelet and leukocyte interaction, and smooth muscle cell function. Under physiological conditions, the endothelium modulates vascular tone by producing and releasing various vasodilator substances, most importantly nitric oxide (NO), and vasoconstrictor substances including endothelin (10, 11). When EC dysfunction occurs, secondary to IR with or without elevated blood glucose levels, vascular homeostasis is disturbed and the process of atherosclerosis ensues (11, 12). EC dysfunction results in these cells expressing adhesion molecules, thus attracting inflammatory cells. Moreover, EC dysfunction disrupts the barrier function of these cells causing the movement of low-density lipoprotein cholesterol (LDL) from vessel lumen into the wall, where it is oxidized to the highly atherogenic ox-LDL. This molecule is then taken up by inflammatory cells that moved from the blood stream to the vessel wall secondary to increased permeability of dysfunctional EC. The uptake of ox-LDL by macrophages results in the formation of foam cells, which aggregate together to form the fatty streak, the earliest abnormality in the atherosclerotic process, and which can be found as early as childhood. An inflammatory reaction is followed by deposition of collagen, gradually transforming the healthy vessel wall into a series of atherosclerotic plaques. Once the atherosclerotic plaque ruptures, it exposes a prothrombotic core resulting in activation of platelets and the protein phase of coagulation, culminating in the formation of the obstructive vascular clot.

Endothelial cell dysfunction plays a role in all stages of the atherosclerotic process from initiation of atherosclerosis to precipitation of thrombosis (10, 13), as summarized in **Figure 1**. Therefore, therapy targeted at improving the health of these cells would help to reduce the risk of atherothrombosis. In diabetes, this includes general measures, such as ensuring good glycemic control, reducing IR, and optimizing blood pressure control. However, there are additional steps that can be undertaken to target specific pathways, which are further detailed below.

Vascular Inflammation and Atherosclerosis in Diabetes

We will concentrate on diabetes-specific pathways for vascular pathology related to IR and elevated glucose levels.

Reduced NO bioavailability and elevated levels of reactive oxygen species (ROS) play fundamental roles in vascular disease in diabetes (**Figure 1**). IR inhibits NO production by decreasing the activity of endothelial NO synthase (eNOS) resulting in reduced vasodilation (14–16). In addition to reduced production of vasodilators, there is an increased production of vasoconstrictors in diabetes. For example, the vasoconstrictor endothelin-1 is involved with endothelial dysfunction and increased plasma levels have been associated with microangiopathy in type 2 diabetes (17). Furthermore, increased arterial stiffness in diabetes has been associated with phenotype switching of vascular smooth muscle cells, a process that appears to be controlled by microRNAs (miRNAs); *mi*R-145 has recently been shown to modulate the phenotypic switch of vascular smooth muscle cells from a contractile to a proliferative state in atherosclerosis (18, 19).

Decreased NO bioavailability has also been related to platelet activation. An *in vivo* study in diabetic mice has demonstrated that inhibiting NO synthase reduced platelet vasodilator-stimulated phosphoprotein (VASP) phosphorylation and increased fibrinogen-platelet binding and expression of P-selectin as well as CD40 ligand. Diabetic mice also exhibited reduced VASP phosphorylation, increased fibrinogen-platelet binding, and enhanced expression of P-selectin/CD40 ligand, which was rescued by endothelial-specific restoration of NO production (20). This emphasizes the importance of NO production by ECs in controlling platelet activation, a process that is compromised in the presence of endothelial dysfunction.

Hyperglycemia in diabetes and elevated levels of free fatty acids enhance ROS production, which in turn compromises NO synthesis *via* a number of cellular mechanisms. More specifically, free fatty acids bind to Toll-like receptor, activating NF-κB, which stimulates inflammation by increasing the expression of the inflammatory molecules interleukin (IL)-6 and tumor necrosis factor (TNF)-α. Moreover, the stimulation of the toll-like receptor induces the phosphorylation of insulin receptor

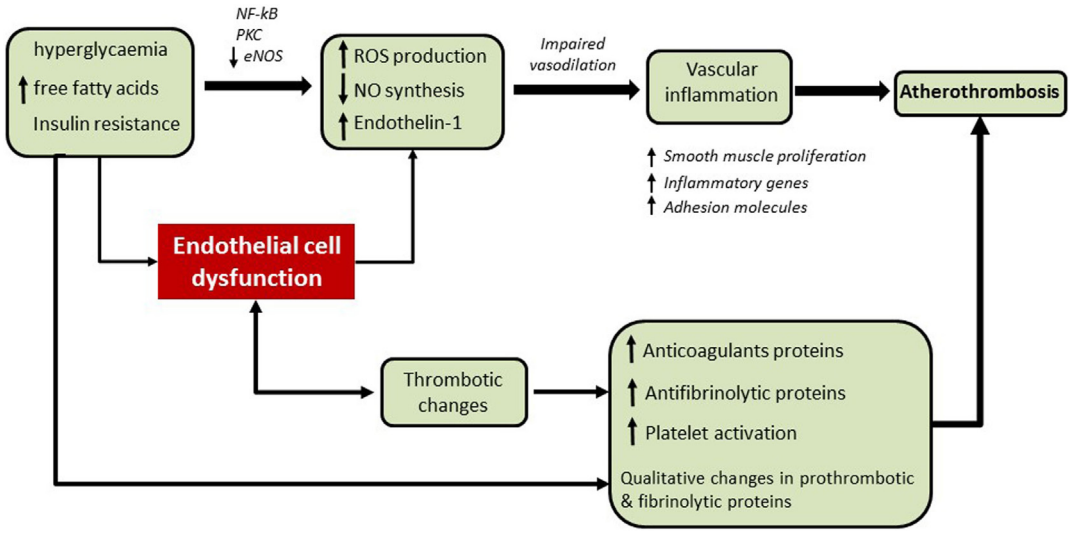

FIGURE 1 | The accelerated vascular pathology and enhanced thrombotic environment in diabetes. Endothelial dysfunction plays a key role in all stages of the atherosclerotic process. In the early stages of the disease, insulin resistance and increased levels of glucose and free fatty acids enhance the production of reactive oxygen species (ROS) and reduce nitric oxide (NO) synthesis *via* several mechanisms including activation of NF-κB and protein kinase C (PKC) signaling and reduction of endothelial NO synthase (eNOS) activity. Endothelial dysfunction contributes to the impairment of vasodilation, expression of adhesion molecules, and further vascular inflammation. In the latter stages of the disease, endothelial dysfunction results in increased platelet activation and a prothrombotic/hypofibrinolytic environment which facilitates vascular occlusion and atherothrombosis.

substrate-1 by c-Jun amino-terminal kinase (JNK) and protein kinase C (PKC) causing downregulation of the PI3-kinase/Akt pathway and the glucose transporter GLUT-4. Suppression of the PI3-kinase/Akt pathway leads to reduced eNOS activity and decreased NO production. Furthermore, the increased oxidative stress and hyperglycemia, stimulate vascular inflammation *via* several cellular mechanisms, including promoting activation of PKC and NF-κB signaling. Secretion of cytokines IL-1 and TNF-α enhances NF-κB activity and production of adhesion molecules by ECs further aggravating the inflammatory process (21, 22).

Figure 2 summarizes the main mechanistic pathways operating to increase vascular inflammation in diabetes.

Thrombotic Changes in Diabetes

The metabolic changes including IR and hyperglycemia are associated with both increased platelet activation and reduced response to antiplatelet therapy, which have been extensively reviewed elsewhere (23–26). Briefly, there are two main types of antiplatelet agents that are currently used for long-term therapy in diabetes. The first targets the thromboxane pathway, represented by aspirin, whereas the second targets the P2Y12 pathway, and this family of drugs currently includes three agents: clopidogrel, prasugrel, and ticagrelor. More antiplatelet agents are in development that target additional pathways (26).

Following myocardial infarction, dual antiplatelet therapy (DAPT) is used employing agents that target the thromboxane and P2Y12 pathways. However, inhibitors of the P2Y12 pathway seem to vary in efficacy. In the TRITON trial, DAPT with aspirin and prasugrel following myocardial infarction was superior to aspirin and clopidogrel but at the expense of increased bleeding events, failing to overall improve clinical outcome. A sub-analysis of diabetes patients, however, has shown reduction in further ischemic events without a significant increase in bleeding risk (27), making prasugrel particularly useful for patients with diabetes. The newer P2Y12 inhibitor ticagrelorhas also shown both biochemical and clinical superiority to clopidogrel when used in combination with aspirin following myocardial infarction, an effect that was observed in both diabetes and non-diabetes patients without an increase in bleeding risk (28–31). Moreover, the PEGASUS-TIMI 54 trial showed that ticagrelor addition to background aspirin therapy in patients with stable coronary artery disease caused significant reduction of cardiovascular risk compared with the placebo group (32). The previously documented reduced efficacy of clopidogrel in diabetes does not seem to apply to the newer agent ticagrelor, showing the progress made at inhibiting P2Y12 pathway in diabetes. In contrast, the inhibition of the thromboxane pathway by aspirin remains an area of debate. Given the short half-life of aspirin and the increased platelet turnover in this condition, the use of twice daily aspirin in diabetes has been advocated. Indeed, clinical studies have shown a significant reduction in platelet reactivity to thromboxane when using aspirin twice daily vs once/day (33–36). However, it remains unclear whether twice daily aspirin affects clinical outcome and studies in this area are lacking.

A relatively recent development is the implication of reticulated platelets in atherothrombotic events (37). These are immature platelets that show resistance to the action of antiplatelet therapy (38, 39). However, the degree of resistance appears to differ comparing antiplatelet agents, with ticagrelor the least affected (40). Interestingly, a recent study has shown that poorly controlled diabetes is characterized by increased circulating reticulated platelets through induction of an

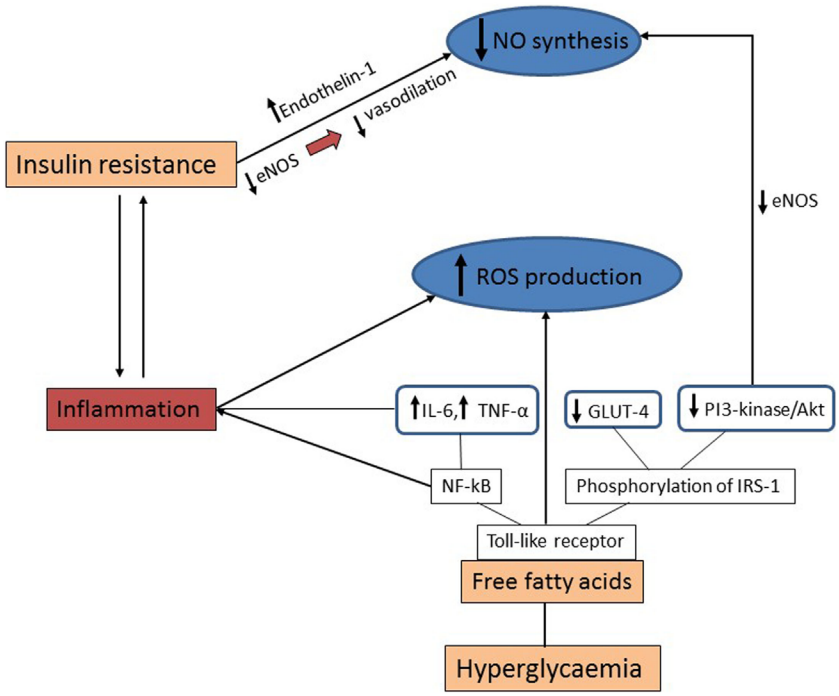

FIGURE 2 | Mechanistic pathways for increased vascular inflammation in diabetes. Insulin resistance inhibits nitric oxide (NO) synthesis by reducing vasodilation *via* decreased activity of endothelial NO synthase (eNOS) together with increased production of vasoconstrictors such as endothelin-1. Hyperglycemia and increased levels of free fatty acids enhance the production of reactive oxygen species (ROS) and reduce NO synthesis *via* several cellular mechanisms. Free fatty acids bind to toll-like receptor which activates the NF-κB pathway and stimulates inflammation by increasing interleukin (IL)-6 and tumor necrosis factor (TNF)-α. Additionally, toll-like receptor stimulates the phosphorylation of insulin receptor substrate-1 (IRS-1) followed by downregulation of the PI3-kinase/Akt pathway and the glucose transporter GLUT-4, consequently resulting in reduction of NO production.

inflammatory pathway that leads to increased thrombopoietin production. A correlation between HbA1c and reticulated platelets was found in patients with T2DM and improving glycemic control, using an agent in the sodium glucose co-transporter-2 (SGLT2) inhibitor family, reduced reticulated thrombocytosis in mice. These findings indicate that one strategy for improved efficacy of antiplatelet agents is to reduce circulating reticulated platelets, which can be achieved by lowering glucose levels using SGLT2 inhibitors. This in turn may help to reduce cardiovascular risk in patients with diabetes (41). Indeed, studies on SGLT2 inhibitors have shown reduction in the composite end point of myocardial infarction, stroke, and cardiovascular death (42) and controlling platelet reactivity may be one of the mechanisms involved.

In addition to enhanced platelet activation, diabetes is associated with increased plasma levels and/or activity of various coagulation factors. The net result is an enhanced susceptibility to forming fibrin networks which are characterized by increased density and resistance to fibrinolysis (43, 44). For example, tissue factor (TF) and factor VII (FVII) levels are increased in diabetes, which in turn explains the enhanced production of thrombin leading to higher risk of clot formation. Moreover, plasma levels of fibrinogen are increased in diabetes, as part of the ongoing low-grade inflammation, which contributes to formation of denser clots. In addition, anticoagulants, such as thrombomodulin and protein C, are reduced in diabetes further predisposing to the prothrombotic environment (43, 45). A key antifibrinolytic protein is plasminogen activator inhibitor-1 (PAI-1), which inhibits conversion of plasminogen to active plasmin. Plasma levels of PAI-1 are increased in diabetes, thereby impairing the fibrinolytic process. It should be noted that patients with type 2 diabetes, but not type 1 diabetes, have increased levels of PAI-1 indicating that IR, rather than hyperglycemia *per se*, drives the increased production of this antifibrinolytic protein, a concept that was confirmed using *in vitro* and *in vivo* studies (9, 44, 46, 47). Therefore, ameliorating IR would have more of an effect on PAI-1 levels than simply improving glycemia in diabetes, highlighting some of the complexities involved in the management of this condition.

In addition to quantitative changes, qualitative abnormalities have been documented in diabetes that increase thrombosis risk. This includes altered fibrinogen posttranslational modifications, including increased glycation and oxidation, resulting in denser fibrin networks (48–51). Also, we have shown that hyperglycemia can directly affect the fibrinolytic system by increasing plasminogen glycation, which impairs conversion to plasmin and adversely affects protein activity. Interestingly, a relatively modest decrease in plasma glucose levels improves plasminogen function, demonstrating the importance of optimizing glycemic control in these patients (52).

An interesting, and potentially clinically relevant, mechanism for impaired fibrin clot lysis in diabetes is focused on increased incorporation of antifibrinolytic proteins into the clot. Alpha 2-antiplasmin is cross-linked into the fibrin clot and it inhibits plasmin by forming a stable complex with this protein. It has been reported that type 1 diabetes patients have increased alpha 2-antiplasmin incorporation into their fibrin clots (47, 53). Similarly, we have shown that complement C3 incorporation into diabetes clots is increased in diabetes and directly prolongs clot lysis (54, 55). Moreover, C3 plasma levels were independent predictors of clot lysis in a large cohort of type 2 diabetes patients (56, 57). Taken together, modulating incorporation of antifibrinolytic proteins into diabetes clots may represent a new therapeutic strategy to reduce thrombotic risk in diabetes. The main mechanisms operating to increase thrombosis risk and may represent future therapeutic targets in diabetes are summarized in **Figure 3**.

From Bench to Bedside

Given the pathophysiological mechanisms involved in vascular pathology in diabetes, there are two main areas to target: slowing the progression of atherosclerosis and reducing the prothrombotic/hypofibrinolytic environment. Therefore, current therapies are directed at improving endothelial function, reducing systemic inflammation, and controlling the enhanced thrombotic environment.

Targeting Atherosclerosis

In general, improving glycemia and reducing IR, which is part of the clinical management strategy in diabetes, have key roles in halting the accelerated atherosclerotic process in this condition.

A number of existing agents, and others in development, target the inflammatory pathway to reduce atherosclerosis risk. Statins, known for their ability to decrease cholesterol levels, have also been shown to affect the inflammatory response and this dual mode of action explains the efficacy of this class of drugs in reducing vascular events in diabetes (58–60).

The initial enthusiasm with preclinical studies involving the inhibitor of phospholipase A2 (PLA2) darapladip quickly faded with the failure of this agent to reduce coronary vascular events. The clinical findings suggest that this enzyme is a biomarker of vascular pathology rather than a factor having a direct role in pathogenesis. Inadequate dosing and off-target effect(s) have also been blamed for failing to demonstrate a clinical benefit with this agent so far (61). More studies are currently ongoing that will fully clarify the role of PLA2 as an agent that modifies the atherosclerotic process (62, 63).

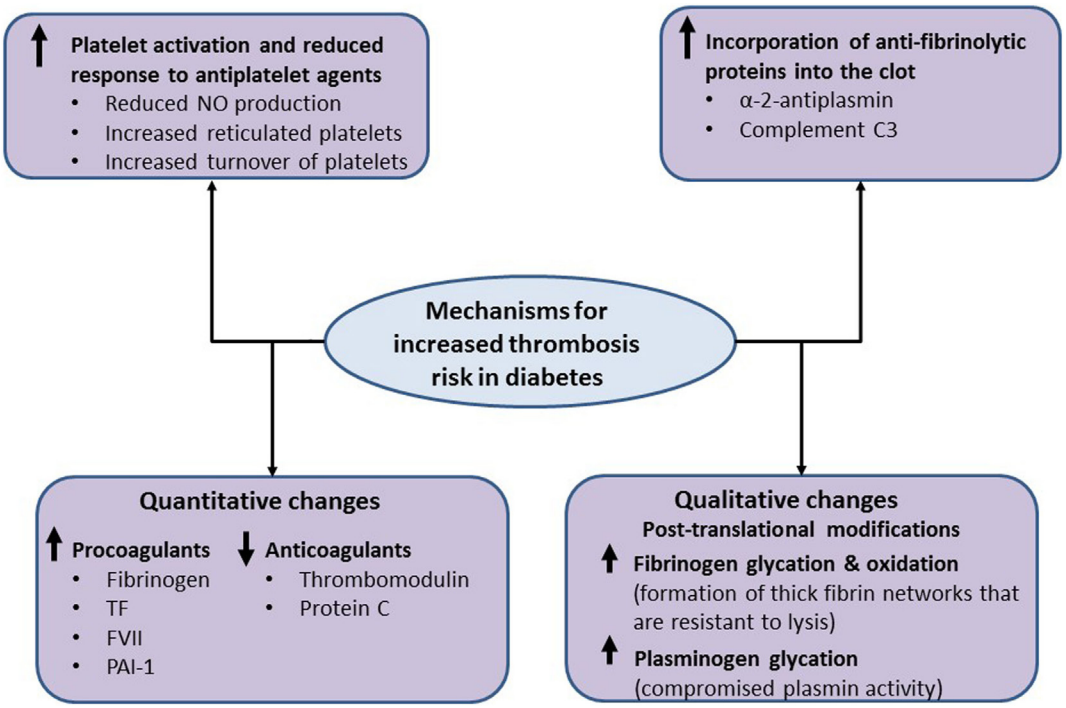

FIGURE 3 | Mechanistic pathways for increased thrombosis risk in diabetes. The metabolic changes in diabetes are associated with increased platelet activation, secondary to reduced nitric oxide (NO) production, an increase in reticulated platelets and increased platelet turnover, which can be only partially controlled by antiplatelet therapy. Diabetes is also associated with increased plasma levels of procoagulants and the antifibrinolytic protein levels, including fibrinogen, tissue factor (TF), factor VII (FVII), and plasminogen activator inhibitor-1 (PAI-1) as well as decreased levels of anticoagulants, including thrombomodulin and protein C. Moreover, qualitative changes in coagulation proteins, including increased oxidation and glycation of fibrinogen, have also been reported in diabetes, which result in the formation of thick fibrin networks that are resistant to lysis. Increased glycation of plasminogen compromises conversion to plasmin and alters protein activity. Finally, increased incorporation of antifibrinolytic proteins, such as α 2-antiplasmin and complement C3, into the clot impairs fibrinolysis and may represent a new therapeutic approach to reduce thrombosis risk in diabetes.

Experimental studies and preclinical models have indicated that IL-1β blockade is effective at preventing coronary artery disease. A recent study involving 10,061 patients with previous myocardial infarction and C-reactive protein levels above 2 mg/l, showed that IL-1 inhibition, using Canakinumab, reduced the composite end point of non-fatal myocardial infarction, non-fatal stroke, and cardiovascular death by 15 and 14% using 150 and 300 mg of this agent, respectively (injected once every 3 months). However, there was no difference in all-cause mortality, which may be related to increased fatal infection in the Canakinumab arm of the study.

Antioxidants have been repeatedly considered as potential drugs for the prevention and therapy of atherosclerosis given the role of ROS in the pathogenesis of atherosclerosis (64). However, clinical studies with antioxidants have been generally disappointing; a well-conducted double-blind, randomized, placebo-controlled study involving 6,144 patients with established CVD has shown that the antioxidant succinobucol (AGI-1067) has no beneficial effect on vascular outcome, dampening the enthusiasm toward such therapy (65). The generally negative results with various antioxidant therapies suggest that targeting a single pathway involved in vascular pathology is perhaps not enough for translation into clinical benefit. The establishment of the European consortium for the study of ROS should further help to shed more light on the role of this pathway as a therapeutic target in vascular pathology, including high-risk conditions such as diabetes (66).

Given the pivotal role of endothelial dysfunction in vascular pathology, a number of agents that improve EC function are currently in clinical use and others are under development. Angiotensin-converting (ACE) inhibitors, angiotensin II-receptor blockers (ARB), some β blockers, and calcium channel blockers, all modulate EC function. However, not all have the same clinical effects; for example, ACEI convincingly provide protection from future vascular events, whereas the case for ARB is less clear despite targeting a largely similar pathway.

Compounds that act directly on guanylate cyclase (sGC), a key enzyme of the NO signaling pathway, have also been investigated in preclinical studies. The therapeutic potential of sGC activators (cinaciguat or ataciguat) and sGC stimulators (riociguat) has been explored in animal models and clinical trials (67). In a recent animal study, it was demonstrated that a sGC activator offered renal protection against the progression of nephropathy induced by type 2 diabetes in obese rats (68). However, it remains unclear whether sGC activators can modulate the risk of vascular occlusion in man, and this remains an area for further research.

While all these agents can improve endothelial dysfunction by lowering blood pressure, it is clear that additional mechanisms operate to explain the difference in vascular outcome (69, 70). It has been reported that the thiazolinedione family of hypoglycemic agents, improve endothelial dysfunction in patients with type 2 diabetes by modulating IR (71, 72). Although pioglitazone treatment showed protection from cardiovascular events in diabetes (73), treatment with rosiglitazone therapy was associated with an increase in vascular events (74). This shows that two agents within the same class can have a different clinical outcome, highlighting the difficulties in treating vascular disease in diabetes.

Experimental agents to improve EC function include eNOS-transcription enhancer and agents that inhibit phosphodiesterase-5 (PDE-5) and sphingosine-1-phosphate (75, 76). It has been shown that the eNOS-transcription enhancer AVE3085 restored endothelial function in a hypertensive rat model suggesting that drugs regulating eNOS may be considered as therapeutic targets (77). Furthermore, PDE-5 inhibitors upregulate eNOS expression and, therefore, NO production. It has been reported that these inhibitors can improve endothelial function and control platelet activation in patients with coronary artery disease and have also been shown to improve vascular relaxation in diabetic rats (75, 78). Another target that gained an interest is sphingosine-1-phosphate, which affects the function of cells involved in the atherosclerotic process, including monocyte attachment and proliferation of smooth muscle cells (79).

Aging and diabetes both cause vascular dysfunction and the existence of both conditions has an additive effect in vascular damage, leading to increased vascular inflammation and cardiovascular risk (80). So far, however, there has been little research on the mechanisms of vascular inflammation due to both aging and diabetes, which would be particularly helpful to address vascular disease in older patients with diabetes.

It has recently been demonstrated that following a coronary ischemic event, a single dose of reconstituted HDL increased cardiac glucose uptake and reduced infarct size in both metabolically normal and insulin-resistant mice (81). While these data are interesting, further studies are needed to clarify the role of this approach in modulating vascular risk in man.

Finally, several studies have investigated the role of miRNAs in endothelial dysfunction in diabetes. It has been suggested that these noncoding, single-stranded RNA molecules which are implicated in key processes such as IR and β-cell function, may contribute to the development of prognostic tools or therapeutic targets of CVD in diabetes (82). For example, miR-155 has been implicated with atherosclerosis *via* modulation of actin cytoskeleton organization in ECs, and it was reported that inhibition of miR-155 can reduce atherosclerotic plaques (83). Platelet miR-223 was reduced in patients with diabetes and in mice which had an effect on platelet function. Platelets from miR-223 knockout mice had increased aggregation and potential for thrombus formation compared to platelets from wild-type mice (84, 85).

The various inflammatory pathways that may represent therapeutic targets to reduce vascular risk in diabetes are summarized in **Figure 4**.

Targeting Thrombosis

The thrombotic potential can be modulated both indirectly and directly. The former includes measures to reduce IR, optimize glycemia, and improve endothelial dysfunction. The latter concentrates on antiplatelet therapy as well as agents that target coagulation proteins.

Indirect Measures to Improve the Thrombotic Environment

Diabetes-specific measures to improve the thrombotic environment concentrate on ameliorating IR and improving glycemia.

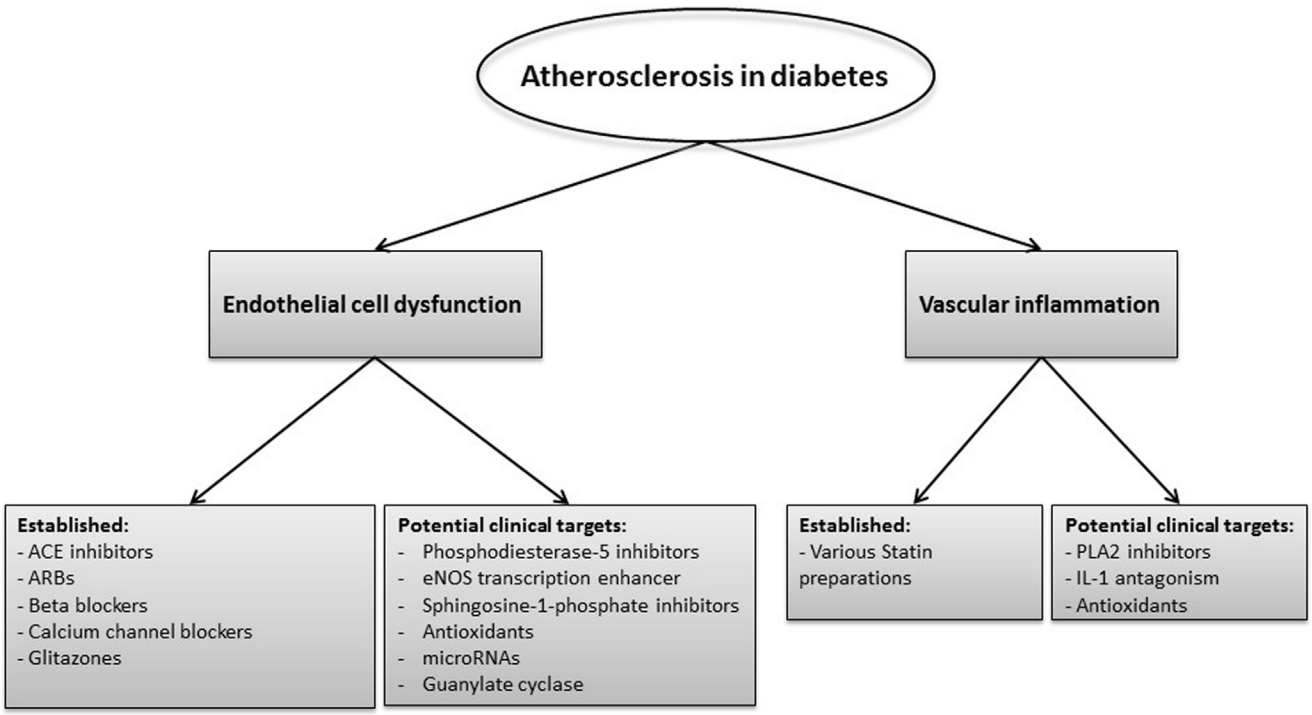

FIGURE 4 | Inflammatory pathways that are currently used or may represent therapeutic targets to reduce vascular risk in diabetes. Current therapies and others in development focus on improving the endothelial cell function and reducing vascular inflammation. Established therapies to target endothelial dysfunction are angiotensin converting (ACE) inhibitors, angiotensin II-receptor blockers (ARBs), beta blockers, calcium channel blockers, and glitazones, whereas agents such as phosphodiesterase-5 inhibitors, endothelial nitric oxide synthase (eNOS) transcription enhancer, and microRNAs represent potential future therapies. Statins are widely used to reduce vascular risk and in addition to their cholesterol lowering effect they reduce vascular inflammation. Other potential targets to reduce vascular inflammation include of phospholipase A2 (PLA2) inhibitors as well as interleukin (IL)-1 antagonists and antioxidants.

This can be achieved by simple measures, such as increased exercise/adhering to a healthy diet through to sophisticated treatment regimen of intensive glycemic control. Improving glycemia reduces platelet activation, modulates fibrin clot structure, and improves efficiency of the fibrinolytic system thus ameliorating the prothrombotic environment in diabetes. However, the short-medium term clinical effects of improving glycemia on vascular events are debatable with studies showing a benefit, no effect and even harm (86–88). The inconsistent results of glycemic studies may be related to precipitation of hypoglycemia with tight glycemic control, which can be prothrombotic. We have shown that an episode of hypoglycemia creates a prothrombotic and hypofibrinolytic environment for at least 1 week (89), explaining the association between hypoglycemia, cardiovascular events, and mortality (90, 91). Therefore, hypoglycemic agents with lower risk of hypoglycemia should be considered in higher vascular risk patients with diabetes. In support of this concept, three relatively recent trials with hypoglycemic agents that are associated with low risk of hypoglycemia have shown improved vascular outcome (92–94). It should be acknowledged, however, that the beneficial effects of these newer hypoglycemia agents are not solely related to avoidance of hypoglycemia and the favorable clinical outcome is likely to be due to modulation of a number of cardiovascular risk factors.

In addition to glycemia, there has been an interest in the antithrombotic properties of omega-3 polyunsaturated fatty acids (n-3 PUFAs). However, a recent study demonstrated that treatment with n-3 PUFAs failed to modulate fibrin clot properties, platelet activation, or inflammation in patients with atherosclerosis and type 2 diabetes (95).

Direct Measures to Improve the Thrombotic Environment

Antiplatelet agents remain a cornerstone in the management of the thrombotic environment in diabetes. However, the best treatment regimen at various stages of the vascular pathology is yet to be determined; this can be divided into primary prevention, secondary prevention immediately following an event and long-term secondary prevention. This topic is reviewed extensively elsewhere (26, 60, 96, 97) and we will only provide here a clinically orientated practical summary.

ANTIPLATELET AGENTS

Primary Prevention

Aspirin irreversibly inhibits cyclo-oxygenase (COX) enzymes, at lower doses exhibiting relative selectivity for COX1, which

is responsible for the synthesis of thromboxane A_2 (TXA_2), a prothrombotic and vasoconstrictive eicosanoid. At higher doses, aspirin can also inhibit COX2, leading to the unwanted reduction in production of prostacyclin (PGI_2) an antithrombotic and vasodilatory agent (98). In addition to platelet inhibition, aspirin appears to affect the fibrin network structure and fibrinolysis, which may also contribute to its clinical antithrombotic effects (99).

Until relatively recently, aspirin was used for primary prevention in patients with diabetes, although there was no clear evidence supporting such an approach. A number of studies investigating aspirin in primary vascular protection in diabetes failed to show a benefit, including PPP trial, JPAD, and POPADAD studies, and therefore, current guidelines do not advocate the use of this agent for primary prevention (100–103). It should be acknowledged that none of these studies was adequately powered to give a definitive answer and results from an ongoing large study are currently awaited that will clarify the use of aspirin for primary prevention in diabetes (ASCEND[2]). The guidelines adopted a pragmatic approach, however, by recommending aspirin use for primary prevention in those at higher risk without clearly defining this group.

One of the issues that remain unresolved is the optimal dosing regimen of aspirin in diabetes. Given the increased platelet turnover in diabetes and the relatively short half-life of aspirin, this agent may fail to provide 24 h coverage (97, 104). Therefore, studies have investigated twice daily administration of aspirin, which appears to improve the platelet inhibitory effect, although the clinical value of this approach remains unclear (34–36). This has been alluded to in joint ESC/EASD guidelines but no management recommendations were made due to lack of evidence (103).

Secondary Cardiovascular Prevention

Aspirin is used in combination with other antiplatelet agents that target the P2Y12 pathway, following acute coronary syndrome (ACS) in diabetes patients and individuals with no diabetes. Of the three agents that target the P2Y12 pathway, both prasugrel and ticagrelor showed superiority to clopidogrel in improving vascular outcome in patients with diabetes (31, 105, 106). A plausible explanation is related to the need to metabolize clopidogrel from the inactive to the active form, which is simpler process with prasugrel and not required at all with ticagrelor.

Dual antiplatelet therapy is usually continued for a period of 1 year after an ACS to be followed by aspirin only therapy that is continued indefinitely. However, recent evidence indicates that longer-term DAPT therapy with aspirin and ticagrelor offers a survival advantage in diabetes patients, unlike those without diabetes (107). The safety and cost effectiveness of this approach requires further analysis and/or studies before routinely recommending long-term DAPT in patients with diabetes (26, 31, 105, 106).

An inherent difficulty in optimizing antiplatelet therapy in diabetes is related to the heterogeneity of this condition and the variable vascular risk according to duration of diabetes, treatment modalities, and presence of microvascular complications. In order to optimize antiplatelet therapy in diabetes, there are a number of questions that remain unanswered, including: (i) Is an alternative dosing of aspirin required in diabetes? (ii) How long to continue treatment with DAT following ACS? (iii) Are different antiplatelet regimen required in different groups of diabetes patients, particularly for primary prevention? (iv) Are additional antiplatelet agents that target other pathways required? Addressing these questions will require large clinical outcome studies which are both time consuming and expensive. Therefore, using surrogate markers, such as platelet activation tests, may provide provisional data justifying clinical outcome studies. In other words, an integrated bench to bedside approach is perhaps the most cost effective way to investigate newer antithrombotic agents or analyze alternative application of existing agents.

AGENTS TARGETING THE COAGULATION PATHWAY

Established Therapies

Proteins of the contact system have been investigated and considered as therapeutic targets due to their important role in the initiation of thrombosis. TF is expressed by exposed vascular smooth muscle cells at the atherosclerotic plaque rupture sites where it triggers the activation of FVII and the initiation of the coagulation cascade. Activation of FIX and FX by the TF/FVIIa complex then leads to the formation of FXa/FVa complex which converts prothrombin into thrombin (108).

Heparin is an indirect inhibitor of FX and prothrombin and is used in diabetes and non-diabetes subjects with ACS. It is mainly used in unfractionated form or as the low-molecular weight form of heparin, named enoxaparin, which has been suggested to be more effective than the unfractionated form of heparin (109). Another indirect inhibitor of FX which was reported to be as effective as enoxaparin or unfractionated heparin is fondaparinux (110). However, unlike heparin, fondaparinux fails to inhibit thrombin.

The recent development of direct oral anticoagulants has further helped by offering a more targeted inhibition of the protein arm of coagulation. Bivalirudin is a direct inhibitor of thrombin which has been reported to be superior to heparin as a therapy for diabetes patients with ACS due to the significant reduction in bleeding complications in some studies (111, 112). The COMPASS study, involving over 27,000 patients with stable coronary artery disease, has recently shown that combination of rivaroxaban (FX inhibitor) with aspirin is superior to aspirin alone in preventing vascular events, an observation that applied to both patients with and without diabetes (113). However, there was a 70% increase in bleeding risk in patients receiving combination therapy, which is a concern and will have implications for the widespread use of such a therapy.

Experimental Therapies

FXII and FXI are involved upstream in the coagulation system and have been considered as potential therapeutic targets for

[2]https://clinicaltrials.gov/ct2/show/NCT00135226.

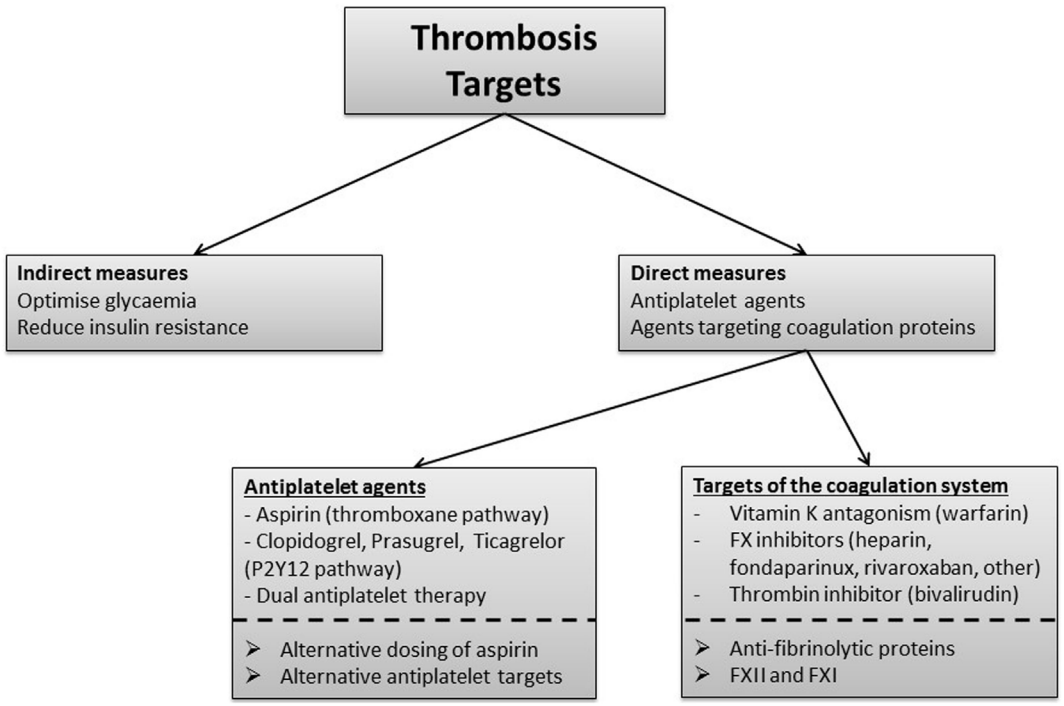

FIGURE 5 | Thrombotic pathways that represent current and potential therapeutic targets in diabetes. Indirect measures to improve the thrombotic environment in diabetes focus on optimizing glycemic control by lowering glucose levels while avoiding hypoglycemia and also by reducing insulin resistance. Antiplatelets and agents that target the coagulation system comprise the main direct measures to manage the thrombotic environment in diabetes. Aspirin targets the thromboxane pathway and is also used in combination with clopidogrel, prasugrel, or ticagrelor which target the P2Y12 pathway. Alternative antiplatelet targets are under consideration. Vitamin K antagonism reduces thrombosis risk but is associated with high risk of bleeding when combined with antiplatelet therapy, and therefore, not routinely used for atherothrombotic disease. Inhibitors of coagulation proteins FX and thrombin, such as heparin and direct oral anticoagulants, represent agents with promise in diabetes while targeting factor (F)XII and FXI requires careful future evaluation. Alternative dosing of aspirin and reduction of incorporation of antifibrinolytic proteins into the clot may represent new approaches to improve clinical outcome in patients with diabetes.

thrombosis prevention. Activation of FXII triggers the initiation of the contact system and FXIIa converts prekallikrein to kallikrein. Kallikrein promotes the generation of additional FXIIa and the activation of FXI. Finally, FXIa activates FIX and promotes FX activation and generation of thrombin. The antithrombotic effects of targeting FXII and prekallikrein have been demonstrated *in vivo* using FeCl3- induced and stenosis-induced models of venous thrombosis (114). Different approaches, such as the use of antibodies, small molecules and aptamers have been developed to target factor XII and FXI but the clinical efficacy and safety of such drugs in man are yet to be determined (115).

A crucial element of a successful antithrombotic therapy is maintaining a clinically acceptable risk/benefit ratio. This requires close understanding of the mechanistic pathways operating to increase thrombosis risk in diabetes. For example, it has been shown that both PI and complement C3 incorporation into diabetes clots is enhanced, which further compromises fibrinolysis (detailed above). Developing therapies that reduce incorporation of these proteins into diabetes clots will facilitate fibrinolysis, thus limiting thrombus formation while keeping bleeding risk to a minimum. Therefore, rather than full inhibition of one pathway, which compromises normal physiology and increases bleeding risk, what is perhaps required is partial inhibition of multiple pathways with a focus on those that show a diabetes-specific abnormality.

Potential treatments and targets to further reduce atherothrombosis risk in diabetes are summarized in **Figure 5**.

CONCLUSION AND THE WAY FORWARD

Research conducted for the prevention and treatment of atherothrombotic disease has seen major advances over the past few decades, which translated clinically into significant improvement in vascular mortality and morbidity. We now have a number of agents that limit the atherosclerotic process and also control the risk of thrombosis. Despite these advances, however, the risk of vascular disease in patients with diabetes remains unacceptably high with relatively poor prognosis following an ischemic event. Therefore, future work is required to develop more effective therapeutic agents to halt the accelerated atherosclerotic process, typically associated with diabetes. Moreover, work is needed to develop antithrombotic strategies that target diabetes-specific pathways, thereby maximizing benefit and limiting the risk of bleeding. These include alternative use of existing agents and development of novel agents. An example of the former is investigating the efficacy of twice daily aspirin regimen in diabetes,

whereas the latter includes development of agents that target the hypofibrinolytic environment, a key abnormality in individuals with deranged glucose metabolism (44).

Finally, we should accept that diabetes is not a single clinical entity but a continuum of different stages of the condition with each having a different vascular risk. Therefore, in order to maximize benefit, future therapies aiming to reduce atherothrombotic risk in diabetes may require stratifying patients into different categories with each group having a "stage-specific" vascular management strategy. Admittedly, this will add to the complexity of clinical therapy, particularly as vascular risk will vary over time in each patient, but this "individualized care" approach is perhaps key to improve long-term outcome in this high-risk population.

AUTHOR CONTRIBUTIONS

NP designed the review, undertook the literature search, and wrote the manuscript and RA designed the review and critically reviewed the manuscript. Both authors agreed to the final manuscript.

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

frontiers in Cardiovascular Medicine

REVIEW
published: 08 January 2018
doi: 10.3389/fcvm.2017.00086

Mechanisms and Consequences of Defective Efferocytosis in Atherosclerosis

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Efficient clearance of apoptotic cells, termed efferocytosis, critically regulates normal homeostasis whereas defective uptake of apoptotic cells results in chronic and non-resolving inflammatory diseases, such as advanced atherosclerosis. Monocyte-derived macrophages recruited into developing atherosclerotic lesions initially display efficient efferocytosis and temper inflammatory responses, processes that restrict plaque progression. However, during the course of plaque development, macrophages undergo cellular reprogramming that reduces efferocytic capacity, which results in post-apoptotic necrosis of apoptotic cells and inflammation. Furthermore, defective efferocytosis in advanced atherosclerosis is a major driver of necrotic core formation, which can trigger plaque rupture and acute thrombotic cardiovascular events. In this review, we discuss the molecular and cellular mechanisms that regulate efferocytosis, how efferocytosis promotes the resolution of inflammation, and how defective efferocytosis leads to the formation of clinically dangerous atherosclerotic plaques.

Keywords: efferocytosis, atherosclerosis, inflammation resolution, macrophages, post-apoptotic necrosis

OPEN ACCESS

Edited by:
Masanori Aikawa, Harvard Medical School, United States

Reviewed by:
Hong Chen, Harvard University, United States
Jianguo Wang, Brigham and Women's Hospital, United States

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

Received: *01 November 2017*
Accepted: *11 December 2017*
Published: *08 January 2018*

Citation:
Yurdagul A Jr., Doran AC, Cai B, Fredman G and Tabas IA (2018) Mechanisms and Consequences of Defective Efferocytosis in Atherosclerosis. Front. Cardiovasc. Med. 4:86. doi: 10.3389/fcvm.2017.00086

Efficient clearance of apoptotic cells, termed "efferocytosis," is an ancient process that evolved to allow organ development, maintain homeostasis, prevent autoimmune disease, and resolve inflammatory insults (1). When efferocytosis functions efficiently, apoptotic cells are cleared before they become necrotic, anti-inflammatory cytokines and pro-resolving lipid mediators are secreted, and the release of immunogenic antigens is prevented. However, when efferocytosis becomes defective, uncleared apoptotic cells undergo post-apoptotic necrosis and release tissue-degrading enzymes, immunogenic epitopes, and pro-inflammatory mediators. Genetically modified mice show that impaired efferocytosis often develop autoimmune or chronic inflammatory diseases (2). Accordingly, there is substantial interest in understanding how efferocytosis becomes defective in chronic inflammatory diseases, such as atherosclerosis. This review will highlight the processes associated with efferocytosis and how these become dysregulated during atherosclerosis.

FINDING AND BINDING APOPTOTIC CELLS

Despite the fact that the macrophage population in most organs and tissues are relatively low compared with other non-immune cells, apoptotic cells are rarely detected in tissues where high levels of cellular turnover are known to occur, indicating that macrophages rapidly mobilize to areas of cell death to expeditiously remove apoptotic corpses (3). Macrophage migration toward apoptotic cells

is guided by chemotactic factors secreted by dying cells either actively in an executioner caspase-dependent mechanism or passively released during self-demise. This class of mediators, known as "find-me" signals, includes the classic chemokine CX3CL1, the lipids sphingosine 1-phosphate and lysophosphatidylcholine, and the nucleotides ATP and UTP (4–7).

After having navigated tissues to arrive at apoptotic-rich areas, macrophages employ a panoply of receptors that bind either directly or indirectly, *via* bridging molecules, to "eat-me" signals displayed on the surface of apoptotic cells (**Figure 1**) (8). While several "eat-me" signals have been identified, including changes in glycosylation at the cell surface or exposure of calreticulin or ICAM-1 epitopes, externalized phosphatidylserine (PtdSer) on apoptotic cells remains the most characterized (9, 10). Macrophages bind PtdSer directly through stabilin-1, stabilin-2, the GPCR brain angiogenesis inhibitor 1 (BAI1), or through the T-cell immunoglobulin and mucin domain family of receptors Tim-1, Tim-3, and Tim-4 (11–14). Alternatively, macrophages may utilize the Tyro3–Axl–Mer (TAM) family of tyrosine kinase receptors, integrins αVβ3 and αVβ5, or CD36 to bind PtdSer indirectly through bridging molecules that interact with PtdSer (3). Gas6 and Protein S bind to TAM receptors, whereas thrombospondin-1 or MFG–E8 link PtdSer to CD36 or integrins αVβ3 and αVβ5, respectively. Some of the PtdSer-relevant receptors have well-characterized signaling capabilities, e.g., MerTK, BAI1, and integrins, while others may function primarily as tethering and adhesion molecules, e.g., the Tim family of receptors and CD36.

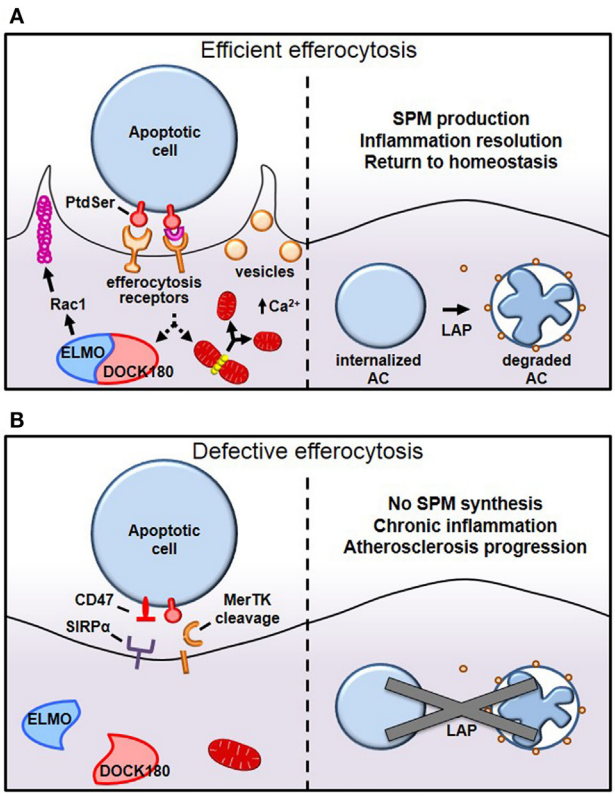

FIGURE 1 | Mechanisms of efferocytosis. **(A)** Macrophages interact with phosphatidylserine (PtdSer) externalized on apoptotic cells either directly or indirectly, through bridging molecules. Many PtdSer receptors stimulate ELMO–DOCK180 interactions to activate Rac1 and polymerize actin around the phagosome. Simultaneously, macrophages trigger Drp1-mediated mitochondrial fission to drive calcium-dependent vesicular trafficking. Once internalized, autophagic machinery is used to conjugate lipids to LC3 bound to phagosomes, which drives phagolysosomal fusion and subsequent apoptotic cell degradation. **(B)** In pathological settings such as advanced atherosclerosis, one or more of these processes can become defective, leading to inefficient clearance of apoptotic cells and subsequent necrosis and inflammation. For example, in advanced atherosclerosis, apoptotic cells can inappropriately express the "don't eat-me" signal CD47, or cell-surface receptors can get proteolytically cleaved, such as with MerTK.

Tethering and internalization are two separate but intimately linked events and operate first through interaction with weak and low avidity "eat-me" signals to ensure adhesion, followed by stereospecific interaction of externalized PtdSer to PtdSer receptors to drive cytoskeletal reorganization around the apoptotic cell. While externalized PtdSer on apoptotic cells binds to receptors on macrophages to mediate tethering, this process alone is insufficient to trigger internalization (15). However, coupling of PtdSer/receptor interaction with other receptors trigger the switch from adhesion to internalization, a process referred to as "tether and tickle" (16). Live cells may also express PtdSer and yet are spared from efferocytosis, primarily because live cells present the "don't eat-me" signals CD31 and CD47 on their cell surface, which actively suppresses efferocytosis. CD31 is expressed on viable cells and cues repulsion or detachment when making homophilic interactions in trans with efferocytes (17). Interestingly, CD31 on macrophages may interact with apoptotic cells using the extracellular matrix protein fibronectin as a bridging molecule (18). When this occurs, integrin α5β1 becomes activated and subsequently promotes phagocytosis of apoptotic cells (18). Therefore, selective CD31 interactions not only prevents accidental internalization of viable cells but may also actively promote efferocytosis (**Figure 1B**). The other major "don't eat-me" signal, CD47, is expressed on live cells and interacts with cell-surface signal-regulatory protein α on macrophages to inactivate myosin assembly and thereby prevent cytoskeletal rearrangement around the phagosome (19).

Internalization of apoptotic cells requires macrophages to dynamically reorganize their actin cytoskeleton to drive F-actin formation around apoptotic cells, forming a so-called phagocytic cup, which then promotes mechanical retraction of the phagosome into the cell (**Figure 1A**) (20). Since the Rho-family of small GTPases, Rac1, Cdc42, and RhoA rearrange the cytoskeleton to mechanically drive migration of cells, it is not surprising that they are also involved in mechanically internalizing phagosomes containing apoptotic cells. Using Forster resonance energy transfer biosensors, it was discovered that these small GTPase family members work in a temporally regulated fashion in which Rac1 and Cdc42 are activated early to facilitate phagocytic cup formation through actin polymerization followed by Rho activation, which drives mechanical retraction and phagosome internalization (21). Constitutive activation of Rac1 may decrease phagocytosis, because Rac1 must be rapidly inactivated to permit engulfment (21). However, when RhoA effectors are inhibited, to tilt the Rac1/RhoA axis toward Rac1 activation, uncontrolled

phagocytosis occurs (22). A shared Rac1 activation pathway that is conserved among several of the apoptotic cell receptors involves association with the adaptor protein ELMO to the RacGEF DOCK180 (23). This ELMO/DOCK180 complex then activates Rac1 to initiate phagocytic cup formation, which then leads to apoptotic cell internalization (24). Accordingly, inhibition of ELMO/DOCK180 signaling prevents efferocytosis (13, 24).

While cytoskeletal remodeling is required for apoptotic cell internalization, the role of membrane trafficking in efferocytosis becomes astoundingly evident given that cells internalize ~50% of their entire surface, yet, plasma membrane surface area does not change as a result of phagocytosis (25). This finding suggests that internal membranes are rapidly recruited to the cell surface to complete closure of the phagosome and to replenish cell membranes utilized during efferocytosis. This concept can be demonstrated in tetanus or botulinum B toxin-microinjected cells, which show defective exocytosis and reduced membrane delivery to incoming phagosomes (26). New work has shown that these critical vesicular trafficking events require efferocytes to undergo Drp1-dependent mitochondrial fission (27). Mitochondrial fission causes endoplasmic reticulum calcium to be released into the cytosol rather than into the mitochondria, and this increase in cytosolic calcium then drives vesicular trafficking (27). Importantly, Drp1-deficient macrophages, which cannot undergo mitochondrial fission upon encountering apoptotic cells, are unable to move vesicles to the site of apoptotic cell attachment, which significantly delays both initial apoptotic cell phagosome sealing and, more notably, compromises the ability of the efferocytes to take up a second apoptotic cell (27).

AC CORPSE DEGRADATION

Once apoptotic cells have been internalized, certain autophagy-related proteins are recruited to conjugate LC3-family proteins to lipids at the phagosome membrane, a process called LC3-associated phagocytosis (LAP) (**Figure 1A**) (28). LAP promotes phagosome fusion to lysosomes to drive hydrolytic degradation of apoptotic cell constituents (28). Importantly, defects in LC3 conjugation to phagosomal membranes delay or even prevent phagosome fusion with lysosomes, resulting in failure to acidify the phagosome and an inability to degrade apoptotic cells (29). After apoptotic cells are degraded in phagolysosomes, macrophages become overloaded with macromolecular constituents and therefore have evolved elegant mechanisms to either use or efflux this cargo. For instance, the burden of cholesterol from degraded apoptotic cells activates members of the peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) families of nuclear receptors and drive ABCA1 and ABCG1 expression, which mediate cholesterol efflux from the cells (30). Furthermore, PPARγ and LXR agonists further enhance efferocytosis (31, 32). To handle the large amount of chromosomal DNA derived from degraded apoptotic cells, macrophage lysosomes contain DNase II that cleaves this phagocytosed DNA. Macrophages lacking DNase II accumulate undigested DNA fragments, and mice lacking DNase II exhibit polyarthritis, an autoimmune disease similar to rheumatoid arthritis in humans (33).

ATHEROSCLEROSIS

Although the last several decades have seen significant medical advances in the diagnosis and treatment of cardiovascular disease, atherosclerosis remains the major cause of morbidity and mortality worldwide (34). Atherosclerosis begins when circulating apolipoprotein B-containing lipoproteins accumulate in focal areas in the subendothelium matrix of medium-sized and large arteries (35). These subendothelial lipoproteins, particularly after oxidation, generate an inflammatory stimulus that drives leukocyte influx into the vessel wall (36–39). Primary among these infiltrating cells are monocyte-derived macrophages, which internalize cholesterol-rich lipoproteins and give rise to foam cells. Foam cells secrete extracellular matrix that further promotes lipoprotein retention as well as pro-inflammatory cytokines that augment the recruitment of additional monocytes, T cells, and neutrophils. In the face of persistent inflammatory stimuli and other cytotoxic factors, many lesional cells become apoptotic. Early on, apoptotic cells are efficiently cleared by neighboring macrophages in an attempt to limit overall lesion cellularity (40). However, efferocytosis can fail as plaques progress, leading to the accumulation of secondarily necrotic cells and the formation of a highly inflammatory "necrotic core" (41–43). Large necrotic cores are a hallmark of advanced atherosclerotic disease and have been associated with the types of atherosclerotic plaque that give rise to heart attack and stroke (44, 45). Therefore, the efficient clearance of dead and dying cells plays a key role in preventing the development of clinically significant atherosclerotic plaques.

MECHANISMS OF IMPAIRED EFFEROCYTOSIS IN ATHEROSCLEROSIS

Why does efferocytosis fail in advanced atherosclerosis? Because efferocytosis is a high-capacity process, it is unlikely that overwhelming lesional apoptosis is the primary cause. Rather, efferocytosis itself becomes defective and/or lesional apoptotic cells become poor substrates for efferocytosis. As an example of the latter, CD47 expression is significantly increased in human atherosclerotic plaque cells, presumably *via* a TNFα-dependent mechanism, and for the reasons explained earlier (19), these cells are poorly internalized by lesional efferocytes (46) (**Figure 1B**). In keeping with this concept, administration of CD47-blocking antibodies to atheroprone mice led to improved lesional efferocytosis and smaller necrotic cores. Other findings suggest that dead cells in lesions express lower amounts of the "eat-me" signal calreticulin (47). For example, *Apoe*$^{-/-}$ mice lacking *Cdkn2b* show decreased levels of calreticulin, and apoptotic bodies from these animals show resistance to being engulfed (47). When fed a Western diet, these mice have an increased overall lesion size as well as increased necrotic core size (47) (**Table 1**). Interestingly, human carriers of the cardiovascular risk allele at the chromosome 9p21 GWAS locus were found to have lower intraplaque expression of calreticulin, suggesting that defective efferocytosis may contribute to cardiovascular disease in these patients (48).

Efferocytosis may also be compromised by competition for apoptotic cell binding. As atherosclerosis progresses, lesions

TABLE 1 | Efferocytosis pathway molecules shown to have a causative role in atherosclerosis.

Molecule	Function	Animal model	Effect on aortic lesion area	Effect on necrotic core size	Other findings	Reference
MerTK	Receptor				↑ ACs ↓ *In situ* efferocytosis	
		$MerTK^{KD}$ $Apoe^{-/-}$ mice	No change	↑	↑ Lesional macrophages	(49)
		$MerTK^{-/-}$ marrow → $Ldlr^{-/-}$ mice	↑	↑	↓ *In situ* efferocytosis	(50)
		$MerTK^{CR}$ $Ldlr^{-/-}$ mice	No change	↓	↑ Collagen cap thickness	(51)
					↑ T regulatory cells ↑ Specialized pro-resolving mediators	
Lipoprotein receptor-related protein 1 (LRP1)	Receptor	Macrophage $LRP1^{-/-}$ marrow → $Ldlr^{-/-}$ mice Macrophage $LRP1^{-/-}$ $Apoe^{-/-}$ mice	↑	↑	↑ ACs ↓ *In situ* efferocytosis ↑ Lesional macrophages ↑ MMP9 levels	(52, 53)
			↑	↑	↑ ACs ↓ *In situ* efferocytosis ↑ Lesional macrophages	(54)
SR-B1	Receptor	$SR\text{-}B1^{-/-}$ $ApoE^{-/-}$ marrow → $Apoe^{-/-}$ mice $SR\text{-}B1^{-/-}$ marrow → $Ldlr^{-/-}$ mice	↑	↑	↑ ACs ↓ *In situ* efferocytosis ↓ Lesional macrophages ↓ Collagen lesion area and cap thickness ↑ ACs ↓ *In situ* efferocytosis ↓ Lesional macrophages ↓ Collagen lesion area and cap thickness	(55)
Tim-1/Tim-4	Receptor	$Ldlr^{-/-}$ treated with Tim-1 or Tim-4 blocking antibodies	↑	Not tested	↑ ACs ↓ *In situ* efferocytosis ↑ Lesional T cells	(56)
Mineralo-corticoid receptor	Non-efferocytosis nuclear receptor	Myeloid $MRKO^{-/-}$ marrow → $Ldlr^{-/-}$ mice	↓	↓	↓ ACs ↑ *In situ* efferocytosis ↓ Lesional macrophages ↓ Foam cell formation ↑ Collagen lesion area	(57)
MFG-E8	Bridging molecule	$MFG\text{-}E8^{-/-}$ marrow → $Ldlr^{-/-}$ mice	↑	↑	↑ ACs ↑ Collagen cap thickness	(58)
C1q	Bridging molecule	$C1q^{-/-}$ $Ldlr^{-/-}$	↑	Not tested	↑ ACs ↑ Lesional macrophages *In vitro*: incubating macrophage cell line with C1q enhances C19-mediated efferocytosis	(59) (60)
Transglutaminase 2 (TG2)	Bridging molecule	$TG2^{-/-}$ marrow → $Ldlr^{-/-}$ mice	↑	↑	↑ Lesional macrophages *In vitro*: $TG2^{-/-}$ macrophages have decreased efferocytosis	(61)
Gas6	Bridging molecule	$Gas6^{-/-}$ $Apoe^{-/-}$ mice	No change	↓	↑ Collagen content	(62)
CX3CL1	Find-me signal	$CX3CL1^{-/-}$ $Apoe^{-/-}$ mice $CX3CL1^{-/-}$ $Ldlr^{-/-}$ mice	Inconsistent change at aortic root, ↓ at brachiocephalic artery	Not tested	↓ Lesional macrophages	(63)
			↓ At aortic root and brachiocephalic artery	Not tested	↓ Lesional macrophages	

(*Continued*)

TABLE 1 | Continued

Molecule	Function	Animal model	Effect on aortic lesion area	Effect on necrotic core size	Other findings	Reference
Fas/Fas ligand	Mediates find-me signaling	Fas$^{-/-}$ Apoe$^{-/-}$ mice Gld Apoe$^{-/-}$ mice	↑ ↑	Not tested Not tested	↑ ACs ↑ ACs ↑ Lesional macrophages ↑ T cells ↓ *In situ* efferocytosis in lymph tissue	(64) (65)
Calreticulin	Eat-me signal	*Cdkn2b*$^{-/-}$ *Apoe*$^{-/-}$ mice have low levels of calreticulin	↑	↑	↓ Collagen content and cap thickness ↓ *In situ* efferocytosis by semi-quantitative measure *In vitro*: loss of CKDN2B leads to ↓ efferocytosis	(47)
CD47	Don't eat-me signal	*Apoe*$^{-/-}$ mice treated with CD47-blocking antibody	↓	↓	↓ ACs ↑ *In situ* efferocytosis	(46)
miR-21	MicroRNA	miR21$^{-/-}$ marrow → Ldlr$^{-/-}$ mice	↑	↑	↑ ACs ↓ Lesional macrophages ↓ Collagen cap area with no change in lesional collagen content *In vitro*: miR21$^{-/-}$ macrophages have ↓MerTK and ↓ efferocytosis	(66) (66, 67)

continue to accumulate lipids and ROS, leading to increased levels of oxidized phospholipids. These lipids can bind to efferocytosis receptors and may compete for apoptotic cell recognition (68). Similarly, autoantibodies against oxLDL and other oxidized phospholipids are able to bind to and potentially mask "eat-me" ligands on the surface of dying cells in the lesions (69, 70). Further, oxLDL increases the expression of and signaling through toll-like receptor 4 (TLR4), leading to increased secretion of the pro-atherogenic cytokines TNFα and IL-1β while reducing the anti-inflammatory cytokines TGFβ and IL-10 (71). This pro-inflammatory environment impairs efferocytosis by reducing the expression of various key efferocytosis molecules, as discussed below, and promotes increased lipid uptake at the expense of phagocytosis (72).

Finally, accumulating experimental evidence has demonstrated that the expression and function of efferocytosis receptors and their bridging molecules are deficient in advanced atherosclerosis. One such example is MerTK and its associated bridging molecule, Gas6. MerTK is expressed by macrophages in both murine and human plaques (50, 51). As lesions progress, MerTK levels on the macrophage surface decline, and this decrease is associated with cleavage of the receptor by the metalloproteinase ADAM17 (**Figure 1B**). Multiple athero-relevant inflammatory stimuli have been shown to promote ADAM17 activity and MerTK cleavage (73–75). Indeed, levels of the soluble fragment of the receptor (solMer) accumulate within the aortas of mice and in human carotid plaques (51). *In vitro,* solMer has been shown to inhibit efferocytosis by competing with Gas6, suggesting that this may amplify the deleterious effects on efferocytosis (73). Using a genetically engineered mouse in which the cleavage domain of MerTK has been rendered resistant, Cai and colleagues demonstrated that cleavage of the MerTK receptor is a causal factor in the development of necrotic cores in atherosclerotic lesions (51). Consistent with this protective role for MerTK activity in atherosclerosis, loss of MerTK, either by genetic deletion or through models in which MerTK has been replaced by a version with an inactive kinase domain, results in increased lesion size and larger necrotic cores (49, 50) (**Table 1**). Interestingly, deletion of the related TAM family member, Axl, in bone marrow cells of *Ldlr*$^{-/-}$ mice did not affect lesional efferocytosis or plaque necrosis in advanced atherosclerosis (76).

Low-density lipoprotein receptor-related protein 1 (LRP1) is a receptor that is activated by calreticulin on the surface of apoptotic cells (77). The macrophage receptor LRP1 can also be downregulated in response to TLR4 signaling and inactivated by ADAM17-mediated proteolytic cleavage (78, 79). Several studies have demonstrated that the loss of *Lrp1* in macrophages or in bone marrow cells leads to increased lesion area and necrotic core size in an *Apoe*$^{-/-}$ mice (52–54) (**Table 1**). A particular bridging molecule that is reduced in atherosclerotic lesions is milk fat globule-epidermal growth factor 8 (MFG-E8), which functions to tether apoptotic cells to efferocytes by interacting with αVβ3 integrins and the transglutaminase 2 (TG2) co-receptor on phagocytes (80, 81). MFG-E8 is expressed in atherosclerotic plaques, but its expression declines in advanced plaque, potentially owing to downregulation by inflammatory stimuli (82). In an *Ldlr*$^{-/-}$ mouse model lacking *Mfge8* in bone marrow cells,

larger plaque area and necrotic cores were observed (58). In addition, *Ldlr*$^{-/-}$ mice lacking TG2 in bone marrow also show increased plaque area and necrotic core size (81). Another bridging molecule, complement component 1q (C1q), has also been shown to be important in atherosclerosis. *In vitro*, macrophages can produce large amounts of C1q, which promotes both cell survival and efferocytosis (60). Loss of C1q from *Ldlr*$^{-/-}$ mice led to larger lesion area and an increase in apoptotic cells, consistent with defective apoptotic cell clearance (59). As another possible mechanism for defective efferocytosis, the pro-inflammatory molecule high-mobility group box 1 (HMGB1) is increased in human and murine atherosclerosis (83, 84), and the secreted form has been shown to interact with integrin αVβ3 and PtdSer to block efferocytosis (85, 86). *Apoe*$^{-/-}$ mice administered an anti-HMGB1 antibody developed smaller atherosclerotic plaques, although necrotic core size was not reported (84). Silencing of HMGB1 in peritoneal macrophages *in vitro* leads to increased efferocytosis, and partially rescues the efferocytosis defect observed in SR-B1$^{-/-}$ macrophages (55).

Recently, microRNAs have been found to have a novel role in the regulation of efferocytosis. Das and colleagues found that macrophages undergoing efferocytosis increase their expression of miR-21 in a TLR4-dependent manner *in vitro* (67). Further, when treated with an miR-21 mimetic *in vitro*, the rate of efferocytosis by bone marrow-derived macrophages increased (67). Transplantation of *miR21*$^{-/-}$ marrow into *Ldlr*$^{-/-}$ mice increased plaque area and necrotic core size. One study reported that loss of miR-21 in macrophages decreases MerTK expression, providing a mechanism for the increased necrotic core size in these mice (66). Additional work is necessary to determine the mechanism by which miR-21 regulates MerTK expression. Another miR, miR-33, is also known to regulate the outcome of atherosclerosis. Murine primary macrophages treated with anti-miR-33 enhanced efferocytosis *in vitro* and treatment of *Ldlr*$^{-/-}$ mice with anti-miR-33 decreased necrotic cores compared with the anti-miR control (87). Together, these results suggest that specific miRs play important roles in regulating efferocytosis in atherosclerosis.

EFFEROCYTOSIS AND INFLAMMATION RESOLUTION

To successfully terminate an inflammatory process, the active process of inflammation resolution is required (88). This process is mediated by various endogenous molecules, including bioactive lipids such as lipoxins, resolvins, protectins, and maresins, which are often referred to as specialized pro-resolving mediators (SPMs); proteins such as annexin A1 and interleukin-10; and gasses such as hydrogen sulfide (88). When the resolution program fails inflammation persists, and this mechanism is now understood to be an underlying factor in the pathogenesis of many chronic inflammatory diseases, including atherosclerosis (89). Emerging evidence has defined an important role for resolution and SPMs in both murine and human atherosclerotic disease (89–91). Gene variants encoding proteins and enzymes necessary for SPM biosynthesis, including 5-lipoxygenase (5-LOX), have been associated with atherosclerosis, stroke, and myocardial infarction in selected populations (92–96). Patients with coronary artery disease have lower plasma SPMs than do healthy patients (97), and one SPM, aspirin-triggered lipoxin A_4, was found to be significantly associated with peripheral and coronary atherosclerosis in humans even after correction for age, sex, and C-reactive protein levels (98). A recent paper showed that stable regions of human atherosclerotic plaque have a higher SPM:leukotriene ratio when compared with more advanced, vulnerable regions having larger necrotic cores and thinner collagen caps (99). Similarly, early murine lesions from Western diet-fed *Ldlr*$^{-/-}$ mice had a higher SPM:leukotriene ratio when compared with advanced lesions (90, 99). In several mouse models of atherosclerosis, treatment of mice with various pro-resolving ligands including annexin 1, Ac2-26, IL-10, resolvin D1 (RvD1), resolvin D2, or maresin 1 decreased lesional necrosis, suggesting improvements in efferocytosis by lesional phagocytes (90, 99, 100–102).

Efferocytosis plays a major mechanistic role in the resolution of inflammation. First, expeditious clearance of dead cells prevents their secondary necrosis. Second, the act of efferocytosis itself triggers several different anti-inflammatory and pro-resolving signaling pathways. Engagement and activation of the TAM family of efferocytosis receptors, including MerTK and Axl, induces the expression of suppressor of cytokine signaling-1 and 3 (SOCS-1 and 3), leading to the inhibition of signaling pathways triggered by cytokines and toll-like receptor ligands (103, 104). Efferocytosis has also been shown to actively increase the secretion of anti-inflammatory cytokines, including TGF-β and IL-10, and decreased secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-8 (105, 106). Further, uptake of apoptotic cells enhances the synthesis of SPMs, while concomitantly reducing the production of pro-inflammatory leukotrienes (107, 108). Recently, a specific mechanistic link between the efferocytosis receptor MerTK and SPM production was revealed (108). In response to engagement of the MerTK receptor, the key biosynthetic enzyme 5-LOX translocates from the nucleus to the cytoplasm, where it drives the production of the pro-resolving mediator lipoxin A_4. When MerTK is inactivated either genetically or proteolytically, 5-LOX is restricted to the nuclear membrane, where it instead favors the production of the pro-inflammatory leukotriene B_4 (108). Mice whose myeloid cells express a cleavage-resistant variant of MerTK (MerTK^{CR} mice) have higher rates of efferocytosis than their wild type counterparts, and macrophages from these mice demonstrate enhanced production of LXA_4 and RvD1. In addition, when *Ldlr*$^{-/-}$ mice are transplanted with bone marrow from MerTK^{CR} mice and fed an atherogenic diet for 16 weeks, the aortas contained an increased SPM:leukotriene ratio (51). The process of resolution in atherosclerosis can also enhance efferocytosis. A recent study showed that administration of RvD1 to Western diet-fed *Ldlr*$^{-/-}$ mice significantly increased the SPM:leukotriene ratio, while also decreasing plaque necrosis and enhancing lesional efferocytosis (99). These studies suggest a positive feedback cycle between resolution and efferocytosis, which, if interrupted, can lead to an amplification loop of inflammation and necrosis that promotes advanced atherosclerotic plaque progression.

SUMMARY AND CONCLUSION

Defective clearance of apoptotic cells in atherosclerotic lesions drives post-apoptotic necrosis of lesional cells and inflammation triggered by the release of cellular debris from these necrotic cells (2). Moreover, active cell signaling programs of inflammation suppression and inflammation resolution in efferocytes are often lost when apoptotic cells are not properly cleared (89). As a result, defective efferocytosis can transform stable, asymptomatic atherosclerotic lesions into necrotic, inflammatory, and non-resolving plaques that are prone to rupture (41). Although we do not know for certain why efferocytosis fails in advanced atherosclerosis, studies thus far suggest complementary mechanisms that involve both poor recognition of lesional apoptotic cells, e.g., due to inappropriate expression of CD47, coupled with defects in the efferocytes themselves; e.g., due to proteolytically cleavage of MerTK (46, 51, 108).

How might this knowledge suggest new types of therapies to prevent atherothrombotic vascular disease? Therapies that lower LDL in the blood are the mainstay of therapy to prevent atherosclerotic disease, and there is reason to posit that this type of therapy can indirectly prevent processes in plaques, such as inflammation and oxidative stress, that may ultimately contribute to defective efferocytosis. However, to the extent that many subjects at risk are not able to lower their LDL to a level low enough to completely suppress atherosclerotic disease, there is a place for complementary therapies (109). For example, recent success of the CANTOS trial demonstrated that lowering inflammation, through administering an anti-IL-1β antibody, successfully reduced recurrent cardiovascular events independently of lipid lowering (110). One type of new approach that may successfully target defective efferocytosis is antibodies that block CD47. However, anti-CD47 antibodies also causes anemia owing to inappropriate clearance of red blood cells (46, 111, 112). Another type of approach is to enhance the function of efferocytes themselves by preventing proteolysis of efferocytosis receptors, e.g., by blocking ADAM17-mediated cleavage of MerTK, or by enhancing the ability of efferocytes to clear multiple apoptotic cells, e.g., by boosting the mitochondrial fission-calcium mechanism that enables macrophages to efficiently ingest secondarily encountered apoptotic cells (27, 51, 108). Yet another approach would be tilting the SPM:leukotriene ratio to favor SPM production, such as through the administration of RVD1, which has been shown to enhance macrophage–apoptotic cell interactions and to increase lesional efferocytosis (51, 99). Finally, glucocorticoids generate anti-inflammatory molecules and are therefore routinely used for managing inflammatory diseases. One such glucocorticoid product, annexin A1, enhances efferocytosis, resolves inflammation, and delays atherosclerosis in mice (113–115). Indeed, the combination enhancing efferocytosis while at the same time restoring resolution mediators in lesions may offer the most promising therapeutic strategy to combat atherosclerotic cardiovascular disease.

AUTHOR CONTRIBUTIONS

All authors contributed to drafting and editing the review. AY designed the graphic in **Figure 1** and AD designed **Table 1**.

FUNDING

This review was supported by the American Federation for Aging Research, Research Grant for Junior Faculty (GF, A16034); American Heart Association Fellow-to-Faculty grant (AD, 17FTF33660643) and Post-Doctoral Fellowship grant (BC, 15POST25620024); and NIH grants T32 HL007343-28 (AY), R00 HL119587 (GF), and R01s HL075662, HL127464, and HL132412 (IT).

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

frontiers in Cardiovascular Medicine

REVIEW
published: 07 December 2017
doi: 10.3389/fcvm.2017.00079

Defective Protein Catabolism in Atherosclerotic Vascular Inflammation

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Vascular inflammation in atheroprone vessels propagates throughout the arterial tree in dyslipidemic patients, thereby accelerating atherosclerotic progression. To elucidate the mechanism of vascular inflammation, most previous studies have focused on inflammation-related signals that are sent in response to vasoactive stimuli. However, it is also important to understand how normal blood vessels become defective and start degenerating. Growing evidence suggests that major protein catabolism pathways, including the ubiquitin-proteasome, autophagy, and calpain systems, are disturbed in atheroprone vessels and contribute to the pathogenesis of atherosclerosis. Indeed, dysregulation of ubiquitin–proteasome pathways results in the accumulation of defective proteins in blood vessels, leading to vascular endothelial dysfunction and apoptosis in affected cells. Impaired autophagy-lysosomal degradation affects smooth muscle cell transformation and proliferation, as well as endothelial integrity and phagocytic clearance of cellular corpses. Dysregulation of the calpain system confers proatherogenic properties to endothelial cells, smooth muscle cells, and macrophages. In this review article, we will discuss the current information available on defective protein catabolism in atheroprone vessels and its potential interrelation with inflammation-related signals.

Keywords: calpain, autophagy, ubiquitin proteasome, nitric oxide, inflammasome, mechanotransduction, apoptosis, efferocytosis

OPEN ACCESS

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

Received: *20 September 2017*
Accepted: *23 November 2017*
Published: *07 December 2017*

Citation:
Miyazaki T and Miyazaki A (2017) Defective Protein Catabolism in Atherosclerotic Vascular Inflammation. Front. Cardiovasc. Med. 4:79. doi: 10.3389/fcvm.2017.00079

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease accompanied by the intimal thickening of systemic arterial walls (1, 2). Rupture of vulnerable plaques as well as thrombotic/embolic occlusion and arterial narrowing can be causal of lethal ischemic disease, including acute coronary syndromes/myocardial infarction (3). Regarding the pathogenic cues that contribute to atherosclerosis, the majority of previous atherosclerosis studies support the hypothesis that atherosclerosis is driven through reactive oxygen species (ROS)-mediated oxidative stress, which leads to the induction of numerous inflammatory elements, such as adhesion molecules in vascular endothelial cells (ECs) and proinflammatory cytokines *via* redox-sensitive transcription factors (2). During prolonged vascular inflammation, degenerative insults, such as increased numbers of apoptotic cells, remodeling of the extracellular matrix (ECM), breakdown of elastic lamella, and the dysfunction of ECs, emerge in atheroprone vessels and accelerate atherosclerosis-related complications (2). Although earlier clinical trials investigated the efficacy of antioxidants, these agents failed to reverse cardiovascular death (4); in contrast, they were effective in ameliorating acute inflammation, including the acute phase of

stroke (5). Therefore, in addition to the mechanisms underlying acute inflammatory insults, it is important to understand how blood vessels shift toward degenerative status in chronic vascular disease. In this regard, accumulating evidence indicates that defects in protein catabolism systems, which consist of a variety of intracellular proteases, critically contribute to inflammatory vascular degeneration. Autophagy, an essential intracellular process mediated by the lysosomal degradation of cytoplasmic components, is detectable in every tissue and mediates the nutrient turnover, particularly in cells under starving conditions. Although this system is involved in sustaining cellular metabolism, homeostasis, and survival (6), autophagic flux was detected in atheroprone vessels (7). Furthermore, the ubiquitin–proteasome system, which acts as a threshold machinery for protein catabolism (8), is regulated through proinflammatory cytokines such as interferon-γ (9), oxidized low-density lipoprotein (LDL) (10), and oxidized cholesterol (11). In addition to their roles in protein degradation, several classes of proteases transduce cellular signaling through their ability to process target proteins in stress-inducing environments. Indeed, calpain, a Ca^{2+}-dependent intracellular cysteine protease, is activated in response to several classes of cytokines, growth factors, lysophospholipids, and physical stresses (12), thereby participating in degenerative vascular disorders (13, 14). In this review, we summarize the recent achievements in the proinflammatory and proatherogenic defects of these protein catabolism pathways. In addition, we will discuss anti-atherosclerosis strategies that target defective protein catabolism.

DEFECTIVE PROTEIN CATABOLISM UNDERLIES ATHEROSCLEROSIS

Autophagy

Autophagy is a self-degenerative process that participates in organelle turnover, and the recycling of cytoplasmic components as well as protein degradation in response to extracellular stresses. Currently, autophagy can be categorized into three classes (6). Microautophagy is a non-selective lysosomal process describing the direct engulfment of cytoplasmic cargos by lysosomes. This process is accomplished by the inward invagination of cargos into the lysosomal membrane. In contrast, chaperone-mediated autophagy enables the selective degradation of cytoplasmic proteins by recognizing chaperone proteins. During this process, lysosomal-associated membrane protein type 2A on the lysosomal membrane recognizes the target proteins *via* their chaperone (e.g., heat shock cognate protein of 70 kDa), allowing internalization of the target protein into lysosomes. Macroautophagy is a process whereby cytoplasmic components are degraded by lysosomes, and it is accompanied by the formation of autophagosomes. These autophagosomes are cytosolic double-layered membrane vesicles, in which cytoplasmic components are separated from the cytoplasmic environment, and which finally fuse with a lysosome where lysosomal digestion occurs. One of the key regulators of autophagy is the kinase mammalian target of rapamycin (mTOR), which negatively regulates autophagy (6). Autophagosome formation is mediated by *Atg* genes, which is regulated by Atg12–Atg5–Atg16L1 and LC3–PE (*Atg8* homolog) complexes (6). It was reported that nutrient supply by macroautophagic protein catabolism sustained anabolic reactions to generate macromolecules, such as nucleic acids, proteins, and organelles. As a result, the nutrient supply can exert a prosurvival mechanism, particularly under starving conditions (15). In contrast, the overactivation of autophagic systems elicits cell death, which may be mediated by a process morphologically distinct from apoptosis (16). The contribution of macroautophagy to tumorigenesis, infection, diabetes, and cardiovascular diseases was described previously (6, 7, 15). In vascular systems, macroautophagy is detectable in macrophages in atherosclerotic lesions as well as in vascular-resident cells, including ECs and vascular smooth muscle cells (VSMCs), where it participates in physiologic and pathogenic responses (6).

Razani et al. reported that macroautophagy was associated with the pathogenesis of atherosclerosis (17). The expression of p62 protein, but not of p62 mRNA, was potentiated in lesional macrophages suggesting insufficient autophagy in these cells. Macrophage-specific deficiency of *Atg5* accelerated the development of atherosclerosis in *Apoe*$^{-/-}$ mice by insufficient autophagy in cells. Furthermore, Liao et al. also reported abnormal expression patterns of LC3 and the presence of p62-positive cells in atheromas in atherogenic mice, indicating impaired autophagy in the lesions (18). Accordingly, macrophage-specific *Atg5* deficiency augmented the expansion of lesional necrotic cores and progression of atherosclerosis. Therefore, insufficient autophagy might decrease the stability of atherosclerotic plaques as well as its pathogenic implications.

Ubiquitin–Proteasome System

The ubiquitin proteasomal systems are part of the central protein catabolism machinery in cells and are involved in the degradation of misfolded proteins, as well as normal protein degradation. These systems are also implicated in cellular events, including antigen presentation in immune cells (19) and the cell cycle (20). The polyubiquitination of target molecules triggers proteasomal degradation, which is driven by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases (21). Following the ubiquitination process, ubiquitinated target proteins are recognized by proteasomal complexes and are proteolytically degraded into peptides. In this process, the 26S proteasome comprising regulatory particle 19S and core particle 20S, has a crucial role in the proteasomal systems (9). Similar to autophagic systems, dysfunctional proteasomes can cause degenerative diseases, such as Alzheimer's disease (22), Parkinson's disease (22), Huntington's disease (22), and atherosclerosis (23, 24). An atherosclerosis study in high fat diet-fed pigs identified that proteasomal activity was elevated in coronary arteries during the progression of atherosclerosis (25). However, in a human atherosclerosis study, the carotid atherosclerotic lesions in subjects complicated with transient ischemic attack or stroke exhibited lower proteasomal activity compared with that in asymptomatic subjects (26). These studies suggest that proteasomal activity declines in advanced atherosclerotic plaques, whereas it is sustained in mild lesions. In addition to these observations, animal experiments with pharmacological proteasomal intervention

have been performed. Herrmann et al. examined the long-term administration of the proteasome inhibitor MLN-273 in high cholesterol diet-fed pigs and identified the elevation of oxidative stress and exacerbation of atherosclerosis (25). Conversely, Wilck et al. reported that a moderate dose of bortezomib (also known as PS-341) led to partial proteasomal inhibition in high cholesterol diet-fed *Ldlr*$^{-/-}$ mice, which reduced oxidative stress and NADPH oxidase 4 expression levels in the aortae, thereby suppressing atherosclerosis (27). Therefore, it hardly defines pathophysiologic roles of proteasomal systems in atherosclerosis. It is well known that macrophage cytotoxicity is prominent in advanced atheromas, while vascular inflammation is sustained throughout moderate-to-advanced atherosclerotic lesions (1). Thus, it is suspected that the contribution of proteasomal systems to the pathogenesis of atherosclerosis is substantially dependent upon the stage of the disease.

Calpain Systems

The calpain family comprises Ca^{2+}-dependent intracellular proteases (28–30). These proteolytic systems are unique because calpains modulate their substrates through limited proteolytic cleavage in addition to proteolytic degradation. The calpain system comprises the endogenous inhibitor calpastatin as well as 15 homologs of the catalytic subunits, and 2 regulatory subunit homologs in mammals (28–30). Calpain catalytic subunits are categorized into conventional and unconventional subtypes (28–30). The conventional subtype consists of two isozymes, calpain-1 and calpain-2, which are ubiquitously expressed in vertebrates (28–30). These are heterodimers of the catalytic subunits, CAPN1 and CAPN2, which form calpain-1 and calpain-2 isozymes, respectively, together with their common regulatory subunit, CAPNS1 (28–30). The substrate selectivity of those conventional calpains is currently unclear, but they exhibit enzymatically distinct properties. Indeed, calpain-1 and calpain-2 require micromolar and millimolar levels of Ca^{2+} for half-maximal activation, respectively. It was reported that a variety of biophysical and biochemical stimuli elevate intracellular Ca^{2+} levels (31–33); therefore, the activity of these protease systems has been the focus of numerous pathophysiologic studies (13, 14). Our previous investigation indicated that oxidized or enzymatically modified LDL or its component, lysophosphatidylcholine, induced calpain-2; accordingly, this molecule is enriched in ECs in human and mouse atheromas (34). Calpain-2 exerts pro-atherogenic roles because the calpain inhibitors calpeptin and ALLM counteracted aortic atherosclerotic development in high cholesterol diet-fed *Ldlr*$^{-/-}$ mice. It was also reported that the calpain inhibitor BDA-410 inhibited atherosclerotic development in angiotensin II-infused *Ldlr*$^{-/-}$ mice (35). Furthermore, angiotensin II- or hypercholesterolemia-induced atherosclerosis in *Ldlr*$^{-/-}$ mice was suppressed by the transgenic overexpression of calpastatin; in contrast, abdominal aneurysmal formation, a complication of angiotensin II-infused mice, was unaltered (36). A study using angiotensin II-infused mice showed that calpain-1 in myeloids and calpain-2 in leukocytes were responsible for atherogenesis (36). Collectively, conventional calpains participate in pathophysiologic regulation in ECs and leukocytes, thereby facilitating atherosclerosis.

Our previous data identified that unconventional calpains also contribute to atherogenesis (37). Expression analysis in *Ldlr*$^{-/-}$ mice showed that *Capn2*, *Capn6*, and *Capn9* were upregulated in atheroprone aortae. *Capn6* deficiency decelerated the progression of atherosclerosis in *Ldlr*$^{-/-}$ mice, while *Capn9* deficiency did not have any effect. Calpain-6 is a non-proteolytic calpain because the cysteine residue in its CysPc domain is substituted with a lysine (28–30). This molecule is expressed preferentially in macrophages in advanced atheromas in humans and mice (37). Interestingly, calpain-6 was originally identified in the skeletal muscle, cartilage, and heart of a murine fetus (38); however, its expression is absent in adult tissues with the exception of the placenta under non-disease conditions (39). Therefore, calpain-6 might be potentiated under developmental and pathogenic conditions. Regarding atherosclerosis, it is likely that calpain-6 is upregulated in macrophages after cells infiltrate into lesions, whereas it is absent in bone marrow cells (37). Furthermore, calpain-6 induction can be reproduced in cultured bone marrow-derived macrophages by cytokine stimulation. In particular, tumor necrosis factor-α markedly induced calpain-6. Atherogenesis appears to be dependent upon myeloid *Capn6* but not upon other cell lineages in mice. Therefore, the proatherogenic transformation of macrophages by calpain-6 is responsible for atherosclerotic disease.

CONTRIBUTION OF DEFECTIVE PROTEIN CATABOLISM TO ATHEROGENIC INFLAMMATION

During the development of atherosclerosis, the dysfunction of ECs and fibrogenicity of VSMCs, as well as cholesterol deposition and proinflammatory responses in macrophages are driven by defective protein catabolism (**Figure 1**; **Table 1**). The cell type-specific contribution of these defects to atherosclerosis is discussed below.

Vascular ECs

Endothelial cells cover the luminal surface of blood vessels and act as a physical barrier to limit the infiltration of active plasma factors through the vascular wall. It is well known that the endothelial barrier is disorganized in atheroprone arteries because of inflammatory insults (1), which accelerate the recruitment of immune cells that subsequently promote proinflammatory responses in the vessels. It is noteworthy that immune cells can also infiltrate atheroprone vascular walls through the vasa vasorum (40). Previous studies identified three major types of interendothelial junctions: tight junctions, gap junctions, and adherence junctions (41). In human umbilical vein ECs, tight junctions are present in up to 20% of the total junctional complexes, while adherence junctions account for approximately 90% (42). Vascular endothelial (VE)-cadherin forms adherence junctions in a Ca^{2+}-dependent homophilic manner (43). Furthermore, vascular permeability in mice was elevated by the administration of anti-VE-cadherin neutralizing antibody, whereas this treatment had no effect on other types of junctions in ECs (44); thus, adherence junctions have a pivotal role in barrier maintenance in ECs. Our previous investigations identified that administration of the calpain

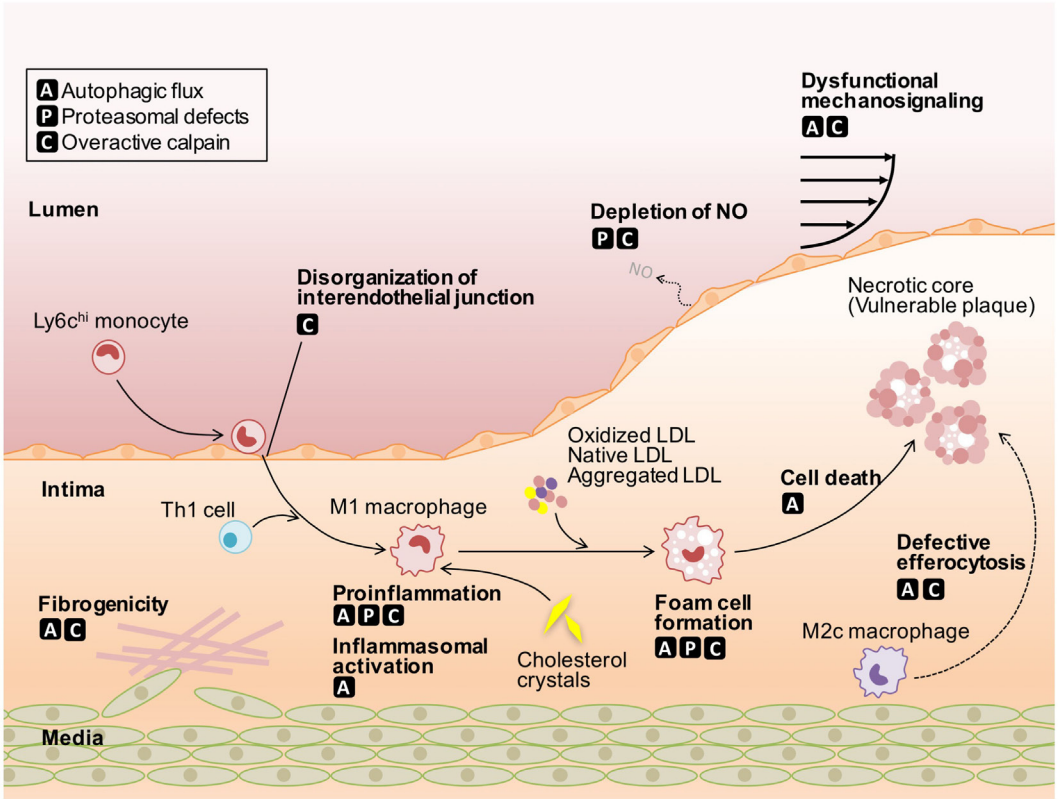

FIGURE 1 | Roles of defective protein catabolism in the pathogenesis of atherosclerosis. Autophagic flux leads to insufficient mechanosignaling in endothelial cells (ECs), impaired phenotypic conversion in vascular smooth muscle cells (VSMCs), proinflammatory actions in macrophages, and defective efferocytosis, thereby accelerating the onset of atherosclerosis. Proteasomal defects induce the depletion of nitric oxide in ECs and harmful proinflammatory responses in macrophages, despite protecting cells from apoptotic and non-apoptotic cell death. Overactivation of conventional calpains leads to impaired EC integrity, VSMC phenotypic conversion, and increased macrophage atherogenicity. Calpain-6 contributes to efferocytic pathways in macrophages. LDL, low-density lipoprotein; NO, nitric oxide.

inhibitors calpeptin and *N*-acetyl-Leu-Leu-methional (calpain inhibitor II) recovered the dysfunction of the EC barrier in dyslipidemic mice (34). Mechanistically, a juxtamembrane domain of VE-cadherin is proteolyzed by calpain-2, thereby dissociating β-catenin from the VE-cadherin complex, leading to the destabilization of adherence junctions. As a result, macrophage infiltration and the subsequent development of atherosclerosis in the dyslipidemic mice were suppressed by pharmacological inhibition of conventional calpains. Calpain proteolyzes E-cadherin and N-cadherin; thus, calpain systems regulate the posttranslational processing of cadherin family proteins (45, 46). In contrast to the calpain family, the contribution of autophagy and proteasomal systems to atherosclerotic endothelial barrier dysfunctions has not been reported to date, although these pathways were reported to participate in the disorganization of tight junctions in septic pulmonary ECs (47) and ischemic cerebral ECs (48).

Endothelial nitric oxide (NO) production is crucial for ensuring vascular integrity. NO reacts with superoxide to neutralize its toxicity; therefore, a reduction of NO bioavailability facilitates ROS-dependent vascular inflammation. Furthermore, uncoupled endothelial nitric oxide synthase (eNOS), which generates superoxide radicals instead of NO, is largely responsible for ROS production even in atheroprone vessels (49, 50), in addition to xanthine oxidase and NADPH oxidase. It was reported that conventional calpains were responsible for physiological NO production in ECs. Indeed, calpain inhibitors *N*-acetyl-leucyl-leucyl-norleucinal (calpain inhibitor I) or calpeptin prevented vascular endothelial growth factor (VEGF)-driven NO production in cultured ECs (51). Furthermore, VEGF accelerated the translocation of calpain to the plasma membrane to form an ezrin-containing molecular complex. Accordingly, AKT, AMP-dependent kinase (AMPK), and eNOSs1179 were phosphorylated through the molecular complex, which is indispensable for NO production in ECs. In addition to VEGF-driven NO synthesis, disorders of NO production under pathophysiologic conditions might be caused by the dysregulation of calpain systems. Yu et al. documented that the administration of calpain inhibitor I increased the activity and protein expression of aortic eNOS in high fat diet-fed rats along with the elevation of NO (52) indicating excessive calpain activity may disturb NO synthesis in ECs. Herrmann et al. reported that in contrast to calpain inhibitors, the chronic administration of the proteasome inhibitor MLN-273 increased oxidative stress and exacerbated the development of atherosclerosis in pigs fed a high cholesterol

TABLE 1 | Protein catabolism defects and atherosclerosis-related events.

	Cell type	Events	Related processes	Reference
Autophagic flux	ECs	NO depletion	Mechanotransduction	(56)
		Endothelial NO synthase uncoupling	Mechanotransduction	(57)
	VSMCs	Fibrogenic response	VSMC transformation to synthetic phenotype	(64)
	Macrophages	Vascular inflammation	Inflammasome secretion of IL-1β	(17)
		Impaired efferocytosis	Efferocytic clearance of dead cells	(18)
Proteasomal defects	ECs	Impaired vasodilation	NO production	(25)
	Macrophages	Proinflammation	NF-κB activation (inhibitor κB degradation)	(76)
		Vascular protection?	Anti-apoptosis	(10)
Overactive calpain	ECs	Barrier dysfunction	VE-cadherin disorganization	(34)
		Endothelial disintegrity	NO depletion	(52)
	VSMCs	Arterial fibrosis	Upregulation of NF-κB signals	(65)
		Age-associated vascular fibrosis	VSMC transformation to synthetic phenotype	(66)
		Vascular calcification	VSMC transformation to synthetic phenotype	(67)
		Carotid restenosis	VSMC transformation to synthetic phenotype	(68)
	Macrophages	Vascular inflammation	Upregulation of NF-κB signals	(36)
		Impaired efferocytosis	mRNA splicing defects (calpain-6)	(37)

ECs, vascular endothelial cells; VSMCs, vascular smooth muscle cells; NO, nitric oxide; IL-1β, interleukin-1β; NF-κB, nuclear factor-κB; VE-cadherin, vascular endothelial-cadherin.

diet (25). *In vitro* experiments further showed that inhibition of the proteasome prevented EC-dependent vasodilation in isolated coronary arteries. Collectively, protein catabolism pathways are critical for endothelial NO production. It appears that proteasomal systems ensure NO synthesis, while calpain systems act as a negative regulator for NO production in atheroprone vessels.

It is widely known that fluid shear stress, the blood flow-generated dragging force tangential to the EC surface, is a critical determinant for atherosclerosis susceptibility in blood vessels. This is because shear stress-dependent pathways in ECs are involved in barrier regulation and NO production (53). Interestingly, aortic arch, coronary artery, and aortic bifurcation, where blood flow is disturbed, are proatherogenic; thus, atherosclerosis susceptibility in blood vessels is largely dependent upon the vascular architecture because of the limited EC mechanotransduction in these regions (54). Yang et al. reported that physiological fluid shear stress sustained substantial autophagic activity in cultured ECs, but was reduced in cells subjected to low shear stress (55). Shear stress-induced eNOS phosphorylation and NO production were markedly blunted by autophagic deficiency (56). Similar to impaired NO production, the loss of autophagic activity potentiates ROS and cytokine production in ECs, suggesting that autophagy may play a suppressive role in eNOS uncoupling. Consistent with these results, autophagic inhibition by 3-methyladenine downregulated eNOS expression in sheared carotid arteries in rabbit, while autophagic activation by rapamycin potentiated eNOS expression (57). In addition to shear stress-dependent autophagic regulation, we previously showed that endothelial calpain-2 was activated in response to physiologic shear stress in cultured ECs (58). This activity was mediated through Ca^{2+} influx and phosphatidylinositol 3-kinase and was responsible for EC alignment in the direction of the blood flow. Interestingly, calpain-2 is enriched in specific vascular regions, such as the lesser curvature of the aortic arch and origin of vascular branch, in normal physiological murine aortae, where the blood flow is structurally disturbed (59). Thus, the autophagic and calpain systems orchestrate endothelial mechanotransduction.

Vascular Smooth Muscle Cells

It was reported that cardiovascular defects such as hypertension and atherosclerosis are strongly related to age-associated alterations in arterial structure and function (60, 61). The hallmark of such disorders is the stiffening of large arteries caused by ECM remodeling and vascular calcification and is triggered by the phenotypic conversion of contractile VSMCs to a synthetic phenotype. Mechanistically, synthetic VSMCs orchestrate elastin fragmentation as well as the synthesis and degradation of collagen through ROS-dependent inflammatory cascades. It was reported that RhoA, TGF-β, Notch, and integrin–matrix pathways modulated SMC differentiation (62) and that platelet-derived growth factor (PDGF) orchestrated the conversion of VSMCs to a contractile state along with autophagic activation (63). The pharmacological intervention of autophagy appears to inhibit the PDGF-induced phenotypic conversion in VSMCs, indicating the contribution of autophagic signals to the conversion. Interestingly, VSMC-specific deficiency of autophagy exacerbated high fat diet-induced atherosclerosis in *Apoe*$^{-/-}$ mice, which was accompanied by a thickening of the fibrous cap (64). Furthermore, the autophagic flux resulted in an increase in collagen content, TGF-β expression, and matrix metalloproteinase-9 in VSMCs. Thus, autophagy in VSMCs is protective in atherogenesis, but augments plaque vulnerability.

In addition to autophagy, calpain systems were reportedly associated with the phenotypic regulation in VSMCs. The transgenic overexpression of calpastatin antagonized arterial fibrosis and hypertrophy in mice infused with angiotensin II (65) by reduced MMP levels and the downregulation of NF-κB signals in arterial media. Similarly, the age-associated overactivation of calpain-1 in VSMCs participated in the upregulation of MMP2 and VSMC motility (66). Furthermore, osteopontin and

osteonectin levels in VSMCs were reduced by the overexpression of calpain-1 in rats, inducing the calcification and fibrosis in the arteries of aged rats (67). Furthermore, VSMC proliferation and collagen synthesis were accelerated during ligation-induced carotid restenosis in mice, which was opposed by the transgenic overexpression of calpastatin (68). Therefore, calpain systems have a pivotal role in inflammation-related fibrogenic responses in VSMCs.

Macrophages

Macrophages exert an essential role in the pathogenesis and progression of atherosclerosis (69). Bone marrow-derived circulating monocytes can be divided into two subsets, LY6C^{hi} and LY6C^{low}, in mice (70–72). The increased number of circulating monocytes in hypercholesterolemic mice is responsible for the expansion of the LY6C^{hi} subset (70), which is a major source of recruited macrophages in atheromas. In the early stage of atherosclerosis, circulating monocytes adhere to luminal ECs and migrate into the intimal layer according to the gradient of cytokines/chemokines (1). Subsequently, monocytes are differentiated into classically (M1) and alternatively (M2) activated macrophage subsets. M1 subset is mainly associated with inflammatory responses in atherosclerotic lesions and is derived from LY6C^{hi} monocytes (70–72). The M2 subset can be further divided into at least 4 subgroups, M2a, M2b, M2c, and M2d, which participate in tissue repair and inflammatory resolution in some instances (73). Foam cells in atherosclerotic lesions are mostly derived from the M1 subset, which is mediated through receptor-dependent and receptor-independent uptake of LDL cholesterol. In contrast, M2c macrophages incorporate cholesterol by engulfing dead cells efferocytically (74). The balance of M1 and M2 polarization can be disrupted in the presence of inflammatory burden in atherosclerotic lesions at least in part by disturbed poly (ADP-ribose) polymerase- or Dll4/Notch-related signals (73). Because cytosolic-free cholesterol exhibits robust cytotoxicity, the excessive incorporation of cholesterol results in cell death in macrophages. Accordingly, the acceleration of cholesterol uptake in macrophages in atheroprone vascular walls increases the number of dying macrophages, thereby forming cholesterol-enriched vulnerable atherosclerotic lesions (75). In addition to their defective cholesterol handling, atheroprone macrophages play central roles in innate immunity, including toll-like receptor and NOD-like receptor-mediated inflammasome signaling, in lesions (69). Although the protein catabolism pathway critically influences both cholesterol handling and inflammatory cascades in cells, cholesterol handling is not discussed in this issue. Please refer to our previous review articles for information on proatherogenic cholesterol handling in macrophages by defective cholesterol catabolism (12).

It was reported that macrophage-specific deficiency of autophagy by the deletion of *Atg5* facilitated atherogenesis in *Apoe*$^{-/-}$ mice (17). Interestingly, *Atg5*-deficient macrophages had increased oxidative stress and enhanced inflammasome secretion of IL-1β. The inflammasome systems in *Atg5*-null macrophages were potentiated by the addition of cholesterol crystals to the culture systems. It is likely that the inflammasome secretion of IL-1β by *Atg5*-null macrophages in atheromas may be potentiated by cholesterol crystals, because they are enriched in atherosclerotic lesions in *Atg5*-deficient mice. Furthermore, necrotic cores and oxidative stress in lesions of *Ldlr*$^{-/-}$ mice were enhanced by the macrophage-specific deletion of *Atg5* (18). Autophagic activity in isolated macrophages was potentiated by proatherogenic stressors, such as 7-ketocholesterol or 1-(palmitoyl)-2-(5-keto-6-octene-dioyl) phosphatidylcholine. Such autophagic activity suppressed apoptotic cell death by the reduction of endoplasmic reticulum stress and oxidative stress by NADPH oxidase 2. Furthermore, atherogenicity in macrophages was also mediated by proteasomal defects. For example, Brand et al. reported that the pharmacological inhibition of proteasomes prevented the proteolytic degradation of inhibitor κBα, an endogenous inhibitor of NF-κB transcriptional systems, by oxidized LDL in monocytes (76). This indicates that proteasomal inhibitors possess anti-inflammatory effects in atherosclerosis. In contrast, it was reported that oxidized LDL-stimulated macrophages showed increased ubiquitination activity, which suppressed apoptosis (10). Similarly, the complex mode of cell death in VSMCs was abrogated by 7-ketocholesterol-indced proteasomal activity (11). Thus, proteasomal activity may be involved in cell survival under stressed conditions, while it participates in cytotoxic inflammatory burden. In addition to the autophagic and proteasomal flux, the participation of calpain systems in inflammatory cascades of macrophages was proposed. It appears that calpain can proteolytically degrade inhibitor κBα, thereby accelerating NF-κB signaling (77); thus, the overactivation of calpain systems potentiates inflammatory responses. The transduction of *CAST* prevented the production of inflammatory cytokines in peritoneal macrophages isolated from high fat diet-fed *Apoe*$^{-/-}$ mice (36). Collectively, macrophages can be susceptible to proatherogenic burden by the overactivation of calpain systems. Whether defective protein catabolism influences macrophage polarity is currently unclear and, therefore, should be studied in the future.

Vulnerable atherosclerotic lesions harbor vast number of inflammatory cells, a necrotic core, and a thin fibrous cap (78). In particular, expansion of the cholesterol-enriched necrotic core by the accumulation of dead cells weakens the physical stability of plaques. It was reported that macrophages engulf and remove cellular corpses in lesions through efferocytic activity, thereby reducing the necrotic core (78). Furthermore, the efferocytic actions of macrophages participate in the reprogramming of cells toward anti-inflammatory phenotypes, leading to the resolution of inflammation. Importantly, the clearance of dead cells by efferocytosis is limited in advanced atheromas, because "eat-me/find-me" signals in apoptotic cells and phagocytosis pathways in macrophages are prevented in the lesions (78). Thus, the coordination of integrated cell death signals in dying cells and phagocytic signals in phagocytes are necessary for efferocytosis. In the case of mammary gland involution in pregnancy, efferocytic activity was accelerated in mammary epithelial cells, resulting in remodeling of the tissues and the production of milk (79). Mammary epithelial-specific *Atg7* deficiency markedly reduced efferocytic activity, Rac1 activity, and MertK expression; thus, autophagic signaling is associated with efferocytosis-associated tissue remodeling. In the case of atherosclerosis, autophagic defects caused by *Atg5* deficiency reduced the efficiency of the

efferocytic clearance of dead cells, leading to an expansion of the necrotic core (18). It is likely that *Atg5* deficiency in dying cells, but not phagocytic macrophages, prevented the efficiency of efferocytosis. Therefore, the autophagic disorder in dying cells or phagocytic macrophages induced defects in efferocytosis, although this needs to be elucidated in the future. While the participation of conventional calpains in efferocytosis has not been reported to date, calpain-6 seems to be involved in this event. Indeed, the efferocytic clearance of dead cells by cultured macrophages was modestly inhibited by *Capn6* deficiency (37). Accordingly, the deletion of *Capn6* resulted in a reduction of necrotic cores in atheromas in $Ldlr^{-/-}$ mice, which was accompanied by the upregulation of Rac1 protein in the cells. Rac1 prevention by a pharmacological inhibitor suppressed efferocytic activity in macrophages (80); therefore, it is interpreted that efferocytic activity in lesional macrophages was prevented by calpain-6, probably through Rac1 downregulation. Because conventional calpains are associated with cellular dynamic processes, such as migration (81) and trafficking of intracellular vesicles (82), it will be interesting to investigate the participation of these calpains in efferocytosis.

TARGETING DEFECTIVE PROTEIN CATABOLISM IN ATHEROSCLEROSIS

As stated above, autophagic disorders cause insufficient mechanosignaling in ECs, impaired phenotypic conversion in VSMCs, inflammatory action in macrophages, and defective efferocytosis, thereby increasing the susceptibility to atherosclerosis (**Figure 1**). Therefore, recovering autophagic activity may be a promising treatment for the atherosclerosis burden. One possible candidate for atherosclerosis intervention is mTOR, a robust autophagic suppressor. mTOR inhibitors normally exhibit proautophagic activity thereby suppressing atherosclerosis (83). For example, atherosclerosis in rabbits fed a high cholesterol diet (84), or in $Apoe^{-/-}$ (85) and $Ldlr^{-/-}$ mice (86) was markedly inhibited by oral administration of the mTOR inhibitor rapamycin. Clinical trials of several mTOR inhibitors for cancer treatment are currently underway (87). Based on our current knowledge, it is expected that the use of mTOR inhibitors will also be beneficial for treating cardiovascular disorders.

Proteasomal activation induces harmful proinflammatory responses in macrophages but protects cells from apoptotic and nonapoptotic cell death, whereas proteasomal inhibition leads to the depletion of EC-derived NO (**Figure 1**). Thus, based on animal experiments, it is likely that inhibiting proteasomes will have a dual action. Accordingly, the clinical applications of proteasomal inhibitors for treating atherosclerosis are currently unclear, even though atherosclerotic diseases can be reversed by moderate levels of proteasomal inhibition (27). In addition to these inhibitors, endogenous activators of proteasomes, PA28, PA200, and PA700, as well as chemical proteasome activators, are currently available (88). The beneficial effects of PA28 overexpression in a neuronal cell model of Huntington's disease were reported (89). Restoring proteasomal activity by activators should be investigated in future atherosclerosis studies.

Both calpain-6 and conventional calpains are involved in atheroprone events in lesional macrophages. Whereas conventional calpains lead to impaired EC integrity, phenotypic conversion of VSMCs and increased macrophage atherogenicity, calpain-6 contributes to cholesterol uptake and efferocytic processes in phagocytic macrophages (**Table 1**). Importantly, the majority of proatherogenic events can be prevented by loss-of-function calpains (12, 30), indicating that targeting calpain systems is a promising approach for atherosclerosis therapy. Clinical trials of candidate compounds for the prevention of conventional calpains are currently underway, especially for neurodegenerative diseases (30). Based on our knowledge from basic research, the repositioning of these agents to the cardiovascular field may be highly beneficial. Isozyme selectivity of available calpain inhibitors, in particular, those regarding unconventional isozymes, are mostly unclear. Furthermore, it is expected that available inhibitors will not block calpain-6 because this molecule exhibits physiologic/pathophysiologic functions through its capacity to bind to other functional molecules, but not through its proteolytic activity. Evaluation of the isozyme selectivity of available inhibitors among unconventional calpains and the development of subtype selective inhibitors will be indispensable for calpain-targeted therapy in the future.

In conclusion, respective protein catabolism pathways, autophagy, proteasomes, and calpain systems, have unique properties under proatherogenic conditions. It appears that autophagy and proteasomes have fundamental roles in cell survival, while calpains appear to be dispensable. In contrast, autophagy and proteasomal systems are limited during the progression of atherosclerosis, while proatherogenic stressors activate calpains in some instances. It is noteworthy that apoptotic cell death was accelerated by the calpain-mediated proteolysis of Atg5 (90). Therefore, exploring the interrelations among the catabolism pathways in atherosclerosis is required for a better understanding of the pathogenic roles of these protein catabolism pathways.

AUTHOR CONTRIBUTIONS

TM conceived, designed, appraised the literature, and wrote the manuscript. AM reviewed and revised the manuscript.

ACKNOWLEDGMENTS

We thank J. Ludovic Croxford, Ph.D., from Edanz Group (http://www.edanzediting.com/ac) for editing a draft of this manuscript.

FUNDING

This study was supported in part by Japan Society for the Promotion of Science KAKENHI Grant Number 17K09869 (to AM), Japan Society for the Promotion of Science KAKENHI Grant Number 15K09418 (to TM), and research grants from Takeda Science Foundation, Banyu Life Science Foundation International, NOVARTIS Foundation, Ono Medical Research Foundation, Japan Heart Foundation & Astellas Grant for Research on Atherosclerosis Update, and SENSHIN Medical Research Foundation (all to TM).

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

frontiers in Cardiovascular Medicine

MINI REVIEW
published: 14 March 2018
doi: 10.3389/fcvm.2018.00022

Long Non-Coding RNAs in Vascular Inflammation

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Less than 2% of the genome encodes for proteins. Accumulating studies have revealed a diverse set of RNAs derived from the non-coding genome. Among them, long non-coding RNAs (lncRNAs) have garnered widespread attention over recent years as emerging regulators of diverse biological processes including in cardiovascular disease (CVD). However, our knowledge of their mechanisms by which they control CVD-related gene expression and cell signaling pathways is still limited. Furthermore, only a handful of lncRNAs has been functionally evaluated in the context of vascular inflammation, an important process that underlies both acute and chronic disease states. Because some lncRNAs may be expressed in cell- and tissue-specific expression patterns, these non-coding RNAs hold great promise as novel biomarkers and as therapeutic targets in health and disease. Herein, we review those lncRNAs implicated in pro- and anti-inflammatory processes of acute and chronic vascular inflammation. An improved understanding of lncRNAs in vascular inflammation may provide new pathophysiological insights in CVD and opportunities for the generation of a new class of RNA-based biomarkers and therapeutic targets.

Keywords: lncRNAs, vascular inflammation, cardiovascular disease, acute inflammation, chronic inflammation

OPEN ACCESS

Edited by:
Ichiro Manabe,
Chiba University, Japan

Reviewed by:
Yuichi Oike,
Kumamoto University, Japan
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *18 November 2017*
Accepted: *28 February 2018*
Published: *14 March 2018*

Citation:
Haemmig S, Simion V and Feinberg MW (2018) Long Non-Coding RNAs in Vascular Inflammation. Front. Cardiovasc. Med. 5:22. doi: 10.3389/fcvm.2018.00022

INTRODUCTION

Accumulating studies highlight that inflammatory processes and traditional cardiac risk factors may cooperatively contribute to vascular disease leading to the development of cardiovascular events (1). A variety of systemic inflammatory diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus, psoriatic arthritis, and medium to large vessel vasculitis are associated with an increased risk of atherosclerotic events and premature cardiovascular disease (CVD) (2). Interestingly, acute inflammation (e.g., sepsis) also significantly increases the risk of future cardiovascular events (3). Although these diseases differ in their autoimmune and/or inflammatory nature, atherosclerosis may represent a common response with local vascular inflammation in subintimal and perivascular layers. Over decades, a progressive inflammatory multistep process in lesion-prone regions of the arterial vasculature develops by different disease-specific upstream insults (1, 4). However, our understanding of the pathophysiological links between systemic inflammatory diseases to vascular inflammation remains poorly understood. The recent recognition that as much as 70–90% of the genome is pervasively transcribed at some point during development has opened new opportunities to address these questions (5–7). Most of those transcripts are non-coding measuring greater than 200 nucleotides in length and display mRNA-like processing properties. This class of non-coding RNA (ncRNA) is known as long ncRNAs (lncRNAs). Whereas the number of ~19,000 human protein-coding genes has plateaued, the number of lncRNAs keeps increasing annually. However, it should be pointed out that lncRNAs have a low cross-species conservation rate and many lncRNAs have extremely low expression

levels per cell (8–10). Nevertheless, lncRNAs have emerged as powerful regulators of nearly all biological processes by mediating epigenetic, transcriptional, or translational control of target genes due to their polyvalent binding properties to RNA, DNA, and protein as well as acting as molecular sponges for other transcripts and miRNAs (11). The subcellular localization pattern can provide additional insights into the mechanistic role for lncRNAs. Other considerations include whether the lncRNA acts in *cis* or *trans* and whether the RNA product itself is essential for fulfilling its function or if its transcription *per se* that underlies its function (12). However, the role of lncRNAs in vascular inflammation and CVD is just emerging (13). This review will summarize recent findings that provide mechanistic and translational insights of lncRNAs in acute and chronic vascular inflammation in the context of CVD.

ACUTE INFLAMMATION

The acute inflammatory response is induced as a first line of defense against microbial infection and other "non-self" stimuli. Antigen-presenting cells express different receptors, of which the Toll-like receptor (TLR) family is best characterized. TLRs are especially sensitive to microbes products such as LPS, lipoproteins, and nucleic acids (14). Once activated, these receptors trigger complex signaling cascades resulting in changes in expression of hundreds of genes involved in immunity and inflammation. TLRs have been implicated in destabilizing plaques leading to atherothrombosis in the vessel wall. For example, TLR4 enhances macrophage responses to lipids and inflammation, whereas TLR2 potentiates inflammation more broadly in the vessel wall in both macrophages and vascular cells, an effect that may lead to superficial erosion of atherosclerotic lesions (15, 16). Recent studies have connected a range of acute inflammatory processes to lncRNA expression and found that lncRNAs can regulate the acute inflammatory response, opening new avenues for exploring pathophysiological insights that may lead to improved disease stage-specific diagnostics and therapeutic interventions.

Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is a conserved lncRNA whose expression correlates with many human cancers. Recent data also indicate its significance in immunity, and specifically in acute inflammation (17). *MALAT1* expression is increased in LPS-activated macrophages (17), cardiac microvascular endothelial cells (CMVEC), and in the hearts of rats with sepsis (18). Knockdown of *MALAT1* increases the LPS-induced expression of TNFα and IL-6 in macrophages. Mechanistically, *MALAT1* interacts with NF-κB in the nucleus to inhibit its DNA binding activity, and consequently decreases the production of inflammatory cytokines (17). *MALAT1* was also found to interact with the polycomb protein EZH2 in CMVECs in response to LPS activation (18). In a recent study, *MALAT1* expression was increased by LPS stimulation in murine cardiomyocytes and in cardiac tissue of a mouse sepsis model (19). *MALAT1* overexpression enhanced TNFα production on LPS-stimulated cardiomyocytes, while *MALAT1* siRNA had an inhibitory effect, *via* serum amyloid antigen 3 (SAA3), an inflammatory ligand that can stimulate IL-6 and TNFα production, as observed in other cells such as endothelial (20) and mouse liver cells (21). Cardiomyocytes transfected with *MALAT1* siRNA were less susceptible to LPS-induced cell apoptosis, suggesting that *MALAT1* induction is a mechanism of cardiomyocyte apoptosis or injury in response to sepsis (19). Collectively, *MALAT1* differentially regulates inflammatory responses in a cell-specific manner.

lincRNA-Cox2, is a lncRNA neighboring the Ptgs2 (Cox2) gene, recently discovered as a key regulator of inflammatory responses mediating both the activation and repression of distinct classes of immune genes (22). LPS stimulation induced *lincRNA-Cox2* expression in both dendritic cells and bone-marrow derived macrophages (BMDM) in a similar pattern as Ptgs2 (22, 23). *lincRNA-Cox2* expression is induced by LPS in a MyD88- and NF-κB–dependent manner and *lincRNA-Cox2* silencing/overexpression in BMDM regulates important immune genes such as TNFα, IL-6, CCL5, SOCS3, and STAT3 (22). Several mechanisms have been described for *lincRNA-Cox2*, including interaction with heterogeneous nuclear ribonucleoprotein (hnRNP) A/B and A2/B1 (22), degradation of IKB-α in the cytosol, and assembly into the SWItch/Sucrose NonFermentable (SWI/SNF) complex, thereby acting as a co-activator of NF-κB or inducing SWI/SNF-associated chromatin remodelling (24, 25). In a recent study, *lincRNA-Cox2* modulated TNFα–induced transcription of the IL-12b gene by promoting the recruitment of Mi-2/nucleosome remodeling and deacetylase (Mi-2/NuRD) repressor complex to the IL-12b promoter region (26). Taken together, rapid activation of *lincRNA-Cox2* may regulate a range of acute inflammatory signaling pathways.

THRIL (TNFα and hnRNPL related immune-regulatory LincRNA) or *linc1992,* is a lncRNA that regulates TNFα expression through a negative feedback mechanism. *THRIL* binds specifically to heterogenous nuclear ribonucleoprotein L (hnRNPL) and forms a functional *THRIL*–hnRNPL complex that regulates transcription of the TNFα gene by binding to its promoter. *THRIL* is also required for expression of many immune-response genes and regulators of TNFα expression. Clinically, *THRIL* expression correlated with the severity of symptoms in patients with Kawasaki disease, an acute inflammatory disease of childhood (27). This study provides strong evidence that *THRIL* is required for induction of TNFα expression and plays an important role in acute inflammation and innate immunity. Further studies will be of interest to verify these findings in other inflammatory contexts, including chronic inflammation such as atherosclerosis or diabetes.

CHRONIC INFLAMMATION

Chronic inflammation is a major contributing factor to vascular events, including atherosclerotic plaque development, plaque erosion, aortic aneurysm, and ischemic myocardial damage. Inflammation disturbs the homeostasis of the endothelium, leading to endothelial dysfunction, which is amongst the earliest processes involved in atherosclerotic initiation (28). The early response is characterised by activation of endothelial cells (ECs), triggered by biochemical (e.g., IL-1β, TNFα, oxLDL, etc.) and biomechanical stimulation in the form of disturbed blood flow [Rev by (29)]. Consequently, expression of adhesion

molecules (e.g., VCAM-1, ICAM-1, E-Selectin) and secretion of membrane-associated chemokines (e.g., MCP-1, fractalkine) fosters the recruitment of monocytes and different types of T cells to the vessel wall (14–17). Chronic endothelial activation leading to the loss of endothelial integrity increases the risk for atherosclerosis. This is often observed in patients with RA, an autoimmune disease that causes chronic inflammation of the joints and systemically in the vasculature (30). A strong relationship exists between RA and atherosclerosis, but causality remains unclear.

Spurlock et al. identified that the expression of the lncRNA *lincRNA-p21* is significantly lower specifically in patients with RA compared to healthy subjects. No dysregulation of *lincRNA-p21* could be observed in systemic lupus erythematosus and Sjörgen's syndrome (31). Interestingly, *lincRNA-p21* could be restored to normal levels in RA patients treated with methotrexate (MTX), which is the most commonly used anti-rheumatic drug with anti-inflammatory properties (32). *In vitro* analysis of Jurkat T cells confirmed the induction of *lincRNA-p21* by MTX (31). Initial work discovered *lincRNA-p21* as a repressor of p53-dependent transcriptional responses. Silencing of *lincRNA-p21* affected the expression of hundreds of genes known to be repressed by p53, which could be rescued by inhibiting p53, suggesting that *lincRNA-p21* functions as a downstream repressor for p53. This transcriptional repression by *lincRNA-p21* is mediated through an interaction with hnRNP-K (33). Because p53 expression levels positively correlated with *lincRNA-p21* expression in RA patients, basal *lincRNA-p21* expression may be p53-independent in PBMCs. In addition, *lincRNA-p21* was initially described to be regulated by p53 in the context of DNA damage response (33). To investigate whether specific inhibition of either ATM or DNA-PKcs, two key upstream regulators of the DNA damage response (34, 35), could restore *lincRNA-p21* or p53 expression, inhibition studies were performed. Indeed, MTX-mediated induction of p53 *and lincRNA-p21* was blocked in Jurkat T cells treated with NU-7441 (i.e., inhibitor for DNA-PKcs), whereas there was no effect using low concentration of KU-55933 (inhibitor for ATM). Furthermore, using *in vitro* NF-κB luciferase reporter assays, silencing of *lincRNA-p21* abrogated MTX-mediated inhibition of NF-κB activity. This effect could be simulated by using the NU-7441 inhibitor, demonstrating a link between *lincRNA-p21* and DNA-PKcs-mediated regulation of NF-κB pathway (31). This finding is consistent with previous reports, demonstrating that DNA-PKcs is a regulator of inflammation by phosphorylating p50, a member of the NF-κB pathway (36). Collectively, these findings suggest that MTX decreases the NF-κB pathway by increasing *lincRNA-p21* levels through a DNA-PKcs-dependent mechanism (31).

Stuhlmüller et al. described high expression levels of the lncRNA *H19* in synovial tissues and isolated synovial macrophages or synovial fibroblasts (SFB) from donor samples of RA patients compared to control subjects. *H19* was also induced in SFB from RA *ex vivo* using multiple pro-inflammatory stimuli such as TNFα, IL-1β, or PDGF-BB. Whether elevated levels of *H19* in RA reflects its role as a biomarker of inflammatory stimuli or as a pathogenic mediator remains unknown (37, 38). Future studies will be required to further define the functional role of lncRNA *H19* in RA pathogenesis and CVD.

Genome wide associated studies (GWAS) have identified the *INK4b-ARF-INK4a* locus located on chromosome 9p21 with multiple single nucleotide polymorphisms (SNPs) linked to coronary artery disease (CAD) (39–41), atherosclerosis (42), aortic aneurysm (43), ischemic stroke (41), type II diabetes (44) as well as specific cancer subtypes (45, 46). The lncRNA *ANRIL* (Antisense Non-coding RNA in the INK4 Locus) lies in opposite direction to the *INK4b-ARF-INK4b* locus, which contains the critical tumour suppressor genes $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ (47). The SNPs associated with CAD do not correlate with well-established CAD risk factors, suggesting that this lncRNA is a novel independent driver for vascular inflammation. Specifically two *ANRIL* transcripts (EU741058 and NR_003529) are significantly increased from patients with CAD in human atherosclerotic plaque tissue as well as peripheral blood mononuclear cells, whereas the most abundant isoform DQ485454 is not differentially expressed (48). Loss-of-function studies reduced cell viability of SMCs for siRNAs targeting exclusively NR_003529 or both NR_003529 and DQ485454 isoforms (49). Moreover, *ANRIL* silencing increased the expression level of the antisense transcripts $p15^{ARF}$ and p16INK4b, both key regulators for senescence, apoptosis, and stem cell self-renewal by the retinoblastoma-p53 pathway abrogating PRC-1/2 binding to their loci (50). Additionally, *ANRIL* binds directly to PRC-1/2 components (i.e., CBX7 and/or SUZ12) supporting its role in regulating epigenetics (51). The multiple splice sites of *ANRIL* may result in isoform-specific effects, thus explaining some paradoxical findings. For example, an interesting isoform is circular *ANRIL* (*cANRIL*), which results from exon skipping events during RNA splicing (52), adding another layer of complexity for the biological understanding of the *ANRIL* locus. *cANRIL* binds to PES1, an essential 60S-preribosomal assembly factor, impairing pre-rRNA processing and ribosome biogenesis in SMCs and macrophages. As a consequence, *cANRIL* induces nucleolar stress and p53 activation, resulting in the inhibition of proliferation and induction of apoptosis, as observed for the linear *ANRIL* (53). Although *ANRIL* is an independent risk factor for CAD, its functional role in vascular inflammation in CVD still requires clarity based upon transcript specificity.

Hu et al. (54) identified increased expression of the lncRNA *RP5-833A20.1* in human foam cells. Gain-of-function studies demonstrated that *RP5-833A20.1* reduced cholesterol efflux and increased inflammatory cytokines, including IL-1β, IL-6, and TNFα in THP-1 macrophages. Mechanistically, *RP5-833A20.1* decreased the expression of NFIA by inducing miR-382–5 p expression. However, the specific mechanism of how *RP5-833A20.1* regulates miR-382–5 p expression for macrophage foam formation and verification of these findings *in vivo* will require further investigation (54).

Recently, Tontonoz et. al. demonstrated that *in vivo* delivery of the liver-expressed liver X receptor-induced lncRNA (*LeXis*) reduced aortic lesion size by Oil-red O staining (55). Since *LeXis* has been previously described to maintain hepatic sterol content and levels of serum cholesterol (56), the adenovirus-

mediated *LeXis* overexpression in the liver was specifically designed using a thyroxine-binding globulin promoter. In line with their previous findings, *LeXis* overexpression reduced total serum cholesterol levels (55). This study raises the possibility for long-term lncRNA therapy in mice. Future studies that can overexpress lncRNAs in the liver or vessel wall may provide a novel therapeutic approach for regulating vascular inflammation in CVD.

Recent studies have illustrated increased *HOTAIR* expression in PBMCs and serum exosomes of RA patients, while lower expression of *HOTAIR* was detected in differentiated osteoclasts and rheumatoid synoviocytes. Overexpression of *HOTAIR* using lentivirus decreased the expression of IL-17, IL-23, IL-1β, and TNFα, and inhibited the activation of NF-κB in LPS-treated chondrocytes in a miR-138 regulated manner (57). These findings are in line with a previous exploratory study where *HOTAIR* expression was significantly reduced in LPS-treated chondrocytes and a RA mouse model (58). However, findings from acute inflammatory states such as sepsis are opposite. Using a mouse model of sepsis, *HOTAIR* expression was significantly

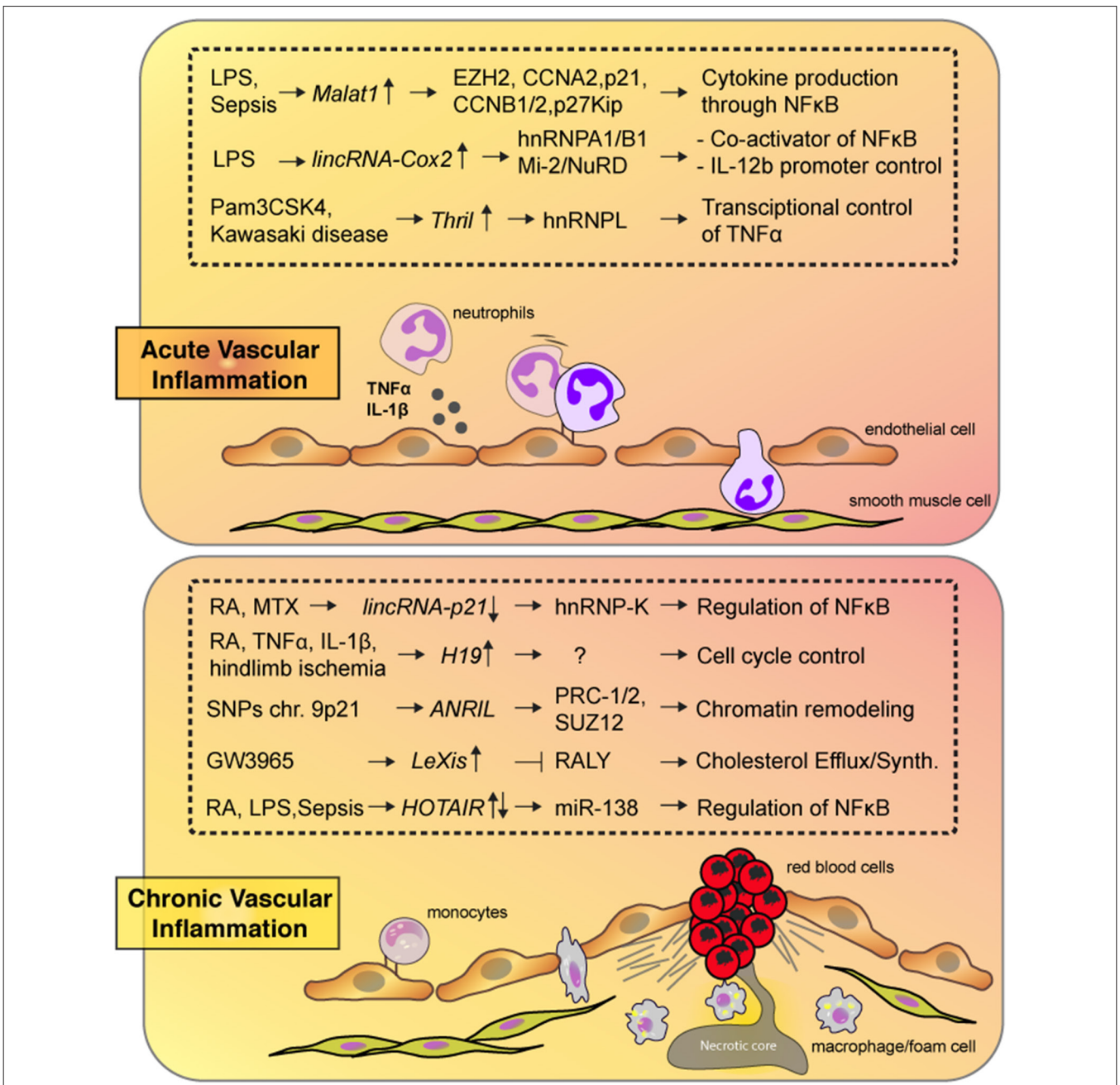

FIGURE 1 | LncRNAs in acute and chronic vascular inflammation. Highlighted lncRNAs from the text are shown along with their targets and biological consequences in response to acute (top) or chronic (bottom) vascular inflammation.

increased in cardiomyocytes. *HOTAIR* silencing improved the cardiac function of septic mice, and markedly decreased TNFα production in the circulation and p65 phosphorylation in cardiomyocytes (59). These studies highlight a cell-type dependent role for *HOTAIR* in acute inflammation. Future studies will be required to identify potential compensatory mechanisms that may be activated in cardiovascular cell types versus chondrocytes after LPS activation.

CONCLUSIONS AND FUTURE DIRECTIONS

Despite current prevention interventions and guideline-based therapeutics, recurrent cardiovascular events after acute coronary syndromes remain elevated at ~10% of patients within one year and ~20% of patients within 36 months of initial presentation (60, 61). Biomarkers for systemic inflammation such as high sensitivity C-reactive protein (hsCRP) has been associated with increased risk for cardiovascular events (62). Although the role of inflammation in atherosclerosis has been identified over 150 years ago by Virchow (63), only recently has the "inflammation hypothesis" in atherosclerosis been specifically tested with an anti-inflammatory drug targeting IL-1β. In the canakinumab anti-inflammatory thrombosis outcomes study (CANTOS), over 10,000 patients with elevated hsCRP at least 1 month post-myocardial infarction were randomized to receive the humanized monoclonal antibody canakinumab to neutralize IL-1β or placebo on top of usual therapy including statins. Impressively, recurrent cardiovascular events were reduced in the canakinumab treatment group independent of changes in lipid levels (64). As statin therapy lowers both LDL-C and inflammation (measured by hsCRP), CANTOS is the first clinical trial showing that lowering inflammation alone, without lowering LDL-C, significantly reduces cardiovascular events. Additional ongoing clinical trials using other anti-inflammatory drugs will likely provide further insights and impact clinical decision-making. For example, MTX is a widely used anti-inflammatory to treat RA patients (32). The cardiovascular inflammation reduction trial (CIRT) trial is currently investigating whether low dose of MTX administration will reduce the risk of cardiovascular events in patients with prior myocardial infraction and either type 2 diabetes or metabolic syndrome, all associated with chronic inflammation (ClinicalTrials.gov Identifier: NCT01594333) (65). Finally, the colchicine cardiovascular outcomes trial (COLCOT) will evaluate the long-term treatment of whether colchicine reduces rates of cardiovascular events in patients after myocardial infarction (ClinicalTrials.gov Identifier: NCT02551094).

Our understanding of the estimated 50,000 human lncRNAs in regulating acute and chronic inflammatory processes in the vasculature remains nascent, although accumulating studies demonstrate that lncRNAs hold great promise as important regulators of vascular inflammation. Apart from their expression profile, functional *in vivo* findings are key to understand their true translational value in acute and chronic inflammation of the vasculature and links with cardiovascular disease states (**Figure 1**). Furthermore, emerging technical advances provide the ability to uncover novel lncRNA interactors (12). *ANRIL* represents a SNP-associated loci, which may bear relevance for CVD and other diseases. (39, 66). Sensitive biomarkers have emerged for chronic inflammation burden such as CRP, SAA, and IL-6 or vascular injury such as sICAM-1, sVCAM-1 and PTX3 (67). However, these two types of biomarkers tend to correlate weakly with each other. Because the expression of some lncRNAs track with stage-specific pathophysiological processes and they can be measured in serum (68), lncRNAs provide new avenues for diagnostics. For example, distinct lncRNAs may shed light on inflammatory subsets associated with systemic autoimmune diseases (e.g., RA, SLE) versus inflammation localized to the vessel wall (e.g., coronary or peripheral artery disease). Recent screening efforts of plasma from patients with CAD revealed a lncRNA named CoroMarker (AC100865.1) that was significantly increased in CAD patients compared to controls (69, 70). It will be of interest to examine whether this lncRNA is specific to CAD or increased in other chronic inflammatory diseases. While their translational value remains to be elucidated, ncRNA-based targeting strategies such as using antisense oligonucleotides (ASO) have already been approved for food and drug administration-approved drugs. Similar to miRNAs, lncRNAs are often differentially regulated in a cell-specific manner or in response to specific pathophysiological stimuli providing unique properties for therapeutic intervention. Challenges remain with the efficiency and specificity of delivery, which may be overcome by chemical modifications and/or nanoparticle carriers (71, 72). For example, ASOs that target liver-specific ligands [e.g., the liver-specific asialoglycoprotein receptor (ASGPR)] appear to confer strong efficacy and reasonable safety (73). Analogous paradigms for ASOs targeting vascular-specific ligands could provide novel therapeutics for vascular inflammation.

Collectively, because lncRNAs provide a new layer of control of protein-coding genes, lncRNAs may hold promise for uncovering novel pathophysiological insights, stage-specific biomarkers, and new targets for vascular inflammation in acute and chronic disease states.

AUTHOR CONTRIBUTIONS

SH and VS researched the data for the article, and significantly contributed to content and writing of the article. MF contributed to the conception of the article, and the writing, reviewing and editing of the draft manuscript.

FUNDING

This work was supported by the National Institutes of Health (HL115141, HL117994, HL134849, and GM115605 to MWF), the Arthur K. Watson Charitable Trust (to MWF), and the Dr. Ralph and Marian Falk Medical Research Trust (to MWF).

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

frontiers in Cardiovascular Medicine

REVIEW
published: 24 January 2018
doi: 10.3389/fcvm.2018.00003

Inflammation in Vein Graft Disease

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Bypass surgery is one of the most frequently used strategies to revascularize tissues downstream occlusive atherosclerotic lesions. For venous bypass surgery the great saphenous vein is the most commonly used vessel. Unfortunately, graft efficacy is low due to the development of vascular inflammation, intimal hyperplasia and accelerated atherosclerosis. Moreover, failure of grafts leads to significant adverse outcomes and even mortality. The last couple of decades not much has changed in the treatment of vein graft disease (VGD). However, insight is the cellular and molecular mechanisms of VGD has increased. In this review, we discuss the latest insights on VGD and the role of inflammation in this. We discuss vein graft pathophysiology including hemodynamic changes, the role of vessel wall constitutions and vascular remodeling. We show that profound systemic and local inflammatory responses, including inflammation of the perivascular fat, involve both the innate and adaptive immune system.

Keywords: cardiovascular disease, bypass graft, saphenous vein, vein graft disease, inflammation, innate immunity, atherosclerosis

OPEN ACCESS

Edited by:
Masanori Aikawa, Harvard Medical School, United States

Reviewed by:
Vasilios Gabriel Athyros, Aristotle University of Thessaloniki, Greece
Christoph Eugen Hagemeyer, Monash University, Australia

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

Received: *02 November 2017*
Accepted: *08 January 2018*
Published: *24 January 2018*

Citation:
de Vries MR and Quax PHA (2018) Inflammation in Vein Graft Disease. Front. Cardiovasc. Med. 5:3. doi: 10.3389/fcvm.2018.00003

INTRODUCTION

Occlusive atherosclerotic disease is a leading cause of mortality and morbidity worldwide. The most commonly used revascularization strategies to unblock or circumvent atherosclerotic lesions are balloon angioplasty (with or without stenting), endarterectomy and bypass surgery. For patients with left main coronary artery disease (CAD), three-vessel CAD and patients with late-stage peripheral artery occlusive disease (PAOD) bypass surgery is the primary standard of care (1–4). For patients receiving a single graft the left internal mammary artery is the graft of choice, since these give the best patency rates (5, 6). However, for bypassing multiple lesions, complex lesions or long diffuse lesions (especially in peripheral artery disease) veins are frequently necessary as a conduit, **Figure 1A**. Among veins the great saphenous vein is the most obvious conduit and is almost exclusively used as graft in patients with PAOD (7). Advantages of the saphenous vein include the length, which allows the use for multiple grafts, its superficial location for easy accessibility and the expendability (after removal of the vein the surrounding tissue is still perfused by other vessels). Unfortunately, patency rates of vein grafts are poor compared to arterial grafts (1). Due to acute thrombosis patency rates of vein grafts decrease with 10% within the first month (1). Intimal hyperplasia and accelerated atherosclerosis lead to a 40% overall patency after 10–20 years, **Figures 1B,C** (8, 9). Risk factors associated with vein graft disease (VGD) include age, race, gender, hypercholesterolemia, diabetes mellitus, and chronic kidney disease (10–14). Also factors associated with the surgery contribute to reduced patency. These include the location and quality of the artery where the bypass will be attached, and quality and handling of the venous conduit. Collection of venous conduits with the so called "no touch technique" in which veins are harvested including perivascular fat improve patency rates (15).

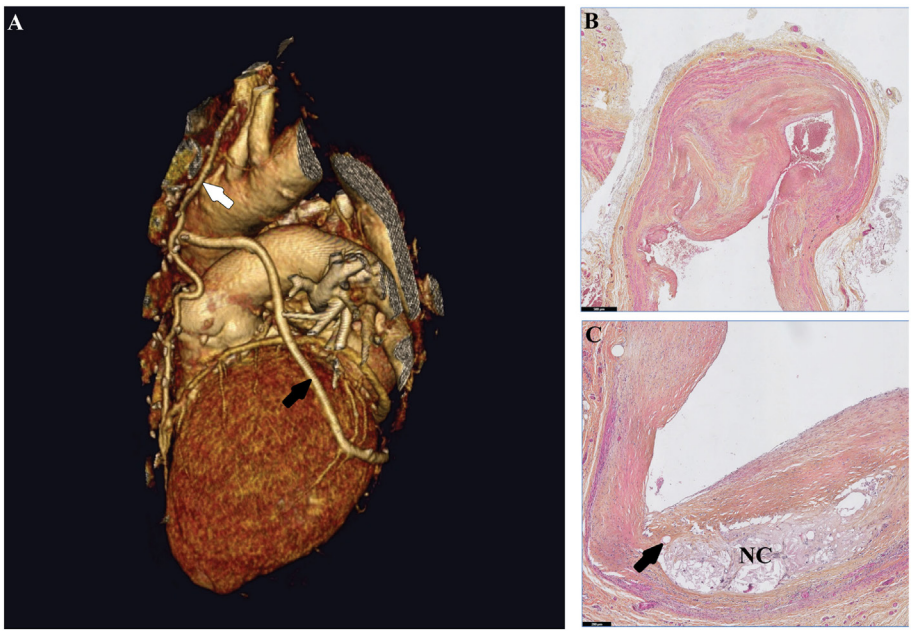

FIGURE 1 | Human vein grafts in macroscopic and microscopic views. **(A)** 3D reconstruction of a heart. In this CT scan, a saphenous vein segment (black arrow) is grafted from the aorta to the ramus circumflexus. The left internal mammarian artery graft (white arrow) is connected to the left anterior descending coronary artery. **(B)** A failed human saphenous vein graft displaying in the intimal hyperplasia, extensive smooth muscle cell accumulation, and extracellular matrix deposition. **(C)** Accelerated atherosclerosis in a human vein graft lesion is characterized by a decellularized necrotic core with cholesterol crystals and calcification (NC) and neovessels (arrow).

In this review, we emphasize the role of inflammatory processes during vein graft remodeling and show how inflammation is involved in all phases leading to VGD, **Figure 2**. Currently, statins and aspirin are the only treatment options recommended for both CAD and PAOD patients (4, 16–18). Although a lot of research is performed on new targets and therapies it is somewhat disappointing that no effective new strategies that prevent VGD have come up. The recent published results of IL1β inhibition with canakinumab resulting in positive effects on atherosclerosis (19) are very encouraging for new studies targeting inflammation in VGD. In this review, we discuss the pathophysiology of vein grafts and the role of inflammatory mediators during this process based on preclinical and clinical research.

VEIN GRAFT REMODELING

Remodeling of the vessel wall of the vein grafts is a crucial process during all subsequent stages of VGD. The initial remodeling event is the distension of the venous segment during surgical harvesting and subsequent controlling for proper ligation of all side branches. Usually this is done by checking the lack of leakage of fluids *via* these side branches when pressure is inflicted on the isolated venous segment, leading to a profound distension of the venous segment. In the next stage, directly after grafting the venous segment in the arterial circulation, the vein graft will be distended again due to the exposure to the arterial blood pressure. Both forms of distension lead to serious damage of the vessel wall (20). Not only endothelial cells become damaged or activated but also the media becomes activated due the distension injury, leading to activation of smooth muscle cells (SMCs) and degradation of several components of the extracellular matrix (ECM) in the media as well as the adventitia. These degradation products of matrix elements like hyaluronic acid, proteoglycans and fibronectin are damage-associated molecular patterns (DAMPs), which can act as endogenous ligands for toll-like receptors (TLRs), thus triggering an initial inflammatory response in vein graft remodeling. Moreover, the ischemia-reperfusion period during and after surgery can lead to generation of DAMPs and as well as reactive oxygen species, resulting in damage of vascular cells and upregulation of cytokines (1). Within the first days to weeks this results in influx of inflammatory cells in the vein graft vessel.

The next step in vein graft remodeling relates to the adaptation of the venous segment to the arterial blood pressure. In the media an arterialization process is initiated based on the proliferation of SMCs. This initially beneficial vascular remodeling process, however, may result in an uncontrolled proliferation and migration of SMCs and even myofibroblasts originating from the adventitia and triggers intimal hyperplasia (1). The concomitant outward remodeling of the vein grafts usually compensates for the pathological lumen loss. However, outward remodeling does not occurs always, resulting in situations in which neointima formation leads to inward remodeling as a result of pathological intimal hyperplasia and lumen loss, **Figure 3** (21, 22). This is often accompanied or even enhanced by infiltration of inflammatory cells, mainly macrophages, into the vein graft wall (23). Moreover, in the later stage of vein graft remodeling, under hypercholesterolemic

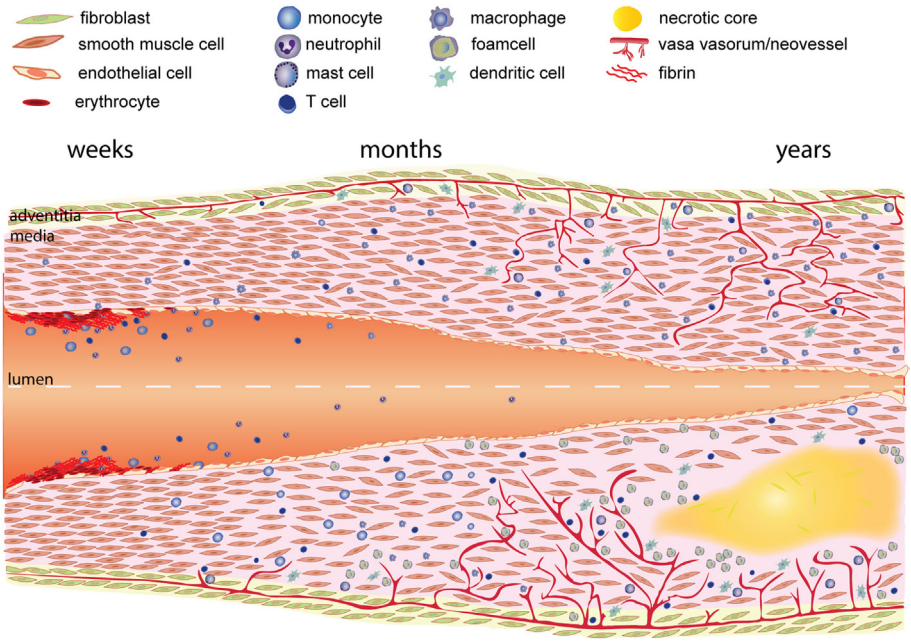

FIGURE 2 | Time course of vein graft development. As a result of the vein graft procedure the endothelial layer is damaged resulting in coverage of the luminal surface by fibrin. White blood cells (neutrophils, monocytes, and lymphocytes) attach and infiltrate the fibrin layer and intima. Next activated smooth muscle cells in the media and fibroblasts in the adventitia are and start migrating toward the intima, forming the intimal hyperplasia. Migration and proliferation of smooth muscle cells is enhanced by growth factors and cytokines released by cells in the vessel wall, and especially inflammatory cells. Growth factors and cytokines also induce extracellular matrix deposition, resulting in further growth of the intimal hyperplasia. The lower part of the figure describes the process of vein graft remodeling as it occurs under atherosclerotic conditions (lower part). Typically macrophages in the vessel wall engulf lipids and become foam cells. Subsequently a necrotic core is formed by dying cells and cholesterol depositions. Hypoxia in the vessel wall induces the growth of plaque neovessels.

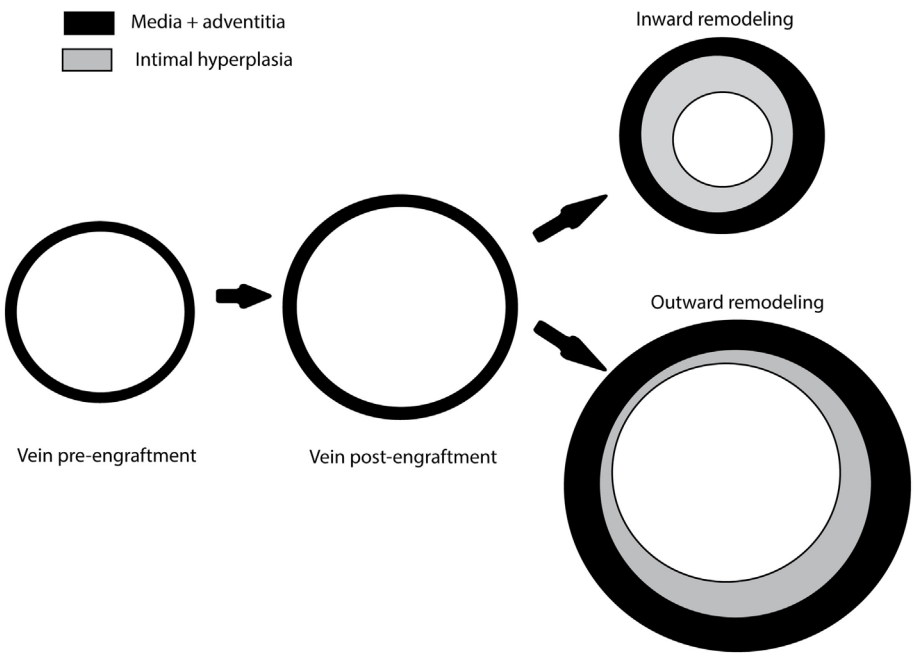

FIGURE 3 | Vein graft remodeling. Damage caused by graft handling and distension during the high-pressure check for leakage as well as implantation in the high blood pressure surrounding of the arterial circulation results in distention of the venous graft. Depending on local and systemic influences like inflammatory factors, this can result in inward remodeling characterized by intimal hyperplasia and a reduced lumen or outward remodeling characterized by moderate intimal hyperplasia and an increased lumen.

conditions, uptake of lipids cause macrophages to turn into foam cells. Macrophage apoptosis leading to necrotic core formation and intraplaque hemorrhage further accelerates the process of VGD by formation of atherosclerotic lesions with an unstable phenotype (1). These accelerated atherosclerotic lesions in the vein grafts represent an end stage in vein graft remodeling which cause long-term (>2 years) vein graft failure (8, 24).

CONTRIBUTION OF VESSEL WALL CONSTITUTIONS TO VGD

Endothelial Cells

One of the first critical events that a vein has to withstand is a period of ischemia followed by reperfusion during and directly after surgery. In addition graft handling also causes damage to the grafts as well as distension that occurs during the high-pressure check for leakage (1). This leads to increased oxidative stress and cytotoxic activation, which on its turn results in endothelial cell loss (25, 26). Remaining endothelial cells can become apoptotic, damaged, or activated, as shown by expression of ICAM 1, VCAM1, and selectins (27–29). Damaged endothelium shows impaired vasorelaxation as a result of reduced endothelial nitric oxide synthase and NO production (30). The increase in oxidative stress and damage to the endothelium is in particularly seen in vein grafts compared to arterial graft (31, 32).

Following endothelial denudation, the ECM components underneath the endothelium such as collagen, elastin, and proteoglycans become exposed and can trigger coagulation processes leading to fibrin deposition on the luminal surface (33). Fibrin formation and fibrin resolution is tightly regulated by thrombosis and fibrinolysis.

Re-endothelialization begins rapidly after the initial damage. Proliferating endothelial cells are observed within the first week after vein graft surgery in experimental models resulting in a nearly intact endothelial lining 4 weeks after the surgery (27, 28, 33, 34). The duration of the re-endothelialization process in humans is not exactly known but it is likely that this takes somewhat more time. The endothelium-dependent relaxation as observed in human vein grafts, indicates that the grafts have seemingly functional endothelial cells (35). It is unknown whether graft endothelial cells in humans originate from the graft, the adjacent arterial tissue or from the circulation progenitor cells or a combination thereof. In a murine vein graft model it was shown that endothelial cell originate primarily from host vasculature instead of the donor vein (36). Interestingly, in humans allografts both host derived and donor derived endothelial cells were found (37).

Both circulating and local (adventitial) progenitor cells have been shown to contribute to re-endothelialization (38–40). Inducible nitric oxide synthase enhances endothelial progenitor cell attachment and differentiation (41). Homing of these progenitor cells is directed by inflammatory-type macrophages and is most probably integrin β3 dependent (42, 43).

Despite the fact that enhancement of re-endothelialization is beneficial in preventing VGD in preclinical studies, no effective therapeutic approaches exist to facilitate this process (1). Therefore, a potential future therapeutic target in which the inflammatory reaction may play a role could be the promotion of endothelial progenitor cell homing to the damaged endothelium in the grafts.

Smooth Muscle Cells

Proliferation and migration of SMCs are key elements in intimal hyperplasia formation. During harvesting and engraftment, SMCs within the vein graft are exposed to ischemia resulting in SMC apoptosis (25, 44, 45). Remaining SMCs can change from a quiescent contractile phenotype to a dedifferentiated, proliferating synthetic phenotype. These cells can migrate from the media to the intima of the graft. Alternatively SMCs may migrate from the anastomosed artery toward the intima of the graft (46, 47). Both arterial and venous SMC have been shown to contribute to the intimal hyperplasia in vein grafts (48, 49). Interestingly, after engrafting of a venous segment in the arterial circulation venous marker Ephrin B4 was decreased pointing toward a loss of venous identity during arterialization (50).

Smooth muscle cells in vein grafts express different growth factors such as PDGF, TGF-β, vascular endothelial growth factor, and endothelin-1, which are major stimulators of intimal hyperplasia formation (51–54). Targeting of growth factors or their receptors in preclinical models interfere with this intimal growth (55–58). Arterial and venous grafts display a different pattern of expression of growth factors and signal transduction pathway factors (45, 48, 59), which might contribute to the lower patency rates of venous grafts. Venous SMCs show enhanced MAPK dependent proliferation in comparison to arterial SMCs (60). SMCs and especially activated SMCs produce cytokines such as tumor necrosis factor alpha (TNFα) and C-C motif chemokine CCL2 (also known as MCP-1) (61, 62), which can increase the Rho/Rac GTPase signaling cascade leading to enhanced SMC migration and proliferation (63).

Veins possess elastin fibers but lack defined external and internal elastic lamina. Therefore, fibroblasts when migrating from the adventitia to the intima in veins encounter little barriers. These adventitial fibroblasts are highly proliferative. Adventitial fibroblasts that acquire a smooth muscle-like phenotype are known to contribute to intimal hyperplasia formation (64, 65).

Adventitial and bone marrow-derived progenitor cells also contribute to the thickening of the vein graft wall (38, 39, 66, 67). Already during vein graft surgery the bone marrow releases functional active $CXCR4^+$ progenitor cells (68). Although a substantial portion of the bone marrow-derived cells express a SMC phenotype, they do not fully acquire the complete SMC lineage phenotype (69). Cytokine dependent activation of Stem Cell Antigen-1^+ positive bone marrow-derived progenitor cells results in enhanced SMC migration and proliferation (70). Vein grafting in mice deficient in CXCR4 resulted in reduced vein graft thickening (71). Also knock down of fibroblast-specific protein-1 in bone marrow cells inhibited intimal hyperplasia (72).

Extracellular Matrix

In the initial phase after vein graft surgery exposure of components of the ECM interact with plasma components and platelets and contribute to the thrombogenic luminal surface of the vein graft. ECM components such as fibronectin, heperansulphate

and other proteoglycans can act as DAMPs which interact with pattern recognition receptors like TLRs, thereby initiating a proinflammatory response, primarily directed by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (73). The ECM component hyaluronic acid is especially found in arterialized vein grafts and contributes to vein graft intimal thickening by inducing SMC proliferation (74). In the first phases of vein graft remodeling, upregulation of TGF-β is linked to increased mRNA expression of collagen I, and collagen III (75, 76). In a rabbit vein graft model late stage remodeling (>1 month) enhanced TGF-β expression was observed which was accompanied by increased fibrosis and reduced matrix metalloproteinase (MMP) 2 and MMP9 activity (77). In rats inhibition of TGF-β signaling resulted in reduced intimal hyperplasia as a result of increased MMP activity (58). The proteases that degrade collagen and other components of the ECM are highly expressed in vein grafts, especially MMP2 and MMP are extensively researched (78–81). MMPs can be secreted by both macrophages and SMC in the vessel wall, however, most interactions with the MMP system results in effects on SMC migration and proliferation and ECM build up. Gene therapeutic approaches in saphenous vein SMCs to silence expression of MMP2 and MMP9 demonstrated reduced SMC migration through a matrigel barrier (82). Moreover, in MMP9 knockout mice the lesion composition was changed due to an increase in collagen content while intimal hyperplasia was similar as in control mice after vein grafting (83). Inhibition of MMPs by the general MMP inhibitor doxycycline resulted in decreased intimal hyperplasia formation in murine vein grafts (80). Overexpression of tissue inhibitors of MMPs (TIMP) 1, 2, and 3 in vein graft models in various experimental animals resulted in intimal hyperplasia formation due to reduced SMC migration and proliferation and inhibition of MMP activity as well as reduced infiltration and migration of inflammatory cells (84–88).

Plasmin, formed by activation of plasminogen by plasminogen activators, can contribute to activation of MMPs and can cleave ECM components, such as laminin and fibronectin. These ECM degradation products subsequently can bind to pattern recognition receptors (89). Failed human vein grafts demonstrated an increased expression of members of the plasminogen activation system (90, 91). The plasminogen activation system consists of two main PAs, urokinase-type PA (uPA) and tissue-type PA (tPA). uPA is essential in extracellular proteolysis, cell migration, and matrix remodeling, while tPA is mainly involved in fibrinolysis (92). In porcine vein grafts overexpression of tPA reduced early vein graft thrombosis (93). Adenovirus mediated overexpression in human saphenous explants of a hybrid protein consisting of the receptor-binding amino terminal fragment (ATF) of urokinase and bovine pancreas trypsin inhibitor (BPTI) was able to potently reduce intimal hyperplasia formation (94). A hybrid protein consisting of TIMP1 and the aforementioned ATF was constructed to inhibit MMP activity locally at the cell surface (95). This construct reduced vein graft thickening and preserved the luminal area (96). A third protein was constructed by combining the three constructs resulting in TIMP1.ATF.BPTI that was capable of inhibiting both plasmin and MMP activity at the cell surface, which effectively reduced vein graft intimal hyperplasia and outward remodeling (97). Plasminogen activator inhibitor 1 (PAI1), another plasminogen activator, showed also clear effects on vein graft remodeling. PAI1-deficient mice showed enhanced intimal hyperplasia due to increased thrombin activity (98).

INFLAMMATION

The immune system plays an important role during all phases in vein graft development (99, 100). Immediately after surgery DAMPs are released which *via* binding to the TLRs activate the cells in the vessel wall resulting in the release of growth factors and cytokines. DAMPS can also activate platelets and thus enhance, due to the platelet expressed adhesion molecules P- and E-selectin, the attachment of circulating leukocytes and subsequent infiltration into the vessel wall (101). Neutrophils are mainly detected on the de-endothelialized lumen within the fibrin layer that is formed there (102). Neutrophils are phagocytes that also produce MMP2 and MMP9, as well as other proteases and a wide array of growth factors with which they can influence neighboring cells in the vessel wall (103). Monocytes enter the vein graft *via* adhesion to the luminal surface or *via* neovessels in the adventitia (104). Macrophage colony stimulating factor turns these invasive monocytes into macrophages. Macrophages represent the vast majority of inflammatory cells in the vein graft wall and by producing and releasing various cytokines and growth factors influence intimal hyperplasia formation (105). Direct or indirect inhibition of macrophages, by targeting macrophage activating factors have been shown to be successful strategies in preventing the inflammatory response and VGD (61, 106, 107). In addition, various types of inflammatory cells seem to be derived from the adventitia, which consists of loose connective tissue, small neovessels, and nerves. Dendritic cells, mast cells, natural killer (NK) cells, T, and B cells are primarily found in the adventitia (108).

The late phase of vein graft development is characterized by oxidized low-density lipoprotein (ox-LDL) retention and subsequent lipid accumulation (24, 34). Phosphorylcholine is one of the neoantigens exposed by LDL oxidation that can elicit an immune response. Passive immunization with anti-phosphorylcholine antibodies resulted in a reduced inflammatory phenotype which prevents vein graft atherosclerosis in a hypercholesterolemic murine model (109). Interestingly, low levels of natural antibodies against phosphorylcholine in humans are associated with VGD (110). Phosphorylcholine is one of the many ligands for TLRs, like the DAMPs that are expressed upon damage to the vein graft wall. TLRs are central in the induction of inflammatory responses in vascular cell types, and can activate inflammatory cells of both the innate and adaptive immune system (1, 73, 111). TLR activation generally lead *via* the myeloid differentiation primary response protein 88 (MyD88) pathway, to activation of NF-κB and results in induction of cytokines (112). In general, proinflammatory cytokines stimulate vein graft remodeling (99, 113–115). These cytokines activate inflammatory cells but also stimulate SMC migration and proliferation as well as activation of endothelial cells.

NF-κB is one of the most important transcription factors for promotion of inflammatory responses in vein graft remodeling (1).

Inhibition of NF-κB resulted in reduced inflammatory responses and attenuation of vein graft thickening in experimental models (116–118). The downstream NF-κB targets CCL2 and TNFα both have been linked to VGD (119, 120). Overexpression of a dominant negative form of CCL2 reduced accelerated atherosclerosis and monocyte invasion in vein grafts in mice on a hypercholesterolemic diet (61). Furthermore, lentivirus mediated overexpression of a shRNA silencing the CCL2 receptor, CCR2, inhibited SMC proliferation and migration and reduced vein graft thickening (121). TNFα is one of the early up-regulated factors in vein graft development. This induction is thought to be a result of the early activation of TLRs. In TNF receptor-1-deficient mice, reduced CCL2 expression and SMC proliferation resulted in reduced vein graft intimal hyperplasia (122). Furthermore, TNF receptor-2-deficient mice also showed reduced vein graft thickening as a result of endothelial cell apoptosis (123). An overview of the various inflammatory factors that are linked to VGD is given in **Table 1**.

IMMUNE MODULATION

Toll-Like Receptors

As indicated above, TLRs play a crucial role in the early inflammatory triggers that initiate vein graft remodeling. Among the first mediators of inflammation in vein grafts are DAMPs such as aggrecan and heat shock proteins (135, 136). Endogenous DAMPs activate TLRs that are expressed by cells in the vein graft wall such as endothelial cells and SMCs (1, 73, 137). Exaggerating this response by applying low dose lipopolysaccharide, a strong TLR4 ligand, topically on the vein graft resulted in a strong induction of the inflammatory response and increased intimal thickening (126). Blocking TLR4 in a murine vein graft model, either by genetic deletion or by lentiviral mediated local shRNA silencing, reduced outward remodeling and intimal hyperplasia formation, due to the suppressed inflammatory responses (73). Ligation of the carotid artery in TLR4-deficient mice showed outward remodeling without intimal hyperplasia formation in the non-ligated artery (111). It is therefore suggested that TLR4 affects hemodynamic adaptations and vascular remodeling independently of intimal hyperplasia formation (1). Inhibition of the TLR4 homolog radioprotective 105 aggravated intimal hyperplasia formation in vein graft by increased proinflammatory macrophage proliferation and enhanced SMC migration and proliferation (127). Comparable results were found in vascular remodeling models for restenosis and arteriovenous fistula (138, 139). Whereas in atherosclerosis models a reduction of atherosclerosis could be observed due to the specific function of RP105 on B cells and inhibition of CCR2 dependent macrophage migration (140, 141). Next to the role of TLRs, other components of the innate immune system such as members of the complement cascade are linked to vein graft remodeling.

TABLE 1 | Inflammatory factors involved in vein graft disease (VGD).

Target/treatment	Effect on VGD	Experimental animal	Reference
Notch ligand delta-like 4	+	Mouse	Koga et al. (107)
Dexamethasone	–	Mouse	Schepers et al. (124)
Annexin A5	–	Mouse	Ewing et al. (125)
Phosphorylcholine antibodies	–	Mouse	Faria-Neto et al. (109)
Il1	+	Mouse	Yu et al. (115)
NF-κB	+	Dog	Shintani et al. (116)
NF-κB	+	Rabbit	Miyake et al. (117)
NF-κB	+	Rat	Meng et al. (118)
MCP-1/CCL2	+	Dog	Tatewaki et al. (119)
MCP-1/CCL2	+	Mouse	Fu et al. (120)
MCP-1/CCL2	+	Mouse	Schepers et al. (61)
CCR2	+	Mouse	Eefting et al. (121)
TNF-R1	+	Mouse	Zhang et al. (122)
TNF-R2	–	Mouse	Zhang et al. (123)
TLR4	+	Mouse	Karper et al. (73)
TLR4	+	Mouse	Nguyen et al. (126)
RP105	+	Mouse	Wezel et al. (127)
C1 inhibitor	–	Mouse	Krijnen et al. (128)
C3	+	Mouse	Schepers et al. (129)
C5a	+	Mouse	de Vries et al. (130)
C5a	+	Mouse	Wezel et al. (131)
Mast cell	+	Mouse	de Vries et al. (130)
Mast cell	+	Mouse	Wu et al. (132)
Natural killer cells	+	Mouse	de Vries et al. (133)
Interferon regulating factor 3 and 7	–	Mouse	Simons et al. (134)

Complement System

The complement cascade is a large family of acute response effector and regulatory proteins that is a prominent member of the innate immunity. Vein graft surgery activates the complement system and continues during the vein graft remodeling process since complement factors are present and produced locally in the vein graft wall (129). Inhibition of the key complement factor C3 resulted in reduced intimal hyperplasia by reducing inflammatory cell influx in murine vein grafts (129). C1inh a natural occurring protease inhibitor of the serpin family and alternative pathway component prevented endothelial cell damage in *ex vivo* perfused human saphenous vein segments and reduced vein graft intimal hyperplasia in a murine model (128). In the same *in vitro* perfusion model it was shown that the endogenous complement inhibitor, the C4b-binding protein, was present in the saphenous vein wall and has protective mechanisms to cellular stress and inflammation (142). C5a is one of the major biologically active components of the complement cascade downstream of C3 and exerts its function including chemotaxis of monocytes and mast cells mainly *via* the canonical C5a receptor. Local application of C5a on the vein graft resulted in increased intimal hyperplasia in a mast cell dependent manner (130). Furthermore, acute application of C5a results in enhancement of plaque disruption (131). Inhibition of complement factors seems to be a very promising strategy for preventing VGD in humans. Most interestingly, the mortality in high-risk surgical patients undergoing CABG surgery was reduced by intravenous administration of an antibody against complement factor C5 (pexelizumab) (143).

Mast Cells

Mast cells are large granular cells that upon activation by IgE, cytokines (TNFα, IL1) and complement factors release granules containing tryptase, chymase, and histamine (144). Vein grafts

are rapidly repopulated with mast cells; it should be noted that resting as well as activated mast cells can be found mainly in the perivascular region of vein grafts but not so much in the vessel wall itself (130, 132, 145). Mast cell-deficient mice showed a reduction in intimal hyperplasia in vein grafts, as well as a general reduction of vascular inflammation (130, 132). Moreover, activation of mast cells locally resulted in more unstable lesions and features of plaque rupture (130). The strong effect of mast cells on lesion instability is also seen in native atherosclerosis (146). Remarkably, in these lesions, most mast cells were found in the close vicinity of plaque neovascularization (146).

NK Cells

Also present in the perivascular region of vein grafts and especially in the adventitia are NK cells (133). Upon activation NK cells secrete lytic granules containing perforin and granzymes and various proinflammatory cytokines (147). The NK cell function is reduced in BALB/C mice due to the lack of crucial NK cell genes of the Ly49 receptor family. When vein graft surgery was performed in BALB/c mice congenic for the C57BL/6 NK gene region, these mice displayed a similar degree of intimal hyperplasia as C57BL/6 mice, while BALB/c mice showed significantly less vein graft remodeling and intimal hyperplasia (133). Furthermore, a decrease in inflammatory cells and interferon-γ expression in the vein graft wall was observed.

Dendritic Cells

Dendritic cells, originating from Ly-6C^{lo} monocytes, are found in all layers of the vein graft and colocalize with T cells as antigen presenting cells (148). In vein grafts dendritic cells are capable of triggering T cells by costimulation of CD40 (149).

The involvement of adaptive immunity members in VGD is less established than the role of the innate immunity. The participation of the adaptive immune system in vascular diseases is clear and the role in atherosclerosis and restenosis is well described (150).

T and B Cells

T and B cells have been identified in vein graft lesions, however, no further characterization of subtypes are performed (37, 151). It has been shown that T cells are capable of proliferation in vein grafts (152). Furthermore, interaction between dendritic cells and T cells in a CD40 dependent manner have been observed in vein grafts (149). However, little is known about the exact function and role of T cells in the pathophysiology of VGD. In a recent study, we demonstrated that downstream TLR signaling *via* interferon regulatory factor (IRF) 3 and 7 results in a protective effect on vein graft remodeling. This is particularly of interest since IRF3 and IRF7 activation leads to expression of type1 interferons, that are subsequently involved in the activation of CD4 and CD8^{+} T cells (134). Further studies to investigate the role of (subtypes) of T as well as B cells in VGD are definitely needed.

PERIVASCULAR ADIPOSE TISSUE (PVAT)

Most blood vessels, including the saphenous vein, are surrounded by PVAT. In the last decades the vasoactive role of PVAT and adipokines derived from PVAT on vascular function are more and more appreciated (153). PVAT harbors numerous amounts of inflammatory cells. Damage to PVAT results in an inflammatory response driven by adipocyte-derived factors such as resistin, leptin, or the cytokines IL-6, TNF-α, and CCL2 (154). Protective effects of adiponectin on NADPH oxidase, superoxide production and NO bioavailability in the vessel wall are reduced after PVAT damage (155). The "no touch" technique of saphenous vein harvesting is in part based on the beneficial effects of preservation of PVAT and PVAT derived leptin (155, 156). Interestingly, PVAT surrounding different blood vessels differs in its response to injury. Different responses are found between PVAT surrounding saphenous veins and internal mammary arteries (157) but also between internal mammary arteries and coronary arteries (158), pointing to a cause of the encouraging patency rates of internal mammary arteries.

ACCELERATED ATHEROSCLEROSIS AND LATE STAGE FAILURE

Comparable to native atherosclerosis, hypercholesterolemia, an import driver of VGD and lipid burden, is clearly associated with vein graft age (159). Analysis of human vein grafts obtained at autopsy has shown that coronary vein grafts undergo rapid atherosclerotic lesion development (24). Lesions in coronary vein graft differ from native lesions in having a more concentric and diffuse appearance. Furthermore, the tendency to rupture and occlude due to thrombosis is very high in these vein grafts (8). Especially, older vein grafts (>2 years) fail most frequently due to accelerated atherosclerosis and rupture of lesions (8, 160, 161). Coronary bypass graft occlusion is clearly associated with presence of necrotic core, calcification and negative remodeling (162). Peripheral vein grafts probably suffer less from accelerated atherosclerosis, since these lesions mostly consist of SMCs (163). Occlusion of peripheral vein grafts is frequently linked to high rates of circulating inflammatory cells (100). Circulating inflammatory cells are now being studied as predictors for VGD; both ratios of platelet-monocyte reactivity or lymphocytes-to-monocytes ratios show correlations with VGD (100, 164).

Foam cell formation is already observed in one year old vein grafts. This is followed by necrotic core development between 2 and 5 years after surgery. Intraplaque hemorrhage, most likely originating from leaky angiogenic neovessels in the lesion is also observed in these advanced lesions (24). Plaque angiogenesis and intraplaque hemorrhage are important causes of plaque destabilization and rupture (165). In vein grafts in hypercholesterolemic mice various features linked to late phase graft failure are observed, including angiogenic neovessels, intraplaque hemorrhage, necrotic cores and rupture (1, 88). Especially the presence of plaque neovessels and intraplaque hemorrhage in this model are interesting, since this is a rare observation in atherosclerotic experimental murine models. Improved lesion stability and decreased plaque rupture could be achieved by up regulation of the MMP inhibitor TIMP-1 (88). Targeting inflammatory factors such as annexin A5, mast cells, complement factors and TLRs are effective strategies to not only inhibit intimal hyperplasia

formation and accelerated atherosclerosis but also to alter plaque composition and reduce plaque rupture (125, 127, 130, 131).

CLINICAL PHARMACOLOGICAL AND SURGICAL INTERVENTIONAL STRATEGIES

Platelet activation and thrombin production are key triggers of early vein graft failure. Antiplatelet therapy starting directly after surgery to prevent early vein graft thrombosis is recommended for both CAD and PAOD patients. Aspirin treatment alone or dual antiplatelet (aspirin and clopidogrel) treatment have been shown to be effective in preventing graft occlusion (166, 167). In both Europe and USA, antiplatelet therapy is recommended to be continued until at least 3 months after the surgery and in some cases indefinitely (4, 17).

Comparable to native atherosclerosis statins are included in the standard of care for patients undergoing vein graft surgery. The mode of actions of statins is primarily cholesterol lowering by inhibiting HMG-CoA reductase but other mechanisms are also described. Statins can improve endothelial function, prevent proliferation of SMCs and decrease activation of macrophages (168, 169). Statin therapy has been proven to prevent vein graft stenosis in both coronary and peripheral grafts (170–172).

A new therapy to prevent VGD is the use of an extravascular support. The extravascular support functions as a protective outer layer of the vein graft, thereby reducing wall tension, activation and stretching of SMCs and endothelial cells (173). Promising results are obtained in *in vitro* and experimental studies (174–177). The recently reported positive preliminary clinical results from a study by Ferrari et al. using an external mesh demonstrate the possibility to improve long-term graft durability (178). The VEST trial showed an improvement in lumen uniformity after external stenting 1 year after CABG surgery in comparison to non-stented vein grafts in the same patients (173, 179, 180). Further elaboration on these studies is needed to solidify the concept of extravascular support on graft patency.

CONCLUSION

The use of vein grafts as a revascularization strategy is still necessary despite the unfavorable patency outcomes. Constrictive remodeling, intimal hyperplasia formation, and unstable atherosclerotic lesions are the main causes of VGD in both coronary and peripheral vein grafts. Histopathological studies of human vein grafts and experimental vein graft models have demonstrated that inflammatory components, especially those from the innate immune system, are crucial in all stages of vein graft development. Additional studies are required to prevent VGD and test new strategies for the treatment of vein grafts. Targeting inflammation either in a broad form or in a very specific has great potential as revascularization strategy for failing grafts.

AUTHOR CONTRIBUTIONS

MV and PQ designed and wrote the manuscript.

FUNDING

This work was supported by a grant from the European union, Horizon 2020 MSCA joint doctoral project, MOGLYNET (project 675527).

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

frontiers in Cardiovascular Medicine

REVIEW
published: 05 February 2018
doi: 10.3389/fcvm.2018.00006

Krüppel-Like Factors in Vascular Inflammation: Mechanistic Insights and Therapeutic Potential

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The role of inflammation in vascular disease is well recognized, involving dysregulation of both circulating immune cells as well as the cells of the vessel wall itself. Unrestrained vascular inflammation leads to pathological remodeling that eventually contributes to atherothrombotic disease and its associated sequelae (e.g., myocardial/cerebral infarction, embolism, and critical limb ischemia). Signaling events during vascular inflammation orchestrate widespread transcriptional programs that affect the functions of vascular and circulating inflammatory cells. The Krüppel-like factors (KLFs) are a family of transcription factors central in regulating vascular biology in states of homeostasis and disease. Given their abundance and diversity of function in cells associated with vascular inflammation, understanding the transcriptional networks regulated by KLFs will further our understanding of the pathogenesis underlying several pervasive health concerns (e.g., atherosclerosis, stroke, etc.) and consequently inform the treatment of cardiovascular disease. Within this review, we will discuss the role of KLFs in coordinating protective and deleterious responses during vascular inflammation, while addressing the potential targeting of these critical transcription factors in future therapies.

Keywords: Krüppel-like factor, Krüppel-like transcription factors, vascular inflammation, atherosclerosis, endothelial cells, vascular smooth muscle cells, macrophages

OPEN ACCESS

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

Received: *03 November 2017*
Accepted: *17 January 2018*
Published: *05 February 2018*

Citation:
Sweet DR, Fan L, Hsieh PN and Jain MK (2018) Krüppel-Like Factors in Vascular Inflammation: Mechanistic Insights and Therapeutic Potential. Front. Cardiovasc. Med. 5:6. doi: 10.3389/fcvm.2018.00006

INTRODUCTION

The role of inflammation in vascular disease is well recognized, involving dysregulation of both circulating immune cells as well as the cells of the vessel wall itself. Upon exposure to noxious stimuli (altered hemodynamics, circulating inflammatory factors, and oxygenation level), the vessel wall undergoes characteristic changes such as endothelial cell (EC) activation and vascular smooth muscle cell (VSMC) proliferation and migration, leading to the presentation of a "sticky" surface attractive to circulating monocytes and other immune cells. In certain contexts (e.g., acute thrombotic occlusion), this inflammation results in beneficial vascular remodeling that maintains proper perfusion to ischemic organs. During chronic insults, such as in dyslipidemia, unrestrained vascular inflammation leads to pathological remodeling that eventually contributes to atherothrombotic disease and its associated sequelae (e.g., myocardial/cerebral infarction, embolism, and critical limb ischemia).

Signaling events during vascular inflammation orchestrate widespread transcriptional programs that affect the functions of ECs, VSMCs, and circulating inflammatory cells. Central to many of these programs is the nuclear factor (NF)-κB signaling cascade. Upon stimulation by inflammatory stimuli [cytokines, oxidized low-density lipoprotein (oxLDL), glucose], regulatory cytosolic protein

IκB is phosphorylated and targeted for ubiquitin–proteosome degradation. This liberates the normally sequestered cofactors p65 and p50 to translocate into the nucleus, where they mediate pro-inflammatory transcription [reviewed in Ref. (1)]. In addition, the coactivator p300 can complex with p65/p50 to stabilize the chromatin structure for effective transcription (2). Outside of NF-κB signaling, regulation of vascular inflammation can also occur through microRNAs (miRs). miRs are non-coding RNAs that regulate post-transcriptional gene expression by inhibiting mRNA translation. Within the scope of vascular inflammation, miRs can either enhance or diminish pathological inflammation, depending on the target genes [reviewed in Ref. (3)]. Multiple facets of vascular cell biology, including NF-κB and miR signaling, are regulated by Krüppel-like factors (KLFs). Within this review, we will discuss the role of KLFs in coordinating protective and deleterious responses during vascular inflammation, while addressing the potential targeting of these critical transcription factors in future therapies.

KLFs BACKGROUND

Originally discovered as homologs to the *Drosophila melanogaster* gene, Krüppel (4), KLFs belong to a family of zinc-finger containing transcription factors with roles in cellular development, differentiation, metabolism, and activation. There are 18 currently predicted mammalian KLFs expressed in various tissues and during periods of development. KLFs share within their C-terminal regions three highly conserved zinc-fingers recognizing a 5′-C(A/T)CCC-3′ consensus sequence often near target genes, though the sequence can occur in distant regions as well such as in enhancers. The amino-terminus functions in transactivation or repression and participates also in protein–protein interactions (5). For many KLFs, there is considerable overlap in gene targets within a single cell type. However, despite the homology of structure, binding sequences, and protein interaction targets, there are also substantial differences in downstream transcriptional effects between KLFs. Several excellent reviews are available discussing sequence homology, chromosomal location, and expression pattern of the KLFs (5, 6). Given their abundance and diversity of function in cells associated with vascular inflammation, understanding the transcriptional networks regulated by KLFs will further our understanding of the pathogenesis underlying several pervasive health concerns (e.g., atherosclerosis, stroke, etc.) and consequently inform the treatment of cardiovascular disease.

EC KLFs

The vascular endothelium acts as an initial sensor and transducer of inflammatory stimuli such as disturbed blood flow, cytokines, oxLDL, and advanced glycation end products, often responding with activation of classical inflammatory cascades which have been elegantly dissected over the past few decades. While brief bouts of inflammation, particular during wound healing, are an appropriate physiologic response, endothelial dysfunction resulting in sustained, chronic inflammation is central to a diverse array of cardiovascular diseases. The transcriptional regulation of endothelial inflammation therefore remains of critical interest. An accumulating body of evidence now exists defining key roles for several KLF transcription factors in the control of vascular inflammation, which we review below (**Figure 1**).

Krüppel-Like Factor 2

Endothelial KLF2 has primarily anti-inflammatory, anti-thrombotic, and anti-migratory functions. As a regulator of inflammation, KLF2 inhibits both the expression of inflammatory cytokines and the production of adhesion molecules that are critical for leukocyte recruitment and extravasation (7–10). Through its binding to the transcriptional coactivator p300/CBP, KLF2 is capable of preventing NF-κB/p300 interaction and subsequent activation of the vascular cell adhesion molecule-1 (VCAM-1) promoter (11) Furthermore, the KLF2-p300 interaction permits KLF2 binding to the endothelial nitric oxide synthase (eNOS) promoter to induce transcription of this vasoprotective enzyme (11). These early studies demonstrated KLF2's ability to influence transcription through direct DNA-binding and or indirect cofactor sequestration mechanisms. In addition to affecting the NF-κB pathway, studies have shown that KLF2's anti-inflammatory effects are also produced through preventing the nuclear translocation of the inflammatory transcription factor ATF2 (9). In response to various stressors, JNK signaling leads to ATF2 nuclear translocation and successive inflammatory transcription. During shear stress, however, KLF2 induces cytoskeletal remodeling that eventually prevents JNK activation, and thus ATF2 translocation (12). Additionally, KLF2 provides protection against oxidative damage in ECs *via* the induction of hemeoxygenase-1 (13). Furthermore, ECs overexpressing KLF2 secrete atheroprotective miRs-143/145 in microvesicles that reduce atherosclerosis by targeting genes critical for VSMC dedifferentiation (*Mmp3*, *Elk1*, *Camk2d*) (14).

Thrombosis associated with atherosclerotic lesions contributes to many of the complications associated with atherosclerosis. Similar to its ability to repress endothelial inflammation, KLF2 modulates anti-thrombotic transcription. KLF2 binds directly to the promoter of thombomodulin-1, thereby increasing transcription of this potent anti-thrombotic and anti-inflammatory factor (8, 15, 16). Additionally, KLF2 inhibits the effects of thrombin-mediated endothelial activation by preventing transcription of thrombin's receptor, PAR-1 (17). *In vivo*, there is a clear association between KLF2 levels and vascular inflammatory disease. While complete knockout of KLF2 is embryonically lethal (18, 19), mice with hemizygous deletion of KLF2 ($KLF2^{\pm}$) are viable. $KLF2^{\pm}$ mice crossed with $ApoE^{-/-}$ mice are more susceptible to atherosclerotic disease compared with $KLF2^{+/+}ApoE^{-/-}$ mice (20). Additionally, post-natal deletion of KLF2 leads to a thrombotic phenotype, while globally overexpressing KLF2 protects mice from thrombus formation in part through the decreased expression of endothelial thrombotic genes (21).

Vascular inflammation is also a major player in the pathogenesis of diabetic vascular disease. Interestingly, hyperglycemia decreases endothelial KLF2 expression *via* FOXO1-dependent transcriptional silencing (22). Moreover, insulin induces KLF2 expression in ECs and KLF2 expression is reduced in the glomerulus of diabetic rats (23). Endothelial KLF2 is also implicated in vascular inflammation seen in neurodegenerative diseases such as Alzheimer's disease. Amyloid beta plaques, a hallmark

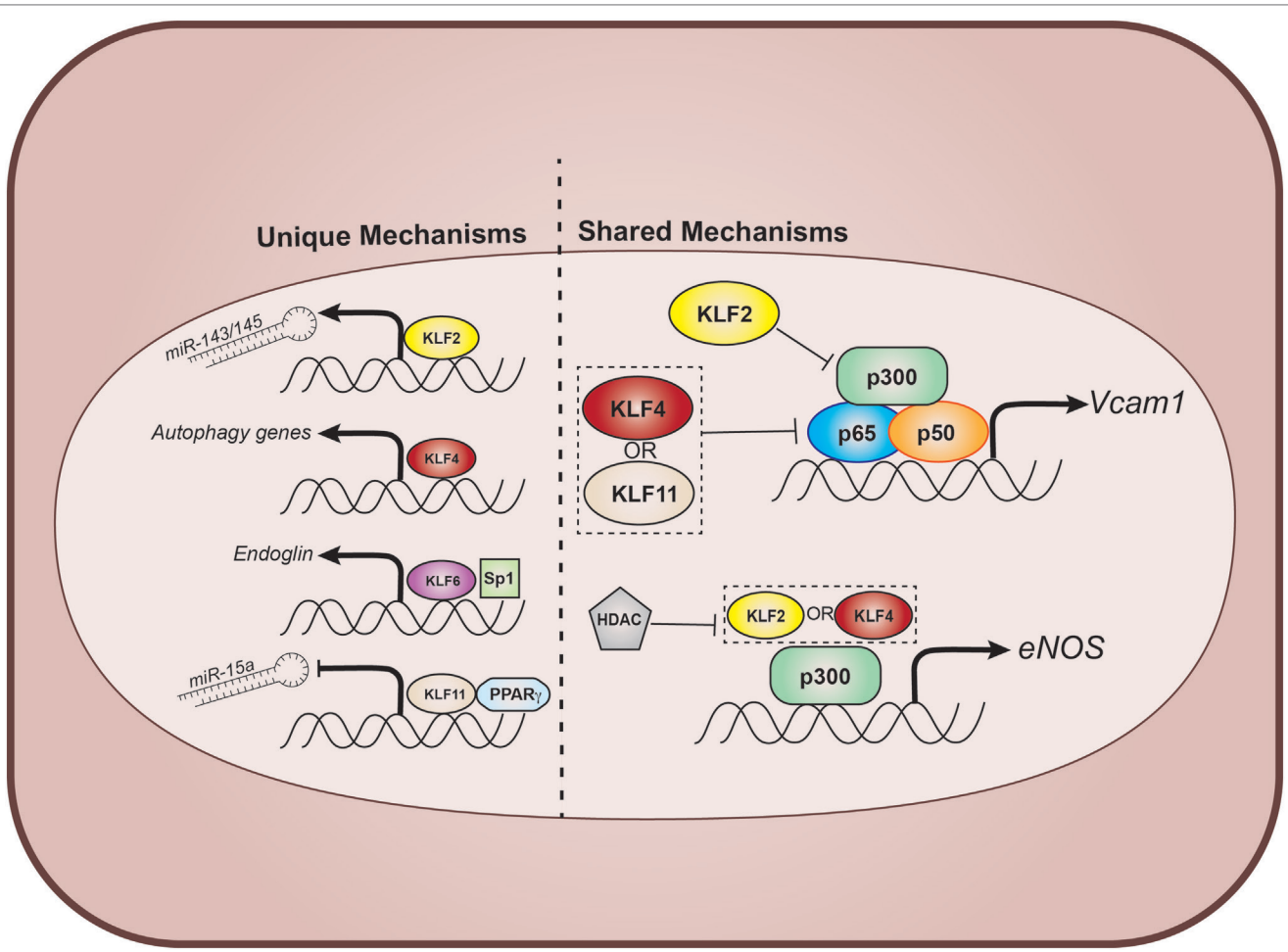

FIGURE 1 | Select effector functions of endothelial KLFs. Within ECs, there are both unique and shared gene targets and binding partners for various KLFs. In general, KLF2, 4, and 11 resist endothelial adhesive transcription by binding to, and inhibiting, multiple cofactors of nuclear factor-κB signaling. Additionally, KLF2 and 4 promote transcription of the vasoprotective factor, eNOS, *via* cooperation with p300. This process is inhibited by recruitment of HDACs to the eNOS promoter. KLFs also affect endothelial function during vascular inflammation through unique transcriptional events that include modulation of miRs and stimulating protective cellular component recycling through autophagy. KLF, Krüppel-like factor; EC, endothelial cell; HDAC, histone deacetylase; VCAM-1, vascular cell adhesion molecule-1; eNOS, endothelial nitric oxide synthase; miR, microRNA; Sp1, specificity protein 1; PPARγ, peroxisome proliferator-activated receptor gamma.

of Alzheimer's, decrease KLF2 levels in cerebral ECs; and overexpression of KLF2 protects against amyloid-induced oxidative stress (24). These studies further demonstrate the protective nature of KLF2 during states of vascular inflammation, expanding the diversity of disease states that would potentially benefit from pharmacological targeting of KLF2.

Vessel hemodynamics strongly influence vascular inflammation and KLF2 is exquisitely sensitive to the biomechanical forces exerted by laminar versus turbulent shear stress. Under conditions of laminar shear stress (LSS), KLF2 is robustly expressed in ECs *in vitro* and *in vivo* (7, 11). Indeed, as recently confirmed by Dekker et al. in humans, KLF2 expression is focally lowered in areas of low LSS such as the bifurcation of the aorta to the iliac and carotid arteries, and this may be downstream of a dual specificity mitogen-activated protein kinase kinase 5(MEK5)/extracellular-signal-regulated kinase 5 (ERK5)/myocyte enhancer factor 2 (MEF2) pathway (7, 25, 26). It has long been known that atherosclerotic lesions have a predilection to form at regions experiencing low LSS, such as bifurcations of the vasculature. Within these so-called "atheroprone" regions, ECs are more likely to become activated and increase production of pro-inflammatory mediators (27, 28).

The extent of KLF2 expression and activity is highly regulated in ECs. As previously mentioned, LSS induces KLF2 expression. The signaling cascade behind this induction has been extensively characterized: In response to LSS, MEK5 is activated, which then phosphorylates ERK5. ERK5 subsequently phosphorylates MEF2 at the KLF2 promoter, leading to KLF2 gene transcription (7, 29). Conversely, KLF2 transcription can be inhibited by tumor necrosis factor alpha (TNF-α) signaling *via* p65 and histone deacetylase (HDAC) 4/5 inhibition of MEF2 (26). p53 also utilizes HDAC5-mediated KLF2 suppression to induce endothelial dysfunction (30). Interestingly, HDAC5 also regulates KLF2's ability to induce transcription of eNOS, implicating HDACs as

regulators of KLF2 function at multiple points (31, 32). Post-transcriptionally, endothelial KLF2 is targeted by microRNA-92a (miR-92a). Low-shear stress and oxidized LDL, factors both associated with atherogenesis, induce miR-92a expression (33, 34). miR-92a is then capable of binding to the 3′-UTR of KLF2, leading to its degradation (33, 35). In fact, targeting miR-92a *in vivo* using an antagomir leads to protection from atherosclerosis, providing a method to indirectly target KLF2 (33). Additional post-transcriptional regulation of KLF2 occurs through PI3K-dependent mRNA stabilization in response to LSS (36).

Krüppel-like factor 2 serves as a prototypical vasoprotective factor as it (1) is induced by EC activating stimuli, (2) resists harmful pro-inflammatory and pro-thrombotic gene transcription, and (3) is associated with protection against vascular inflammatory disease.

Krüppel-Like Factor 4

Krüppel-like factor 4 shares many transcriptional targets and protective functions with KLF2 in ECs. Like KLF2, KLF4 is induced during LSS (37, 38). Moreover, KLF4 expression is repressed under turbulent flow as a result of DNA methyltransferase-mediated methylation within the KLF4 promoter (39). The same mechanism also silences endothelial KLF3 under turbulent flow, an anti-inflammatory KLF that is less well characterized in ECs (40). Downstream transcriptional effects of KLF4 are similar to those seen in KLF2. For instance, a KLF4–p65 interaction inhibits VCAM-1 induction, reducing leukocyte homing (38, 41). KLF4 also regulates expression of eNOS. In multiple studies, overexpression or knockdown of EC KLF4 leads to increased or decreased eNOS production, respectively (38, 42, 43). KLF4 overexpression also increases transcription of anti-thrombotic factor thrombomodulin as well *via* a physical association with its cofactor, p. 300 (38). A novel and fascinating role of endothelial KLF4 was recently discovered in the context of endothelial inflammation and cholesterol flux. KLF4 induces the expression of cholesterol-25-hydroxylase (Ch25h) and liver X receptor (LXR) in ECs, which contribute to reverse cholesterol transport out of the vascular wall and inhibition of endothelial inflammasome activation, both protective against atherosclerosis (44).

In addition to its role in maintaining an anti-adhesive and anti-thrombotic endothelium, KLF4 also modulates intrinsic EC health. Autophagy is a conserved process by which cells recycle damaged organelles and misfolded proteins. Disrupted autophagy has been associated with multiple age-related phenotypes such as metabolic dysfunction, neurodegeneration, and cardiovascular disease [reviewed in Ref. (45)]. A recent study identified a role for endothelial KLF4 in regulating autophagic genes (46). Endothelial overexpression of *Klf4* protected vessels from vascular aging, an effect that is likely largely due to enhanced autophagy (46). Interestingly, this study also demonstrated an inverse correlation between the age of vessels and KLF4 expression in humans.

The essential role of endothelial KLF4 in vascular health has been demonstrated in multiple *in vivo* models. Endothelial-specific knockout of *Klf4* using a VE-Cadherin driven *Cre*-Lox system results in significantly enhanced atherosclerotic lesions when backcrossed onto the *Apoe*$^{-/-}$ mouse line (38). Additionally, EC-Klf4 KO exhibited increased thrombotic capacity. Conversely, endothelial-driven overexpression of *Klf4* is protective against atherosclerosis. Conversely, endothelial-driven overexpression of Klf4 is protective against atherosclerosis and thrombosis (38). Outside of atherothrombotic disease, vascular inflammation can also negatively affect renal arteries during instances of ischemia. Endothelial KLF4 is vasoprotective in this context as demonstrated in a model of hematopoietic deletion of *Klf4* during ischemia–reperfusion injury (47). Mice lacking KLF4 demonstrated exacerbated renal injury as a result of increased adhesion molecule expression on ECs with consequent immune cell invasion (47). This mechanism was also at play in a model of carotid artery injury. Interestingly, loss of endothelial KLF4 resulted in enhanced proliferation of both EC and neointimal VSMCs, as mediated by increased immune cell presence (41). In another disease model of pathological vascular remodeling, KLF4 levels are decreased in the lungs of patients with pulmonary artery hypertension (PAH) (42). Loss of endothelial KLF4 is associated with increased hypertension and pulmonary artery vascularization, in part through enhanced expression of endothelin-1 (ET-1) and decreased eNOS expression (42).

Like KLF2, KLF4 is post-transcriptionally regulated by miRs. Specifically, both KLF2 and KLF4 are inhibited by the "atheromiR," miR-92a (33, 35). Additionally, however, KLF4 is negatively regulated by miR-103 (48). In mice with endothelial-specific deletion of miR processing machinery, Dicer, there is a decrease in miR-103-mediated KLF4 suppression; this increase in KLF4 subsequently restrains NF-κB-driven CXCL1 and macrophage infiltration in atherosclerotic lesions (48).

Endothelial KLF2 and KLF4 have remarkably similar functions; they respond to many of the same stimuli, share gene targets and have a high degree of similarity in amino acid sequence. Early studies on endothelial KLF4 function noted overlapping functions between the two phylogenetically close factors (37, 49). Indeed, loss of one allele of *Klf2* leads to a compensatory increase in *Klf4*, while a single allele of either *Klf2* or *Klf4* is sufficient to rescue lethality in a double *Klf2*/*Klf4* knockout mouse, suggesting genetic redundancy of functions central to endothelial function and identity (20, 50). Indeed, the double *Klf2*/*Klf4* knockout mouse demonstrates loss of endothelial integrity and hemostatic dysfunction, as well as the loss of an endothelial-like transcriptome.

Krüppel-Like Factors 5 and 6

Unlike KLF2 and 4, endothelial KLF5 and 6 are associated with vascular inflammation and remodeling that is largely deleterious. While KLF5 is largely considered to be a major effector of VSMC function (see below), there is evidence that it mediates endothelial chemotactic function. Specifically, knockdown of endothelial KLF5 *in vitro* reduces TNF-α-induced expression of key monocyte chemoattractant protein, MCP-1 (51). While the *in vivo* implications of this phenomenon are unclear, there is ample evidence implicating MCP-1 in many forms of vascular inflammation including atherogenesis, diabetic vascular disease, and vascular occlusion (52–54).

Largely implicated in cancer biology, KLF6 also has documented roles in ECs. KLF6 is an early response factor to vascular injury that induces transcription and processing of the

pro-angiogenic factor endoglin, a member of the TGF-β receptor superfamily member (55). Mechanistically, KLF6 interacts with related transcription factor specificity protein 1 (Sp1) to bind to the endoglin promoter (56). While endoglin's role in angiogenesis has been extensively characterized, it is also implicated in leukocyte trafficking during vascular inflammation (57). Endothelial KLF6, therefore, may promote immune cell infiltration during vascular injury. In addition to regulating endoglin, KLF6 also induces expression of activin receptor-like kinase 1 (ALK1), another member of the TGF-β receptor family (58). KLF6–Sp1 interactions mediate *Alk1* transcription during endothelial denudation, and KLF6 heterozygotes exhibit reduced neointimal formation in response to vascular injury, a mechanism that is proposed to be through reduced ALK1 levels (58). While additional studies regarding endothelial KLF5 and 6 need to be completed, both factors seem to promote vascular inflammation and remodeling in response to injury.

Krüppel-Like Factor 11

Krüppel-like factor 11 is also highly expressed in ECs and is involved in regulating vascular inflammation. While *Klf2* expression is ultimately inhibited by TNF-α, KLF11 is induced as a result of inflammation, and similar to interactions seen with endothelial KLF4, KLF11 binds to p65 to inhibit transcription of NF-κB target genes such as VCAM-1 and E-selectin resulting in less leukocyte adhesion to ECs during vascular inflammation (59). This allows KLF11 to serve as an inflammatory-responsive factor to reduce excessive endothelial activation. In fact, loss of KLF11 in a model of cerebral ischemia results in enhanced inflammation and worse outcome (60). Endothelial KLF11 is regulated, in part, by the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptor proteins, which includes three mammalian isoforms: α, β, and γ. Administration of a PPARγ agoinst leads to increased KLF11 expression in cerebral vascular ECs (61). Interestingly, KLF11 also serves as a coregulator of PPARγ target genes *via* physical interaction of the two proteins at PPAR responsive elements (61). This interaction results in repression of pro-apoptotic miR-15a, increasing EC survival and conferring protection against cerebrovascular ischemia. These two studies demonstrate a role of endothelial KLF11 in regulating vascular inflammation during ischemic events. In addition to being responsive to PPARγ, *Klf11* transcription is also induced by PPARα (62). Administration of PPARα agonist causes KLF11 induction and its subsequent binding and inhibition of the ET-1 promoter. This phenomenon further bolsters KLF11's role as a vasoprotective factor through its ability to inhibit endothelial inflammation and vasoconstriction.

VSMC KLFs

Vascular smooth muscle cells, along with collagen and elastin, form the medial layer of blood vessels and regulate vasomotor tone to maintain proper hemodynamic pressure throughout the vascular system. In the absence of noxious stimuli, VSMCs express numerous mature markers including smooth muscle α-actin, SM22α, and smoothelin (63). When challenged by growth factors, inflammation, or injury, VSMCs undergo phenotypic switching: cells dedifferentiate, losing mature VSMC markers and regaining the ability to proliferate, migrate, and synthesize extracellular matrix proteins. This can lead to pathological vessel remodeling, ultimately resulting in obstruction of proper blood flow. Multiple KLFs modulate VSMC phenotype switching in the face of vascular injury and inflammation (**Figure 2**).

Krüppel-Like Factor 4

Vascular smooth muscle cell KLF4 maintains cells in a dormant state by binding to and recruiting the potent anti-proliferative protein p53 to the $p21^{WAF1/Cip1}$ (*Cdkn1a*) promoter/enhancer, consequently increasing transcription of *Cdkn1a*, a cell cycle inhibitor (64, 65). Further, viral overexpression of KLF4 in both VSMC and balloon-injured rat carotid arteries leads to increased expression of a panel of anti-proliferative genes such as p57 and GADD45β (66). *In vivo* implications of KLF4's anti-proliferative effects are seen in a carotid artery ligation model using conditional KLF4 knockout: KLF4 KO mice demonstrate enhanced neointimal formation as a result of dysregulated VSMC proliferation (64).

Given VSMC KLF4's role in suppressing proliferation, KLF4 also plays a somewhat counterintuitive role in promoting dedifferentiation of VSMCs. KLF4 coordinates multiple molecular events to repress markers of VSMC maturity in the context of pathological vessel remodeling. KLF4 binds directly to the TGF-β control element (TCE) to inhibit transcription of smooth muscle α-actin and SM22α (64, 67). Moreover, KLF4 recruits inhibitors of mature VSMC marker transcription such as ELK-1 and HDACs to TCEs (68). Recent evidence indicates that VSMC dedifferentiation during vascular injury not only decreases markers of mature VSMCs, but also causes VSMCs to express markers associated with macrophages, myofibroblasts, and mesenchymal stem cells. Utilizing elegant lineage tracing experiments, Shankman et al. showed that KLF4 is necessary for VSMCs to gain genetic characteristics of other cell types within atherosclerotic lesions (69). Evidence for this transition from VSMC to a "macrophage-like" cell type was recapitulated *in vitro* using cholesterol loaded *Klf4*-sufficient VSMCs, an effect that was lost in *Klf4* mutant cells (69). These studies demonstrate KLF4's importance in VSMC phenotype switching during pathological remodeling. Modulating KLF4 expression may provide an important therapeutic avenue to adjust VSMC phenotype and disease progression.

Mature, uninjured VSMCs express very low levels of KLF4 both *in vitro* and *in vivo* (67, 70); however, KLF4 expression is significantly increased in injured VSMCs, contributing to subsequent dedifferentiation. Oxidized phospholipids, which are associated with atherosclerotic burden, induce both KLF4 mRNA transcription and KLF4 nuclear translocation in VSMCs (71). Additionally, carotid artery ligation-induced vascular injury leads to a swift increase in VSMC KLF4 expression that is associated with repression of smooth muscle α-actin (64). Mechanistically, this occurs through the reduction of miR-143/145, which normally inhibit KLF4 expression to promote VSMC maturation (72). Cigarette smoke is another well-characterized stimulant of vascular inflammation. Cigarette smoke extract has been shown to induce *Klf4* transcription, enhance KLF4 binding at the promoters of VSMC differentiation genes, and increase KLF4-driven epigenetic changes that are associated with transcriptional repression (73).

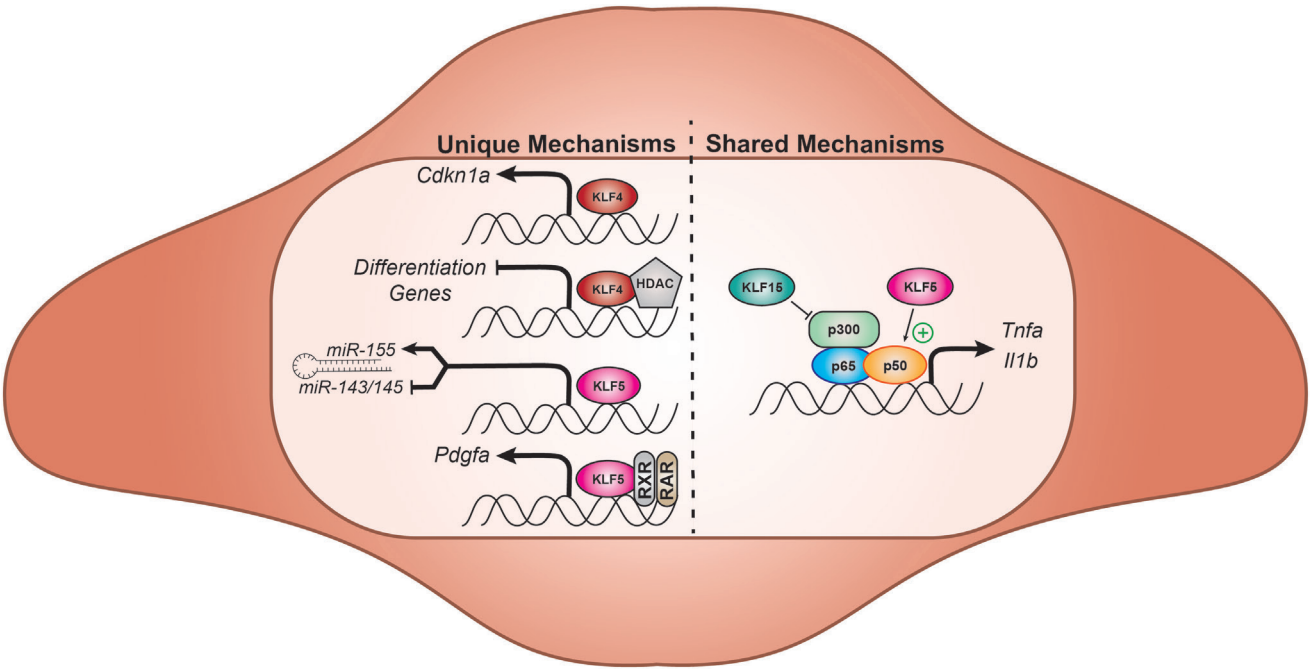

FIGURE 2 | Select effector functions of vascular smooth muscle KLFs. KLFs differentially affect VSMC differentiation, proliferation, and inflammation through a multitude of mechanisms. KLF5 and 15 exhibit reciprocal regulation of nuclear factor-κB signaling within VSMCs by binding to cofactors p50 and p300, respectively. KLF5 also promotes proliferation and dedifferentiation by modulating important driver of VSMC dedifferentiation, platelet-derived growth factor-A (PDGF-A), and differentially regulating miR transcription. While KLF4 also promotes dedifferentiation, it resists VSMC proliferation through the transcription of CDKN1A. KLF, Krüppel-like factor; VSMC, vascular smooth muscle cell; HDAC, histone deacetylase; miR, microRNA; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1 beta; RXR, retinoid X receptor; RAR, retinoid acid receptor; Cdkn1a, cyclin-dependent kinase inhibitor 1a.

Krüppel-Like Factor 5

Krüppel-like factor 5 modulates processes that engender pathological remodeling of the vasculature: like KLF4, KLF5 promotes VSMC dedifferentiation; however, dissimilarly, KLF5 also promotes cellular proliferation. KLF5's importance in phenotype switching in the face of vascular injury has been shown both *in vitro* and *in vivo*. Overexpression of KLF5 leads to reduced expression of VSMC maturity markers myocardin and smooth muscle α-actin while the converse occurs with KLF5 knockdown (74). Concordantly, *in vivo* experiments show that in wild-type mice, vascular injury causes a decrease in smooth muscle α-actin and smooth muscle myosin heavy chain (MHC), whereas this effect is not seen in mice heterozygous for KLF5 (75).

Krüppel-like factor 5's exerts its pro-inflammatory effects through two main mechanisms: complex formation with unliganded retinoid acid receptor (RAR)/retinoid X receptor (RXR) and recruitment of NF-κB subunit p50. KLF5-RAR/RXR complex binds to the promoter of platelet-derived growth factor-A (PDGF-A), a potent inducer of VSMC proliferation and dedifferentiation (75, 76). Administration of a synthetic retinoid abolishes KLF5-RAR/RXR interaction, resulting in decreased KLF5 transcriptional activity; whereas, administration of an RAR antagonist stabilizes KLF5 transcriptional effects (75, 77). Furthermore, this mechanism has been shown *in vivo* to govern VSMC proliferation in times of injury. Both pharmacological KLF5 inhibition *via* the administration of an RAR agonist and genetic loss of one *Klf5* allele diminishes neointimal formation and vascular remodeling in models of AngII infusion or femoral artery cuff injury, indicating a critical role of KLF5 in VSMC proliferation in response to injury (77). In addition to KLF5-RAR/RXR complex formation, KLF5 recruits p50 to influence VSMC phenotype toward proliferation and dedifferentiation, as well as augment VSMC inflammatory transcription (78, 79). In VSMCs from diabetic patients, increased *KLF5* and inducible nitric oxide synthase expression leads to augmented nitrated-KLF5, which possesses a heightened ability to interact with p50 and subsequently enhance TNF-α and interleukin-1 beta (IL-1β) expression (79). Interestingly, estradiol competes with KLF5 for p50 binding and can inhibit KLF5-p50-mediated transcription of inflammatory genes (79).

Krüppel-like factor 5 has been found to be upregulated in both human lung biopsies and isolated human pulmonary artery smooth muscle cells from patients with PAH, a vascular remodeling disease process (80). Using a hypoxic PAH model, Li et al. demonstrated that KLF5 serves as an upstream regulator of hypoxia inducible factor 1-alpha activity (81). Loss of KLF5 abrogated hypoxia-induced vascular remodeling partly through upregulating proliferation factors (e.g., cyclin B1 and D1) and downregulating apoptosis factors (e.g., bax, bcl-2, cleaved caspase-3, and cleaved caspase-9) (80, 81). A similar effect is seen in cardiomyocytes in response to ischemia/reperfusion injury, further emphasizing a conserved role of KLF5 in promoting proliferation and cell survival (82).

Similar to other KLFs, VSMC KLF5 both regulates and is regulated by the transcription of miRs. miR-145 is highly expressed in differentiated VSMCs and is important in maintaining cellular differentiation: expression of miR-145 is associated with upregulation of smooth muscle α-actin, calponin, and smooth muscle-MHC and reciprocal downregulation of KLF5 (72, 74, 83). In injured vessels such as those seen in atherosclerotic lesions, however, expression of miR-145 is downregulated within VSMCs (74, 84). In the absence of vascular injury, miR-145 directly targets the 3′-UTR of KLF5 to inhibit it. Following injury, PDGF inhibits miR-145 expression, thus attenuating KLF5 degradation and consequently suppressing transcription of differentiation markers (see above) and myocardin, a modulator of differentiation genes (74). In addition to being regulated by miRs, KLF5 also controls the expression of the pro-inflammatory miR-155. When VSMCs are exposed to oxLDL, KLF5 is induced, resulting in decreased anti-inflammatory miRs-143/145 and increased miR-155 (85). Interestingly, KLF5-mediated miR-155 production leads to secreted miR-155 in exosomes that are capable of destroying endothelial tight junctions and enhancing atherosclerotic progression (85).

Krüppel-Like Factor 15

Krüppel-like factor 15 serves largely as a protective factor in many aspects of cardiovascular biology including inhibiting cardiomyocyte hypertrophy, regulating cardiac lipid metabolism, and establishing circadian control of ventricular rhythm (86–88).

Vascular smooth muscle cell KLF15 primarily acts as a protective factor against vascular inflammation and disease by resisting VSMC proliferation and inflammation. Similar to other KLFs, VSMC KLF15 interacts with the histone acetylase, p300. Direct binding to p300 prevents acetylation of NF-κB member p65, thus limiting transcription of NF-κB target genes and inflammation. Both rat aortic VSMCs exposed to oxidized phospholipids and human atherosclerotic tissue demonstrate markedly decreased KLF15 expression, suggesting that KLF15 plays an important role in atherogenesis (89). Orthotopic carotid artery transplantation from Klf15$^{-/-}$ mice into *Apoe*$^{-/-}$ mice results in significantly enhanced intimal hyperplasia and inflammatory cell infiltrate. Additionally, these VSMCs express higher levels of inflammatory proteins such as VCAM-1, MCP-1, and MMP3. These results have been recapitulated in smooth muscle-specific deletion of *Klf15* on the *Apoe*$^{-/-}$ background. When rat aortic smooth muscle cells are exposed to PDGF-BB, a stimulator of VSMC proliferation and migration, *Klf15* mRNA expression is reduced (90). Interestingly, in KLF15-deficient VSMCs, *Pdgf* transcription is enhanced, demonstrating a feed-forward loop that permits VSMC proliferation and inflammation by decreasing KLF15 levels (89). In humans and mice, KLF15 deficiency is associated with cardiomyopathy and aortic aneurysm (91). Additionally, humans with ruptured intracranial aneurysms exhibit diminished KLF15 expression while expressing elevated levels of inflammatory genes (92).

MONOCYTE/MACROPHAGE KLFs

In addition to cells of the vessels themselves, circulating immune cells and their infiltration into the vascular wall are paramount to the initiation and propagation of vascular inflammation. There is ample research implicating both innate and adaptive immune cells in the progression of atherosclerosis [reviewed in Ref. (93, 94), respectively]. Comparable with their role in vascular cells, KLFs have divergent functions in myeloid cell-derived inflammation, capable of either repressing or promoting inflammatory processes (**Figure 3**).

Krüppel-Like Factor 2

Originally studied in the context of acute inflammation and bacterial sepsis, KLF2 is a central regulator of monocyte inflammatory activation (95, 96). KLF2 resists inflammation within macrophages *via* recruitment of NF-κB cofactors away from the promoters of inflammatory genes. Among these factors bound by KLF2 are p300 and p300/CBP-associated factor (PCAF) (95, 97, 98). Given the inflammatory potential of KLF2 knockout macrophages, one would predict that loss of myeloid KLF2 would be associated with increased vascular inflammation and atherosclerosis. This is, indeed, the case as mice with myeloid-specific KLF2 deletion on the *Apoe*$^{-/-}$ background exhibit increased atherosclerosis with increased vascular oxidative stress (99). This is the result of increased adhesive potential of KLF2 knockout neutrophils and macrophages. Interestingly, a similar effect is seen with dendritic cell (DC)-specific KLF2 knockout. Loss of KLF2 in DCs aggravates atherosclerosis as a result of enhanced T-cell activation and heightened inflammatory cytokine production (100). Together, these studies demonstrate a central role of KLF2 in maintaining quiescence in circulating myeloid cells; a role it serves in ECs as well.

Krüppel-like factor 2 is itself controlled by atherogenic stimuli. When anti-inflammatory macrophages are challenged with oxLDL, they shift to a pro-inflammatory state *via* the downregulation of KLF2 (101). There is also a link between low-immune cell KLF2 levels with increased risk of cardiovascular disease in humans. Monocytes from patients with atherosclerosis exhibit less *Klf2* expression than healthy controls, indicating that the inflammatory state associated with low-KLF2 translates to atherosclerotic disease (98).

Krüppel-Like Factor 4

Just as KLF2 and KLF4 have overlapping functions in ECs, this is also the case in monocytes/macrophages. While KLF2 regulates inflammatory activation of monocytes, KLF4 regulates macrophage polarization from the pro-inflammatory ("M1") state to the anti-inflammatory ("M2") state (102). Like KLF2, KLF4 recruits p300 and PCAF away from the promoter of inflammatory genes, resisting the M1 polarization state. Complementarily, KLF4 promotes the M2 state by cooperating with critical M2 transcription factor STAT6 to induce transcription of traditional M2 genes through induction of MCP-1-induced protein (102, 103). While STAT6 is largely responsible for anti-inflammatory polarization in macrophages, CREB is another transcription factor that limits resists inflammation (104). In addition to interacting with STAT6 to modulate anti-inflammatory transcription, KLF4 also interacts with CREB to increase transcription at the apoE promoter in macrophages, ultimately resulting in atheroprotection (105–107). Myeloid KLF4, therefore, resists

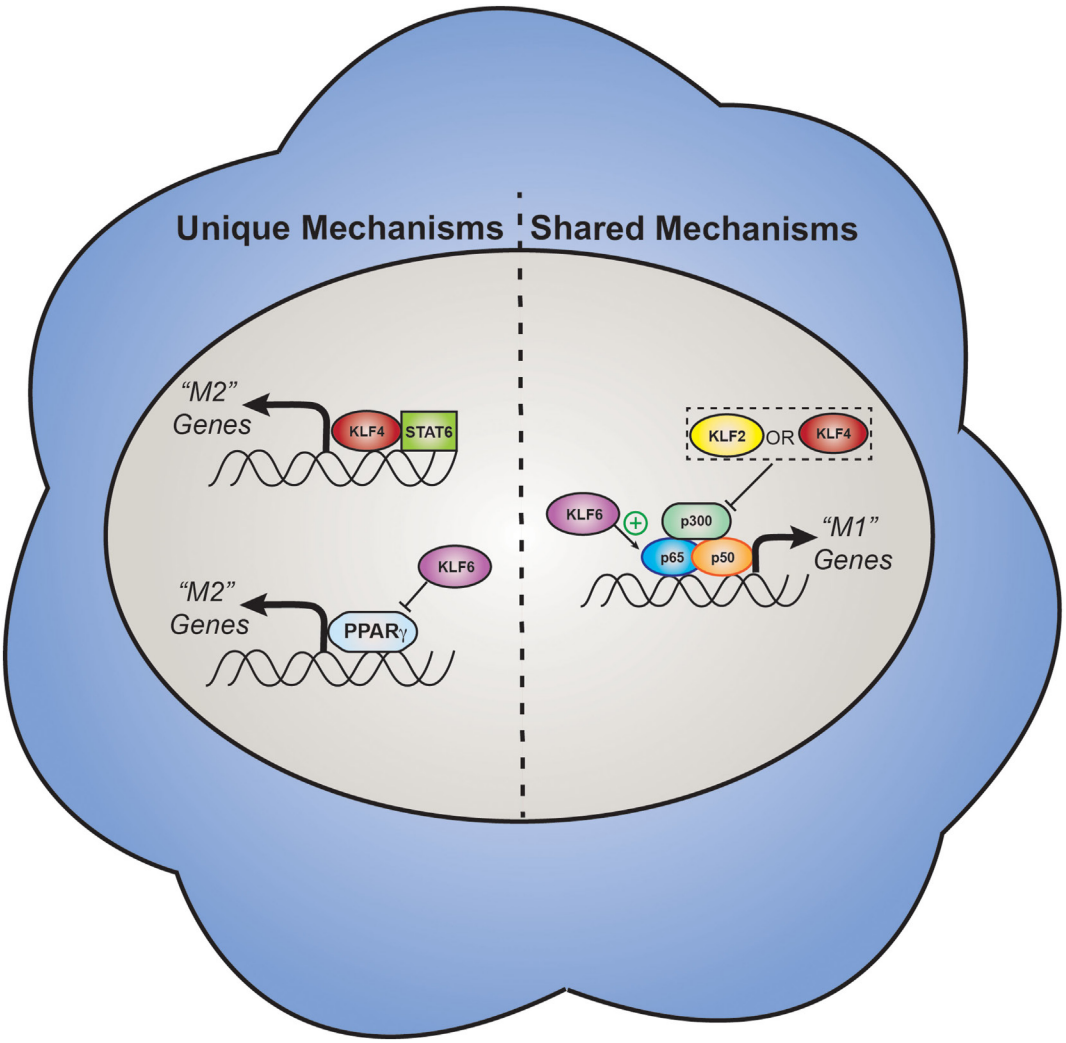

FIGURE 3 | Select effector functions of myeloid KLFs. Myeloid KLFs affect polarization of cells to a pro-inflammatory ("M1") or anti-inflammatory ("M2") state. KLF2 and 4 inhibit M1 polarization by negatively regulating nuclear factor (NF)-κB-mediated transcription. Additionally, KLF4 promotes M2 polarization through its interactions with STAT6, a prominent transcription factor in M2 skewing. KLF6 promotes M1 polarization through cooperation with NF-κB cofactor p65 and through its inhibition of PPARγ-mediated M2 transcription. KLF, Krüppel-like factor; miR, microRNA; PPARγ, peroxisome proliferator-activated receptor gamma; M1, pro-inflammatory classical macrophage activation; M2, anti-inflammatory alternative macrophage activation; STAT6, signal transducer and activator of transcription 6.

inflammation and is largely a protective factor against vascular inflammation. Moreover, loss of myeloid KLF4 is associated with augmented atherosclerosis, and macrophages deficient in KLF4 display increase inflammation in response to oxidized phospholipids (108). While KLF4-mediated Ch25h and LXR expression drives reverse cholesterol transport in ECs and macrophages, there is also evidence implicating Ch25h and LXR in KLF4-mediated M2 polarization (44). It is evident that KLF4 regulation of macrophage polarization and its role in preventing vascular inflammation is exceedingly complex and likely involves multiple downstream regulators.

Krüppel-Like Factors 5 and 6

The increased inflammatory drive associated with endothelial KLF5 and 6 is paralleled by that of macrophage KLF5 and 6. Overexpression of KLF5 increases the ability of macrophages to migrate and proliferate (109). This contributes to worsened intimal hyperplasia following carotid ligation in KLF5 overexpressing mice. This is in contrast to the protective effect afforded by myeloid-specific KLF5 deletion. Interestingly, pro-inflammatory stimuli stabilize KLF5 protein *via* various post-translational modifications. TNF-α increases KLF5 sumoylation and decreases ubiquitination to stabilize the protein and prevent degradation (109, 110). KLF5's responsiveness to inflammatory stimuli, along with its ability to propagate macrophage-mediated inflammation, contributes to its deleterious role in vascular inflammation.

Krüppel-like factor 6 expression is also responsive to pro-inflammatory stimuli. KLF6 increases when macrophages are stimulated with M1-driving stimuli and decreases with M2-driving stimuli (111). Additionally, KLF6 impacts both M1

and M2 gene transcription. KLF6 is required for optimal binding of p65 binding to its promoters, and importantly, through its interaction with p65, KLF6 promotes transcription of NF-κB targets (112). Additionally, KLF6 suppresses B cell lymphoma 6 expression, which leads to increased pro-inflammatory gene expression and increased macrophage motility (113). Conversely, KLF6 binds to PPARγ and prevents it from inducing M2 gene transcription (111). It is evident that KLF6 is a dynamic regulator of macrophage polarization.

Krüppel-Like Factor 14

Within the past 5 years, KLF14 has been extensively studied in its role in lipid and cholesterol metabolism. Given that aberrant nutrient handling, obesity, and type 2 diabetes are risk factors for atherosclerosis, it is unsurprising that multiple genetic variants involving the *KLF14* gene have been implicated in the development of atherosclerotic disease. While the genetic associations of *KLF14* variants on metabolic disease have been extensively studied (114), the role of KLF14 in macrophages is less well characterized. Recent work by Wei and colleagues has begun to parse out details on how KLF14 contributes to atherogenesis. They found that *Apoe*$^{-/-}$ mice aortas had elevated levels of *Klf14* on either high-fat diet or standard chow (115). This increase in Klf14 expression was associated with elevated pro-inflammatory cytokines in circulation: *Klf14* adenoviral knockdown ameliorated this effect. Importantly, overexpression of *Klf14* in a macrophage cell line led to increased inflammatory cytokine production as well as total cholesterol and cholesteryl ester content, a classic signature of atherogenic foam cells (116). Mechanistically, KLF14-mediated inflammation seems to be dependent on p38 MAPK and ERK1/2 signaling leading to increased cytokine release (115). Together, this work provides evidence that KLF14 may play a causal role in modulating inflammation associated with atherosclerosis, further implicating it in metabolic disease.

KLFs IN OTHER CIRCULATING IMMUNE CELLS

In addition to regulating differentiation, activation, and polarization of monocytes, KLFs also shape lymphocyte and DC function. While there is a paucity of studies investigating KLF-driven lymphocyte processes in vascular inflammation, there is extensive evidence demonstrating the importance of KLFs in lymphocyte biology that can be extrapolated to the context of vascular disease (**Figure 4**).

Krüppel-Like Factor 2

Like in monocytes, KLF2 expression maintains T cells in a quiescent state. KLF2 is expressed in naïve, effector, and memory T cells (117, 118) and its loss causes single-positive, resting T cells to spontaneously activate and apoptose in the spleen and lymph nodes (18, 19). In CD8+ T lymphocytes, KLF2 levels decrease upon stimulation of T-cell receptors and its expression is reestablished after treatment with IL-2 or IL-7 (118). In contrast, CD4+ T lymphocytes demonstrate a transient increase in KLF2 expression upon stimulation that is associated with increased IL-2 production (119). KLF2 is essential in the expression of T-cell migration factors such as $S1P_1$, cluster of differentiation 62 ligand (L-selectin) (CD62L), and β_7 integrin (Itgb7), allowing T cells to traffic to sites of vascular inflammation or draining lymph nodes (120, 121). Furthermore, statin-induced KLF2 expression in effector T cells reduces inflammation in a myocarditis model, an effect that is likely related to diminished interferon-γ production (122).

In addition to the proatherogenic functions of effector T cells, regulatory T cells (Tregs) play an important role in suppressing vascular inflammation (123). In the presence of oxLDL, Tregs restore *endothelial* KLF2 to protect the vasculature from inflammation (124). Within Tregs, forkhead box P3 (FoxP3), a lineage-specific transcription factor, is under direct control of KLF2 (125). Loss of KLF2 prior to FoxP3 induction results in impaired Tregs differentiation, while loss of KLF2 after FoxP3 induction does not affect this process. Pabbisetty et al. also demonstrated that stabilization of KLF2 protein through statin administration or by genetic deficiency of E3 ubiquitin ligase SMURF1 results in enhanced Treg production.

Within B lymphocytes, KLF2 appears to be important in determining cellular identity. Higher KLF2 expression is associated with B1 B cells in the periphery versus follicular or marginal zone B cells. Concordantly, inactivation of KLF2 in B cells leads to a decrease in B1 B cells with a concurrent increase in marginal zone B cells (126, 127). Similar to T cells, loss of KLF2 in B cells is also associated with less CD62L and Itgb7 expression, resulting in impaired B-cell trafficking (126, 128). Finally, KLF2 also plays a role in regulating the DC response during vascular inflammation. As is seen with monocytes and neutrophils (99), loss of KLF2 in DCs increases inflammatory cytokine production, DC tissue infiltration, and T-cell activation in atherogenic *Ldlr*$^{-/-}$ mice (100). Together, these studies further demonstrate that KLF2 largely opposes inflammatory activation in circulating immune cells.

Krüppel-Like Factor 3

Within B cells, KLF2 and KLF3 have opposing effects and compete for the same gene targets. While KLF2 is associated with a B1 B-cell differentiation pattern (with lower levels associated with follicular and marginal zone cells), KLF3 expression favors marginal zone B-cell development (129). Additionally, KLF2 and KLF3 compete for occupancy of the *Itgb7* promoter: while KLF2 promotes expression of Itgb7 and, thus, migration, KLF3 leads to downregulation of Itgb7 and impaired homing ability of lymphocytes (130). Interestingly, KLF2 and 3 differentially regulate KLF3 expression itself. KLF3 negatively regulates its own expression through direct binding to the KLF3 promoter (130). Conversely, loss of KLF2 in B cells results in decreased expression of KLF3 (i.e., KLF2 increases KLF3 expression) (128). The interplay between these two factors is critical for B-cell differentiation and function.

Krüppel-Like Factor 4

Given its well-defined role in maintaining self-renewing capabilities of stem cells, it is unsurprising that loss of KLF4 expression is necessary for proper T-cell development. Remarkably, KLF4 is

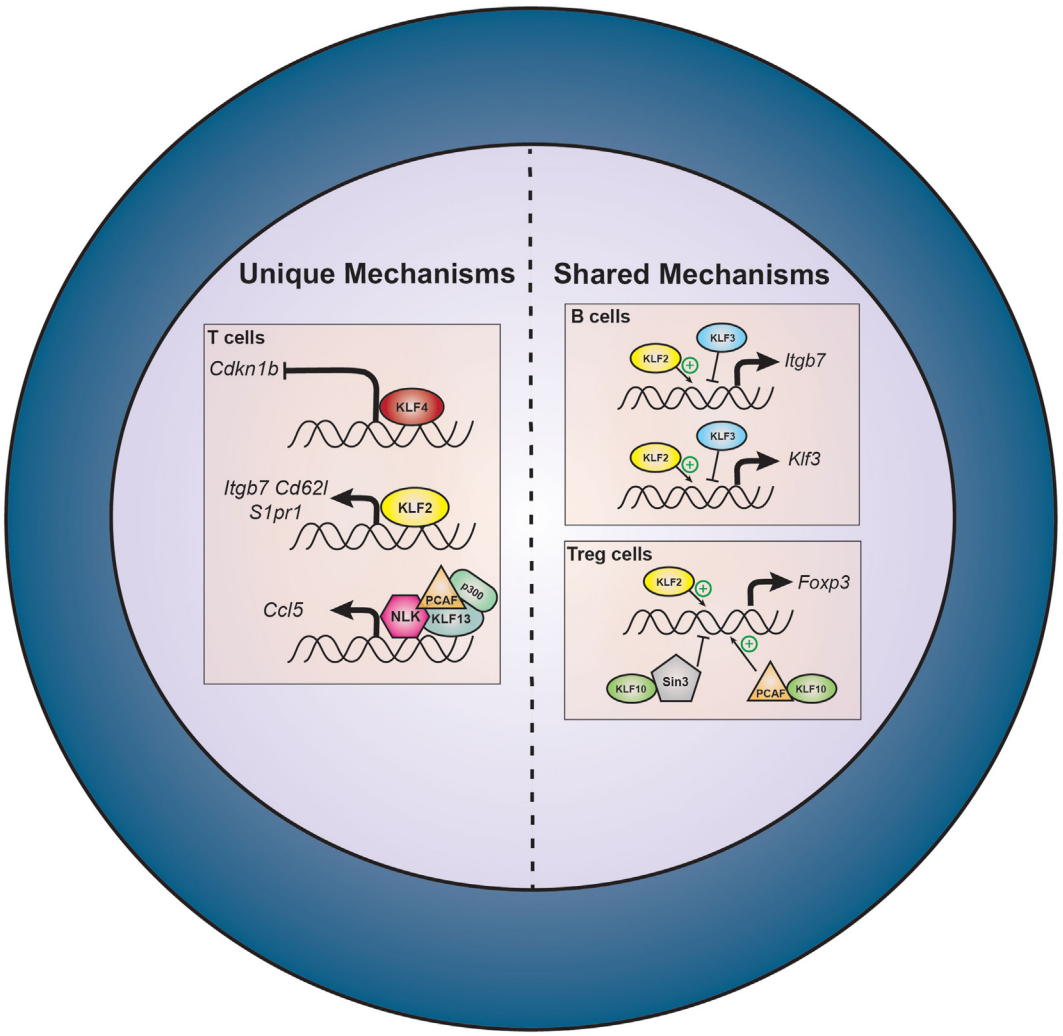

FIGURE 4 | Select effector functions of lymphocyte KLFs. Lymphocyte KLFs largely dictate cellular identity, proliferation, and survival. KLF2 and KLF3 oppose each other's function at the *Itgb7* and *Klf3* promoters. In regulatory T cells (Treg), corepressor/coactivator interactions determine the transcriptional effects of KLF10. KLF, Krüppel-like factor; Itgb7, β_7 integrin; Foxp3, forkhead box P3; Sin3, represents Sin3-histone deacetylase complex (Sin3-HDAC); PCAF, P300/CBP-associated factor; Cdkn1b, cyclin-dependent kinase inhibitor 1b ($p27^{Kip1}$); Cd62l, cluster of differentiation 62 ligand (L-selectin); S1pr1, sphingosine-1-phosphate receptor 1 ($S1P_1$); Ccl5, chemokine ligand 5 (RANTES); NLK, Nemo-like kinase.

the only Yamanaka factor that is downregulated throughout each step of T-cell differentiation (131). This attenuation is required for the transition from double negative (DN)2 to DN3 as evidenced by diminished T-cell differentiation at this stage during forced KLF4 overexpression (131). While loss of KLF4 is critical to T-cell *differentiation*, DN T-cell population proliferation is maintained through KLF4 activity: KLF4 binds to and inhibits the promoter of cyclin-dependent kinase inhibitor 1b/$p27^{Kip1}$, releasing inhibition of CDK-mediated proliferation (132). Interestingly, this is contrary to how KLF4 interacts with $p27^{Kip1}$ in VSMCs [(66); see below], demonstrating cell-type specific functions of KLF4 in regulating proliferation. Within B cells, KLF4 is lowly expressed in the most immature stages but is increased throughout B-cell maturation (133). Upon activation, however, mature B cells decrease KLF4 levels. Additionally, KLF4 appears to be important in promoting B-cell proliferation through the activation of cyclin D2 (133). KLF4's role in DC biology closely mirrors that seen in monocyte/macrophages. IRF4-expressing DCs are important in promoting type 2 helper T-cell (Th2) response, and KLF4 is required for this interaction (134). Additionally, loss of KLF4 in pre-DCs leads to fewer IRF4-expressing DCs. Together, the DC and monocyte data suggests that KLF4 strongly favors the "anti-inflammatory" polarization of immune cells and its expression may be a potential target to reduce deleterious vascular inflammation.

Krüppel-Like Factor 10

Krüppel-like factor 10 plays an important role in establishing Treg identity through FoxP3 expression while also directly promoting Treg function through TGFβ1 production. Indeed, forced overexpression of KLF10 in CD4+ CD25− (non-Treg) T cells induces both *Foxp3* and *Tgfb1* expression while downregulating

markers of Th1 and Th2 cells (Tbet and Gata3, respectively) (135). Conversely, loss of KLF10 in CD4+ CD25− cells enhances Th1 and Th2 differentiation. In response to Treg stimulating factor TGFβ1, KLF10 transactivates both FoxP3 and TGFβ1 promoters, representing a positive feedback loop of Treg function (135). Important in vascular inflammation, the addition of KLF10 knockout CD4+ CD25− T cells promoted atherosclerosis in ApoE$^{-/-}$/scid/scid mice *via* increased leukocyte accumulation and inflammatory cytokine production (135). Recent studies have provided mechanistic insight on how KLF10 regulates FoxP3 transcription. Within Tregs, KLF10 recruits PCAF to the FoxP3 promoter, leading to acetylation and subsequent activation of the FoxP3 promoter (136). Remarkably, KLF10 also associates with the corepressor Sin3-HDAC to repress FoxP3 transcription. A study by Xiong et al. demonstrated that PCAF disrupts KLF10/Sin3 interactions to allow PCAF-mediated FoxP3 acetylation through its interaction with KLF10 (137). The authors of this study posit that KLF10 interacts with Sin3-HDAC in the dominant state while post-translational modifications in KLF10 downstream of lymphocyte signaling is required to favor PCAF/KLF10 interactions. Along these lines, KLF10 interaction with the FoxP3 promoter appear to be dependent on Itch-mediated ubiquitination of KLF10 in a degradation-independent manner (138). While this study did not investigate how ubiquitination of KLF10 affects PCAF or Sin3 interactions, ubiquitination of KLF10 promoted FoxP3 expression suggesting that this mechanism may contribute to KLF10's interaction with PCAF.

Krüppel-Like Factor 13

Krüppel-like factor 13 also demonstrates complex interactions with acetyl transferases/deacetylases to regulate activation of T cells. RANTES (or chemokine ligand 5) is a classically expressed gene late in T-cell activation whose blockade is associated with diminished atherosclerosis (139). KLF13 promotes RANTES expression through the recruitment of a "enhancesome" that consists of various kinases and acetyltransferases. Specifically, Nemo-like kinase is recruited to phosphorylate the H3 histone on the RANTES promoter. Following this, PCAF and CBP/p300 are recruited to acetylate H3 and allow for ATP-dependent chromatin remodeling and RNA Polymerase II binding (140, 141). In addition to promoting RANTES expression in T cells, KLF13 also promotes apoptosis by binding to the promoter of anti-apoptotic factor BCL-X_L and reducing its expression (142). The authors of this study suggest that KLF13-mediated repression of BCL-X_L occurs through the recruitment of Sin3-HDAC to the promoter, as is seen in the context of other genes inhibited by KLF13 (143).

PHARMACOLOGICAL MODULATION OF KLFs

Current therapies for atherosclerosis largely target mechanisms known to activate vascular inflammatory cascades such as dyslipidemia (statins), disturbed flow (anti-hypertensives), and activated circulating inflammatory cells (aspirin). Given the importance of these stimuli in the pathogenesis of atherosclerosis and thrombosis, understanding molecular mediators of vascular inflammation is imperative in developing novel agents against cardiovascular disease.

While accomplishing specificity in targeting KLFs will likely be difficult, multiple compounds act upstream of KLFs, thereby modulating their expression and function (**Figure 5**). Below, we summarize a few modulators of KLF biology, with special emphasis on those that affect multiple different KLFs important in vascular inflammation.

Krüppel-Like Factor 2

Numerous pharmacological agents induce KLF2. Notably, the prominent lipid-lowering statins are potent inducers of KLF2 expression in ECs and circulating immune cells *via* MEF2 (144–146). Studies in mouse have demonstrated a potential role for statin-induced KLF2 expression in protecting against diabetic vascular reactivity and inflammation, as well as myocarditis (122, 147). These studies indicate the widespread anti-inflammatory properties of statins through the modulation of KLF2. In addition to statins, phenol compounds such as tannic acid and resveratrol, are capable of inducing endothelial KLF2 and preventing inflammation (148, 149). Acting *via* sirtuin 1 and MEK5/MEF2-dependent mechanisms, resveratrol induces the expression of KLF2-dependent atheroprotective genes (149). While the benefits of chronic resveratrol therapy in humans are still under investigation, it has been attributed with increasing lifespan and the prevention of multiple age-related diseases in small mammals [(150, 151)]. Additional work needs to be done, however, to determine the relative contribution of KLF modulation in resveratrol's protective qualities.

Therapeutic proteasome inhibitor Bortezomib has also been demonstrated to induce KLF2 in multiple cell types (21). Normally prescribed to combat multiple myeloma, Bortezomib treatment at non-myelosuppresive doses is actually thromboprotective, in part, through KLF2 induction (21).

Krüppel-Like Factor 4

As with KLF2, KLF4 expression is induced by statin use. Utilizing a MEK5/ERK5 axis, statin-induced KLF4 expression leads to increased transcription of genes associated with anti-thrombosis, vasodilation, and hemostasis while increasing apoptosis resistance and decreasing inflammatory potential in ECs (43). Additionally, in a model of renal ischemia–reperfusion injury, statins protected against injury in a KLF4-dependent manner (47). Given their widespread use in patients at risk for cardiovascular disease, statins represent a tool to further understand the importance of KLFs in regulating vascular inflammation in humans.

Vascular smooth muscle cell KLF4 is also induced by multiple pharmacological agents including rapamycin and cyclosporine A (CSA). Rapamycin is a known inhibitor of cell proliferation *via* induction of $p27^{kip1}$ (152) and has long been used in drug-eluting stents to prevent restenosis *via* VSMC proliferation (153). Within VSMCs, rapamycin inhibits mammalian target of rapamycin (mTOR), which subsequently increases KLF4 production (66). Interestingly, overexpression of KLF4 results in increased $p27^{kip1}$ production and inhibition of VSMC proliferation. These results suggest that rapamycin and VSMC KLF4 enhance each other's activities in the regulation of VSMC proliferation. CSA is an

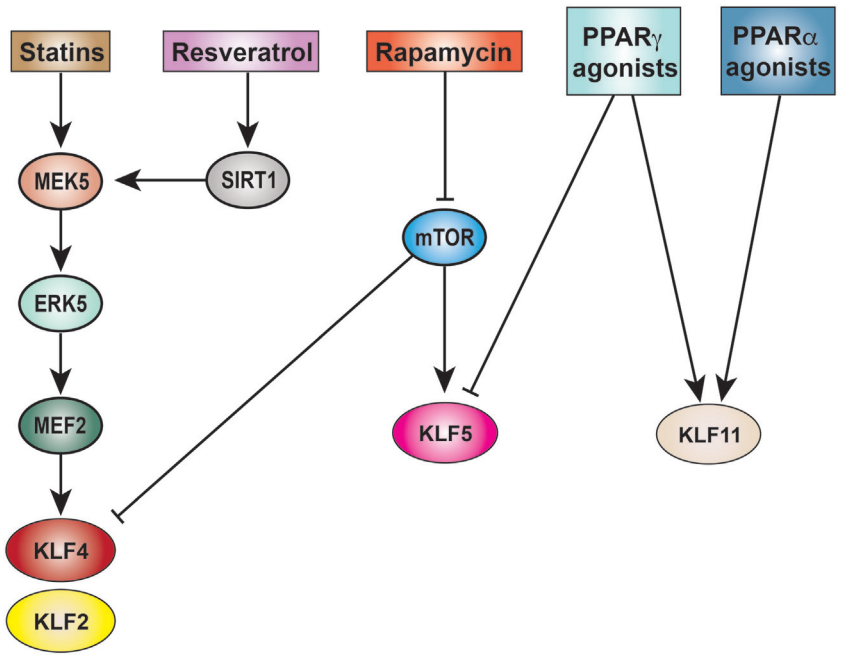

FIGURE 5 | Pharmacological targeting of KLFs in vascular inflammation. There is substantial crossover in the mechanisms of drugs targeting the induction/inhibition of KLFs in vascular inflammation. Both statins and resveratrol augment KLF2 through MEF2-mediated transcription, the same pathway used in statin-mediated KLF4 induction. Rapamycin has differential effects on KLF4 and KLF5 expression, eventually stimulating KLF4 production while inhibiting KLF5. Inhibition of KLF5 is also achieved through use of PPARγ agonists (e.g., rosiglitazone). KLF11 is induced through PPAR signaling, exhibiting increased expression in response to agonists to both PPARγ and PPARα (e.g., fenofibrate). KLF, Krüppel-like factor; MEK5, dual specificity mitogen-activated protein kinase5; ERK5, extracellular-signal-regulated kinase 5; MEF2, myocyte enhancer factor 2; SIRT1, sirtuin 1; mTOR, mammalian target of rapamycin; PPAR, peroxisome proliferator-activated receptor.

immunosuppressant used in inhibiting lymphocyte proliferation that upregulates VSMC KLF4 production, resulting in anti-proliferative and phenotype switching effects (154).

Krüppel-Like Factor 5

Contrary to its inductive effect on endothelial KLF2, resveratrol has been shown to decrease TGF-β-mediated KLF5 transcription (155). Through its inhibition of the Akt-mTOR pathway, resveratrol is capable of blocking KLF5-driven VSMC dedifferentiation, thereby preventing intimal hyperplasia. Additionally, targeting this TGF-β/phospho-Akt/phospho-mTOR/KLF5 axis with Akt inhibitor LY249004 or mTOR inhibitor rapamycin also decreases KLF5 levels. As previously mentioned, retinoid agonists and antagonists can also diminish and augment KLF5 activity, respectively, by targeting processes downstream of KLF5-mediated transcription. A recent study demonstrated that the PPARγ agonist, rosiglitazone, is capable of reducing VSMC proliferation by suppressing KLF5 expression (156). While PPAR agonists have differential effects on KLF expression (see KLF11 below), their importance in modulating KLF activity cannot be understated as they represent critical modulators of vascular inflammation. Interestingly, there is also evidence that the traditional Chinese medicine Tongxinluo inhibits macrophage KLF5 transcription and blocks PI3K/Akt signaling to prevent KLF5 sumoylation (109).

Krüppel-Like Factor 10

Given its importance in Treg homeostasis, targeting KLF10 is a potential therapeutic option to combat vascular inflammation. Interestingly, a screen investigating small molecule *inhibitors* of KLF10 identified multiple compounds that are able to prevent conversion of CD4+ CD25− T cells to CD4+ CD25+ Tregs (157). While this was done in the context of reducing Treg effects in immunosuppression seen in cancer, it is feasible that a similar screen can be utilize to identify small molecule *activators* of KLF10 to be used in inflammatory conditions.

Krüppel-Like Factor 11

Krüppel-like factor 11 is under the transcriptional control of PPAR nuclear receptors and its expression and activity can be indirectly targeted through the use of PPAR agonists. The PPARα ligand fenofibrate stimulates KLF11 transcription and, therefore, inhibits ET-1 production (62). In addition, fenofibrate has demonstrable beneficial effects in preventing diabetic microvascular complications (158). Taken together, KLF11 targeting may serve as a potential mechanism of vascular protection during PPARα agonist use. Pioglitazone, a PPARγ agonist, has cytoprotective properties in cerebrovascular ECs *in vitro* and *in vivo* (61). In the absence of KLF11, however, these effects are lost, indicating a dependency of pioglitazone on KLF11.

CONCLUDING REMARKS

Vascular inflammation is central to the pathogenesis of a wide array of debilitating conditions, especially those most prominent in Western society. Inflammatory responses in the vessel wall and circulating cells are governed, in part, through the action of select transcriptional regulators with a body of evidence pointing to the KLFs as having such a role. As critical regulators of the vascular inflammatory response in multiple tissue types, future investigations of the KLFs utilizing whole transcriptome approaches will provide valuable information regarding the breadth of KLF influence as well as potential interactions among them; these promise to be complex, as the shared consensus sequence 5′-C(A/T)CCC-3′ is prevalent throughout the genome. Additionally, the KLFs may represent attractive targets for therapeutic intervention; this will require further exploration, as the targeting of zinc-finger transcription factors remains non-trivial. Ultimately, mechanistic and therapeutic insights in KLF biology will advance our understanding of the complex signaling networks at play during vascular inflammation.

AUTHOR CONTRIBUTIONS

DS, LF, PH, and MJ contributed conception of the manuscript. DS wrote the first draft of the manuscript. DS, LF, PH, and MJ contributed to manuscript revision. All authors read and approved submitted version.

FUNDING

This work was supported by the National Institute of Health (NIH) grants R01DK111468, R35HL135789, and R01HL086548 (to MJ), T32GM007250 (to DS, LF, and PH), F30AG054237 (to PH), T32HL134622 (to LF), and F30HL139014 (to DS).

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

frontiers in Cardiovascular Medicine

REVIEW
published: 11 June 2018
doi: 10.3389/fcvm.2018.00069

Krüppel-Like Factors in Metabolic Homeostasis and Cardiometabolic Disease

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

Received: *27 December 2017*
Accepted: *21 May 2018*
Published: *11 June 2018*

Citation:
Oishi Y and Manabe I (2018) Krüppel-Like Factors in Metabolic Homeostasis and Cardiometabolic Disease. Front. Cardiovasc. Med. 5:69. doi: 10.3389/fcvm.2018.00069

Members of the Krüppel-like factor (KLF) family of transcription factors, which are characterized by the presence of three conserved Cys_2/His_2 zinc-fingers in their C-terminal domains, control a wide variety of biological processes. In particular, recent studies have revealed that KLFs play diverse and essential roles in the control of metabolism at the cellular, tissue and systemic levels. In both liver and skeletal muscle, KLFs control glucose, lipid and amino acid metabolism so as to coordinate systemic metabolism in the steady state and in the face of metabolic stresses, such as fasting. The functions of KLFs within metabolic tissues are also important contributors to the responses to injury and inflammation within those tissues. KLFs also control the function of immune cells, such as macrophages, which are involved in the inflammatory processes underlying both cardiovascular and metabolic diseases. This review focuses mainly on the physiological and pathological functions of KLFs in the liver and skeletal muscle. The involvement of KLFs in inflammation in these tissues is also summarized. We then discuss the implications of KLFs' control of metabolism and inflammation in cardiometabolic diseases.

Keywords: KLF, metabolism, liver, muscle, macrophage

INTRODUCTION

The Krüppel-like factors (KLFs) belong to a family of zinc-finger containing transcription factors. KLFs regulate diverse biological processes in mammalian tissues, including cell proliferation, differentiation and survival, and tissue development. KLFs are also crucially involved in the maintenance of systemic and tissue homeostasis (1, 2). To date, 18 KLFs have been identified, though the *KLF18* gene is likely a pseudogene (3). All KLFs contain three conserved Cys_2/His_2 zinc-fingers within their C-terminal domains (**Figure 1**) and bind to similar consensus sequences (CACCC-, GC-, or GT- box elements) located within the promoters and enhancers of target genes. Despite their binding to similar sequences, their target genes and functions are diverse and highly context-dependent. But what are the mechanisms that enable KLFs to have distinct and context-dependent functions? First, KLF expression is often highly dependent on cellular and environmental contexts, such as cell type and environmental cues. Second, their varied N-terminal

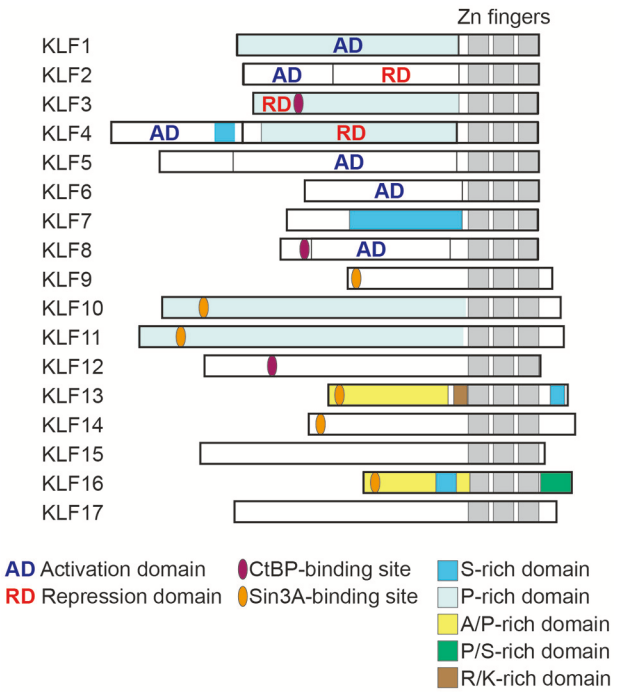

FIGURE 1 | Structure of human KLF proteins. All KLF members have homologous C-terminal DNA binding domains that are composed of three C2H2 zinc fingers. By contrast, their N terminal regions are highly diverse and may contain activation and repression domains and various protein interaction domains. For instance, KLF3, 8 and 12 all contain CtBP-binding motifs, while KLF9, 13, 14, and 16 all contain Sin3A-binding motifs. *KLF18* gene is closely related to *KLF17*, though *KLF18* may not be expressed (pseudogene) (3). Modified from Kim et al. (4).

sequences, interactions with other transcription factors and coregulators, and post-translational modifications contribute to the distinct and context-dependent function of each KLF (**Figure 1**) (4). For instance, it is likely that interactions with other factors and the status of the chromatin opening, which is also context-dependent, limit a KLF's binding to a small subset among the numerous CACCC motifs within the genome. Although there is much still to be learned about the precise mechanisms underlying their functional diversity, previous studies using genetically engineered mouse and cell models have experimentally demonstrated that each KLF family member has distinct functions. Among these diverse functions, control of metabolism appears to be evolutionally conserved among a number of KLFs (5). For example, two of the three KLFs expressed in *C. elegans* are important for lipid metabolism (6).

Metabolic abnormalities such as obesity and diabetes are the major risks for cardiovascular disease, as multiple pathways link metabolic alterations to cardiovascular disease development. For instance, dyslipidemia, such as high low-density lipoprotein (LDL) cholesterolemia, triggers, and promotes atherogenic processes within the arterial wall. Insulin resistance is also involved in vascular pathology and heart failure (7). Mediators secreted from metabolic tissues, including adipokines, myokines, and hepatokines from fat, muscle and liver, respectively, have all been shown to affect cardiometabolic pathologies (8).

Chronic inflammation is another key mechanism that links cardiovascular and metabolic diseases (9). Chronic inflammation is not only a common and pivotal mechanism underlying the initiation and development of both cardiovascular diseases, such as atherosclerosis, and metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD) and diabetes, it also mechanistically connects these diseases. One example is the impact of adipose tissue inflammation on cardiometabolic disease. Visceral obesity induces chronic inflammation within visceral adipose tissue, which may promote cardiometabolic pathology, such as atherosclerosis and type 2 diabetes, in part by promoting inflammation within the affected tissue through release of inflammatory cytokines and free fatty acids from the inflamed adipose tissue (9, 10). Several clinical trials of drugs targeting inflammatory pathways have highlighted the pathological and therapeutic importance of inflammation in cardiometabolic disease (11–13).

In this review, we focus on the roles of KLFs in metabolic regulation and their contributions to metabolic and cardiovascular diseases. Our particular focus is on the physiological and pathological functions of KLFs in the liver and skeletal muscle because these organs are the major sites of regulation of systemic metabolism, and metabolic defects in these organs are essential to type 2 diabetes (14). We will also discuss the impact of KLF regulation in these organs from the perspective of the metabolic modulation and inflammation that promote cardiometabolic disease. Although we do not cover it here, adipose tissue also plays a key role in systemic metabolic regulation and is a pivotal contributor to the development of cardiometabolic disease. Readers are referred to recent reviews on the functions of KLFs in white and brown adipose tissue (15, 16).

KLFS IN LIVER BIOLOGY AND PATHOBIOLOGY

KLF6 Is Essential for Liver Development

KLF6 plays a central role in fetal liver development (17). Liver organogenesis in mammals depends on vascular and hematopoietic development (18, 19). In mice, for example, systemic deletion of *Klf6* is lethal by embryonic day (E) 12.5, and is associated with markedly reduced hematopoiesis, poorly organized yolk sac vascularization, and an apparent lack of the liver. Consistent with the phenotype in early embryos, $Klf6^{-/-}$ embryonic stem (ES) cells display hematopoietic defects following differentiation into embryoid bodies. Moreover, $Klf6^{-/-}$ ES cells fail to differentiate into hepatocytes (20). Deletion of *Klf6* in zebrafish independently confirmed that KLF6 is essential for development of endoderm-derived organs, including liver.

KLFs Control Glucose and Amino Acid Metabolism in the Liver

The liver is a major site of insulin action in adults, as glucose uptake, glycolytic metabolism, and gluconeogenesis all occur there. KLF15 is abundantly expressed in the liver, and its expression is increased by food deprivation and is reduced by feeding. KLF15 regulates gluconeogenesis-related genes such as *Pck1*, which encodes phosphoenolpyruvate carboxykinase (PEPCK) in mice (21). Genetic deletion of *Klf15* results in fasting hypoglycemia due to abnormal gluconeogenesis and defects in the use of amino acids as sources of gluconeogenic substrates. *Klf15* deletion also decreases hepatic expression of genes encoding gluconeogenic and amino acid catabolic enzymes (22, 23). For instance, in the $Klf15^{-/-}$ liver, reductions were observed in the expression of alanine aminotransferase 1 (*Alt1*), proline dehydrogenase (*Prodh*), tryptophan 2,3-dioxygenase (*Tdo2*), and 4-hydroxyphenylpyruvic acid dehydrogenase (*Hpd*) genes, which catabolize alanine, proline, tryptophan, and tyrosine, respectively (23). Alanine aminotransferase catalyzes conversion of alanine to the gluconeogenic substrate, pyruvate. As expected, the ability to utilize exogenous alanine for glucose production is disrupted in $Klf15^{-/-}$ mice. These findings demonstrate that KLF15 as an important regulator of gluconeogenesis and amino acid catabolism in the liver (**Figure 1**). Interestingly, KLF15 is involved in the action of metformin, one of the first-line medications for the treatment of type 2 diabetes (23). Metformin suppresses gluconeogenesis in hepatocytes by reducing KLF15 levels through enhancement of its ubiquitination and degradation and downregulation of its mRNA.

KLF6 is also involved in glucose metabolism in the liver. KLF6 transactivates *GCK*, which encodes glucokinase, a rate-limiting enzyme for hepatic glucose utilization and a major regulator of blood glucose homeostasis (24). In liver tissue from humans with NAFLD, expression of the full-length KLF6 isoform and glucokinase are correlated, suggesting KLF6 regulates *GCK* under pathological conditions in the liver. Glucokinase facilitates post-prandial extraction of blood glucose by liver by promoting glucose storage as glycogen through glycogenesis or as lipid through lipogenesis (25, 26). Indeed, hepatic overexpression of *GCK* in rodents reduces blood glucose and induces hypertriglyceridemia and hepatic steatosis. In addition, in humans *GCK* mRNA levels were associated with markers of de novo lipogenesis and the triglyceride content of liver tissues over a wide range of steatosis levels (27). Also reported, however, were conflicting data indicating that *KLF6* and *GCK* mRNA levels were downregulated in advanced steatosis as compared with mild steatosis (24). Although the reason for the conflicting observations is not immediately clear, expression of *KLF6* and *GCK* may be differentially regulated in mild vs. advanced steatosis. Nevertheless, these findings suggest KLF6 is involved in the development of NAFLD, in part by controlling *GCK* expression. However, there is a need of further analysis of its pathological actions and regulation at different stages of the progression of NAFLD, and we will discuss its contribution to non-alcoholic steatohepatitis (NASH) development in the following section.

KLFs Control Lipid Metabolism in the Liver

Regulation of lipid metabolism is another important function of the adult liver. In addition to its functions in glucose metabolism, KLF15 also contributes to hepatic lipid metabolism. *Klf15* deletion ameliorates hepatic insulin resistance induced by a high-fat diet (HFD) without affecting the endoplasmic reticulum (ER) stress or hepatic inflammatory responses that typically accompany insulin resistance (28). Adenovirus-mediated hepatic *Klf15* knockdown or systemic *Klf15* deletion increases hepatic levels of ER stress markers in mice fed with a HFD. Interestingly, HFD-induced systemic and hepatic insulin resistance is ameliorated by genetic interventions targeting *Klf15*. Moreover, while an ER stress activator, tunicamycin, induces liver steatosis and insulin resistance, these hepatic responses are much reduced in $Klf15^{-/-}$ mice. By contrast, *Klf15* inhibition increases JNK phosphorylation and proinflammatory cytokine expression. Thus, *Klf15* deletion uncouples hepatic insulin resistance and steatosis from ER stress and inflammation in HFD-induced obesity. Although the precise mechanism remains unknown, Jung et al. proposed that KLF15 activates mTORC1 signaling. In $Klf15^{-/-}$ mice, enhanced fatty acid oxidation presumably due in part to inhibition of mTORC1 may protect the liver from steatosis (29, 30). These observations suggest KLF15 is a crucial contributor to the regulation of hepatic metabolism, and its perturbation leads to alterations in HFD-induced hepatic pathology.

Takeuchi et al. very recently showed that KLF15 plays an important role in the switching between lipogenesis and gluconeogenesis during fasting (31). Through a series of experiments examining *in vivo* promoter activity, these investigators identified KLF15 as a regulator of *Srebf1*, which encodes SREBP-1, a transcription factor that controls cellular lipid metabolism. One of the two SREBP-1 isoforms, SREBP-1c, is particularly involved in controlling genes required for lipogenesis, including *Fasn* (fatty acid synthase) and *Acc* (acetyl-CoA carboxylase) (32). Fasting markedly reduces hepatic expression of *Srebf1*, while *Klf15* expression is increased. During fasting, upregulated KLF15 interferes with LXR/RXR-dependent transactivation of *Srebf1* transcription by forming a complex with LXR/RXR and a corepressor RIP140 (31). Because KLF15 promotes gluconeogenesis during fasting (see above), induction of KLF15 through fasting rapidly switches hepatic metabolism from lipogenesis in the fed state to gluconeogenesis in the fasting state.

KLFs Contribute to NAFLD Development

In addition to its essential role in fetal liver development, KLF6 is involved in the pathogenesis of liver steatosis and fibrosis (33). Expression of KLF6 is reportedly increased during early hepatic fibrosis in response to liver injury induced by CCl_4 administration in rats (34). KLF6, which is induced early after injury, transcriptionally activates transforming growth factor β1 (TGF-β1) and TGF-β receptors in hepatic stellate cells (35). Activation of TGF-β signaling in stellates cells upregulates genes involved in fibrosis, such as those encoding extracellular matrix, plasminogen activator inhibitor and platelet-derived growth factor receptors (33). An association between TGF-β1 and liver

fibrosis was confirmed by the observation that liver-specific, forced expression of TGF-β1 induces liver fibrosis associated with increased expression of collagen I in hepatic stellate cells (36).

Liver steatosis is frequently observed in heavy drinkers but also in conditions such as obesity, diabetes, and dyslipidemia. Non-alcoholic fatty liver is one of the two types of NAFLD and can progress into NASH, which is characterized by inflammation and fibrosis in addition to steatosis. *Klf6* and *Tgfb1* are upregulated during the progression of rat models of NASH (37), and hepatocyte-specific *Klf6* deletion is protective against HFD-induced liver steatosis and insulin resistance, which suggests KLF6 contributes to NASH development (38). KLF6 also acts at the post-transcriptional level to upregulate peroxisome proliferator-activated receptor α (PPARα) expression. PPARα controls several genes that promote insulin resistance and NASH, including *Trib3*, whose expression is correlated with that of *KLF6* in liver tissue from humans with NAFLD (38). However, the actions of PPARα in the context of NASH are much more complex. For instance, while *Ppara* deletion reportedly enhances HFD-induced NASH pathology (39), a conflicting observation has also been reported (40). In line with the anti-fibrotic function of PPARα, a recent whole genome profiling study identified pro-fibrogenic dermatopontin (encoded by *Dpt*) as a potential downstream mediator of KLF6 in NASH (41). PPARα activation reduced *Dpt* expression, possibly by decreasing *Klf6* and *Tgfb1* expression. KLF6 thus appears to be profibrogenic in the liver, but its interactions with PPARα are complex, and their effects in NASH may be context-dependent.

Hepatic *KLF6* expression is associated with more advanced stages of NAFLD in humans (42), and the presence of KLF6 splicing variants that antagonize the full-length form increases the complexity of its role in the ailment (43). A single nucleotide polymorphism (*KLF6–IVS1-27G>A*, rs3750861) that promotes alternative splicing of KLF6 into the dominant-negative variant was shown to negatively associate with the level of NASH-related fibrosis (42), supporting the notion that full-length KLF6 promotes fibrosis. But although both experimental and clinical findings suggest KLF6 promotes NASH, KLF6 levels correlate negatively with steatosis levels in NAFLD samples (24). This suggests KLF6 may have different functions at different stages of NASH development. Because KLF6 also affects stellate cells and macrophages, during NASH development it may be involved in a variety of pathological processes, including hepatocyte metabolism, myofibroblastic activation of stellate cells, fibrosis, and inflammation. Consequently, KLF6's roles during NASH development are likely complex and cell- and disease stage-dependent.

KLF2 is also involved in hepatic steatosis and is significantly elevated in the livers of obese mice. Adenovirus-mediated overexpression of *Klf2* induces accumulation of triglycerides in lean mice, while silencing *Klf2* ameliorates liver steatosis in obese ob/ob mice. KLF2 acts by directly upregulating expression of CD36, which mediates hepatic triglyceride accumulation (44).

KLFs Involved in the Regulation of Macrophages in Liver and Artery Disease

In addition to hepatocytes, recent studies have revealed the crucial contributions made by non-hepatocytes to the control of metabolism and to pathology in the liver (45, 46). In particular, liver macrophages play critical roles in metabolism, inflammation, fibrosis, and repair. Macrophages exhibit diverse phenotypes and functions in response to environmental cues (47). The M1/M2 classification was established based mainly on observations of cultured macrophages *in vitro* and has been used to group the variety of macrophage phenotypes into two subgroups (48, 49). Exposure to TLR ligand or Th1 cytokines, such as TNF-α, and IFN-γ, activates macrophages into the proinflammatory, M1 phenotype. M1 macrophages express proinflammatory cytokines and reactive oxygen species. By contrast, Th2 cytokines, such as IL-4 and IL-13, induce the M2 phenotype, though other factors are likely involved in *in vivo* settings. M2 macrophages are known to be essential for parasite clearance and have also been shown to promote resolution of inflammation and fibrosis (50, 51).

Although the M1/M2 dichotomy has been widely used, it is now clear that it does not adequately encompass the diversity and plasticity of macrophage phenotypes and functions. For instance, M1 and M2 markers can be expressed simultaneously in macrophages *in vivo* (52). Moreover, macrophages *in vivo* must respond to numerous signals other than the model stimuli used *in vitro* and express very divergent transcriptomes. In addition, while proinflammatory M1 macrophages are often said to promote inflammation and damage tissue, they are also essential for resolution and regeneration in several injury models, such as muscle injury, and regeneration (53). Consistent with that observation, M1 macrophage-derived inflammatory cytokines, such as TNF-α, have been shown to positively regulate healing and regeneration. As such, the simple M1/M2 dichotomy based on expression of a few marker genes cannot predict the functions of macrophages within complex inflammation and healing processes *in vivo*. In that regard, although several KLF members have been shown to be important for M1/M2 activation, mainly in *in vitro* settings, less clear are their regulatory functions during macrophage activation in *in vivo* inflammatory settings.

Several KLF members are known to be involved in the activation of macrophages (**Figure 3**). KLF4 is important for the IL-4-induced M2 phenotype in macrophages, while deletion of *Klf4* enhances expression of M1 macrophage-related genes, demonstrating that KLF4 promotes M2 polarization of macrophages (54, 55). KLF2 inhibits NF-κB-dependent proinflammatory activation of macrophages (56, 57). By contrast, KLF6 acts cooperatively with NF-κB to promote proinflammatory gene expression and to suppress M2 marker gene expression, thereby promoting M1 polarization (58, 59). KLF6 also promotes macrophage motility and recruitment, in part by suppressing BCL6 (60). These results suggest KLF6 promotes proinflammatory activation of macrophages. However, myeloid-specific deletion of *Klf6* promoted aortic inflammation in a mouse model of aortic aneurysm (61). In that study, KLF6 negatively regulated production of GM-CSF, which is critical

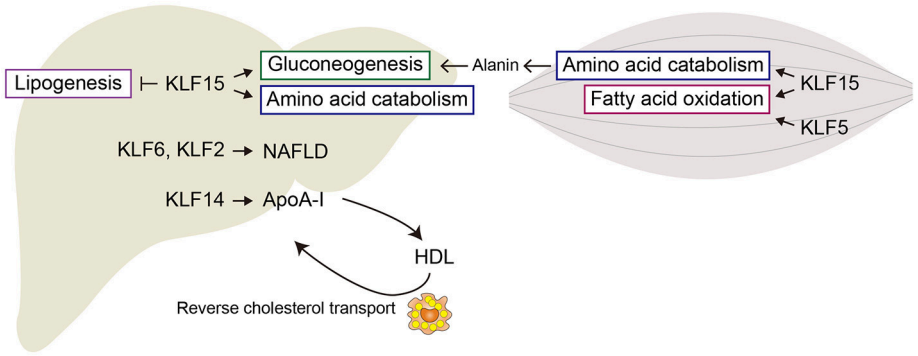

FIGURE 2 | Metabolic regulation by KLFs in liver and skeletal muscle. Shown are major metabolic pathways regulated by KLFs. In liver, KLF15 enhances gluconeogenesis and amino acid metabolism, but inhibits lipogenesis. KLF2 and KLF6 are involved in the development of steatohepatitis and liver fibrosis. In skeletal muscle, KLF15 regulates amino acid catabolism and fatty acid oxidation. KLF5 is also involved in the regulation of fatty acid oxidation.

for aneurysm development and inflammation. These conflicting findings highlight the need for more studies of macrophage KLF functions in *in vivo* inflammatory models.

Myeloid-specific *Klf4* deletion augments HFD-induced obesity, insulin resistance and adipose tissue inflammation (54), which is indicative of the systemic metabolic impact of KLF4-dependent regulation of macrophage activation. In addition, a pivotal contribution of KLF4-dependent regulation of macrophage function to cardiovascular disease is exemplified by the observation that myeloid *Klf4* deficiency augments vascular inflammation and atherosclerotic lesion formation in $Apoe^{-/-}$ mice (62). A surprising function of KLF4 in atherosclerosis was also recently reported (63). KLF4 promotes phenotypic modulation of smooth muscle cells (SMCs) from a highly differentiated state to a less differentiated "synthetic" phenotype. Lineage tracing studies identified phenotypically modulated SMCs that express macrophage markers within atherosclerotic plaques, indicating that SMCs are able change their phenotype, even becoming macrophage-like. KLF4 appears to regulate this transition to a macrophage-like phenotype. Collectively then, whereas KLF4 in myeloid-lineage cells appears to be atheroprotective, KLF4 in SMCs is proatherogenic. These results point to differential functions of KLF members in different cell types in pathology. Moreover, recent studies also showed that myeloid-lineage cells can acquire SMC-like phenotypes (64, 65). These findings question the lineages of traditionally identified SMCs and macrophages and the functions that have often been assigned to them (e.g., anti- and pro-atherogenic).

KLF14 Regulates Reverse Cholesterol Transport

Within the liver, macrophages modulate hepatocyte metabolism during homeostasis and during development of NAFLD. They also play a key role in the chronic inflammation contributing to NASH. In mouse models of diet-induced NAFLD, the liver-resident macrophages, Kupffer cells, increase hepatic triglyceride accumulation, and proinflammatory cytokine production and suppress fatty acid oxidation and insulin sensitivity (66). HFD-induced obesity also increases numbers of monocyte-derived macrophages in the liver, where they promote inflammation and insulin resistance (67). Han et al. recently showed that a nuclear receptor, retinoic-acid-related orphan receptor α (RORα) induces M2 polarization by activating KLF4 in Kupffer cells, and that myeloid-specific deletion of *Rora* aggravated HFD-induced hepatic steatosis and inflammation (68). Interestingly, M2-polarized Kupffer cells produce IL-10, which reduces lipid accumulation and lipotoxicity in hepatocytes, suggesting liver macrophages control both inflammation and metabolism. In that regard, perturbing M2 activation of Kupffer cells through *Ppard* deletion reportedly impairs hepatic fatty acid oxidation and promotes hepatic steatosis (69), though the precise mechanism by which hepatic metabolism is regulated remains unknown. Macrophages are thus integral to the control of hepatic lipid metabolism, and KLF4 appears to be a critical regulator of these macrophages.

KLF14 expression is reduced in the livers of dyslipidemic model mice, and hepatic overexpression of human *KLF14* increased plasma high density lipoprotein (HDL) cholesterol levels by inducing apolipoprotein A-I (ApoA-I), a major protein component of HDL particles (70). Conversely, loss of *Klf14* decreased HDL cholesterol levels in the liver. KLF14 transactivates hepatic *Apoa1* transcription.

Epidemiological studies have shown an inverse association between plasma HDL cholesterol levels and coronary heart disease (71). ApoA-I is primarily responsible for reverse cholesterol transport (RCT), through which cholesterol is transported from peripheral tissues back to the liver by HDL (72). RCT is thought to be a major mechanism underlying the anti-atherogenic effects of HDL cholesterol. The crucial initial step in RCT from atherosclerotic lesions is efflux of cholesterol from macrophages to extracellular acceptors, namely HDL. Upregulation of hepatic *Klf14* expression may thus protect against atherosclerosis (**Figure 1**).

Guo et al. used drug screening to identify perhexiline as an activator of *Klf14* expression (70). Perhexiline is a prophylactic

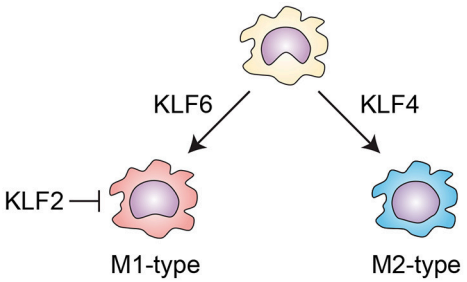

FIGURE 3 | KLFs control macrophage activation. KLF4 is important for the IL-4-induced M2 phenotype in macrophages. By contrast, KLF6 promotes proinflammatory gene expression and suppresses M2 marker gene expression, thereby promoting M1 polarization. KLF2 inhibits NF-κB-dependent proinflammatory activation of macrophages.

antianginal agent thought to act by inhibiting mitochondrial carnitine palmitoyltransferase-1 (CPT-1). Administration of perhexiline increases HDL cholesterol and ApoA-I levels and suppressed atherosclerosis in *Apoe*$^{-/-}$ mice. Because genome-wide association studies have shown that genetic variations near the *Klf14* locus are associated with HDL cholesterol levels, coronary heart disease, and metabolic syndrome (70), therapeutic intervention targeting KLF14 is an attractive strategy for the treatment and prevention of human dyslipidemia and atherosclerosis.

KLFS IN SKELETAL MUSCLE BIOLOGY AND PATHOBIOLOGY

KLFs Control Muscle Regeneration and Development

Skeletal muscle is the dominant organ for locomotion, postural maintenance, and energy metabolism in mammals. It is the largest organ in non-obese subjects and a major site of insulin- and exercise-stimulated glucose disposal (73, 74), with a remarkable capacity for repair and regeneration in response to injury. During the course of embryonic development, mesenchymal progenitor cells originating from the somites undergo a multistep differentiation process to form skeletal muscle (75). Muscle satellite cells are myogenic precursor cells formed during embryonic development, and are also present in a quiescent state within adult muscle. In response to muscle damage, satellite cells are activated and assume a myoblast identity. Satellite cell-derived myoblasts undergo differentiation and fusion to form myotubes that replace the damaged myofibers (76, 77). The molecular mechanism that controls muscle regeneration recapitulates many aspects of the process of muscle development and many of the transcription factors that control embryonic myogenesis contribute to adult regenerative myogenesis (77, 78).

Skeletal muscle repair is conducted through activation, proliferation, and differentiation of satellite cells, a population of muscle stem cells that reside within a niche between the basal lamina and the sarcolemma of associated muscle fibers. A family of four myogenic regulatory factors (MRFs) govern early skeletal muscle development and also control the postnatal muscle regeneration program (77, 79, 80). These MRFs include the myogenic basic-helix-loop-helix type transcription factors MyoD and Myf5, which bind to regulatory regions of skeletal muscle-specific genes, where they determine myogenic fate and initiate the differentiation cascade (77). Thereafter, MyoD acts in cooperation with myogenin and MRF4 to increase expression of late target genes through a feed-forward mechanism that regulates terminal differentiation (81). MRFs also interact with other transcription factors, including MEF2 in myogenesis (77, 82), and recent studies indicate the involvement of several KLFs.

KLF7 is critical for maintaining satellite cell quiescence *in vitro* through activation of p21 expression (83). KLF7 expression is increased by TGF-β and Notch signaling, which controls satellite cell quiescence and myoblast arrest. KLF7 thus appears to be important for maintenance of satellite cell quiescence, though this needs to be tested in *in vivo* settings.

We found that KLF5 regulates muscle differentiation and regeneration by directly controlling muscle-specific genes in cooperation with MyoD and MEF2 (77). During muscle regeneration after injury caused by cardiotoxin injection, expression of KLF5 is upregulated in the differentiating myoblasts and newly formed myofibers, and the expressed KLF5 is recruited to MyoD binding sites. Interestingly, association of MyoD with its binding sites is greatly reduced in the absence of KLF5, which is consistent with close cooperation between these two transcription factors. Satellite cell-specific *Klf5* deletion using the *Pax7-CreER* line delays and impairs muscle regeneration, confirming the role of KLF5 in muscle repair. Notably, inflammation and fibrosis are enhanced in injured muscle tissues from satellite cell-specific *Klf5*$^{-/-}$ mice (77), which highlights the close interplay between myocyte differentiation and regulation of inflammation during muscle regeneration after injury (84).

Fusion of myoblast into multi-nucleated fibers are necessary for the maturation and differentiation of myotubes. It is reported that KLF2 and KLF4 are upregulated in differentiating muscle cells and promote muscle cell fusion (85). During muscle differentiation of C2C12 myoblasts, *Klf2* and *Klf4* are upregulated by ERK5, a member of extracellular signal-regulated kinase (ERK) family, at least in part through activation of Sp1, which transactivates *Klf2* and *Klf4* transcription. KLF2 and KLF4 in turn transactivate *Npnt*, which encodes nephronectin, a mediator required for muscle fusion. The MEK5-ERK5-Sp1-KLF2/4-nephronectin pathway is thus crucial for muscle cell fusion and myotube differentiation of C2C12 cells, though the function of this pathway remains to be tested *in vivo*.

KLF3 was identified as a factor that enhance muscle creatine kinase expression by binding to a GC-rich sequence in the muscle creatine kinase (*Mck*) promoter, in concert with serum response factor (SRF) (86). In addition, their chromatin immunoprecipitation (ChIP) assays indicated that KLF3 binds to the promoter regions of myosin heavy chain IIa (*Myh2*), *Six4*, skeletal α-actin (*Acta1*), and calcium channel receptor α-1 genes

in cultured muscle cells, suggesting KLF3 controls muscle genes, though its actions during muscle development and regeneration have not been directly tested.

Collectively, these results demonstrate that several KLF members play important roles in the differentiation of C2C12 and satellite cells. However, with the exception of KLF5 in muscle regeneration, their *in vivo* functions are less clear. Future studies could further address the functions of KLFs during muscle regeneration and embryonic development by, for example, deleting selected *Klf*s from myogenic precursor cells using *Pax3* and *Pax7* promoter-driven cre lines.

KLFs Control Muscle Metabolism

KLF15 is a critical regulator of skeletal muscle nutrient catabolism and a key determinant of exercise capacity (22, 87, 88). KLF15 expression is low during development and robustly induced during postnatal maturation (87). Nonetheless, *Klf15* knockdown does not interfere with muscle differentiation of C2C12 cells (89), and the skeletal muscle in *Klf15*$^{-/-}$ mice exhibit no clear developmental defects (87). In addition, KLF15 controls expression of slow-type myosin heavy chain (*Myh7*), suggesting it contributes to myofiber typing (89), though *Klf15*$^{-/-}$ mice exhibit no changes in their fiber type compositions (87).

As in the liver, KLF15 in muscle regulates genes involved in amino acid and lipid metabolism. *Klf15* expression in both skeletal muscle and liver is upregulated by fasting (21, 31, 90). During starvation, amino acids derived from muscle proteins are degraded to ketoacids, which are metabolized to pyruvate by PEPCK and pyruvate kinase. Alanine aminotransferase is active in skeletal muscle, so much of the pyruvate produced is transaminated to alanine. The alanine is then transported to the liver and used for gluconeogenesis (**Figure 2**). Branched-chain amino acids (BCAAs) are the major donors of amino groups for alanine synthesis (91). In *Klf15*$^{-/-}$ skeletal muscle, expression of *Bcat2*, encoding mitochondrial branched-chain aminotransferase 2, which catalyzes the first step in the oxidation of BCAAs, is downregulated (22). This is consistent with the observation that KLF15 activated during fasting enhances the flux of BCAA-derived carbons into the gluconeogenic pathway. It thus appears that KLF15 acts collaboratively in both the liver and skeletal muscle to promote glucose production in the liver.

Klf15 is also induced in both mouse and human muscle by acute endurance exercise (87). *Klf15*$^{-/-}$ mice exhibit diminished endurance capacity during treadmill running, which is indicative of the importance of KLF15 for endurance exercise performance. In the absence of KLF15, the soleus muscle (slow twitch muscle), which uses predominantly lipid oxidation for energy production, has reduced capacity for repetitive contraction, whereas fast-twitch function is largely unaffected. In *Klf15*$^{-/-}$ soleus muscle there is dysregulation of genes involved in fatty acid partitioning/transport, fatty acid oxidation and lipid storage. As expected from these alterations, *Klf15*$^{-/-}$ muscle exhibits reduced lipid utilization. These results indicate that KLF15 controls the transition from carbohydrate catabolism to higher reliance on lipid oxidation during endurance exercise (87, 92).

It is previously demonstrated that the involvement of KLF5 in fatty acid metabolism in skeletal muscle (93). We found that heterozygous *Klf5* knockout mice show resistance to diet-induced obesity accompanied by increased systemic energy expenditure. Under basal conditions, SUMOylated KLF5 associates with transcriptionally repressive regulatory complexes containing unliganded PPARδ and co-repressors, which inhibit expression of the lipid oxidation genes *Cpt1b*, *Ucp2,* and *Ucp3*. Upon agonist stimulation of PPARδ, deSUMOylation of KLF5 is induced and the unSUMOylated KLF5 associates with transcriptional activation complexes containing liganded PPARδ and coactivators, which then transactivates lipid oxidation genes.

Drosatos et al. recently showed that KLF5 directly regulates *Ppara*, which controls genes involved in lipid metabolism in cardiomyocytes (94). Cardiomyocyte-specific *Klf5* deletion induces cardiac dysfunction and lipid accumulation in aging mice. In models of diabetes, cardiac expression levels of *Klf5* and *Ppara* were correlated, suggesting involvement of KLF5 in diabetic cardiomyopathy. While the function of the KLF5-PPARα pathway in skeletal muscle is unknown at the present, these findings suggest that interactions between KLF5 and PPARs are important for regulation of lipid metabolism in muscle.

KLFs Control Skeletal Muscle Growth and Pathological Wasting

Recent studies suggested KLF15 is involved in regulating muscle mass in both physiological and pathophysiological conditions. Skeletal muscle loss, so called muscle atrophy, occurs under various conditions, including prolonged disuse, sepsis, cachexia, starvation, type 2 diabetes, and aging (95). Muscle mass reflects the dynamic balance between anabolic and catabolic processes, and muscle atrophy in adult tissues occurs when the rate of protein degradation exceeds that of protein synthesis (96). mTORC1 is one of two protein complexes that contain mammalian target of rapamycin (mTOR), which controls protein synthesis in response to growth factors, energy status, oxygen, and amino acids, especially BCAAs (97). Protein degradation is primarily mediated via two major pathways: the ubiquitin-proteasomal pathway and autophagic/lysosomal pathway (96).

Glucocorticoids affect metabolism in skeletal muscle, and the effect of their prolonged use is muscle atrophy. Glucocorticoid (dexamethasone) induces *Klf15* as well as atrophy-related genes (collectively termed "atrogenes") such as *Fbxo32* (atrogin-1), *Trim36* (MuRF1), *Foxo1* (FoxO1), and *Mstn* (myostatin) in muscle, but does not affect *Klf15* expression in the liver. Glucocorticoid-induced *Klf15* expression is mediated by direct transactivation of *Klf15* transcription by glucocorticoid receptor (GR) (88). KLF15 transactivates *Fbox32* and *Trim36* expression in cooperation with FoxO1 in response to dexamethasone, and the adenovirus-mediated overexpression of *Klf15* induces expression of atrogenes (88), although conflicting data have been reported (98). As with fasting, KLF15 transactivates *Bcat2* transcription and enhances BCAA catabolism in response to dexamethasone. Amino acids, particularly BCAAs, activate mTOR, while overexpression of KLF15 suppresses mTOR activity and causes muscle atrophy. It thus appears that

KLF15 acts in concert with other mediators to control expression of atrogenes and BCAA degradation in response to prolonged use of glucocorticoids. The GR-KLF15 axis promotes muscle breakdown and nutrient transfer from muscle to the liver under stressful conditions associated with excess levels of glucocorticoids. However, chronic dexamethasone induces muscle atrophy in *Klf15*$^{-/-}$ as well as wild-type mice (98), which means chronic dexamethasone can also induce muscle atrophy independently of KLF15. Moreover, while adenovirus-mediated strong overexpression of *Klf15* in muscle induces expression of atrogenes (88), dexamethasone-induced upregulation of atrogenes is not affected by deletion of *Klf15* (98). Furthermore, moderate overexpression of *Klf15* in skeletal muscle via a transgene did not induce atrogenes or muscle atrophy (98). Consequently, KLF15's role in atrogene expression remains unclear. Further studies will be needed to clarify the functional involvement of KLF15 in muscle wasting, though results of earlier studies suggest KLF15 may control muscle metabolism in both physiological and pathological settings.

Whereas excessive or sustained glucocorticoid exposure induces muscle atrophy, moderate or transient use of glucocorticoid enhances muscle performance in both animals and humans (98). Moreover, increases in endurance exercise capacity were observed in wild-type mice receiving a single dose of dexamethasone, but no such increase was observed in *Klf15*$^{-/-}$ mice. Comparing transcriptomes in muscle tissues from wild-type and *Klf15*$^{-/-}$ mice treated with a single dose of dexamethasone, Morrison-Nozik et al. found that KLF15 is important for induction of genes related to amino acid and lipid metabolism, but not atrophy-related genes (98). It appears that low-dose and/or transient glucocorticoid induces KLF15 that controls metabolic genes without inducing atrogenes. A separate study comparing the effects of daily and weekly administrations of glucocorticoid found that *Klf15* expression is increased by weekly administration of glucocorticoid, but not by daily administration (99). Interestingly, histone marks at the GR binding site on *Klf15* indicate that while weekly glucocorticoid activate the enhancer, a daily regimen inhibited it, despite strong recruitment of GR to the region. This suggests GR exerts highly context-dependent regulatory effects on *Klf15* and muscle gene transcription.

The physiological importance of KLF15 is also suggested in the context of muscular dystrophy and muscle repair. *Klf15* deletion exacerbates the dystrophic phenotype in *mdx* mice, a model of Duchenne muscular dystrophy, while 5-fold overexpression of *Klf15* ameliorates the phenotype (98). Low-dose or weekly administration of glucocorticoid suppresses the dystrophic phenotype, while daily glucocorticoid promotes muscle atrophy in *mdx* mice (98, 99). A weekly regimen of glucocorticoid, but not a daily regimen, also improves muscle repair following cardiotoxin-induced acute muscle injury (99). It appears that KLF15 is involved in maintaining muscle physiology in response to a variety of stresses, including exercise, muscle injury, fasting, and glucocorticoid, presumably through regulation of muscle metabolism. However, excessive activation or dysregulation of KLF15 may lead to muscle wasting in response to reduced mTOR activity due to altered BCAA metabolism and possibly induction of atrophy-related genes, though its pathological mechanism needs to be further analyzed.

A recent study showed that KLF15 also critically controls metabolic genes in the heart (100). *Klf15* deficiency alters the circadian oscillation of the expression of a large number of genes, particularly genes related to metabolism. KLF15 appears to regulate genes involved in lipid and amino acid catabolism, presumably to support ATP production during the active phase. Based on the effects of cardiomyocyte-specific disruption of the cellular clock, Van Laake et al. proposed that "the cardiomyocyte clock promotes oxidative metabolism at the sleep-wake transition (in anticipation of increased energetic demand upon awakening), augments nutrient storage toward the end of the awake period (in anticipation of the upcoming fast during the sleep period), and increases cellular constituent turnover at the beginning of the sleep period (facilitating repair/renewal of the myocardium prior to awakening)" (101). Accordingly, the circadian control of cardiac metabolism by KFL15 may also regulate the shift toward cardiac remodeling and repair during the inactive phase by regulating metabolism as well as the genes involved in remodeling and growth (100, 102). In addition to physiological cardiovascular regulation (e.g., heart rate and blood pressure), time-of-day-dependent changes are also observed in diseases (e.g., arrhythmia, sudden cardiac death, and myocardial infarction). In that regard, KLF15 controls diurnal expression of Kv channel interacting protein 2 (KChIP2), a critical subunit required for generating the transient outward potassium current (103). *Klf15* deletion causes loss of diurnal QT variation, abnormal repolarization, and greater susceptibility to ventricular arrhythmias. It thus appears KLF15 is a key regulator of time-dependent cardiac physiological and pathological events.

Systemic deletion of *Klf10* results in hyperplasia and hypertrophy of both slow (soleus) and fast (extensor digitorum longus) muscles and leads to glycolytic hypertrophy in mice, regardless of muscle type (104). In addition, overexpression of *Klf10* suppresses differentiation of C2C12 myoblasts (105). *Klf10* is induced by TGF-β signaling, and KLF10 expression is upregulated by myostatin, a member of TGF-β family that inhibits myogenesis. These findings suggest KLF10 acts as a mediator of myostatin's negative effects on myogenesis and muscle growth, though this idea needs to be directly tested in *in vivo* settings.

CONCLUSION AND FUTURE PERSPECTIVES

KLFs control a variety of processes in the liver and skeletal muscle (**Table 1**). In addition to their roles in development, their functions in the control of metabolism are essential for both local tissue and systemic metabolic homeostasis in the context of dynamic variation in the supply of and demand for energy. In that regard, KLF15 is well-studied for its coordinated actions in the maintenance of systemic metabolism. During fasting, KLF15-mediated upregulation of BCAA metabolism in skeletal muscle and upregulation of gluconeogenesis in the liver coordinate the flux of substrates for gluconeogenesis from muscle to liver to

TABLE 1 | Functions of KLFs in liver, skeletal muscle, and macrophages.

	Liver	Skeletal muscle	Macrophage
KLF2	↑Steatosis (*Cd36*)	Myotube fusion (*Npnt1*)	↓M1 activation
KLF3		?Differentiation (*Mck*)	
KLF4		Myotube fusion (*Npnt1*)	↑M2 activation
KLF5		Differentiation/regeneration Fatty acid oxidation (*Cpt1b*, *Ucp3*)	
KLF6	Liver development ↑Fibrosis (*Tgfb1*, *Tgfbr1* in SCs) ↑Steatosis (PPARα) ↑Glucose disposal (*Gck*)		↑M1 activation
KLF7		Quiescence of SCs (*Cdkn1*)	
KLF10		↓Myogenesis	
KLF14	↑Reverse cholesterol transport (*Aopa1*)		
KLF15	↑Gluconeogenesis ↑Amino acid catabolism ↓Lipogenesis (*Srebf1*)	↑Amino acid catabolism ↑Fatty acid oxidation	

The physiological and pathological functions of KLF members are listed, as are their major targets. In the liver column, the indicated targets are regulated in hepatocytes, except Tgfb1 and Tgfbr1, which are regulated in satellite cells (SCs). KLF3 is suggested to control muscle differentiation, thought this has not been formally tested.

maintain blood glucose levels (106). The coordination of KLF15 function in metabolism is also suggested by its involvement in the circadian regulation of nitrogen (107). In *Klf15*$^{-/-}$ mice, the circadian rhythmicity in circulating amino acid and urea levels is markedly altered. That *Klf15* expression exhibits circadian rhythm in both liver and muscle suggests KLF15 coordinately controls oscillatory amino acid metabolism. These findings have established KLF15 as a critical physiological regulator of liver and muscle metabolism. Accordingly, dysregulation of KLF15 may contribute to liver and muscular pathologies by affecting metabolism. Similarly, alterations in metabolism resulting from dysregulation of other KLFs may be involved in the development of metabolic disease, though this idea remains largely untested.

In addition to their function in metabolic regulation, KLFs may also contribute to metabolic diseases by affecting a variety of other biological processes. For instance, the regulation of inflammation by KLFs via macrophage activation likely contributes to metabolic diseases. In liver inflammation, control of stellate cell activation by KLF6 is important for fibrosis. In addition, regulation of muscle stem cell maintenance and differentiation by KLFs may contribute to sarcopenia and pathological muscle wasting. As such, studies of the cell-specific functions of KLFs would be important for elucidating how KLFs coordinately regulate various cells to maintain tissue and metabolic homeostasis, and how such regulation is involved in metabolic disease development.

Alterations in KLF-dependent regulation of metabolic organs such as the liver, skeletal muscle and adipose tissue likely contribute to the development of cardiovascular diseases via multiple pathways, including insulin resistance, dyslipidemia, and proinflammatory cytokine production within adipose tissue. It is also very likely that KLF-mediated regulation of metabolism within cardiovascular cells is crucially involved in cardiovascular disease development. For instance, KLF5 and KLF15 have been shown to control lipid metabolism within cardiomyocytes, which is essential for maintenance of cardiac homeostasis, at least in part by interacting with nuclear receptors (94, 108, 109). Recent studies have also revealed the crucial involvement of endothelial metabolism in physiological and pathological angiogenesis (110). KLFs may also control endothelial and SMC function through metabolic regulation. This remains to be tested, however.

In this article, we presented an overview of the involvement of KLFs in the hepatic inflammation and fibrosis that leads to NASH. Although we did not cover it in this review, it has been suggested that inflammation contributes to the muscle atrophy seen in both type 2 diabetes and sarcopenia (95, 111). KLFs are also likely involved in muscle inflammation, but their specific functions remain largely unknown (77). Another important unexplored aspect of KLFs is their actions at the crossroad of metabolism and immunity (immunometabolism) (112, 113). It is becoming increasingly clear that the regulatory pathways governing metabolism and inflammation are tightly linked. For instance, on the one hand visceral obesity induces chronic inflammation within visceral adipose tissue, which in turn contributes to the development of cardiometabolic diseases, such as atherosclerosis and type 2 diabetes, in part by promoting inflammation in the affected tissues. On the other hand, regulation of cellular metabolism is integral to the inflammatory and regulatory activation of immune cells (114). It has not yet been well-addressed, but systemic and/or local metabolic disturbances may alter immune cell activities by modulating their cellular metabolism (113, 114). Metabolism and immunity are thus intricately connected at the cell, tissue and system levels. Future studies will need to address how KLF-dependent regulation of metabolism in various cells types, including immune cells, contribute to inflammatory processes in cardiometabolic tissues.

An important functional characteristic of many KLF members, which requires further study, is that their target genes can be different in different cellular and environmental contexts. For instance, ChIP-sequencing showed that KLF5 binding sites in C2C12 myotubes and 3T3-L1 adipocytes differ, and only a small fraction of sites are common between the two cell types (**Figure 4A**). Moreover, even in the same C2C12 cells, differentiation alters KLF5 bindings sites (**Figure 4B**) (77). Likewise, KLFs may control different sets of genes in the same cells in response to different microenvironments. An example is that renal injury reduces homeostatic KLF5 binding to the *Cdh1* promoter, and instead KLF5 is recruited to the *S100a8* and *S100a9* promoters in renal collecting duct epithelial cells (118). Furthermore, even at the same promoter, post-translational modifications may modulate KLF function. For example, SUMOylation switches KLF5 function from an activator to a repressor (93). Such context-dependent changes in KLF functions need to be further analyzed, particularly in pathological settings. In that regard, because recent ChIP-sequencing experiments

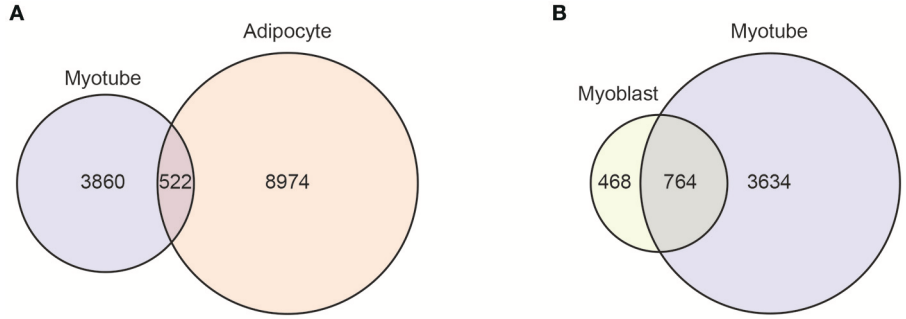

FIGURE 4 | Context-dependent differential KLF5 binding. Venn diagrams showing the overlap between the KLF5 peaks in C2C12 myotubes and 3T3-L1 adipocytes **(A)** and in C2C12 myoblasts and myotubes **(B)**. KLF5 binding peaks were detected using published ChIP-sequencing data (GSE80812 and GSE56872). For myotube peaks, KLF5 ChIP-sequencing of C2C12 cells differentiated for 5 days was used (77). 3T3-L1 cells were treated with an adipocyte differentiation cocktail for 4 h and used in an early differentiated state (115). Reads were mapped to the mm9 genome using STAR (116). Peak calling and annotation was performed using HOMER version 4 (117). Peaks that overlapped blacklisted regions (ENCODE Project Consortium, 2012) or simple repeat regions were removed. Homer-identified peaks with scores $\geq$ 20 were used as high confidence binding sites (77).

showed that KLF members bind to thousands of genomic sites, it will be important to analyze context-dependent changes in genome-wide binding of KLFs. Currently, ChIP-sequencing data are available for only a few KLFs in limited cell types. In addition, for most KLF bindings sites, it remains unclear whether KLF binding has regulatory importance (e.g., enhancer activity). Genome-wide interrogation of enhancer activation states and the binding of other transcription factors along with transcriptomic analysis, including enhancer RNAs, will provide us with a better understanding of the regulatory functions of KLF members. Another important issue is how the genome-wide target genes of KLF control biological processes as a network. Although studies have so far identified a small number of key targets of KLFs, it is very likely that the target genes that can be identified through ChIP-sequencing and other genome-wide technologies will also play important roles.

As we have discussed in this review, KLFs control a variety of biological processes within metabolic tissues. Although each KLF member appears to have a distinct set of functions, they may also cooperate and/or compete in control of the same process. For instance, KLF5 and KLF15 regulate lipid metabolism in skeletal and cardiac muscle, and KLF6 and KLF15 regulate glucose metabolism in the liver. KLF2/4/6 are involved in macrophage activation. Future studies will need to address how multiple KLF members coordinate metabolism and inflammation. Together, findings from transcriptomics, epigenomics and metabolomics studies may reveal the potential interlinks between KLFs.

Because of their crucial involvement in cardiometabolic diseases, extensive efforts have been made to develop pharmacological agents with which to modulate KLF function. Metformin and perhexiline are two mentioned in this review. Up to now, compounds reported to alter KLF function have acted on pathways affecting KLF expression and post-translational modification, degradation, and transcriptional activity. However, the interactions between KLFs and nuclear receptors are a particularly attractive target in the context of cardiometabolic diseases, as KLFs interact with nuclear receptors to control cellular metabolism and inflammation (119). For instance, we previously showed that KLF5, which contributes to the control of lipid metabolism within cardiomyocytes, interacts with RARα and PPARδ, and its activity can be modulated by ligands for those nuclear receptors (93, 120). This supports the idea that KLFs can be therapeutically targeted using nuclear receptor ligands.

In conclusion, KLFs are important contributors to both homeostasis and pathology in metabolic tissue. They also control metabolism in both cardiovascular and immune cells, as well as immune cell activation. These diverse functions of KLFs may converge at the development of cardiometabolic disease via complex interplay at the cellular, tissue and systemic levels. A better understanding of the KLF-regulated networks that control communications between cells, tissues, and systems could promote development of drugs targeting these disease pathways.

AUTHOR CONTRIBUTIONS

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

FUNDING

This study was supported in part by the Joint Usage/Research Program of MRI, TMDU, Grant-in-Aid for Scientific Research (17K09589 to YO, 16H05295, 17KT0047 to IM) and Grant-in-Aid for Scientific Research on Innovative Areas Stem Cell Aging and Disease (17H05636 to YO, 17H05632 to IM) from the MEXT Japan; JP17gm5910021h0001 (to YO), JP17gm0610011h0404, and JP17gm5010002 from Japan Agency for Medical Research and Development, AMED (to IM); and grants from MSD Foundation, Daiichi Sankyo Foundation of Life Science, Mochida Memorial Foundation for Medical and Pharmaceutical Research,

Daiichi Sankyo Foundation of Life Science, Mitsui Life Social Welfare Foundation, and Nakatomi Foundation (to YO); Takeda Science Foundation, Ono Medical Research Foundation, and SENSHIN Medical Research Foundation (to YO and IM); and Tokyo Biochemical Research Foundation, Suzuken Memorial Foundation, Novartis Foundation for the Promotion of Science, Naito Foundation, and Uehara Memorial Foundation (to IM).

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

frontiers in Cardiovascular Medicine

REVIEW
published: 22 February 2018
doi: 10.3389/fcvm.2018.00012

Source of Chronic Inflammation in Aging

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Aging is a complex process that results from a combination of environmental, genetic, and epigenetic factors. A chronic pro-inflammatory status is a pervasive feature of aging. This chronic low-grade inflammation occurring in the absence of overt infection has been defined as "inflammaging" and represents a significant risk factor for morbidity and mortality in the elderly. The low-grade inflammation persists even after reversing pro-inflammatory stimuli such as LDL cholesterol and the renin–angiotensin system (RAS). Recently, several possible sources of chronic low-grade inflammation observed during aging and age-related diseases have been proposed. Cell senescence and dysregulation of innate immunity is one such mechanism by which persistent prolonged inflammation occurs even after the initial stimulus has been removed. Additionally, the coagulation factor that activates inflammatory signaling beyond its role in the coagulation system has been identified. This signal could be a new source of chronic inflammation and cell senescence. Here, we summarized the factors and cellular pathways/processes that are known to regulate low-grade persistent inflammation in aging and age-related disease.

Keywords: hyper coagulation, cell senescence, inflammation, aging, IGFBP-5

OPEN ACCESS

Edited by:
Masanori Aikawa,
Harvard Medical School,
United States

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

Received: *11 October 2017*
Accepted: *09 February 2018*
Published: *22 February 2018*

Citation:
Sanada F, Taniyama Y, Muratsu J, Otsu R, Shimizu H, Rakugi H and Morishita R (2018) Source of Chronic Inflammation in Aging. Front. Cardiovasc. Med. 5:12. doi: 10.3389/fcvm.2018.00012

INTRODUCTION

At present, chronic inflammation is thought to be a risk factor for a broad range of age-related diseases such as hypertension, diabetes, atherosclerosis, and cancer. (1) Although age-related diseases may be partially preventable with lifestyle modifications, including diet, the burdens of unhealthy aging associated with lifestyle are increasing, both in developed and developing regions. Therefore, the elucidation of the sources and cellular pathways/processes of chronic inflammation is an urgent task. There are several possible factors that initiate and maintain a low-grade inflammatory response. These include aging, unbalanced diet, low level of sex hormones, and smoking. In contrast to young individuals, aged individuals have consistently elevated levels of inflammatory cytokines, especially interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (2), which may induce muscle atrophy and cancer through DNA damage. Visceral fat tissue from obese individuals can also produce both IL-6 and TNF-α, affecting systemic metabolism (3, 4). The accumulation of macrophages in visceral fat seems to be proportional to body mass index and appears to be a major source of low-grade persistent, systemic inflammation and insulin resistance in obese individuals (5, 6). Cigarette smoke contains multiple inducers of inflammation, especially reactive oxygen species. Indeed, chronic smoking increases production of several pro-inflammatory cytokines such as IL-6, TNF-α, and interleukin-1β (IL-1β) (7). Smoking also increases systemic

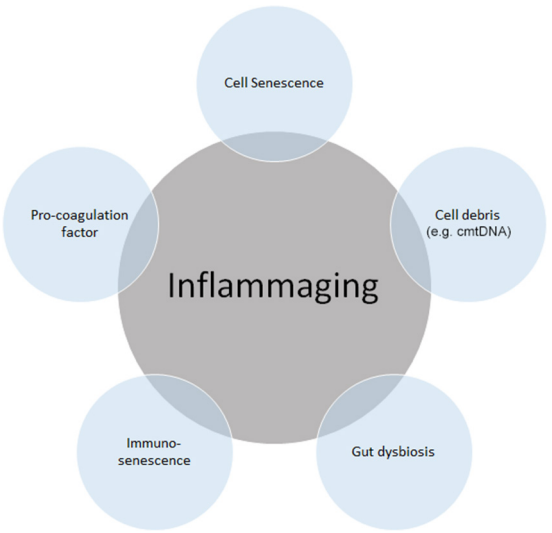

FIGURE 1 | Source of "inflammaging". Among the main causes of inflammaging, the accumulation of cell senescence, pro-coagulation factors, cell debris such as circulating mitochondrial DNA (cmtDNA), gut dysbiosis, and immune senescence is known to be the main causes of inflammaging. Inflammaging can also be influenced by many other factors, including age itself, reactive oxygen species (ROS), and those not directly related to inflammation, such as microRNAs (miRs) and agalactosylated N-glycans.

inflammation and is an independent risk factor for several lifestyle-related diseases. Other inciting factors such as mental stress and periodontal disease have been reported. Importantly, the low-grade inflammation persists even after reversing the pro-inflammatory stimuli such as LDL cholesterol, the renin–angiotensin system (RAS), and smoking. These findings can be explained by the discovery of senescent associated secretory phenotype (SASP) and immunological imprinting.

In this narrative review, we aimed to review the sources of chronic inflammation during aging (**Figure 1**). The cellular pathways/processes that are known to regulate the DNA damage response are also discussed.

SOURCE OF CHRONIC INFLAMMATION IN AGING

Acute inflammation is indispensable for immune responses to invading pathogens or acute traumatic injuries. This process enables repair and cell turnover in multiple tissues. In contrast, chronic inflammation normally causes low-grade and persistent inflammation, leading to tissue degeneration. (8). Chronic, low-grade inflammation is a crucial contributor to various age-related pathologies and natural processes in aging tissue, including the nervous and the musculoskeletal system (9). Many tissues in the elderly are chronically inflamed, and inflammatory cytokines such as IL-6, IL-1β, and TNF-α are known to weaken the anabolic signaling cascade, including insulin and erythropoietin signaling, leading to the development of sarcopenia (10). The possible sources of chronic inflammation during aging, termed "inflammaging", have been described previously (9).

Cell Debris or Immunoglobulin Accumulation

Debris and immunoglobulin accumulation due to inappropriate cell elimination systems in aging trigger the innate immune system activation leading to persist inflammation. Glycosylation represents the most frequent post-translational modification of proteins. Protein-linked sugar chains play a variety of specific roles in the "fine-tuning" of interactions between cells and between molecules (11, 12). High-throughput analysis of the N-glycome, i.e., the sugar chains N-linked to asparagine, revealed new candidate biomarkers of natural aging, such as N-glycans devoid of galactose residues on their branches, in a variety of human studies (13–15) comparing healthy elderly people, centenarians, and their offspring, as well as in experimental animal models (16–18), such as the calorie-restricted mice model. These agalactosylated biantennary structures mainly decorate Asn297 of the Fc portion of IgG (IgG-G0) and are present in patients affected by progeria syndromes as well as a several autoimmune/inflammatory diseases. IgG-G0 exerts a pro-inflammatory effect through various mechanisms, including the lectin pathway of complement, binding to Fcγ receptors and formation of autoantibody aggregates. Similarly, the age-related accumulation of IgG-G0 activating the immune system can contribute to inflammaging. On the other hand, among the complex determinants of aging, mitochondrial dysfunction has attracted attention for some time. The consequences of age-related failing mitochondrial quality control include the release of mitochondria-derived damage-associated molecular patterns (DAMPs). Mitochondrial DAMPs, especially cell-free circulating mitochondrial DNA, have recently become the subject of intensive research because of their possible involvement in conditions associated with inflammation, such as aging and degenerative diseases (19, 20). Through their bacterial ancestry, these molecules contribute to increasing an inflammatory response by interacting with receptors similar to those involved in pathogen-associated responses.

The Gut Mucosa and Microbiota of Elderly People

The barrier of the oral and gut mucosa against bacterial invasion deteriorates with age. Periodontal disease has also demonstrated to cause chronic low-grade inflammation (12). The gut microbiota of elderly people displays decreased diversity (21, 22). The abundance of anti-inflammatory microbiota, such as members of *Clostridium* cluster XIVa, *Bifidobacterium* spp., and *F. prausnitzii* are diminished in aged individuals (23). Toward R et al. demonstrated supportive data that the level of *Bifidobacterium* is inversely correlated with serum levels of inflammatory cytokines, such as TNF-α and IL-1β. Conversely, inflammatory and pathogenic microbiota, including *Streptococcus* spp., *Staphylococcus* spp., *Enterococcus* spp. and *Enterobacter* spp., are increased with age (23). Changes in the gut microbiota diversity in aged people might increase susceptibility

to infectious agents by pathobionts colonization. Unique stool microbiota profiles were evident between healthy "community-dwelling elderly" and subjects with "residential long-term care" (24). These differences might be due to the disparate consumption of food in various groups, suggesting a new therapeutic target for prolonged healthy life years.

Cell Senescence

Cellular senescence is defined as irreversible cell cycle arrest driven by a variety of mechanisms, including telomere shortening, genotoxic stress, mitogen stimuli, and inflammatory cytokines, that result in the activation of the p53 tumor suppressor and/or the cyclin-dependent kinase inhibitor p16 (25). It is evident that the number of senescent cells in several organs increases with age; these cells secrete multiple inflammatory cytokines, generating low-grade inflammation. This phenotype of senescent cells is termed the senescence-associated secretory phenotype or SASP, which recently has been proposed as the main origin of inflammaging in both aging and age-related diseases such as atherosclerosis, cancer, and diabetes (26–28). Increasing evidence has suggested that the clearance of senescent cells in animal models attenuates the progression of age-related disorders, including atherosclerosis and osteoarthritis (29–31). These data strongly support the hypothesis that senescent cell clearance, reprogramming of senescent cells, and the modulation of pro-inflammatory pathways related to the acquisition of SASP might be pursued as potential anti-aging strategies for combating age-related diseases and expanding the health span of humans.

Immunosenescence

"Immunosenescence", which is the age-related dysregulation of an innate immune system, is characterized by persistent inflammatory responses (32). Immunosenescence increases the susceptibility to malignancy, autoimmunity, and infections; decreases the response to vaccinations; and impairs wound healing (33, 34). Conversely, chronic inflammatory disease can accelerate the "immunosenescence" process. The mechanisms that underlie this persistent aging-associated basal inflammation remain incompletely understood but seem to involve changes in the numbers and functions of innate immune cells. Changes in the expression of pattern recognition receptors (PRRs), activation of PRRs by endogenous ligands associated with cellular damage, and unusual downstream signaling events of PRRs activation have been implicated to induce chronic cytokine secretion. Thus, together with cell senescence, dysregulation of immunological imprinting mediated by trained innate immunity might also contribute to persistent low-grade inflammation that occurs even after the initial stimulus has been removed.

Coagulation and Fibrinolysis System

Increased coagulation and fibrinolysis activity in the elderly has recently been implicated in enhanced inflammation through the protease-activated receptor, PAR (35–37) leading to age-related diseases such as atherosclerosis and lung fibrosis (38). The plasma concentrations of coagulation factor V, VII, VIII, and IX, have been reported to increase in healthy humans in conjunction with the physiological processes of aging (39, 40). In addition, fibrinogen (coagulation factor I) levels, a primary risk factor for thrombotic disorders proved in several clinical studies, has been shown to increase with advancing age. Additionally, we have recently identified that coagulation factor X is locally synthesized at high levels in human atherosclerotic plaques, specifically in endothelial cells, smooth muscle cells, and inflammatory cells (41). Thus, based on these observations, increased levels of plasma and local coagulation factors during physiological aging might account for the higher cardiovascular risk observed in the elderly. Additionally, a clinical trial, ATLAS ACS 2–TIMI, 51 investigators showed that the direct coagulation factor Xa inhibitor, rivaroxaban, reduced the risk of the composite endpoint of death from cardiovascular causes, myocardial infarction, and stroke in patients with a recent acute coronary syndrome event (42). Although the mechanism has not been elucidated, activation of the coagulation cascade following fibrinogen activation may increase thrombosis, and elevated levels of coagulation factor Xa and thrombin could enhance the inflammatory response through PAR-1/2, in addition to its roles in coagulation and the fibrinolysis system (43). Interestingly, PAR-1/2 signaling induced by coagulation factor Xa (FXa) and the fibrinolytic factor plasmin has been shown to increase insulin-like growth factor binding protein-5 (IGFBP-5) expression (37, 41, 44, 45), which plays decisive roles in cell senescence and inflammation. Kojima et al. demonstrated that IGFBP-5, a downstream mediator of signal transducer and activator of transcription 3 (STAT3), regulates interleukin-6 (IL-6)-induced reactive oxygen species production, subsequent DNA damage response, and senescence of fibroblast cells (46). As shown in the study by Yasuoka et al., IGFBP-5 induces a fibrotic phenotype by activating MAPK signaling and nuclear EGR-1 translocation that interacts with IGFBP-5 and promotes fibrotic and inflammatory gene transcription (47). Consistent with previous reports, our recent study demonstrated that FXa stimulation of smooth muscle cells, endothelial cells, and endothelial progenitor cells enhances cellular senescence through the early growth response-1 (EGR-1)-IGFBP-5-p53 pathway (37). These data imply that inflammaging, hyper-coagulability, and cell senescence might share a common pathway that is regulated by IGFBP-5 (**Figure 2**). Intriguingly, our recent experiment showed that the FXa- and IGFBP-5-positive areas were similarly distributed within human atherosclerotic plaques (41). These finding strongly suggest that locally produced coagulation factor Xa in atherosclerotic plaques might induce IGFBP-5 expression, enhancing cellular senescence with SASP, although the involvement of thrombin in this process is undeniable (48).

CONCLUSIONS

Ideally, inflammation should subside immediately after elimination of the pathogen and insult to allow normal tissue to be rebuilt. However, low-grade persistent inflammation occurs in the majority of older people, leading to degeneration of several organs. There is strong evidence that the development of age-related multi-factorial conditions such as cancer,

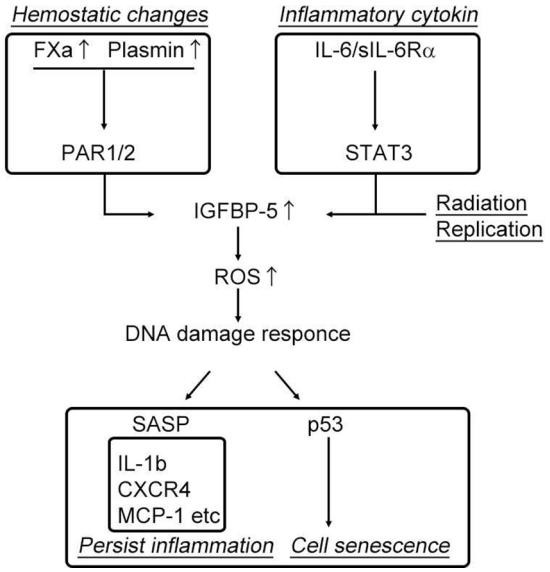

FIGURE 2 | Activation of the coagulation cascade induces cell senescence and persistent inflammation.

cardiovascular disease, Alzheimer's disease, type II diabetes, frailty, sarcopenia, and osteoporosis is associated with low-grade elevations of circulating inflammatory mediators. Considering that aging is a complex process that results from a combination of environmental, genetic, and epigenetic factors, focusing future work on interventions addressing selectively destroying senescent cells, namely, "senolytic therapies" in the aging host rather than by treating symptoms of disease or attempting to block the effects of the multi-source of inflammaging, will offer improved therapeutic opportunities (49–52).

AUTHOR CONTRIBUTIONS

FS and YT performed experiment and organized manuscript. JM, HS, and RO performed experiments. HR and RM supervised this project.

FUNDING

This work was partially supported by a Grant-in-Aid from the Organization for Pharmaceutical Safety and Research, a Grant-in-Aid from the Ministry of Public Health and Welfare, a Grant-in-Aid from Japan Promotion of Science, special coordination funds from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government, and research funding from Bayer Yakuhin, Ltd.

ACKNOWLEDGMENTS

We would like to thank Blure May Kanako and Kana Shibata for providing technical support and all members of the Clinical Gene Therapy group at the Osaka University Graduate School of Medicine.

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Conflict of Interest Statement: RM received research funding from Bayer Yakuhin, Ltd. Other authors have no conflicts of interests.

frontiers in Cardiovascular Medicine

REVIEW
published: 05 March 2018
doi: 10.3389/fcvm.2018.00018

Vascular Senescence in Cardiovascular and Metabolic Diseases

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In mammals, aging is associated with accumulation of senescent cells. Stresses such as telomere shortening and reactive oxygen species induce "cellular senescence", which is characterized by growth arrest and alteration of the gene expression profile. Chronological aging is associated with development of age-related diseases, including heart failure, diabetes, and atherosclerotic disease, and studies have shown that accumulation of senescent cells has a causative role in the pathology of these age-related disorders. Endothelial cell senescence has been reported to develop in heart failure and promotes pathologic changes in the failing heart. Senescent endothelial cells and vascular smooth muscle cells are found in atherosclerotic plaque, and studies indicate that these cells are involved in progression of plaque. Diabetes is also linked to accumulation of senescent vascular endothelial cells, while endothelial cell senescence *per se* induces systemic glucose intolerance by inhibiting skeletal muscle metabolism. A close connection between derangement of systemic metabolism and cellular senescence is also well recognized. Aging is a complex phenomenon, and there is no simple approach to understanding the whole process. However, there is accumulating evidence that cellular senescence has a central role in the development and progression of various undesirable aspects of aging. Suppression of cellular senescence or elimination of senescent cells reverses phenotypic changes of aging in several models, and proof-of-concept has been established that inhibiting accumulation of senescent cells could become a next generation therapy for age-related disorders. It is clear that cellular senescence drives various pathological changes associated with aging. Accordingly, further investigation into the role of this biological process in age-related disorders and discovery of senolytic compounds are important fields for future exploration.

Keywords: cellular senescence, p53, atherosclerosis, heart failure, diabetes, senolysis

OPEN ACCESS

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

Received: *24 October 2017*
Accepted: *21 February 2018*
Published: *05 March 2018*

Citation:
Katsuumi G, Shimizu I, Yoshida Y and Minamino T (2018) Vascular Senescence in Cardiovascular and Metabolic Diseases. Front. Cardiovasc. Med. 5:18. doi: 10.3389/fcvm.2018.00018

INTRODUCTION

In aging societies, the discrepancy between the total lifespan and the healthy lifespan is becoming a major problem. Chronological aging is associated with a higher prevalence of age-related diseases, including heart failure, diabetes, and atherosclerotic disorders with or without various comorbidities, resulting in impairment of the quality of life by limitation of normal activities. Thus, aging is associated with several undesirable processes. The mechanisms of aging and age-associated disorders are complex, and thus cannot be comprehended by a simple approach. However, recent

studies have indicated a pivotal role of cellular senescence in the progression of age-related disorders (1–5). Back in the 1960s, Hayflick et al. demonstrated that fibroblasts have limited potential to replicate (6), indicating that aging also occurs at the cellular level. Such aging of cells is currently described as "cellular senescence". Senescent cells become enlarged and flattened. In association with proliferative arrest, alterations of gene expression by these cells lead to secretion of pro-inflammatory molecules (7). This is known as the senescence-associated secretory phenotype, and it results in chronic sterile inflammation that promotes tissue remodeling. Senescent cells have been found in various organs of animal models, as well as in elderly humans and persons with age-related disorders. There is evidence that cellular senescence in the vasculature, termed "vascular senescence", is crucially involved in the pathogenesis of cardiovascular and metabolic disorders. Vascular senescence has been reported to promote atherosclerosis (8), systolic cardiac dysfunction (9, 10), and systemic metabolic dysfunction (11). In this review, we delineate the role of cellular senescence in the diseases associated with aging, focusing on vascular senescence in cardiovascular and metabolic disorders, and discuss the potential usefulness of therapies targeting senescent cells.

Role of Cellular Senescence in Aging and Age-Related Diseases

Diverse deleterious changes of cells and tissues accumulate with the progression of aging, leading to a decline of physiological activity and an increased risk of death. Organ function deteriorates with chronological aging, and this biological process is characterized by cellular senescence, changes of intercellular communication, mitochondrial dysfunction, and deregulation of nutrient sensing (1). It is well known that DNA damage, telomere shortening, oncogenic stress, and exposure to high levels of reactive oxygen species (ROS) all occur with chronological aging, and signaling via the p53 pathway has been reported to increase under these conditions (12). The p53 protein is a transcriptional factor with a crucial role in maintenance of genomic stability that mediates the coordination of DNA repair, cell cycle regulation, apoptosis, and cellular senescence. Because p53 is involved in suppression of tumorigenesis, it has been described as the "guardian of the genome" (13). In addition to its role in the repair of DNA damage and maintenance of genomic stability, studies have indicated that p53 contributes to a broad spectrum of biological processes, such as cell metabolism, autophagy, antioxidant defenses, and angiogenesis (13–15). Accordingly, p53 signaling is thought to have a central role in cellular senescence. Somatic cells have a finite lifespan and eventually enter a state of irreversible growth arrest termed "replicative senescence." Telomeres are repetitive nucleotide sequences located at the terminals of mammalian chromosomes that undergo incomplete replication during cell division, resulting in telomere shortening. Because telomeres are essential for chromosomal stability and DNA replication, DNA damage is recognized when telomere shortening exceeds the physiological range and this triggers cellular senescence, mainly via the p53 or p16 signaling pathways. "Stress-induced premature senescence" is another type of cellular senescence that is triggered by various stress signals, including DNA damage induced by oxidative stress or irradiation, constitutive activation of mitogenic stimuli, oncogenic activation, and metabolic stress. It is also mediated via the p53 or p16 signaling pathways. Preference for one pathway over the other depends on the cell type and also varies among species (16, 17). In humans, telomere dysfunction activates either p53 or p16 signaling, while only p53 signaling is activated in rodents (18). It is generally accepted that p53 signaling is primarily activated by DNA damage and telomere dysfunction, while p16 signaling is primarily linked to mitogenic stress, chromatin disruption, and general cellular stress (16, 17, 19) (**Figure 1**).

It was reported that p53 is increased in the failing heart, in aged vessels, and in the visceral fat of patients with obesity or heart failure. Studies have indicated a pathological role of p53-induced cellular senescence in aging and age-related disorders, including heart failure, atherosclerotic disease, obesity, and diabetes (10, 11, 15, 20–24). However, there is controversy about the role of p53 in aging and age-related diseases (3, 25, 26). In some settings, p53 signaling has been shown to have a beneficial effect by suppression of aging. Matheau and colleagues reported that Trp53/Cdkn2a transgenic mice were resistant to carcinogenesis and had a longer median lifespan (27). It was also reported that Trp53 transgenic ("Super p53") mice displayed resistance to carcinogenesis without any signs of premature aging, and these mice showed normal glucose tolerance on a standard diet (28–30). Furthermore, Baker et al. found that loss of p53 or p21 accelerated cellular senescence in the adipose tissue and skeletal muscle of BubR1 progeroid mice (31). They also reported that $p19^{Arf}$, acting upstream of p53, suppressed senescence and aging in the same progeroid model (32). Taken together, these various reports suggest that the p53/p21 signaling pathways regulate cellular senescence in a context-dependent manner.

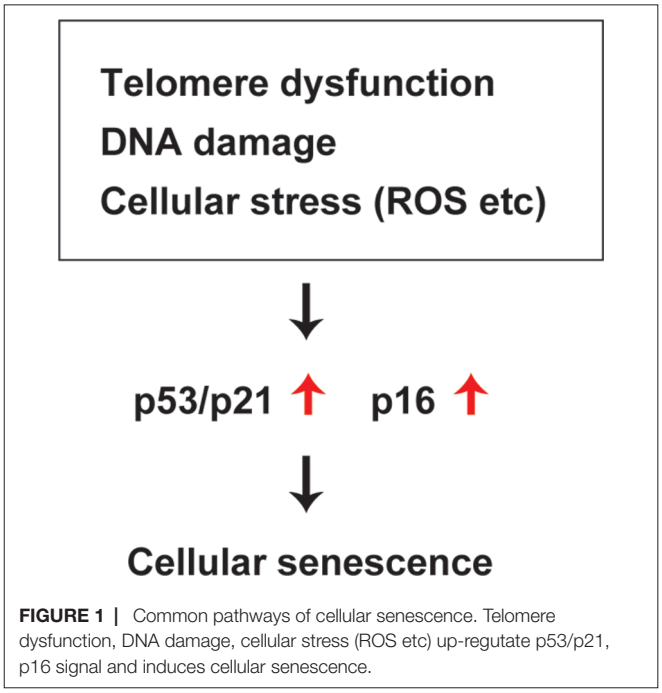

FIGURE 1 | Common pathways of cellular senescence. Telomere dysfunction, DNA damage, cellular stress (ROS etc) up-regutate p53/p21, p16 signal and induces cellular senescence.

Interestingly, it was recently reported that elimination of senescent cells by genetic manipulation inhibited age-related degenerative changes in several organs of mice, such as the heart and kidneys (33). Other studies have identified several pharmacological agents that selectively damage and remove senescent cells, and these compounds have been described as "senolytic agents". For example, an inhibitor of anti-apoptotic proteins (ABT263) depletes senescent bone marrow hematopoietic stem cells and senescent muscle cells in a chronological aging model, leading to rejuvenation of these tissues (34). The gene expression profile of senescent cells is shifted toward a pro-inflammatory phenotype associated with the secretion of biologically active molecules (senescence-associated secretory phenotype). *In vitro* studies have shown that exposure of young fibroblasts to senescent fibroblast promotes senescence of the young cells via a gap junction-mediated process, which has been described as the "bystander effect" (35). Studies have shown that senescent cells damage their local environment and promote tissue remodeling in age-related disorders, suggesting that inhibition of cellular senescence and/or elimination of senescent cells could be potential next generation therapies for diseases associated with aging.

Biological Markers of Cellular Senescence

Biological markers reflecting direct evidence of cellular senescence have not yet been identified, but several markers are used to indirectly detect senescent cells, among which senescence-associated beta-galactosidase (SA-β-gal) activity is the most common. Lysosomal beta-galactosidase activity is normally detected at a low pH (usually around pH 4), but becomes detectable at a higher pH (pH 6) in senescent cells due to marked expansion of the lysosomal compartment (36). Other established markers of cellular senescence include high expression of p53, p16, p21, p38 mitogen-activated protein kinase (p38MAPK) and γH2AX, reflecting the activation of DNA damage responses (4, 37–40). In addition, high mobility group A (HMGA) proteins or heterochromatin markers, including HP1 and tri-methylated lysine 9 histone H3 (H3K9me3), are recognized as molecular markers of senescence-associated heterochromatin foci and are considered to indicate cellular senescence (40).

Cardiac Aging Predisposes to Heart Failure

Heart failure has a high prevalence among the elderly (41). The prognosis of severe heart failure is still unacceptably poor, and there is an urgent need to find better therapies for this condition. Age-related heart failure develops in persons without established risk factors, such as hypertension, obesity, diabetes, or atherosclerotic diseases (42, 43). Heart failure without systolic dysfunction is classified as heart failure with a preserved ejection fraction (HFpEF), and occurs in approximately half of all patients with heart failure. HFpEF is prevalent among the elderly and lack of specific therapy for this type of heart failure is a major clinical problem. The mechanism of HFpEF is still not fully understood, although there is evidence of cardiac endothelial cell remodeling being involved in its onset and progression (44). It was also reported that coronary microvascular endothelial inflammation is critically involved in the pathology of HFpEF (45), while a recent study indicated a causative role of senescent signaling in this disorder (46). Thus, the physiological aging process seems to increase susceptibility to the onset of heart failure, considering that the prevalence of heart failure increases with age. Various studies have indicated that cellular senescence is critically involved in the pathology of heart failure, as described below.

Vascular Senescence and Heart Failure

Endothelial Cell Senescence

Although the role of cellular senescence in the failing heart is still not fully understood, a number of studies have suggested a pathological influence on heart failure. The cardiac level of p53 is increased in a murine model of left ventricular pressure overload, leading to suppression of myocardial angiogenesis that results in capillary rarefaction, tissue hypoxia, and cardiac dysfunction (15). Chronic sterile inflammation develops in the failing heart, and it is now well accepted that such inflammation is one of the mechanisms underlying cardiac remodeling (47). It was recently demonstrated that activation of p53 signaling in vascular endothelial cells induces cardiac inflammation and remodeling in a murine model of left ventricular (LV) pressure overload (10). Expression of p53 by capillary endothelial cells in the left ventricle increases in response to LV pressure overload, leading to elevated expression of intercellular adhesion molecule (ICAM)−1 by these cells that promotes infiltration of macrophages and cardiac inflammation. Conversely, depletion of p53 from capillary endothelial cells results in suppression of ICAM-1 expression and cardiac inflammation with improvement of cardiac dysfunction. Activation of the sympathetic nervous system occurs in heart failure and is associated with a poor prognosis (48). It was reported that the sympathetic nervous system/ROS axis increases p53 expression by endothelial cells in a murine model of LV pressure overload (10). In another study, depletion of p53 from endothelial cells improved capillary rarefaction and cardiac function, while suppressing cardiac fibrosis and remodeling (9). These findings indicate that endothelial p53 signaling suppresses angiogenesis, thereby promoting capillary rarefaction in the failing heart. Inhibition of p53 in endothelial cells could potentially become a next generation therapy for patients with heart failure and a reduced ejection fraction. As mentioned above, about half of all heart failure patients have HFpEF with a preserved ejection fraction. There are several established risk factors for HFpEF, including overweight/obesity, hypertension, diabetes, and aging. Cardiomyocyte hypertrophy and interstitial fibrosis develop in patients with HFpEF, leading to incomplete myocardial relaxation and increased wall stiffness (49–51). It is generally accepted that coronary microvascular inflammation is central to the pathogenesis of HFpEF (45), and it was recently demonstrated that endothelial cell senescence also makes a contribution. When mice with accelerated senescence were fed a high-fat, high-salt diet, both endothelial cell senescence and inflammation increased in cardiac tissue, along with the typical hemodynamic and structural changes of HFpEF (46). Considering that cellular senescence induces

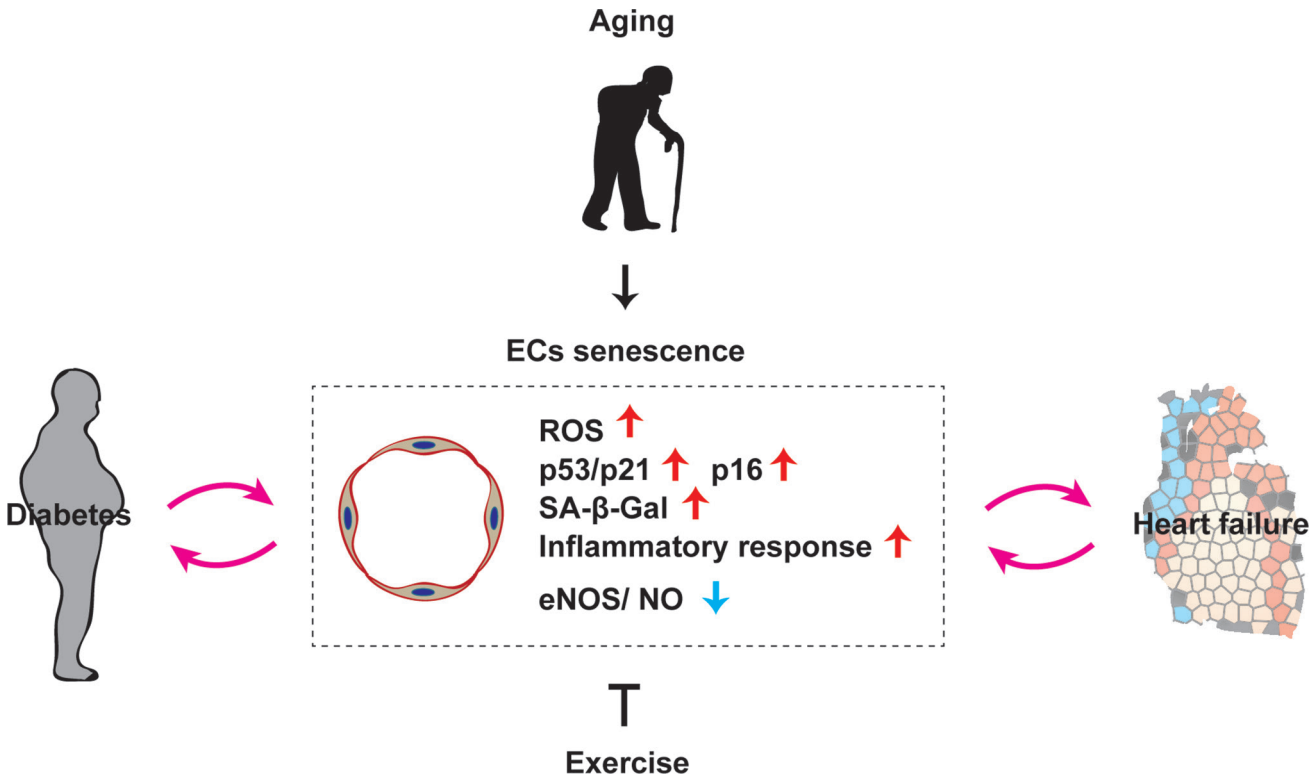

FIGURE 2 | Role of endothelial senescence in cardio-metabolic disease. Chronological aging induces vascular endothelial cell senescence (EC senescence as characterized with an increase of ROS, p53/p21, p16, SA-β-Gal, inflammatory response, and reduced eNOS/NO level). EC senescence has a pivotal role in the progression of diabetes and heart failure. Exercise has a potential to suppress ECs senescence.

vascular dysfunction and inflammation, it seems reasonable that it would also promote pathologic changes of HFpEF. Accordingly, suppression of endothelial cell senescence may be a therapeutic option for this currently untreatable disorder (**Figure 2**).

VASCULAR AGING PREDISPOSES TO ATHEROSCLEROTIC DISEASES

Structural and Functional Changes of Aging Arteries

Coronary artery disease and stroke are associated with arterial dysfunction. Arterial remodeling occurs with aging, even in the absence of cardiovascular disease and cardiovascular risk factors. While aging is a physiological process and not a pathological condition, studies indicate that aging *per se* is linked with vascular remodeling that predisposes to cardiovascular disease. Aged arteries are characterized by an increase of the intima/media thickness ratio, which was reported to increase by 2- to 3-fold from 20 to 90 years of age (52, 53). Vascular smooth muscle cells switch from the "contractile" to "synthetic" phenotype with aging and this change contributes to intimal thickening, which is associated with increased arterial permeability and leads to development of atherosclerotic disease. The arterial media also becomes thicker with aging and its cellularity decreases simultaneously (54). Moreover, the length and circumference of the aorta increase with aging (55), and these structural changes reflect increased collagen production and a corresponding decline of the elastin content (56). In association with such changes, aged vessels show reduced compliance, reduced elasticity/distensibility, and increased stiffness, resulting in a higher systolic blood pressure and lower diastolic pressure (57). Medial calcification is another characteristic of aged vessels. In association with other age-related disorders like hypertension, dyslipidemia, and diabetes, such vascular remodeling increases susceptibility to atherosclerotic vascular diseases. In elderly patients, atherosclerotic plaques tend to become larger and vascular stenosis becomes more severe over time. Aged rabbits fed a high fat diet developed more severe atherosclerotic lesions compared to young animals on the same diet. Therefore, it is well accepted that aging *per se* promotes the pathogenesis of atherosclerotic disorders, and studies have suggested that cellular senescence has a critical role in this process.

Vascular Senescence in Arterial Diseases

ROS and chronic low-grade sterile inflammation are two major contributors to the progression of age-related vascular dysfunction. Senescent cells accumulate in the arteries with aging irrespective of whether or not a person has age-related vascular disorders (58–61). Along with aging, vascular tissues of rodents and humans show elevation of the levels of p16,

p21, phosphorylated p38, and double-stranded DNA breaks, in association with high SA-β Gal activity (62–65). It was reported that expression of p53 and p21 is increased in the arteries of elderly persons, together with structural breakdown of telomeres known as telomere uncapping (61). Both telomere length and telomerase activity were found to be reduced in endothelial progenitor cells from patients with coronary heart disease (66). In patients with chronic heart failure, telomere attrition was identified in circulating leukocytes (67). Moreover, the leucocyte telomere length displays an inverse association with the risk of coronary heart disease independently of conventional vascular risk factors (68). Accordingly, it is generally accepted that telomere length and telomerase activity are involved in human cardiovascular disease (69). Interestingly, senescent cells are increased in the coronary arteries of patients with ischemic heart disease, but not in the internal mammary arteries (58). Endothelial cells and vascular smooth muscle cells (VSMCs) from patients with abdominal aortic aneurysm (AAA) have the phenotypic features commonly observed in senescent cells (60). Hypertension is an established risk factor for atherosclerotic diseases, and it was reported that binding of p53 to the p21 promoter is increased in the arteries of hypertensive patients. While telomere length is comparable between patients with hypertension and controls, telomere uncapping is 2-fold higher in hypertensive patients (70). A murine model of genomic instability demonstrated senescence of endothelial cells and VSMCs in the aorta, along with impaired vasodilation, increased vascular stiffness, and hypertension (71). In hypertensive rats treated with deoxycorticosterone acetate and salt, overexpression of p16 was detected in the coronary arteries (72). Aortic p16 expression was elevated in another model of hypertension (mice administered an endothelial nitric oxide synthase inhibitor) (73), indicating the existence of a vicious circle between cellular senescence and hypertension. Thus, studies have shown that senescent cells accumulate in the vessels of patients with atherosclerosis, hypertension, aneurysms, diabetes, and intimal hyperplasia (**Figure 3**), so the role of endothelial cell, VSMC and immune cell senescence in arterial diseases is discussed next.

Endothelial Cell Senescence in Arterial Diseases

Endothelial cells are critically important for maintaining vascular homeostasis and are involved in various biological functions, including angiogenesis, blood pressure regulation, coagulation, and systemic metabolism. Aged endothelial cells develop a dysfunctional phenotype that is characterized by reduced proliferation and migration, decreased expression of angiogenic molecules, and low production of nitric oxide (NO), which is synthetized by NO synthase (NOS) and mediates vasodilatation. In dysfunctional endothelial cells, NO production is generally reduced due to low NOS activity. This change is associated with impairment of endothelium-dependent dilatation (EDD), which is reported to predict future cardiovascular events. Dysfunctional endothelial cells develop pro-oxidant, pro-inflammatory, vasoconstrictor, and prothrombotic properties, and studies indicate that cellular senescence has a pathological role in such phenotypic aging.

Senescent endothelial cells have been found in atherosclerotic plaque (58). An autopsy study of patients with ischemic heart disease revealed that SA-β-gal activity is increased in the coronary arteries, but not in the internal mammary arteries. In the coronary arteries, SA-β-gal activity is high in cells located on the luminal surface (probably endothelial cells). ICAM-1 is also increased in senescent human aortic endothelial cells, while both endothelial nitric oxide synthase (eNOS) and NO activity are reduced in these cells compared to young cells. Importantly, these pathological phenotypic changes induced by replicative senescence were suppressed by activation of telomerase reverse transcriptase in aged human aortic endothelial cells, indicating that telomere shortening induces endothelial cell senescence and has pathological consequences in atherosclerotic diseases (58). In patients with AAA, telomeres are significantly shorter and oxidative DNA damage is more severe in endothelial cells from the aneurysmal region (60). In atherosclerotic mice, disturbance of flow in the ascending aorta and aortic arch promotes endothelial cell senescence, and *in vitro* studies have indicated that aberrant

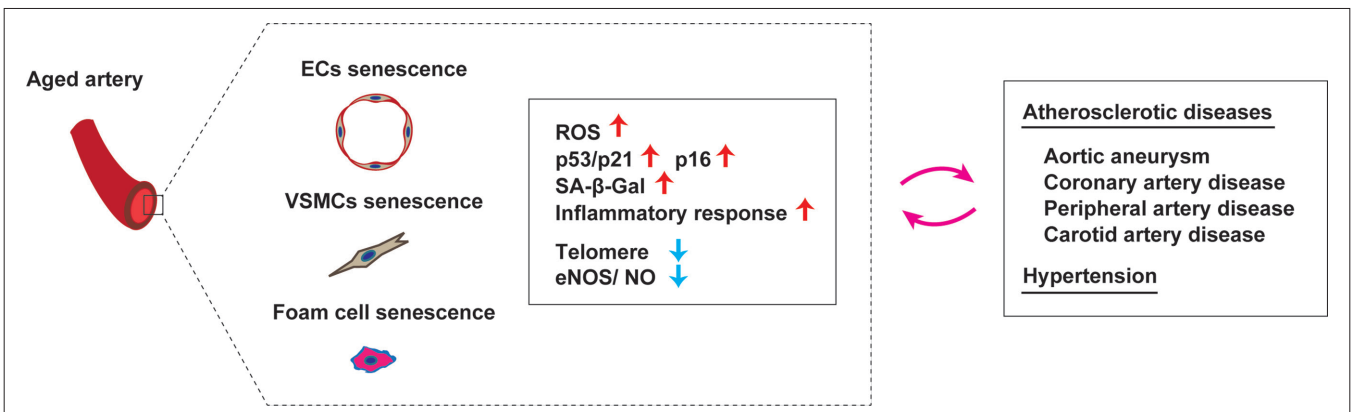

FIGURE 3 | Cellular senescence in aged arteries. Aged arteries are characterized by accumulation of senescent vascular endothelial cells (EC), senescent vascular smooth muscle cells (VSMCs), and senescent foam cells. Accumulation of these senescent cells are associated with an increase of ROS, p53/p21, p16, SA-β-Gal, inflammatory response, telomere attrition and reduced eNOS/NO level). Senescence of these cells promotes pathological changes in atherosclerotic diseases and also has a role in development of hypertension.

flow is a signal inducing cellular senescence (74). One of the problems related to an increase of senescent cells is development of the senescence-associated secretory phenotype, which is characterized by production of pro-inflammatory cytokines with a causal role in tissue remodeling (7). In human arterial endothelial cells with replicative senescence, levels of H_2O_2 and O_2^- are high and NO production is reduced. High ROS levels in senescent endothelial cells are thought to accelerate senescence. Aging is reported to be linked with increased circulating levels of pro-inflammatory cytokines, such as interleukin-6, tumor necrosis factor alpha, and monocyte chemoattractant protein-1 (75). It is highly possible that accumulation of senescent endothelial cells in the arteries of elderly persons induces chronic sterile inflammation and vascular remodeling, increasing susceptibility to atherosclerotic diseases. Controversy exists as to whether physical activity is associated with telomere length, since physical activity is positively correlated with telomere length in some studies, but not in other studies (76). It is generally accepted that physical activity improves vascular structure and function in humans and rodents. Bioavailability of NO declines with aging in association with elevation of ROS levels, while these changes are ameliorated or reversed by physical activity. Recently, it was shown that older individuals who performed exercise had lower levels of p53, p21, and p16 in endothelial cells from the brachial arteries and antecubital veins compared to sedentary older individuals, indicating that physical activity suppresses senescence of human vascular cells (77). Thus, there is evidence that physical activity improves vascular function, but further studies are needed to identify the detailed molecular mechanisms involved (**Figure 2**).

Vascular Smooth Muscle Cell Senescence in Arterial Diseases

Functional changes of VSMCs occur with aging, partly due to deregulation of TGF-β signaling, and these cells undergo transformation from a "contractile" to a "synthetic" phenotype. It was reported that aged SMCs show enhancement of inducible NOS (iNOS) activity, as well as higher expression of ICAM-1 and angiotensinogen in response to stress (78, 79). Intimal thickening develops with aging, partly due to increased production of collagen and a corresponding decrease of elastin (56). Generally, intimal thickening is widespread and concentric in the aorta, while it is eccentric in the coronary arteries. Intimal thickening is a preatherosclerotic lesion because topographic correspondence has been demonstrated between the sites of intimal thickening and atherosclerotic plaques. Telomeres are shorter in cells from atherosclerotic plaque in the human aorta compared to normal vessels. Telomeres are also shorter in VSMCs from the fibrous cap of atheroma compared to VSMCs from the normal vascular media, and these cells are positive for SA-β-gal staining along with elevated p16 and p21 expression. It was reported that oxidative stress induces DNA damage in VSMCs and suppresses telomerase activity, leading to telomere shortening and cellular senescence that contribute to acceleration of atherosclerotic disorders (80). In addition, senescence of VSMCs was reported in apolipoprotein E null mice treated with angiotensin II (81). Smooth muscle 22α is an actin-binding protein that is known as a marker of smooth muscle cell senescence. Recently, it was shown that smooth muscle 22α promotes angiotensin II-induced cellular senescence by suppressing Mdm-2-mediated degradation of p53 (82). In addition, Cafueri et al. reported that VSMCs from the aneurysms of AAA patients display telomere attrition and marked oxidative DNA damage (60). Senescent VSMCs have been identified in atherosclerotic lesions of patients with coronary artery disease, AAA, and peripheral artery disease (58). It was also reported that 18% of VSMCs from carotid artery plaques are positive for p16 and p21, in association with detectable SA-β-gal activity (80). Furthermore, SA-β-gal positive VSMCs in carotid plaques express interleukin-6, suggesting that senescent VSMCs have a senescence-associated secretory profile and a causative role in the progression of atherosclerotic disorders (83). Senescent VSMCs show dysregulated production of pro-inflammatory cytokines, growth factors, and extracellular matrix modifiers, accelerating the process of vascular remodeling. Some inducers of senescence are common to VSMCs and vascular endothelial cells, such as ROS and angiotensin II (84, 85), while hypoxic stress was reported to inhibit senescence by promoting telomerase activity (86). It was recently found that senescent VSMCs in atherosclerotic plaque display loss of telomeric repeat-binding factor-2 (TRF2), a protein localized in the telomeres. TRF2 overexpression reduces DNA damage, accelerates DNA repair, and suppresses cellular senescence *in vitro*, while introduction of TRF2 into loss of function mutants results in the opposite phenotype. Studies using transgenic mice have shown that VSMC-specific loss of TRF2 function increases atherosclerosis and necrotic core formation *in vivo*, while these pathological changes are suppressed in mice with VSMC-specific gain of TRF2 function (87). These results clearly indicate that inhibition of VSMC senescence is extremely important for suppressing the progression of atherosclerotic diseases.

Immune Cell Senescence in Arterial Diseases

In human vascular cells, telomere length shows a strong inverse correlation with aging, and telomere shortening is linked with cardiovascular disease and diabetes (88, 89). Elderly individuals with short telomeres in leukocyte DNA are reported to have a higher mortality rate, partly attributable to increased death from heart disease (90). Monocytes from patients with atherosclerosis exhibit increased production of ROS and various pro-inflammatory cytokines, including MCP-1, IL-6, IL-1β, and TNF-α (91). It was recently demonstrated that senescent intimal foam cells accumulate in atherosclerotic lesions and act as the key drivers of atheroma formation. Importantly, specific deletion of these senescent cells by genetic or pharmacological approaches has been shown to reverse atherosclerosis in mice (92). According to another report, cellular senescence mediated by $p16^{INK4a}$ promotes pro-inflammatory phenotypic changes in macrophages (93). These studies indicate that leukocyte senescence is involved in the progression of atherosclerotic plaque, suggesting that suppression of leukocyte senescence may be an important approach for combating atherosclerotic diseases.

Vascular Senescence in Metabolic Syndrome

Chronic sterile inflammation of visceral fat develops in patients with heart failure, obesity, and/or diabetes, and is well accepted to have a central role in inducing systemic insulin resistance and progression of metabolic disorders. It was previously reported that p53 induces inflammation of visceral adipose tissue in murine models of obesity or heart failure, which is involved in the progression of these age-related diseases (21, 24, 94). Occurrence of cellular senescence in visceral fat was reported to result in deterioration of systemic metabolic health. Capillaries have a crucial role in metabolically active organs, including skeletal muscle and brown adipose tissue. Capillarization of skeletal muscle was reported to increase in older adults after exercise training, and this increase of capillaries leads to enhancement of systemic insulin sensitivity (95). It was recently demonstrated that vascular endothelial cell senescence induces systemic metabolic dysfunction (11) (**Figure 2**). Conversely, it has been well documented that obesity is associated with vascular senescence. In metabolically unhealthy persons with obesity and/or diabetes, insulin/insulin receptor/insulin receptor substrate/phosphoinositide 3-kinase/Akt signaling is down-regulated in vascular cells, while insulin receptor/son of sevenless/growth factor receptor bound protein/mitogen-activated protein kinase (MAPK) signaling is enhanced. This is known as "selective insulin resistance", and it mediates pro-atherosclerotic responses by activation of MAPK signaling. It was reported that activation of MAPK signaling by hyperinsulinemia and selective insulin resistance induces vascular remodeling through vasoconstriction, proliferation, and vascular cell migration (96). Various studies have indicated the existence of a vicious circle between vascular senescence and metabolic syndrome, as discussed below.

Endothelial Cell Senescence in Metabolic Syndrome

Capillary network formation is critically important for morphogenesis and maintenance of homeostasis, while vascular dysfunction induces organ malfunction and systemic metabolic disorders (97, 98). In animal studies, diabetes has been shown to induce vascular cell senescence. For example, endothelial cell senescence develops in the aortas of Zucker diabetic rats and hyperglycemic mice or rats (99–101). These reports indicate that hyperglycemia induces cellular senescence, while there is also evidence that cellular senescence *per se* promotes systemic metabolic dysfunction. It is widely accepted that skeletal muscle contributes to glucose disposal, so maintaining skeletal muscle homeostasis is crucial for systemic metabolic health. Metabolic stress induces accumulation of lipids and chronic sterile inflammation in skeletal muscle, contributing to development of systemic insulin resistance (102, 103). It was recently demonstrated that endothelial cell senescence suppresses skeletal muscle metabolism, leading to systemic glucose intolerance. Metabolic stress induced by dietary obesity increases p53 expression in the vascular endothelium (11), while endothelial cell-specific depletion of p53 reduces both visceral and subcutaneous fat volumes and improves systemic glucose intolerance. It is generally accepted that eNOS has a protective role in the cardiovascular system, which is mainly mediated by production of NO. It was reported that eNOS up-regulates skeletal muscle expression of peroxisome proliferator-activated receptor-γ coactivator-1α, a master regulator of mitochondrial biogenesis and cell metabolism, while this up-regulation is suppressed by p53. Down-regulation of p53 expression in vascular endothelial cells promotes glucose uptake by skeletal muscle through up-regulation of glucose transporter-1 expression in endothelial cells, and contributes to better systemic metabolic health. These findings indicate that suppression of endothelial cell senescence is important for maintenance of systemic metabolic health (11).

Potential Next Generation Therapies Targeting Senescent Cells for Cardiovascular and Metabolic Disorders

It was recently established that selective depletion of senescent cells ("senolysis") reverses phenotypic changes of aging (33, 34, 104–106). Accumulation of senescent cells promotes chronic sterile inflammation in the visceral adipose tissue of patients with obesity and elderly persons. In a murine model of premature aging, elimination of p16-positive senescent cells contributed to suppression of the aging phenotype in several organs, including epididymal/inguinal white adipose tissue, the heart, and the kidney (33). Several agents causing selective depletion of senescent cells (senolytic activity) have been identified. It was reported that an anticancer agent (ABT263) has a senolytic effect by selectively removing p16-positive senescent cells from the bone marrow via apoptosis, leading to rejuvenation of hematopoietic stem cells during aging (34). In addition, Xu et al. showed that depletion of senescent cells in aged mice preserved adipogenesis and increased insulin sensitivity (104). Zhu et al. showed that administration of another senolytic therapy (dasatinib + quercetin: D + Q) significantly improved systolic cardiac function and reduced the left ventricular end-systolic dimension in 24-month-old mice (105). Treatment with D + Q also improved vasomotor function in aged mice, as well as reducing aortic calcification and osteogenic signaling in hypercholesterolemic mice (107). Regarding genetic approaches, elimination of p16-positive senescent cells from plaques suppressed pathologic changes in low-density lipoprotein receptor-deficient mice (92). Senescent VSMCs show hypermetabolic changes, with increased glycolysis and oxygen consumption. Administration of 2-deoxyglucose causes greater depletion of senescent VSMCs than control VSMCs *in vitro*, but clinical trials investigating the anticancer effect of 2-deoxyglucose have identified the issue of toxicity (108). Depletion of specific components to alter cell metabolism has now attracted attention in the field of anticancer therapy, including the search for senolytic agents targeting cell metabolism. There is evidence that elimination of senescent cells by administration of senolytic agents has the potential to become a next generation therapy for cardiovascular disorders (109, 110). Suppression of cellular senescence is another possibility. Sirtuin1 (SIRT1) is one of the most promising molecules to be studied in relation to suppression of aging. Activation of SIRT1-signaling was reported to prolong the lifespan of rodents, while overexpression of SIRT1 in VSMCs or vascular endothelial cells suppresses senescence and extends the survival of these cells. Resveratrol activates SIRT1, and administration of resveratrol was reported to prevent arterial

TABLE 1 | Senolytic agents and their molecular targets.

	Senolytic agents	Target	Effective type of senescent cells	Effective for aging mouse model	Effective for CVD mouse model	Ongoing clinical trials	Reference
1	Dasatinib	Dependence receptor/Src kinase/Tyrosine kinase	Irradiated human preadipocytes, human lung fibroblasts (IMR-90), MEFs, mouse preadipocytes (adipose-derived stem cells)	Yes [irradiation, progeria (*Ercc1*−/Δ), chronological aging]	Yes [atherosclerosis ($ApoE^{-/-}$)]	(D + Q); chronic kidney disease, idiopathic pulmonary fibrosis, hematopoietic stem cell transplantation	(105, 107, 125–129)
2	Quercetin	Bcl-2 family, p53/p21/serpine, PI3K/AKT signaling	Irradiated HUVECs, human lung fibroblasts (IMR-90), mouse bone marrow-derived stem cells, MEFs	Yes [irradiation, progeria (*Ercc1*−/Δ), chronological aging]	Yes [atherosclerosis ($ApoE^{-/-}$)]	(D + Q); chronic kidney disease, idiopathic pulmonary fibrosis, hematopoietic stem cell transplantation	(105, 107, 125–129)
3	Navitoclax (ABT-263)	Bcl-2 family (Bcl-2, Bcl-xl, and Bcl-w)	Human lung fibroblasts (IMR-90, WI-37; irradiated, RAS induction, replicative), irradiated human renal epithelial cells, MEFs	Yes (irradiation, chronological aging)	N/A	N/A	(34, 126)
4	TW-37	Bcl-2 family (Bcl-2, Bcl-xl, and Mcl-1)	MEFs passaged in high oxygen condition (20%)	N/A	N/A	N/A	(126)
5	ABT-737	Bcl-W and Bcl-XL	Human lung fibroblasts (IMR-90)	Yes (irradiation)	N/A	N/A	(125)
6	Fisetin	PI3K/AKT signaling	Irradiated HUVECs	N/A	N/A	N/A	(130)
7	A1331852	Bcl-2 family (Bcl-XL)	Irradiated human lung fibroblasts (IMR-90), HUVECs	N/A	N/A	N/A	(130)
8	A1155463	Bcl-2 family (Bcl-XL)	Irradiated human lung fibroblasts (IMR-90), HUVECs	N/A	N/A	N/A	(130)
9	Foxo4-DRI	Foxo4-p53 interaction	Irradiated human lung fibroblasts (IMR-90, WI-38), foreskin fibroblasts (BJ cells)	Yes [doxorubicin, progeria (*Xpd*TTD/TTD), chronological aging]	N/A	N/A	(106)
10	Alvespimycin (17-DMAG)	HSP90	*Ercc1*−/− MEFs	Yes [progeria (*Ercc1*−/Δ)]	N/A	N/A	(131)
11	Tanespimycin (17-AAG)	HSP90	*Ercc1*−/− MEFs	N/A	N/A	N/A	(131)
12	Geldanamycin	HSP90	*Ercc1*−/− MEFs	N/A	N/A	N/A	(131)
13	Piperlongumine	p53/p21 & Bcl-2 family (PUMA)	Irradiated human lung fibroblasts (WI-38)	N/A	N/A	N/A	(132)
14	Panobinostat	Histone deacetylase	Chemotherapy-induced senescent NSCLC and HNSCC cells	N/A	N/A	N/A	(133)

MEF: mouse embryonic fibroblast, HUVEC: human umbilical vein endothelial cell, NSCLC: non-small cell lung carcinoma, HNSCC: head and neck squamous cell carcinoma, D + Q: combination of dasatinib and quercetin, CVD: cardiovascular disorder, N/A: not applicable.

wall inflammation and elevation of the pulse wave velocity by dietary obesity (111). Moreover, activation of SIRT1 attenuates arterial stiffness and hypertension in Klotho-haplodeficient mice (112). SIRT1 expression and activity are decreased in the VSMCs of patients with AAA, together with vascular cell senescence and elevated p21 expression, while SIRT1 inhibits p21-induced cellular senescence and contributes to suppression of vascular inflammation (113). Another study showed that calorie restriction up-regulates SIRT1 expression in vascular smooth muscle cells, and reduced the incidence of AAA (114). These results indicate that activation of SIRT1 in VSMCs may potentially prevent the progression of AAA (113, 114). Nicotinamide adenine dinucleotide (NAD) is a coenzyme involved in cell metabolism, redox reactions, and DNA repair, and it is well known to suppress aging (115). Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme for conversion of nicotinamide to nicotinamide mononucleotide, enabling subsequent biosynthesis of NAD^+. Nampt overexpression was reported to suppress senescence of VSMCs through a process mediated by SIRT1 signaling (116). In patients with aortic dilation, an inverse relationship between Nampt expression by VSMCs and the diameter of the ascending aorta was recently identified, and the authors concluded that NAD^+ biosynthesis in the aortic media is important for protection against DNA damage and premature VSMC senescence (117). Systemic metabolic dysfunction is reported to induce cellular senescence in endothelial cells. Aged mice with systemic glucose intolerance and hyperinsulinemia show elevation of aortic NADPH oxidase-2 (Nox2) expression, while *in vitro* glucose and insulin challenge increases Nox2 and ROS levels in coronary microvascular endothelial cells, promoting cellular senescence along with elevation of p53 (118). Furthermore, hyperlipidemia associated with aging enhances mitochondrial oxidative stress and induces plaque instability in $ApoE^{-/-}$ mice (119). It was previously reported that Akt, which acts downstream of insulin signaling, negatively regulates the lifespan of human endothelial cells via p53/p21 signaling (120). Dietary intake of rapamycin (an inhibitor of mTOR, a molecule that acts downstream of the insulin signaling pathway) was shown to reverse age-related vascular dysfunction and oxidative stress, in association with reduced arterial expression of the senescence marker p19 (121). In the LEADER trial, a glucagon-like peptide 1 analogue (liraglutide) reduced the death rate from cardiovascular disease in patients with type 2 diabetes, as well as decreasing nonfatal myocardial infarction and nonfatal stroke (122). Another glucagon-like peptide 1 analogue (exenatide) showed beneficial vascular effects, partly via enhancing adiponectin production, and suppressed oxidative stress and inflammation in the vascular plaques of $ApoE^{-/-}$ mice (123). Taken together, these studies indicate that, in addition to use for the inhibition of systemic metabolic disorders, suppression of cellular senescence and/or elimination of senescent cells could become next generation therapies for cardiovascular disorders.

Conclusion and Future Directions

This review outlined the pathological role of vascular senescence in cardiovascular disease and metabolic disease. Both capillaries and arteries are critically important for delivery of nutrients and oxygen to the organs/tissues for maintenance of physiological function. Vascular endothelial cells and VSMCs have a crucial role in vascular homeostasis. Senescence of vascular cells promotes the development of age-related disorders, including heart failure, diabetes, and atherosclerotic diseases, while suppression of vascular cell senescence ameliorates phenotypic features of aging in various models. Recent findings have indicated that specific depletion of senescent cells reverses age-related changes. Considering that suppression of cellular senescence is associated with a risk of tumorigenesis, specific depletion of senescent cells may be a more promising approach to the treatment of age-related diseases. An issue that remains to be explored is the potential side effects of such treatment. For example, Demaria and colleagues found that genetic removal of senescent cells delayed wound healing in mice (124). We also need to identify the best senolytic agents, and optimize the dosage and administration and combinations for treatment of various conditions. Potential gender differences are another important research topic. Although the biological networks contributing to maintenance of homeostasis are extremely complex, it seems reasonable to explore senolytic agents that can act on specific cellular components or tissues. Several clinical trials of senolytic agents are currently ongoing. Survivors of hematopoietic stem cell transplantation are prone to premature aging, and one pilot clinical study is designed to test whether D + Q can suppress aging in these patients (Clinical Trials. Gov Identifier: NCT02652052). Another clinical trial is testing whether D + Q reduces pro-inflammatory cells obtained by skin biopsy in patients with idiopathic pulmonary fibrosis (Clinical Trials. Gov Identifier: NCT02874989). Furthermore, a clinical trial is ongoing to determine whether D + Q can reduce the senescent cell burden and frailty in patients with chronic kidney disease, as well as improving the function of adipose tissue-derived mesenchymal stem cells (Clinical Trials. Gov Identifier: NCT02848131). So far, only D + Q has been assessed in the clinical setting, and none of the current clinical trials are testing whether senolytic agents can inhibit cardiovascular disorders. However, depletion of senescent cells was demonstrated to suppress pathological progression of atherosclerotic plaque in rodents, suggesting that senolytic agents could become a next generation therapy for cardiovascular disorders (**Table 1**).

AUTHOR CONTRIBUTIONS

GK, IS, YY, TM wrote the manuscript.

FUNDING

This work was supported by a Grant-in-Aid for Scientific Research (Grant number 17H04172, 25870127), a Grant-in-Aid for Scientific Research on Innovative Areas (Grant number 26115008), and a Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT, Grant number 15K15306) of Japan and grants from the Takeda Medical Research Foundation, the Japan Foundation for Applied Enzymology, the Takeda Science Foundation, the SENSHIN Medical Research Foundation, the Terumo Foundation, the Manpei Suzuki Diabetes Foundation, the Naito Foundation, and the NOVARITIS foundation (to T.M.).

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

frontiers in Cardiovascular Medicine

MINI REVIEW
published: 27 June 2018
doi: 10.3389/fcvm.2018.00078

Tissue Specific Origin, Development, and Pathological Perspectives of Pericytes

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Pericytes are mural cells surrounding blood vessels, adjacent to endothelial cells. Pericytes play critical roles in maturation and maintenance of vascular branching morphogenesis. In the central nervous system (CNS), pericytes are necessary for the formation and regulation of the blood-brain barrier (BBB) and pericyte deficiency accompanies CNS diseases including multiple sclerosis, diabetic retinopathy, neonatal intraventricular hemorrhage, and neurodegenerative disorders. Despite the importance of pericytes, their developmental origins and phenotypic diversity remain incompletely understood. Pericytes express multiple markers and the origin of pericytes differs by tissue, which may cause difficulty for the identification and understanding of the ontogeny of pericytes. Also, pericytes have the potential to give rise to different tissues *in vitro* but this is not clear *in vivo*. These studies indicate that pericytes are heterogeneous in a tissue- and context- dependent manner. This short review focuses on recent studies about identification of pericytes, heterogeneous origin of pericytes during development and in adults, and the differentiation capacity of pericytes, and pericytes in pathological settings.

Keywords: pericyte, development, differentiation capacity, origin, heterogeneity

OPEN ACCESS

Edited by:
Masanori Aikawa,
Harvard Medical School,
United States

Reviewed by:
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James B. Hoying,
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *14 April 2018*
Accepted: *07 June 2018*
Published: *27 June 2018*

Citation:
Yamazaki T and Mukouyama Y (2018) Tissue Specific Origin, Development, and Pathological Perspectives of Pericytes. Front. Cardiovasc. Med. 5:78. doi: 10.3389/fcvm.2018.00078

INTRODUCTION

Pericytes are mural cells surrounding blood vessels and embedded within the basement membrane of the vasculature and adjacent to endothelial cells (1). Pericytes cover microvessels such as arterioles, venules and capillaries, while large-diameter vessels like arteries and veins are covered by vascular smooth muscle cells (VSMCs), the other type of mural cells. Mural cells are known to play fundamental roles in vascular network formation: pericyte coverage is critical for vascular stability and structure. In the central nervous system (CNS), pericytes play an essential role in the maturation and maintenance of the blood brain barrier (BBB) (2–4), which is established by the interaction between the microvessels (endothelial cells and pericytes) and surrounding astrocytes within the neurovascular unit. The BBB is a diffusion barrier which blocks the inflow of various molecules and toxins from blood to brain, but not all CNS vessels equally contribute to the BBB: several areas of the brain including the pituitary gland, pineal gland, subventricular zones and choroid plexus are not protected by the BBB. The diversity of the BBB architecture and function remains largely unexplored. Given that pericyte density, morphology, and function vary in different vascular beds (5), these observations may reflect their heterogeneous characteristics. In this review, we give an overview of pericytes with

a focus on their heterogeneity. A more objective definition of different subsets of pericytes may help to shed light on novel therapeutic approaches for neuro-vascular diseases associated with pericyte loss such as stroke and Alzheimer's disease.

IDENTIFICATION OF PERICYTES

Morphological characteristics of pericytes were defined by electron microscopy: pericytes possess a cell body with a prominent nucleus and contain a small amount of cytoplasm with several long processes covering the endothelial wall. Pericytes are embedded in the basement membrane where they interact with endothelial cells: pericyte-endothelial cell interaction enhances basement membrane assembly (**Figure 1**).

Although the ultrastructural characteristics of pericytes has been well studied using electron microscopy, pericytes remain a relatively poorly defined cell type, without highly specific markers available for their identification (6). The following markers have been used as pericyte markers, but no general pan-pericyte molecular marker has been discovered because of their heterogeneous distributions and functions in various tissues (2, 5, 7). We should note that these markers are also expressed by other cell types including perivascular fibroblasts, VSMCs and macrophages. Chondroitin sulfate proteoglycan4/neural glial antigen 2 (NG2) is expressed on the surface of pericytes during angiogenesis (8, 9), but is also expressed on glial precursor O2A cells in the CNS, which generate either oligodendrocytes or astrocytes *in vitro*. Platelet-derived growth factor receptor beta (PDGFRβ) (10, 11) is one of the most widely studied molecular marker expressed in pericytes. PDGF-B/PDGFRβ signaling is essential for pericyte proliferation and recruitment to blood vessels. Alpha smooth muscle actin (αSMA) (12), desmin (13), and vimentin (14) are contractile filaments. Regulator of G protein signaling 5 (RGS5) (15, 16) is a GTPase activating protein for Giα and Gqα, and the expression pattern of RGS5 overlaps with expression pattern of NG2 and PDGFRβ. CD146, also known as melanoma cell adhesion molecule (MCAM) has been used as a marker for pericytes and VSMCs as well as endothelial cells (17). Recent studies suggest that CD146 regulates PDGFRβ activation and involves in BBB integrity (18) and is essential for pericyte recruitment (19). CD13/aminopeptidase N (APN) (20) is a membrane-bound metalloprotease which was originally identified as a myeloid cell marker (21). CD13 has been used as a surface marker for brain pericytes (22, 23). Overall, current immunohistochemical approaches to identify pericytes use antibodies against these proteins, depending on tissue and microvessel types. The expression of these markers varies depending on developmental stages, organs, pathological situations, and *in vitro* or *in vivo* conditions. Therefore, anatomical characteristics combined with at least two molecular markers are important to define pericytes. Indeed, recent studies have revealed that double *PDGFRβ-EGFP* and *NG2-DsRed* fluorescence reporter expression patterns are valuable for pericyte/mural cell identification of the CNS tissues (24, 25). Although the expression of αSMA is known as a marker for both pericytes and VSMCs, capillary pericytes do not express αSMA (12): capillary pericytes in embryonic skin express only NG2 and PDGFRβ but not αSMA (26). In contrast, capillary pericytes in tumors do express αSMA (27).

Genetic mouse models including transgenic markers, fluorescent reporters and lineage tracing lines are valuable tools to trace the pericyte lineage during development and in pathological conditions: nuclear β-galactosidase reporter (28) as well as emerging fluorescent reporter and lineage tracing lines using the promoter of *PDGFRβ* (24, 29), *NG2* (24, 30, 31), *or Tbx18* (32) are available. Alternatively, a fluorescent Nissl dye specifically labels brain pericytes and enables the imaging in the live mouse (33).

DEVELOPMENTAL ORIGIN OF PERICYTES

The developmental origin of pericytes is heterogeneous, and much remains to be deciphered. Most commonly described, and best understood is their origin from mesenchymal stem cells (34). Chick-quail chimera analysis (35, 36) and genetic lineage tracing experiments using neural crest-specific *Cre* recombinase lines such as *Wnt-1-Cre* and *Sox10-Cre* mice in combination with a *Cre*-mediated reporter line demonstrate that neural crest contributes to pericytes in the face, brain, and thymus (36–40). Using similar genetic lineage tracing experiments, the origin of pericytes in the gut (41), lung (42), and liver (43) in mice has been traced to the mesothelium, a single layer of squamous epithelium. In the heart, the epicardial mesothelium gives rise to coronary pericytes and VSMCs (44–46). Recent studies have demonstrated that some endocardium also contributes to coronary pericytes (47). These studies clearly indicate that the origin of pericytes is heterogeneous in a tissue- and context- dependent manner.

We have recently revealed that myeloid progenitor cells differentiate into a subset of pericytes in the ectoderm-derived skin and brain during development (**Figure 2**) (26). Using high-resolution whole-mount imaging and a series of genetic lineage tracing experiments with hematopoietic cell-specific *Vav-Cre* and myeloid cell-specific *CD11b-Cre* lines in combination with a *Cre*-mediated fluorescent reporter line, we found that the developmental sources of pericytes are heterogeneous and some pericytes are derived from myeloid progenitors in the developing skin and brain. Mutant mice lacking myeloid lineage exhibit defective pericyte development. Moreover, TGF-β promotes the differentiation of myeloid progenitors into pericytes *in vitro* and *in vivo*. In a similar line of research, some $CD31^+$ $F4/80^+$ macrophages contribute to cerebrovascular pericytes during embryogenesis (48). Insight into the heterogeneous origins of pericytes will have important implications for understanding the establishment of the organ-specific vascular networks during embryonic angiogenesis. Moreover, whether pericytes of different origins have different functions in these tissues remains to be elucidated.

It is an intriguing question of whether pericytes of heterogeneous origins at embryonic stages remain in adult: one possible scenario is that a subtype of pericytes may become

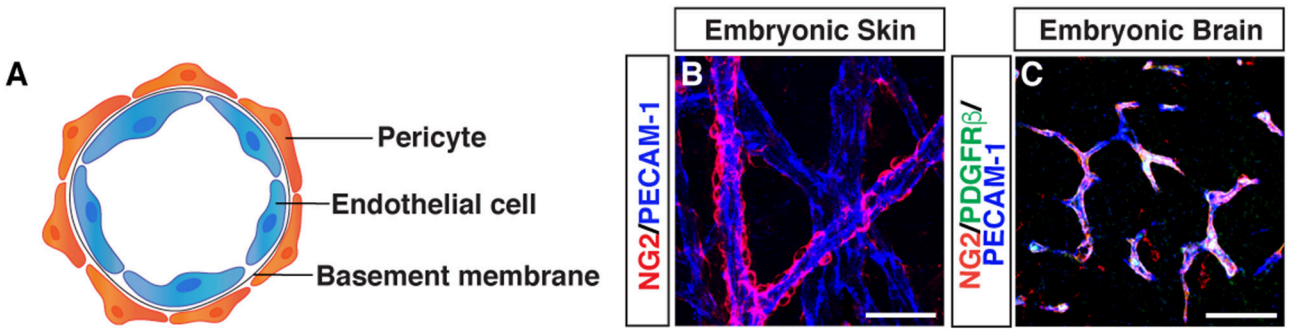

FIGURE 1 | Morphology of pericytes. **(A)** Schematic image of pericyte-endothelial cell interaction. **(B)** Whole-mount immunostaining of mouse embryonic skin with antibodies to pericytes (NG2, red) and endothelial cells (PECAM-1, blue) at embryonic day (E) 15.5. Pericytes are semicircular shape and cover blood vessels. **(C)** Triple immunostaining of E15.5 mouse embryonic brain with antibodies to pericytes (NG2, red; PDGFRβ, green) and endothelial cells (PECAM-1, blue). Scale bars, 50 μm.

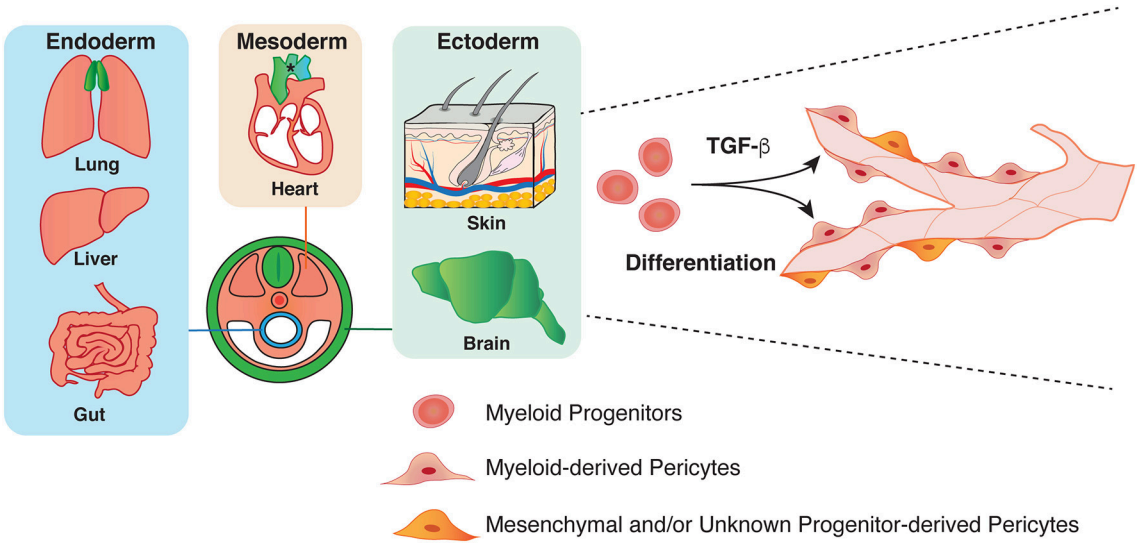

FIGURE 2 | Heterogeneity in the origin of pericytes. Pericytes in both endoderm and mesoderm-derived organs, such as lung, liver, gut, and heart have mesoderm origin (orange). Pericytes in the aorta region have several origins: secondary heart field, neural crest, and somite (asterisk). Previous studies have demonstrated that ectoderm-derived neural crest give rise to pericytes in brain and thymus (green). Our group has recently shown that mesoderm-derived myeloid progenitor cells differentiate into pericytes in ectoderm-derived organs such as skin and brain through TGF-β signaling during development.

dominant in adult. Moreover, adult tissue-resident progenitors contribute to pericytes in pathological situations. Indeed, mesenchymal stem cells generate pericytes after radiation therapy (49), while mesenchymal tumors such as bone and soft tissue sarcomas can be derived from pericytes (50). Glioblastoma stem cells generate pericytes to support tumor growth (51). Defining the origin of adult tissue pericytes needs the development of a cell-type specific inducible *Cre* recombinase line such as *CreER*. Transient *Cre* activity produces fluorescent reporter positive cells at a defined developmental time point and then allows for the tracking of their progeny in adults. The inducible lineage tracing experiment also allows us to examine whether pericytes of different origins could differentially contribute to the neovascularization processes in pathological conditions such as tumor angiogenesis and wound healing.

PERICYTES IN ADULT AND DISEASES

Pericytes have been reported as a component of stem cell niches and mesenchymal stem cells. In the bone marrow, $NG2^+$ pericytes in arterioles promote hematopoietic stem cell (HSC) quiescence and are important for HSC maintenance (52). Likewise, $NG2^+$/$Nestin^+$ pericytes associate with portal blood vessels in fetal liver and are required for the HSC niche (53). In the adult brain, neural stem cells (NSCs) located in the largest germinal region of the forebrain, the ventricular-subventricular zone (V-SVZ). Endothelial cells and pericytes in the V-SVZ form the NSC niche, and V-SVZ pericytes secrete diffusible factors that increase the proliferation and enhance neuronal differentiation (22). *In vitro* differentiation capacity of pericytes into mesenchymal cell types (e.g., adipocytes,

chondrocytes, osteoblasts, fibroblasts, VSMCs) has been proven in a multitude of studies (54–58). Pericytes facilitate repair process after myocardial infarction in the heart (59, 60), while pericytes regenerate injured and dystrophic skeletal muscles (61). *In vivo* lineage tracing experiments have reported pericytes as progenitors of white adipocytes (62), follicular dendritic cells (63), odontoblasts (mesenchyme-derived dentine producing cells) (64), and skeletal muscle (61). Differentiation capacities of pericytes into neurons, astrocytes, and oligodendrocytes have been also reported (56). In addition, pericytes have been reported to play a major role as fibroblast progenitors in fibrotic responses (65).

Cardiac pericytes have been studied as a therapeutic target after injury. Cardiac pericytes account for up to 5% of the total non-cardiomyocyte cell population (66). Recent studies identified microvascular pericytes in the human ventricular myocardium and demonstrated that human cardiac pericytes express mesenchymal stem/stromal cell markers including CD44, CD73, CD90, and CD105 (58). Indeed, cardiac pericytes have the capacity to differentiate into mesodermal lineage: osteo-, chondro-, and adipogenesis, but no potential for skeletal myogenesis *in vitro* (58). Many cardiac diseases are associated with fibrosis, an accumulation of fibroblasts and an excess of extracellular matrix proteins which may affect the architecture and function of the organ or the tissue. Pericytes have been shown to contribute to fibrosis organ-dependently (67): $NG2^{+}$/$Nestin^{-}$ type 1 pericytes are recruited and accumulated in the ischemic interstitial space around fibrotic tissue but do not contribute to fibrosis (67).

However, a series of lineage tracing experiments with *Tbx18CreERT2* line, which is selectively expressed by pericytes and VSMCs in multiple adult organs, has revealed that adult pericytes of heart, brain, skeletal muscle and fat depots do not behave as multipotent progenitors in aging and different pathological situations such as a high-fat diet and injury. In addition, pericytes do not contribute to fibroblasts in fibrotic responses (32). These results are in contrast to the previous studies that demonstrated the multipotent potential of pericytes. The discrepancies might be explained by the differing methods used in the studies to identify pericytes as well as the different behavior of *in vitro* and *in vivo* pericytes. The population of pericytes which can differentiate into mesenchymal cell types may vary by organ and developmental stage *in vivo*. It is also possible that pericyte differentiation requires stem cell-like cells of non-pericyte origin (64).

CONCLUSION

The absence of any single marker to identify pericytes complicates the study of the ontogeny and the differentiation capacity of pericytes. Much remains to be elucidated regarding how and when the origin of pericytes in an organ is determined and whether pericytes have potential for differentiation *in vivo*. There are questions about how pericytes derived from different origins behave when they co-exist in a tissue: do they show identical gene expression patterning and behavior in adult tissues under normal and pathological conditions? Future studies using pericyte-specific lineage tracing mice and precise gene-expression profiling will pave the way for the understanding of the spatial-temporal pericyte behavior *in vivo* and may contribute to new therapeutic strategies for diseases associated with pericyte loss and/or dysfunction.

FUNDING

YM is supported by the Intramural Research Program of the National Heart, Lung, and Blood Institute (HL005702-11). TY was supported by the Japan Society for the Promotion of Science (JSPS) NIH-KAITOKU.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

We thank K. Gill for editorial help and laboratory management, and R. Reed for administrative assistance.

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

frontiers in Cardiovascular Medicine

REVIEW
published: 05 January 2018
doi: 10.3389/fcvm.2017.00088

Cathepsin S As an Inhibitor of Cardiovascular Inflammation and Calcification in Chronic Kidney Disease

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Cardiovascular disease (CVD) is responsible for the majority of deaths in the developed world. Particularly, in patients with chronic kidney disease (CKD), the imbalance of calcium and phosphate may lead to the acceleration of both vascular and valve inflammation and calcification. One in two patients with CKD are reported as dying from cardiovascular causes due to the resulting acceleration in the development of atherosclerosis plaques. In addition, CKD patients on hemodialysis are prone to aortic valve calcification and often need valve replacement before kidney transplantation. The lysosomal proteases, cathepsins, are composed of 11 cysteine members (cathepsin B, C, F, H, K, L, O, S, V, W, and Z), as well as serine proteases cathepsin A and G, which cleave peptide bonds with serine as the amino acid, and aspartyl proteases D and E, which use an activated water molecule bound to aspartate to break peptide substrate. Cysteine proteases, also known as thiol proteases, degrade protein *via* the deprotonation of a thiol and have been found to play a significant role in autoimmune disease, atherosclerosis, aortic valve calcification, cardiac repair, and cardiomyopathy, operating within extracellular spaces. This review sought to evaluate recent findings in this field, highlighting how among cathepsins, the inhibition of cathepsin S in particular, could play a significant role in diminishing the effects of CVD, especially for patients with CKD.

Keywords: cathepsin S, cardiovascular disease, chronic kidney disease, calcification, atherosclerosis, aortic valve

OPEN ACCESS

Edited by:
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Reviewed by:
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Vasilios Gabriel Athyros, Aristotle University of Thessaloniki, Greece

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

Received: *02 November 2017*
Accepted: *14 December 2017*
Published: *05 January 2018*

Citation:
Sena BF, Figueiredo JL and Aikawa E (2018) Cathepsin S As an Inhibitor of Cardiovascular Inflammation and Calcification in Chronic Kidney Disease. Front. Cardiovasc. Med. 4:88. doi: 10.3389/fcvm.2017.00088

INTRODUCTION

Cathepsins are lysosomal proteases that breakdown peptide bonds linked to specific amino acids. They are known for their key role in protein turnover and significantly contribute to the breakdown of the extracellular matrix (ECM). More importantly, cathepsins play a crucial role in various conditions that involve large biological systems such as autoimmune disease, cardiac repair, cardiomyopathy, heart valve disease, and atherosclerosis.

The cathepsin family, cysteinyl, in specific, is known to date to be composed of 11 members in humans including cathepsins B, C, F, H, K, L, O, S, V, W, and Z. Cathepsin S is thought to be a particularly potent cysteine protease cleaving elastin and generating bioactive elastin peptides, leading to the promotion of cardiovascular inflammation and calcification. Beyond boosting inflammation as a part of its own mechanistic process, cathepsin S is also released by smooth muscle cells

and macrophages as a systemic response to inflammation in a continuous recursive feedback loop.

Vascular inflammation, in particular, is a characteristic feature in the initiation and progression of atherosclerotic lesions. Vascular inflammation that is caused by atherosclerosis is described by widespread changes and remodeling of the ECM within the arterial wall, both in the degradation of the matrix and cellular phenotypic changes. Compounded by chronic inflammation and a consistent imbalance in calcium phosphate serum levels, patients with chronic kidney disease (CKD) face a deleterious feedback loop. This harmful combination further accelerates and exacerbates the evolution of atherosclerosis in patients with CKD. Studies on atherosclerosis-associated inflammatory cytokines have also shown that cathepsin expression and activity are amplified within vascular cells (1).

Also affecting patients with CKD, aortic valve calcification is highlighted as a major clinical problem in this population, where calcification can lead to the progression of aortic valve stenosis. Patients with CKD on hemodialysis are prone to developing aortic valve calcification and likely to need valve replacement before undergoing kidney transplantation. Of further importance is the fact that no current treatment options are available for patients with aortic valve stenosis besides valve replacement. Further, recent clinical trials have shown no benefit to statin therapy in halting the progression of aortic calcification and stenosis (2), leaving health professionals and patients with little to no options.

Within this context, one in two patients with CKD are reported as dying from increased cardiovascular burden particularly resulting from an acceleration in the development of calcification and atherosclerotic plaque (3). Hence, the acceleration in the development of calcification and atherosclerosis in patients with CKD is a leading factor in the simultaneously higher cardiovascular risk, the likelihood of developing cardiovascular disease (CVD), and ultimately death from cardiovascular causes. Thus, interventions that could support in halting or reducing calcification and atherosclerosis, especially for patients with CKD is of desperate need.

The inhibition of cathepsin S has been highlighted as a promising intervention in reducing plaque development and diminishing the effects of CVD, especially for patients with CKD (4). This is especially critical considering the lack of treatment options for patients with aortic valve calcification that risk progression to stenosis and the need for valve replacement. Cathepsin S inhibition has been shown in evaluations of various cathepsins and inhibitors as a promising target. Through this review, we hope to better understand the role of cathepsin S in CVD inflammation and calcification in CKD. More specifically, we will analyze inquiries into cathepsin S mechanism of action, its role in various processes of inflammation, calcification, and renal disease, and identify cathepsin S inhibitors that have been gaged as potential and promising treatment targets.

MATERIALS AND METHODS

A review was conducted through a search of the PubMed, Medline, the Cochrane Library, and Google databases. The following keywords were used: "cathepsin S," "cardiovascular disease," "chronic renal disease," "cathepsin S inhibitor," "renal disease," "calcification," and "atherosclerosis." The keywords were combined to reach the relevant results.

A review of the titles and abstracts was undertaken to find those that matched the keywords of the search. Following the review of titles and abstracts, the authors identified six categories of interest and organized the articles based on this classification: "treatment by cathepsin S inhibitor," "cathepsin S and renal disease," "cathepsin S mechanism," "cathepsin S and calcification," "cathepsin S and inflammation," and "miscellaneous."

The initial review of the databases yielded a total of 41 articles. Of those 41, 13 articles were categorized under "cathepsin S mechanism," 12 under "cathepsin S and inflammation," 7 articles were categorized under "treatment by cathepsin S inhibitor," 6 were as "cathepsin S and renal disease," one under the category "cathepsin S and calcification," and two under "miscellaneous,"

Categories	Number of articles	Publications
Cathepsin S mechanism	13	Aikawa et al. (3), Wu et al. (5), Chen et al. (6), Moran et al. (7); Pauly et al. (8), Qin et al. (9), Lv et al. (10), Pai et al. (11), Cheng et al. (1), Sasaki et al. (12), Shi et al. (13), Liuzzo et al. (14), and Shi et al. (15)
Cathepsin S and inflammation	12	Lafarge et al. (16), Reiser et al. (17), Naour et al. (18), Stenvinkel et al. (19), Lutgens et al. (20), Rodgers et al. (21), Liu et al. (22), Basalyga et al. (23), Go et al. (24), Sukhova et al. (25), Jobs et al. (26), and Jobs et al. (27)
Treatment by cathepsin S inhibitor	7	Ahmad and Siddiqi (28), Rupanagudi et al. (29), Payne et al. (30), Jadhav et al. (31), Hilpert et al. (32), Figueiredo et al. (4), and Samokhin et al. (33)
Cathepsin S and renal disease	6	Huang et al. (34), Steubl et al. (35), Carlsson et al. (36), Jobs et al. (37), Smith et al. (38), and Inker et al. (39)
Cathepsin S and calcification	1	Kumar et al. (40)
Miscellaneous	2	Hewitt et al. (41), and Nadkarni et al. (42)

RESULTS

Proposed Mechanism of Cathepsin S Action

Researchers have sought to understand the role of cathepsin S in CVD and systems by evaluating the molecule's potential mechanisms of action. One of the systemic mechanisms recently studied is cathepsin S's role in cardiovascular inflammation and calcification especially for patients faced with CKD.

Aikawa et al. (3) demonstrate through *in vivo* experiments that elastin degradation induced by cathepsin S accelerates arterial and aortic valve calcification in a CKD model. The findings indicate a mechanism of action where cathepsin S contributes to the cleavage of the elastin matrix in atherosclerotic lesions and inflamed valves, and in the disruption of the tissue layer, mesenchymal cells [vascular smooth muscle cells (SMCs) or valvular myofibroblasts] proliferate and calcify. The researchers

highlight that CKD patients with systemic mineral imbalance or hyperphosphatemia experience further acceleration of calcification. The investigators conclude that future interventions in calcification-prone individuals should target inflammation and phosphate imbalance to reduce the effects of negative feedback loops related to elastin degradation and calcification, and incorporate the selective inhibition of cathepsin S as a treatment target.

On the other hand, in an arterial medial calcification (AMC), a different type of vascular calcification, uremic mouse-model fed a high-phosphate diet, Pai et al. (11) suggest that even though elastin degradation is a necessary factor in the cascade leading to AMC, it may not be sufficient on its own to induce calcification. The researchers indicate that in their findings although elastin degradation did occur in uremic mice on a normal phosphate diet, they did not develop AMC and thus elastin degradation would not be sufficient to induce medial calcification. The researchers instead indicate that phenotypic changes and loss of vascular SMCs would be both "necessary and sufficient" culprits to induce AMC and not elastin degradation alone.

Nevertheless, Simionescu et al. (43) in a study looking at the role of fibroblasts in medial vascular calcification find that calcified nodules are formed in the presence of elastin degradation products and transforming growth factor (TGF)-β1, especially when used together. In this way, the investigators show that elastin degradation peptides, highly active biologic products known as matrikines, can induce calcification of mesenchymal cells *in vitro*. These findings suggest that elastin degradation could in fact induce calcification.

Additionally, in a murine model with cathepsin S inhibitor E64d, Chen et al. (6) find that cathepsin S plays a part in the signaling of TGF-β1, myofibroblast differentiation, and ECM creation and modulation in myocardial infarction (MI). The researchers describe how cathepsin S participates in regulating scar formation in the infarcted myocardium and preserve left ventricular function after experimentally induced MI. Further, Shi et al. (13) find that growth of new blood vessels is abnormal during wound repair in a cathepsin S-deficient mouse model, despite regular endothelial and fibroblast growth factor levels. Their results show that when cathepsin S activity is inhibited, the formation of microtubules is reduced, suggesting an essential role of cathepsin S in ECM degradation during vessel formation and repair.

Sasaki et al. (12) suggest that cathepsin S derived from macrophages are involved in the mechanisms that lead to the vulnerability of atherosclerotic plaque with increased levels of cathepsin S found in plaque. Knowing of angiotensin II as a player in vascular homeostasis, the researchers elucidate to angiotensin II type 1 receptor (AT1) blocker, olmesartan, as maintaining the stability of plaques, while simultaneously suppressing cathepsin S and macrophage activity. The authors then suggest the use of olmesartan as a treatment intervention in reducing cardiovascular consequences.

Cheng et al. (1) in a comprehensive review of the literature on the contributions of cathepsins in atherosclerosis-based vascular disease find strong evidence implicating cathepsins in related mechanisms of action. The researchers highlight that feasibility studies looking at cathepsins as diagnostic tools have shown promising results in the use of cathepsins S and L, and the endogenous inhibitor cystatin C as biomarkers for determining coronary artery disease and the formation of aneurysms.

In abdominal aortic aneurysm (AAA), often described for its wide degradation of the aortic wall matrix, remodeling and further rupturing of the wall, Qin et al. (9) in a murine model, provide evidence of cathepsin S's role in the formation of AAA and suggest cathepsin S as a new therapeutic target for preventing AAAs in humans. Additionally, Lv et al. (10) test plasma samples of 476 male patients with AAA and 200 age-matched male controls as part of an ongoing randomized trial of more than 50,000 men aged 65–74 in Denmark. The researchers find in this population a correlation between plasma cathepsin S, cystatin C, aortic diameter, and the lowest ankle–brachial index. Their results suggest these parameters could be used as biomarkers for assessing arterial diseases and AAA, highlighting that more advanced cases of AAA would contain higher levels of cathepsin S in the AAA lesions as well as in circulation.

Important to highlight is that investigators suggest future interventions in calcification-prone individuals target inflammation and phosphate imbalance, together with the selective inhibition of cathepsin S as a treatment target (3, 11). Additionally, after review of evidence on cathepsins in atherosclerosis-based vascular disease, and feasibility studies of cathepsins as potential diagnostic tools, Cheng et al. (1) conclude that cathepsins S and L and endogenous inhibitor cystatin C could be used as biomarkers in determining the existence of coronary artery disease and the formation of aneurysms clinically.

Cathepsin S Inhibition

Other researchers have also inquired about various cathepsin S inhibitors as treatment for cardiovascular disorders. Ahmad and Siddiqi (28) perform simulations of the molecule's docking and dynamics to better understand the molecular mechanism of action of the cathepsin S inhibitor RO5444101. The researchers demonstrated a selectivity of this inhibitor for cathepsin S rather than cathepsin L1/L, cathepsin L2/V, and cathepsin K, claiming that the cathepsin S compound is more stable and involves more protein–molecule interactions. Figueiredo et al. (4) found that the systemic inhibition of cathepsin S by the compound RO5444101 attenuated the progression of atherosclerotic lesions in high-fat high-cholesterol fed apolipoprotein E-deficient nephrectomized mice. The authors conclude that the cathepsin S inhibitor accomplishes this through the simultaneous reduction in immunoreactivity of cathepsin S, elastin degradation, plaque size, macrophage accumulation, growth differentiation factor-15, and alkaline phosphatase activity. These results suggest a potential role of cathepsin S in the treatment of atherosclerosis in patients with CKD by possibly reducing the progression of atherosclerotic lesions.

In addition, Rupanagudi et al. (29), in an experimental mouse model of systemic lupus erythematosus and lupus nephritis, find that cathepsin S inhibition by RO5461111 shows therapeutic benefits. The inhibition of cathepsin S significantly reduced the excessive autoimmune response found in this disease presentation by blocking the assembly of major histocompatibility complex class II molecules in T and B cells. The investigators suggest that the

inhibition of cathepsin S protects in the progression of lupus nephritis and could be useful in other autoimmune diseases.

Further, Payne et al. (30) assess the tolerability of safety of the cathepsin S inhibitor, LY3000328 in a phase 1, placebo-controlled study. The researchers use a single escalating dose ranging from 1 to 300 mg with 21 healthy male volunteers and find that the compound was quickly cleared from plasma within 12–13 h. They also found that the compound produced a transient decrease in plasma cathepsin S activity followed by a more prolonged increase in plasma cathepsin S mass. With this, the authors suggest that future studies include longer post-dose measurements to assess activity and impact. Additionally, Jadhav et al. (31), using an experimental CaCl2-induced AAA mouse model, report that cathepsin inhibitor LY3000328 binds to subsites without forming covalent interactions. The researchers report that among the tested compounds, LY3000328 was selected for clinical development, and may provide a new clinical treatment for AAA.

Hilpert et al. (32) further account the development of "Potent and Selective Cathepsin S Inhibitors Containing Different Central Cyclic Scaffolds." In a transgenic mouse model of antigen presentation (DO10.11), the authors show a reduction in the production of the immune system cytokine interleukin (IL)-2 by one of the cathepsin S inhibitors studied through dose-dependent studies. Finally, Samokhin et al. (33) investigate the effects of a specific cathepsin S inhibitor in atherosclerotic plaque progression in an apoe–deficient mice fed a high-fat diet. The inhibition of cathepsin S showed protection of atherosclerotic activity. The researchers indicated that this protection is depicted in the decrease of atherosclerotic plaque size, the number of elastin lamina breaks, the numbers of plaque macrophages and buried fibrous caps. To highlight is that the researchers found a 36 and 68% reduction in plaque size, 60 and 75% in elastin breaks in males vs. females, respectively, and caphepsin S-deficient mice showed a decrease of close to 90%. These results further demonstrate the potential of cathepsin S inhibition as therapy.

The described research has looked at various cathepsin S inhibitors such as RO5444101, RO5461111, LY3000328, among others, to understand mechanism, evaluate clearance from plasma, test toxicity, and investigate whether these inhibitors may be good options as clinical treatment targets. Researchers have also demonstrated how the inhibition of cathepsin S decreased the size of atherosclerotic plaques showing cathepsin S's potential role as a therapeutic option.

Cathepsin S and Inflammation

In terms of the association between cathepsin S and inflammation, Jobs et al. (27) investigate in a community-based cohort of elderly men, whether there is an existing association between serum levels of cathepsin S and markers of inflammation mediated by cytokines. The authors then find that higher levels of cathepsin S were associated with higher C-reactive protein and higher serum IL-6 levels, both inflammatory markers. This association was persistent at a reassessment after 7 years from the initial baseline levels acquired. The researchers find that an interplay between cathepsin S and inflammation markers are present even in normal-weight individuals.

Further, in two independent cohorts of elderly men and women, Jobs et al. (26) sought to evaluate associations between circulating cathepsin S levels and mortality, considering that experimental studies have suggested the connection between cathepsin S activity and the development of CVD through the increase in the formation of and destabilization of atherosclerotic plaque. The researchers find that higher serum cathepsin S levels were associated with an increased mortality risk in a linear regression model. Further, in one of the independent cohorts, the researchers found that cathepsin S was independently associated with CVD and cancer, and suggest future studies should evaluate cathepsin S's potential clinical utility.

Lafarge et al. (16), in a review of current literature, attempt to understand further the links between obesity, metabolic disease, and CVD. Knowing that adipose tissue produces a number of pro-inflammatory factors, the group find that the gene encoding cathepsin S is one of the most unregulated in the adipose tissue of obese subjects. Further, the researchers also add that cathepsin S is positively correlated with body mass index. To conclude, they highlight that future inquiries are needed to establish whether cathepsin inhibitors could be beneficial in reducing metabolic and cardiovascular comorbidities in the obese.

Additionally, Naour et al. (18) in a prospective study with two independent cohorts of obese females find that when looking at cathepsin S, L, and K, that obese subjects have a twofold increase in cathepsin S in adipose tissue, as compared to normal-weight control subjects, and an increased rate of cathepsin S release in adipose tissue. However, it remains unknown whether the inhibition of cathepsin S in obesity can reduce cardiovascular risk or improve the metabolic status of obese patients.

Cathepsin S and Renal Disease

Aikawa et al. (3) demonstrate that CKD accelerates cathepsin S-induced atherosclerotic and aortic valve inflammation and calcification in apolipoprotein-deficient mice. On the connection between cathepsin S and renal disease, a study by Steubl et al. (35) finds that as glomerular filtration rates decline, cathepsin S and markers of inflammation-related endothelial dysfunction increase. This indicates that cathepsin S activity increases with CKD progression, suggesting that cathepsin S may be a therapeutic target to prevent cardiovascular complications in CKD. Huang et al. (34) reveal a role of cathepsin S in epidermal growth factor receptors (EGFR) signaling regulation. EGFR expression has been reported as increased in various tumors of the bladder, colon, ovarian, and kidney. Thus, the researchers argue for the clinical evaluation of cathepsin S and EGFR tyrosine kinase inhibitors in combination.

Further, Carlsson et al. (36) in a longitudinal cohort study of 207 patients undergoing hemodialysis found that cathepsin S and L were associated with receptors for tumor necrosis factors. The researchers conclude that the high levels of endostatin, cathepsins S and L, and their associations with tumor necrosis factors warrant further studies within this population exploring mortality, and pathways involved in end-stage renal disease. Additionally, Smith et al. (38) analyze in 200 patients with stages 3 and 4 CKD and a subgroup of 65 patients, elastin-derived peptides, their endogenous inhibitors, and aortic pulse wave velocity

over a 36-month period. The researchers find that higher serum elastin-derived peptide levels were independently associated with increased all-cause mortality.

Cathepsin S and Calcification

Cardiovascular calcification is described as a disease resulting from the disarray of an individual's mineral metabolism where the buildup of minerals such as calcium and phosphate can lead to the vessel hardening and disruption of normal physiological processes (3). At the population level, a growing burden of epidemiologic factors such as aging, hypercholesterolemia, and renal insufficiency have led to an increased prevalence of arterial and aortic valve calcification.

Further, of important clinical concern has been the progression of calcification to even more debilitating conditions such as plaque rupture or aortic valve stenosis. Of additional concern is the fact that there are currently no treatment options available for reducing or avoiding these advanced conditions, beyond valve replacement (2).

In a study looking at the role of fibroblasts in medial vascular calcification, Simionescu et al. (43) find that calcified nodules are formed in the presence of elastin degradation products and TGF-β1. The researchers show that elastin degradation peptides can induce calcification of mesenchymal cells *in vitro*. These findings suggest that elastin degradation could in fact induce calcification of vascular SMCs and valvular myofibroblasts and thus mediate calcification.

A few other studies have also suggested that in the presence of mineral discrepancy, cathepsin S can participate in cardiovascular calcification where extracellular vesicles could then serve as loci for microcalcifications (44–49). The above described studies as well as studies by Aikawa et al. (3) and Figueiredo et al. (4) suggest the role of cathepsin S in cardiovascular calcification associated with mineral imbalance found in diabetes and CKD.

DISCUSSION

Multiple studies have indicated that cathepsin S activity increases with the progression of CKD and have highlighted cathepsin S inhibition as a therapeutic target in the prevention of cardiovascular complications for this patient population. Patients with CKD face a deleterious feedback loop composed and compounded by chronic inflammation and a consistent imbalance in calcium phosphate serum levels. This harmful combination accelerates and exacerbates the evolution of atherosclerosis in patients with CKD. In atherosclerotic lesions, cathepsin S, one of the most potent mammalian elastases, is highly expressed. Macrophages are then set in action as an essential part of the innate immune response, further releasing high levels of cathepsin S.

With the progression of kidney failure in patients with CKD, uremia and uremic toxins, produced during the deterioration of multiple biological functions could lead to the apoptosis and early damage of vessel wall. Extracellular vesicles could then serve as loci for microcalcifications (44–49). However, in the face of altered mineral metabolism, this could also lead to a set of pathological reactions including calcification of medial SMCs and elastin. Damaged kidneys and abnormal hormone levels in CKD cause calcium and phosphorus levels in the blood to be out of balance. This disruption and potential damage more commonly occurs in people with kidney failure receiving hemodialysis, who are more

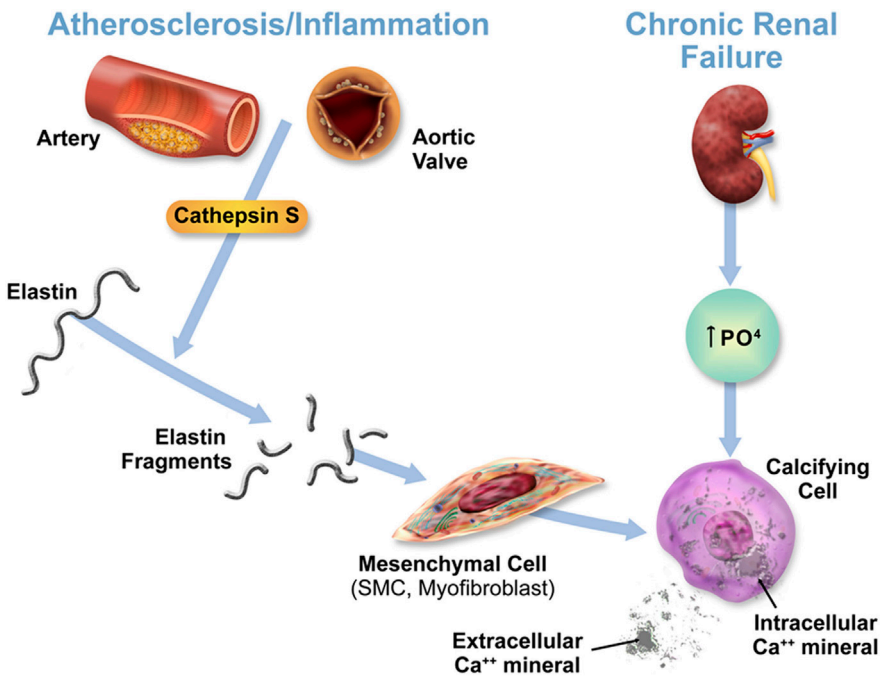

FIGURE 1 | Acceleration of cardiovascular calcification through cathepsin S-associated elastin degradation in a CKD milieu. This figure is reproduced from Aikawa et al. (3), with the author's permission.

prone to aortic valve calcification and stenosis, needing valve replacement before undergoing kidney transplantation. This in itself could lead to increased cardiovascular mortality for those with CKD. Elevations in serum calcium and phosphate levels, such as what occurs with patients under dialysis with CKD, or as a result of calcium–phosphate binder use, or vitamin D treatment (11), can lead to devastating circumstances, and is even more critical for this patient population.

Cathepsin S, through the cleavage of elastin and generation of bioactive elastin peptides, boosts cardiovascular inflammation and calcification. Changes in mineral imbalance, along with the elevation of cathepsin S, may lead to the transformation of mesenchymal cells, including vascular SMCs and interstitial cells to an osteochondrogenic, or stiffened phenotype, thus accelerating calcification even further (**Figure 1**) (3). Through its role in cardiovascular calcification in its association with calcium and phosphate imbalance, cathepsin S is thus involved in the feedback loop between these two biological processes predominant in CKD.

Therefore, as demonstrated in these investigations, cathepsin S may play a deleterious role and be the culprit for the initiation of calcification. This transition to a calcified state could be induced either *via* increased elastolytic activity and production of elastic peptides that directly induce smooth muscle cell or interstitial cell differentiation toward osteogenic bone-like phenotype, or *via* the induced release of extracellular vesicles or apoptotic bodies that could serve as loci resulting in dystrophic calcification (50).

Aortic valve calcification through the hardening of the valve reduces the movement of aortic valve leaflets, impacting and weakening cardiac function. Because mature aortic valves have an elastin-rich, multilayered structure and can develop inflammatory lesions that recapitulate features of atherosclerotic plaques, researchers have proposed that similar mechanisms of cathepsin S-associated elastin degradation contribute to the development of calcific aortic valve disease (3).

Facing this deleterious feedback loop compounded by chronic inflammation and a consistent imbalance in calcium–phosphate serum levels, patients with CKD are found in a harmful combination that would further accelerate and exacerbate the evolution of cardiovascular calcification (51, 52). Therefore, researchers have suggested that the early diagnosis and intervention toward interfering the progression of aortic valve calcification could provide immense clinical benefits.

The studies evaluated in this review have provided further evidence of the potential of the inhibition of cathepsin S as intervention toward reducing plaque development and diminishing the effects of CVD especially for patients with CKD.

AUTHOR CONTRIBUTIONS

BS: data analysis and manuscript writing; JF: data analysis; EA: critical review of the manuscript, supervision, and funding.

FUNDING

EA is supported by National Institutes of Health (NIH) grants R01HL 114805, R01HL 136431, and R01HL 119798.

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

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 16 October 2018
doi: 10.3389/fcvm.2018.00144

Activation of Toll-Like Receptor 9 Impairs Blood Flow Recovery After Hind-Limb Ischemia

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OPEN ACCESS

Edited by:
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Reviewed by:
Jürgen Bernhagen, Ludwig-Maximilians-Universität München, Germany
Andrew James Murphy, Baker Heart and Diabetes Institute, Australia

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

Received: *21 December 2017*
Accepted: *25 September 2018*
Published: *16 October 2018*

Citation:
Nishimoto S, Aini K, Fukuda D, Higashikuni Y, Tanaka K, Hirata Y, Yagi S, Kusunose K, Yamada H, Soeki T, Shimabukuro M and Sata M (2018) Activation of Toll-Like Receptor 9 Impairs Blood Flow Recovery After Hind-Limb Ischemia. Front. Cardiovasc. Med. 5:144. doi: 10.3389/fcvm.2018.00144

Background: Peripheral artery disease causes significant functional disability and results in impaired quality of life. Ischemic tissue injury releases various endogenous ligands for Toll-like receptors (TLRs), suggesting the involvement of TLRs in blood flow recovery. However, the role of TLR9, which was originally known as a sensor for bacterial DNA, remains unknown. This study investigated the role of TLR9 in blood flow recovery in the ischemic limb using a mouse hind-limb ischemia model.

Methods and Results: Unilateral femoral artery ligation was performed in TLR9-deficient ($Tlr9^{-/-}$) mice and wild-type mice. In wild-type mice, femoral artery ligation significantly increased mRNA expression of TLR9 in the ischemic limb ($P < 0.001$) and plasma levels of cell-free DNA (cfDNA) as determined by single-stranded DNA (ssDNA) ($P < 0.05$) and double-stranded DNA (dsDNA) ($P < 0.01$), which are endogenous ligands for TLR9, compared with the sham-operated group. Laser Doppler perfusion imaging demonstrated significantly improved ratio of blood flow in the ischemic to non-ischemic limb in $Tlr9^{-/-}$ mice compared with wild-type mice at 2 weeks after ligation ($P < 0.05$). $Tlr9^{-/-}$ mice showed increased capillary density and reduced macrophage infiltration in ischemic limb. Genetic deletion of TLR9 reduced the expression of TNF-α, and attenuated NF-κB activation in ischemic muscle compared with wild-type mice ($P < 0.05$, respectively) at 3 days after the surgery. ODN1826, a synthetic agonistic oligonucleotide for TLR9, or plasma obtained from mice with ischemic muscle promoted the expression of TNF-α in wild-type macrophages ($P < 0.05$), but not in $Tlr9^{-/-}$ macrophages. ODN1826 also activated NF-κB signaling as determined by the degradation of IκBα in wild-type macrophages ($P < 0.05$), but not in $Tlr9^{-/-}$ macrophages. In vitro experiments using human umbilical vein endothelial cells demonstrated that TNF-α, or conditioned medium obtained from wild-type macrophages treated with ODN1826 accelerated cell death as determined by MTS assay ($P < 0.05$ and $P < 0.01$, respectively).

Conclusion: Our results suggest that ischemic muscle releases cfDNA, which activates TLR9 and enhances inflammation, leading to impairment of blood flow recovery in the ischemic limb. cfDNA-TLR9 signaling may serve as a potential therapeutic target in ischemic limb disease.

Keywords: hind-limb ischemia, blood flow recovery, Toll-like receptor 9, inflammation, macrophage

INTRODUCTION

Peripheral artery disease (PAD), due to partial or complete obstruction of the arteries, is one of the most common manifestations of atherosclerosis, which is associated with increased risk of cardiovascular events (1). Collateral vessel development is a physiological response in ischemic tissues to compensate reduced blood flow. Previous studies indicated that an inflammatory process regulates neovascularization and subsequent blood flow recovery (2). In this process, the immune system plays a pivotal role (3). Exogenous ligands such as microbial-associated molecules activate macrophages through Toll-like receptors (TLRs), whereas endogenous molecules derived from damaged and dead host cells also activate macrophages though TLRs and promote sterile inflammation by inducing cytokines (4). Ischemia induces cellular and tissue damage (5), causing the release of cellular debris which contains various endogenous ligands for TLRs. In fact, several studies reported that TLR-2, 3 and 4 participate in blood flow recovery in ischemic tissues by modulating inflammation and angiogenesis (6–11). However, the role of TLR9 in blood flow recovery in ischemic tissues remains unknown. TLR9 recognizes exogenous DNA fragments which contain unmethylated CpG-DNA and plays a role in the innate immune system (12, 13). In addition, accumulating evidence has revealed that TLR9 has a broad range of functions in pathophysiological conditions. TLR9 recognizes cell-free DNA (cfDNA) released from degenerated tissues/cells and activates inflammatory cells including macrophages, leading to the development of sterile inflammation-associated diseases such as autoimmune diseases, insulin resistance, and others (14–16).

Therefore, in this study, we hypothesized that TLR9 activation by cfDNA released from ischemic tissues promotes inflammation and modulates blood flow recovery in the ischemic limb. To test our hypothesis, we induced hind-limb ischemia in wild-type and TLR9-deficient ($Tlr9^{-/-}$) mice and compared blood flow recovery and the degree of inflammation in the ischemic limb between these strains of mice. Also, we investigated the role of TLR9 in the link between inflammation and blood flow recovery in ischemic tissue.

Abbreviations: cfDNA, cell-free DNA; CM, conditioned medium; dsDNA, double-stranded DNA; HUVEC, human umbilical vein endothelial cells; PAD, peripheral artery disease; qPCR, Quantitative real-time RT-PCR; ssDNA, single-stranded DNA; TLR, Toll-like receptor; $Tlr9^{-/-}$, TLR9-deficient.

MATERIALS AND METHODS

Animals

C57BL/6 (wild-type) mice and $Tlr9^{-/-}$ mice (C57BL/6 background) were obtained from Japan SLC Inc. and Oriental BioService, Inc., respectively. Mice were maintained under a 12-h light/dark cycle, with a standard diet and water *ad libitum*. All experimental procedures conformed to the guidelines for animal experimentation of Tokushima University. The protocol was reviewed and approved by our institutional ethics committee under No. T29-96.

Hind-Limb Ischemia by Ligation of Femoral Artery and Blood Flow Monitoring

Unilateral hind-limb ischemia was induced in wild-type mice and $Tlr9^{-/-}$ mice as described previously (17). Briefly, the animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The proximal and distal portions of the femoral artery and the distal portion of the saphenous artery were ligated. The arteries and all side branches were dissected free and excised. Blood flow in limbs was measured using a laser Doppler blood perfusion monitor (Moor LDPI) pre- and post-operatively and on days 7 and 14. Blood flow recovery in the ischemic limb was determined by the ratio of perfusion in the ischemic limb to the untreated limb.

Immunohistochemical Staining

Capillary density in the ischemic limb was measured as described previously (17). At 14 days after femoral artery ligation, mice were sacrificed by intraperitoneal injection of an overdose of pentobarbital. The whole limbs were fixed in methanol overnight, and the ischemic muscles were embedded in paraffin. Sections (5 μm) were de-paraffinized and incubated with an anti-CD31 antibody (clone MEC13.1, BD Pharmingen). Antibody distribution was detected with the avidinbiotin-complex technique and a Vector Red Alkaline Phosphatase substrate kit (Vector Laboratories). Nuclei were stained with hematoxylin. Six different fields from each section were randomly selected, and visible CD31-positive capillaries were quantitated with a FLOVEL Filing System (FLOVEL Company, Ltd.). Capillary density was expressed as the number of capillaries per field or per muscle fiber.

Accumulation of macrophages in ischemic limb was also examined by immunohistochemistry. Sections were incubated with anti-Mac3 antibody (BD Pharmingen). Development was performed by the combination of HRP-conjugated secondary antibody and ImmPACT DAB substrate (Vector Laboratories). Sections were counterstained with hematoxylin. Four different

fields from each section were randomly selected, and Mac3-positive cells were quantitated with CellSens (OLYMPUS). Accumulation of macrophages was determined as the number of macrophages per field or per muscle fiber.

Cell Culture

Thioglycollate-stimulated peritoneal macrophages were collected with cold PBS from age-matched female wild-type mice or *Tlr9*$^{-/-}$ mice at the age of 8-12 weeks and cultured in DMEM containing 10% FBS at 37°C in a humidified incubator in 5% CO_2 and 95% air. At 24 h after plating, isolated peritoneal macrophages were used for experiments. Peritoneal macrophages were stimulated with ODN1826 or control-ODN1826 (GeneDesign, Inc.), synthetic oligonucleotides that contain unmethylated CpG, for indicated time. We collected plasma from wild-type mice at 3 days after femoral artery ligation or sham-operation. Wild-type or *Tlr9*$^{-/-}$ macrophages were stimulated with these plasma for 4 h.

Human umbilical vein endothelial cells (HUVEC) were purchased from Life Technologies and grown in EGM-2 (Lonza) at 37°C in a humidified incubator in 5% CO_2 and 95% air. HUVEC (passages 4-6) were used for experiments.

Assessment of Cell Viability

HUVEC were plated in 96-well plates and grown to 40∼50% confluence in EGM-2, and then stimulated with or without human recombinant TNF-α for 24 h in EBM-2 containing 1% FBS. Cell viability of HUVEC was determined by MTS assay using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. The percentage of absorbance was calculated against that of non-stimulated cells. Cell viability of HUVEC was also examined after treatment with conditioned medium (CM) of macrophages. Wild-type macrophages or *Tlr9*$^{-/-}$ macrophages were pretreated with ODN1826 or control-ODN1826 (100 nM) under serum-starved conditions for 24 h, and then cells were cultured for another 24 h in a starvation medium without synthetic oligonucleotides. CM was collected after centrifugation and filtration through a 40-μm mesh to remove cell debris. Cell viability was determined by MTS assay after 72-h incubation.

Measurement of cfDNA in Plasma

cfDNA in plasma was extracted using a QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The concentrations of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) in extracted cfDNA were measured using a QuantiFluor ssDNA System (Promega) and Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies), respectively, according to the instructions.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

RNA extracted from muscles and cells with an illustra RNAspin RNA Isolation Kit (GE Healthcare) was used for cDNA synthesis using a QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time RT-PCR (qPCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers on an Mx3000P (Agilent Technologies). Data are expressed in arbitrary units normalized by β-actin. The sequences of primers used in this study were as follows: TNF-α, sense 5′-accctcacactcagatcatcttc-3′ and antisense 5′-tggtggtttgctacgacgt-3′; F4/80, sense 5′-tgc atctagcaatggacagc-3′ and antisense 5′-gccttctggatcCATttgaa-3′; β-actin, sense 5′-CCTgagcgcaagtactctgtgt-3′ and antisense 5′-gctgatccacatctgctggaa-3′.

Western Blot Analysis

Protein lysates were isolated from tissues or cells using RIPA buffer (Wako Pure Chemical Industries, Ltd.) containing a protease inhibitor cocktail (Takara Bio Inc.) and phosphatase inhibitors (Roche LifeScience). Proteins were separated by SDS-PAGE and transferred to polyvinilidine difluoride membranes (Hybond-P; GE Healthcare). The membrane was blocked in 5% BSA for 1 h at room temperature, followed by incubation with a primary antibody against either total-Iκ Bα (Cell Signaling Technology), TNF-α (abcam), α-Tubulin (MBL), or β-actin (Sigma) at 4°C overnight. After blots were washed in TBS containing 1% Tween-20, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Chemicon) for 1 h. Expression of α-Tubulin or β-actin was used as an internal control to confirm equivalent total protein loading. Antibody distribution was visualized with ECL-plus reagent (GE Healthcare) using a luminescent image analyzer (LAS-1000, Fuji Film).

Statistical Analysis

Data were expressed as mean ± SEM. Comparisons between two groups were made by unpaired Student's *t* test. Comparisons of multiple groups were made by one-way analysis of variance (ANOVA) followed by Tukey test. $P < 0.05$ was considered statistically significant.

RESULTS

Femoral Artery Ligation Increased Expression of TLR9 in Ischemic Limb and Endogenous Ligand for TLR9 in Blood

To determine the participation of TLR9 in blood flow recovery of the ischemic limb, we examined the expression of TLR9 and circulating level of TLR9 ligand at 3 days after femoral artery ligation in wild-type mice. Results of qPCR demonstrated that ligation of the femoral artery markedly increased *Tlr9* expression in the ischemic limb compared to the sham operated group (**Figure 1A**). Induction of hind-limb ischemia in wild-type mice also increased the plasma levels of dsDNA and ssDNA, internal ligands for TLR9, compared with the sham-operated group (**Figures 1B,C**).

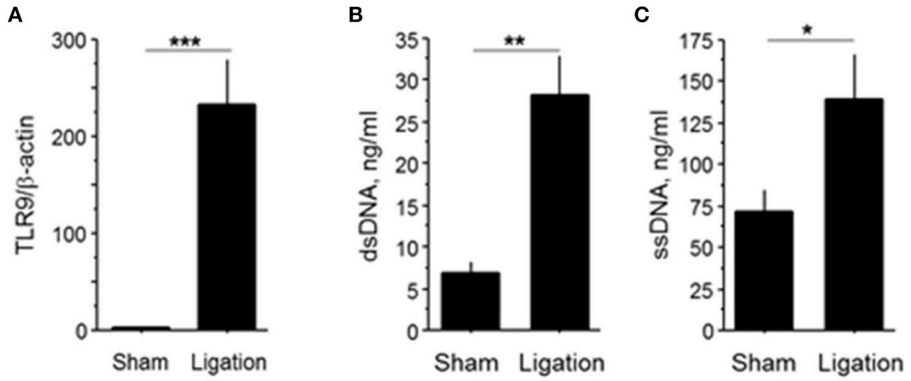

FIGURE 1 | Femoral artery ligation increased TLR9 expression in ischemic limb and circulating level of endogenous TLR9 ligand. **(A)** Femoral artery ligation increased TLR9 expression in the ischemic limb at 3 days after surgery in wild-type mice ($N = 7$, per group). **(B,C)** Induction of ischemia increased plasma circulating level of cfDNA as determined by dsDNA and ssDNA at 3 days after surgery in wild-type mice ($N = 7$, ligated group; $N = 8$, sham operated group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. All values are mean ± SEM.

TLR9 Deficiency Increased Blood Flow Recovery and Capillary Density in Ischemic Limb

Wild-type mice and *Tlr9*$^{-/-}$ mice were subjected to femoral artery ligation, and serial blood flow measurements were performed by laser Doppler imaging. *Tlr9*$^{-/-}$ mice showed better blood flow recovery than wild-type mice (**Figure 2A**). To clarify the role of TLR9 in the process of blood flow recovery, we examined capillary density in the ischemic limb by histological analysis. The results of CD31 staining showed that capillary density per square meter or per muscle fiber in the ischemic limb in *Tlr9*$^{-/-}$ mice was higher than that in wild-type mice at 14 days after ligation (**Figure 2B**). We also found that *Tlr9*$^{-/-}$ mice showed reduced infiltration of macrophages, one of the key players in the development of inflammation in the ischemic limb at 14 days after surgery, compared with wild-type mice (**Figure 2C**).

TLR9 Deficiency Reduced Inflammatory Response in Ischemic Limb

We examined the effect of TLR9 deficiency on the inflammatory process in the ischemic limb. The results of qPCR demonstrated that *Tlr9*$^{-/-}$ mice had reduced expression of TNF-α and F4/80, a macrophage marker, in the ischemic limb at 3 days after surgery compared with wild-type mice (**Figure 3A**). The results of western blotting confirmed reduced expression of TNF-α in *Tlr9*$^{-/-}$ mice at the protein level (**Figure 3B**). In addition, the results of western blotting using ischemic limbs showed that TLR9 deficiency significantly suppressed the degradation of IκBα compared with wild-type mice, indicating reduced activation of NF-κB signaling, one of the regulator TNF-α expression, in the ischemic limb in *Tlr9*$^{-/-}$ mice (**Figure 3C**).

Activation of TLR9 Promoted Expression of TNF-α in Macrophages

Inflammation associated with macrophage activation plays a pivotal role in blood flow recovery in the ischemic limb. Therefore, we examined the effect of TLR9 activation in macrophages. The results of *in vitro* experiments using peritoneal macrophages demonstrated that TLR9 activation by ODN1826, a synthetic oligonucleotide which activates TLR9, promoted the expression of TNF-α in wild-type macrophages, but not in *Tlr9*$^{-/-}$ macrophages (**Figure 4A**). We further stimulated these macrophages with plasma obtained from mice which received femoral artery ligation or sham-operation. Wild-type macrophages increased TNF-α expression in the presence of plasma from mice with ischemic muscle compared with that from sham-operated mice, however, *Tlr9*$^{-/-}$ macrophages did not show this response (**Figure 4B**). The ligation of TLR9 agonist to wild-type macrophages significantly activated NF-κB signaling as determined by the degradation of IκBα, however, this response was not observed in *Tlr9*$^{-/-}$ macrophages (**Figure 4C**).

TLR9-Mediated Macrophage Activation Attenuated Cell Viability of Endothelial Cells

To investigate the effect of TLR9-mediated macrophage activation on endothelial cell, we treated HUVEC with the CM obtained from wild-type macrophages or *Tlr9*$^{-/-}$ macrophages treated with ODN1826. CM obtained from wild-type macrophage treated with a TLR9 agonist significantly reduced the viability of HUVEC determined by MTS assay, although CM from *Tlr9*$^{-/-}$ macrophage did not have any effect (**Figure 5A**). HUVEC treated with TNF-α also showed attenuated cell viability (**Figure 5B**). These results suggest that TNF-α, produced by macrophages via TLR9 activation, accelerated cell death of endothelial cells, leading to the suppression of blood flow recovery in ischemic limb.

DISCUSSION

In this study, we demonstrated that femoral artery ligation increased TLR9 expression in the ischemic limb and circulating

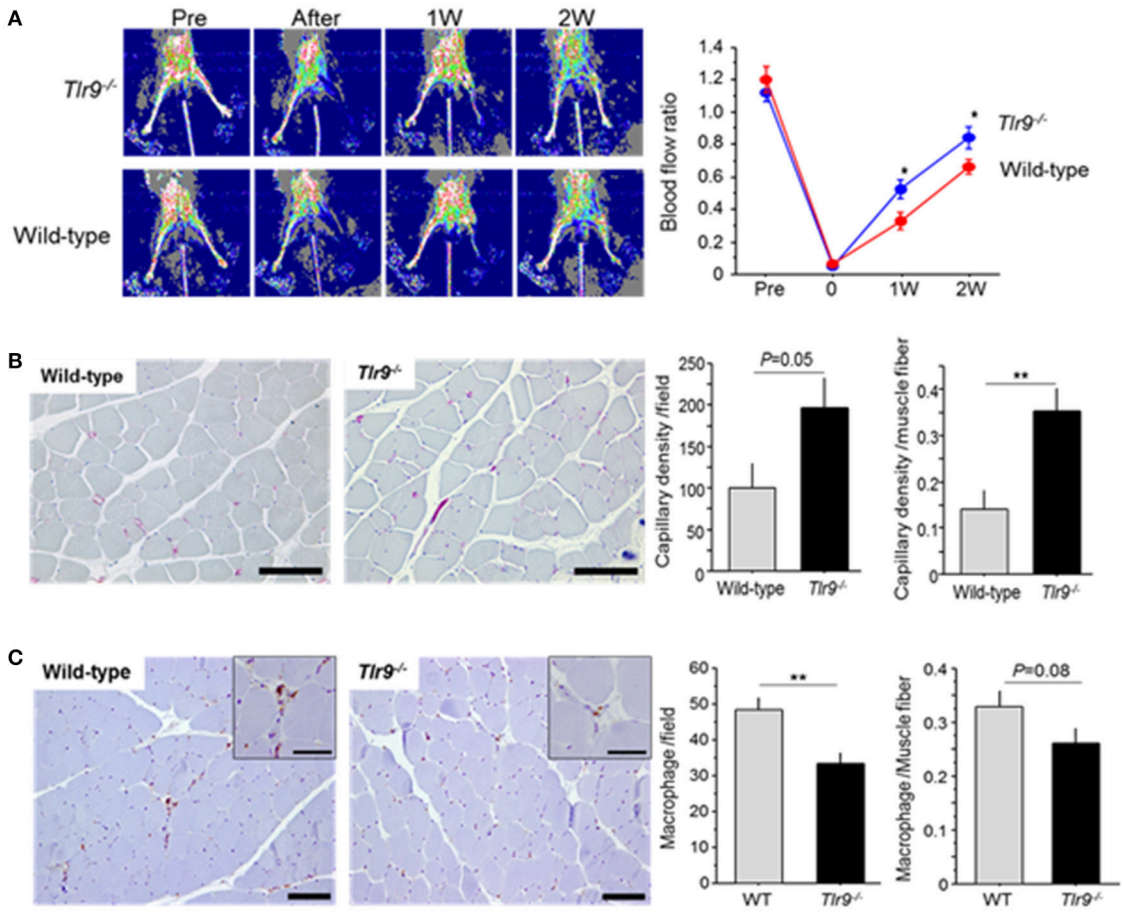

FIGURE 2 | TLR9 deficiency increased blood flow recovery and reduced macrophage infiltration in ischemic limb. **(A)** Wild-type mice and $Tlr9^{-/-}$ mice were subjected to hind-limb ligation surgery, and serial blood flow in the ischemic limb was monitored. In laser Doppler perfusion images, blue color represents lower perfusion and red color represents higher reperfusion. The ratio of flow (ischemic limb/non-ischemic limb) was quantitated. $Tlr9^{-/-}$ mice showed better blood flow recovery compared with wild-type mice ($N = 17$, per group). **(B)** Capillary density in gastrocnemius muscle was determined by immunohistochemical staining. Representative images of CD31 staining (red; CD31 positive) are shown. Capillary density was higher in $Tlr9^{-/-}$ mice compared with wild-type mice ($N = 9$, per group). Bar; 100 μm. **(C)** Infiltration of macrophages into the ischemic limb was examined by immunohistochemistry. $Tlr9^{-/-}$ mice showed reduced macrophage infiltration as determined by Mac3 staining at 14 days after surgery compared with wild-type mice. ($N = 9$, per group). Bar; 100 μm. Insets are larger magnification (Bar; 50 μm). $^{*}P < 0.05$ and $^{**}P < 0.01$. All values are mean ± SEM.

cfDNA, an endogenous ligand for TLR9, in plasma. The results of *in vivo* experiment demonstrated that genetic deletion of TLR9 increased capillary density in ischemic muscle and promoted blood flow recovery. Genetic deletion of TLR9 attenuated inflammation as determined by TNF-α expression in the ischemic limb. The results of *in vitro* experiments using macrophages and HUVEC indicated that TLR9-mediated macrophage activation attenuated cell viability of HUVEC. These results suggest that TLR9 activation caused by cfDNA released from ischemic muscle promotes inflammation, leading to deterioration of blood flow recovery.

Accumulating evidence has suggested the involvement of the innate immune system in the pathophysiology of various diseases (18–21). In this process, host-derived internal ligands initiate and promote sterile chronic inflammation. Ischemic or damaged tissue releases various molecules, such as fatty acids, nuclear proteins, and nucleic acids, which can activate pattern recognition receptors including TLRs as endogenous ligands (22). cfDNA is one of these internal ligands which activates TLR9. Previous studies have reported the association of circulating cfDNA level with the pathophysiology and/or severity of chronic inflammatory diseases, including severe cardiovascular diseases, and suggested the role of cfDNA as a biomarker of several chronic inflammatory diseases (16, 23, 24). Our results showed that hind-limb ischemia increased TLR9 expression in ischemic muscle and the circulating level of cfDNA, suggesting that cfDNA-TLR9 signaling participates in the process of blood flow recovery.

A number of previous studies reported the contribution of TLR signaling to neovascularization or blood flow recovery after ischemia. However, its roles are still under debate. TLR4 is one of the most studied TLRs from the perspective of blood flow recovery after ischemia. Several studies reported that the absence

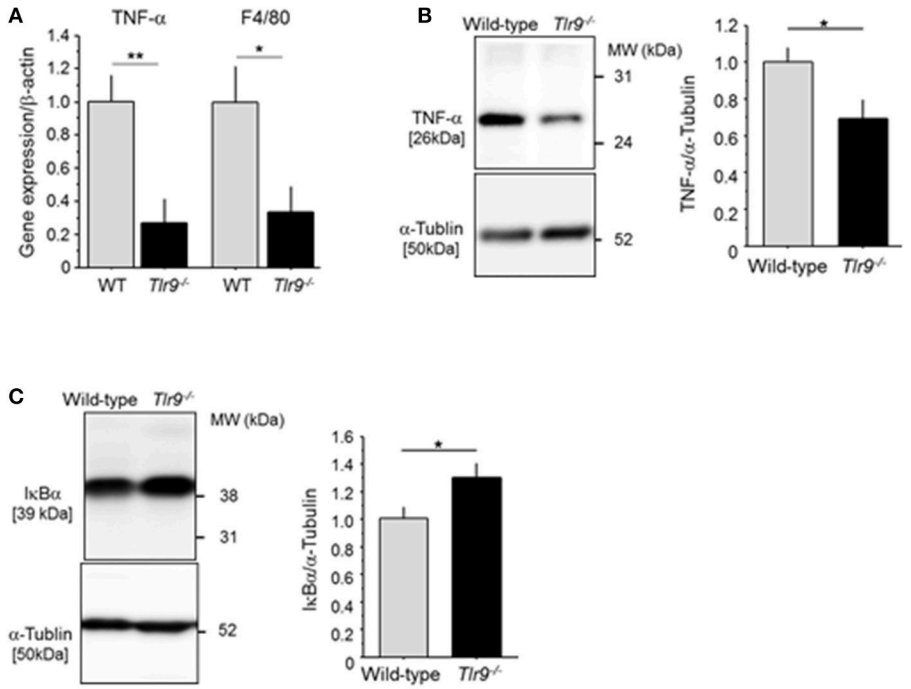

FIGURE 3 | TLR9 deficiency reduced inflammation in ischemic limb. **(A)** The result of qPCR analysis demonstrated that the expression of TNF-α and F4/80 in ischemic limb at 3 days after surgery was lower in $Tlr9^{-/-}$ mice than in wild-type mice ($N = 6$-7, per group). **(B)** Protein level of TNF-α in ischemic muscle in $Tlr9^{-/-}$ mice was lower than that in wild-type mice ($N = 4$-6, per group). **(C)** The degradation of IκBα in the ischemic limb was significantly suppressed in $Tlr9^{-/-}$ mice compared with wild-type mice, suggesting suppression of NF-κB signaling in $Tlr9^{-/-}$ mice ($N = 4$-6, per group). $^*P < 0.05$ and $^{**}P < 0.01$. All values are mean ± SEM.

of TLR4 attenuates collateral formation and tissue perfusion after arterial occlusion (10, 25). In addition, lipopolysaccharide, an exogenous TLR4 ligand, stimulates collateral artery formation after arterial occlusion (11, 26). These studies indicated that TLR4-mediated inflammation resulted in proliferation of collateral arteries and improvement of blood flow recovery. On the contrary, recent studies demonstrated that activation of TLR4 impairs blood flow recovery after ischemia. Suppression of TLR4-mediated inflammation is associated with muscle recovery and rapid blood flow recovery in TLR4-deficient mice (11, 27). Also, lack of CD180, a specific inhibitor of the TLR4-mediated inflammatory response, accelerated inflammation and retarded blood flow recovery after ischemia (6, 28). These studies indicated that unrestrained inflammation impaired blood flow recovery. Therefore, adequate inflammation is important for promoting blood flow recovery in ischemic tissues.

Few studies have reported the role of TLR9 signaling in blood flow recovery in the ischemic limb, although several previous studies examined its role in angiogenesis using different models. One study reported that TLR9 signaling stimulated wound healing by enhancing blood flow (29). On the other hand, other studies demonstrated that TLR9 activation reduced vessel sprouting from aortic rings and hemangiogenesis and lymphangiogenesis in a suture-induced corneal angiogenesis model (30). Also, another *in vitro* study reported that TLR9 activation with a lower concentration of ODN1826, a TLR9 agonist, stimulated angiogenesis, although activation with a higher concentration of ODN1826 impaired angiogenesis (31). This study suggested dual roles of ODN1826 in angiogenesis. This discrepancy might have been due to the difference in models used in these studies; however, adequate control of TLR9 activation seems to be essential for angiogenesis.

In our present study, we demonstrated that genetic deletion of TLR9 reduced TNF-α expression in the ischemic limb. We also found that TLR9 activation by ODN1826 promoted TNF-α expression in macrophages, one of the key players in blood flow recovery in the ischemic limb. Furthermore, plasma obtained from mice with ischemic muscle increased TNF-α expression in wild-type macrophages but not in $Tlr9^{-/-}$ macrophages. These results suggested that TLR9 activation by endogenous ligands released from ischemic muscle promotes TNF-α expression in macrophages, regulating blood flow recovery in ischemic limb. In this study, genetic deletion of TLR9 reduced the degradation of IκBα in ischemic muscle, suggesting suppression of NF-κB signaling. Previous studies have reported that TLR signaling pathways activate NF-κB, which regulates the expression of inflammatory cytokines including TNF-α (32, 33). As well as other TLRs, TLR9 signaling also activates this pathway (16). In fact, we demonstrated that the ligation of TLR9 agonist activates NF-κB signaling in wild-type macrophages as determined by the degradation of IκBα, but not in by $Tlr9^{-/-}$ macrophages in this study. Our results suggested the link between TLR9 and NF-κB,

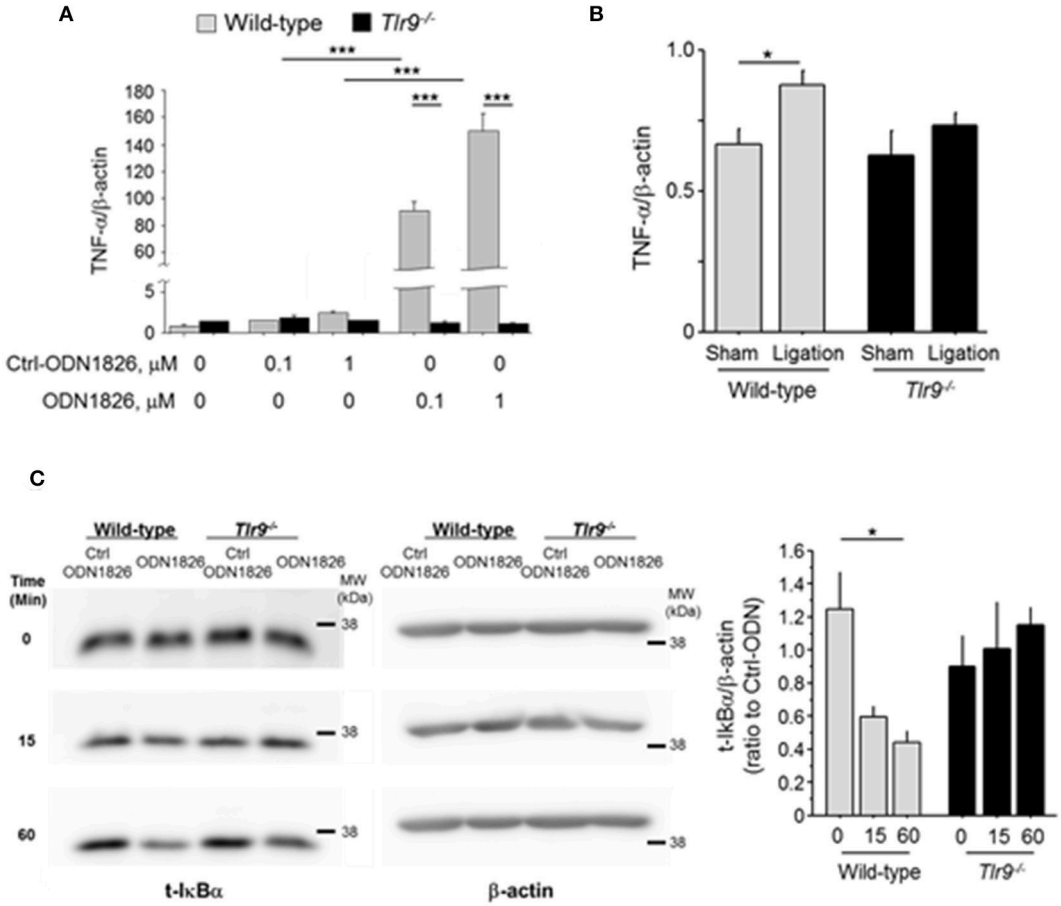

FIGURE 4 | TLR9 activation promoted TNF-α expression in macrophages. **(A)** Activation of TLR9 by ODN1826 (0.1-1.0 μM) for 4 h promoted the expression of TNF-α in wild-type macrophages but not in *Tlr9*$^{-/-}$ macrophages ($N = 4$, per group). **(B)** Wild-type or *Tlr9*$^{-/-}$ macrophages were treated with plasma obtained from wild-type mice which received femoral ligation or sham operation for 4 h. Plasma from mice with ischemic muscle increased TNF-α expression in wild-type macrophages compared with that from sham-operated mice, however, plasma from mice with ischemic muscle did not show this response in *Tlr9*$^{-/-}$ macrophages ($N = 4$-6, per group). **(C)** ODN1826 promoted degradation of IκBα in wild-type macrophages in time dependent manner, suggesting the activation of NF-κB signaling. In *Tlr9*$^{-/-}$ macrophages, this response was not observed. $^{*}P < 0.05$ and $^{***}P < 0.001$. All values are mean ± SEM.

at least partially, although further studies are needed to elucidate precise signaling in ischemic muscle. Previous studies reported that TNF-α has two contradictory functions on endothelial cells (34–36). This contradiction has been partially explained by tissue TNF-α concentration (34). That is, a low TNF-α concentration promotes, but a high TNF-α concentration inhibits angiogenesis. Previous *in vitro* studies, a higher concentration of TNF-α reduced cell viability of endothelial cells (34, 36). The result of our experiment is consistent with these studies. Furthermore, in this study, CM obtained from wild-type macrophages activated by a TLR9 agonist attenuated cell viability of HUVEC, although CM from *Tlr9*$^{-/-}$ macrophage did not have any effect. These results suggest that suppression of TNF-α expression in TLR9-deficient macrophages is associated with higher capillary density and better blood flow recovery through inhibition of endothelial cell death. Combined with the results of previous studies, the results of our study suggest that well-controlled activation of TLR9 is important for blood flow recovery in the ischemic limb by regulating TNF-α expression. Several clinical studies have demonstrated elevated plasma level of TNF-α in patients with PAD (37, 38). Further studies are required to determine the role of cfDNA-TLR9 signaling in inflammation and blood flow recovery in ischemic tissues, which might provide a novel therapeutic strategy for PAD.

There are several limitations in this study. The time point we examined blood flow, capillary density, and inflammatory status in the ischemic limb was limited. *In vitro* experiments were also performed at limited time point. Other cell types or signaling pathways might also have roles in different time points. Second, we focused on pro-inflammatory activation of macrophages via TLR9, however, we did not perform macrophage-specific investigation in our *in vivo* studies. Therefore, other cell-types might also contribute to the results. Last, the results of our *in vivo* study suggested that TLR9-deletion promotes blood flow recovery in ischemic muscle. However, functional analysis was not performed in this study.

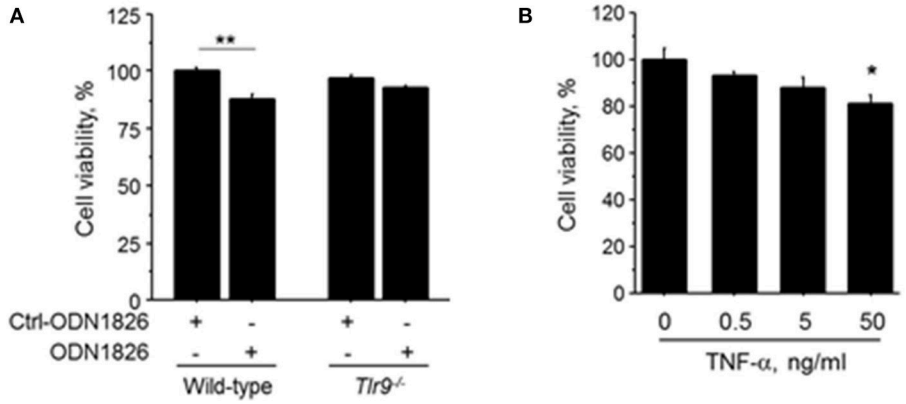

FIGURE 5 | TLR9-induced macrophage activation accelerated cell death of HUVEC. **(A)** HUVEC were treated with the CM obtained from wild-type or $Tlr9^{-/-}$ macrophages treated with ODN1826 or control-ODN1826. At 72 h after treatment, the viability of HUVEC determined by MTS assay was significantly reduced by CM obtained from wild-type macrophage activated by ODN1826, although the viability of HUVEC was not affected by CM obtained from $Tlr9^{-/-}$ macrophage ($N = 5$, per group). **(B)** Stimulation with TNF-α for 24 h decreased cell viability in HUVEC as determined by MTS assay ($N = 4$-6, per group). $^{*}P < 0.05$ and $^{**}P < 0.01$. All values are mean ± SEM.

Inflammation regulates blood flow recovery in ischemic tissues. The results of our present study suggested that TLR9-deletion attenuates inflammatory responses and promotes blood flow recovery in ischemic muscle. These results indicate that strict control of inflammation and appropriate expression of TNF-α through TLR9 signaling in ischemic tissues are critical for blood flow recovery. Regulation of cfDNA-TLR9 signaling could be a potential therapeutic strategy for regulation and acceleration of blood flow recovery in ischemic limb injury.

AUTHOR CONTRIBUTIONS

SN, KA, and DF designed and performed the experiments, interpreted the results, and prepared the manuscript. YaH, KT, YoH, SY, and TS assisted with *in vivo* experiments. KK, HY, and MiS contributed to data interpretation and critical reading of the manuscript. MaS interpreted the data and prepared the manuscript. All authors discussed the results and commented on the manuscript.

FUNDING

This work was partially supported by Japan Society for the Promotion of Science KAKENHI grants (grant 16K09517 to DF; grants 16H05299 and 26248050 to MaS), the Uehara Memorial Foundation (to MaS), the Fugaku Trust for Medicinal Research (to MaS), Takeda Science Foundation (to DF and to MaS), Banyu Life Science Foundation International (to DF), and a Katsunuma Scholarship (to SN).

ACKNOWLEDGMENTS

All authors are grateful to H. Kato and Y. Sugawara (Tokyo University), and E. Uematsu and S. Okamoto (Tokushima University) for their expert technical assistance.

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Conflict of Interest Statement: The Department of Cardio-Diabetes Medicine, Tokushima University Graduate School, is supported in part by unrestricted research grants from Boehringer Ingelheim, Tanabe-Mitsubishi, Kowa, and Actelion.

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

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 22 December 2017
doi: 10.3389/fcvm.2017.00084

An Interleukin-6 Receptor Antibody Suppresses Atherosclerosis in Atherogenic Mice

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IκBNS is a nuclear IκB protein which negatively regulates nuclear factor-κB activity. We demonstrated that IκBNS deficiency accelerates atherosclerosis in LDL receptor-deficient (LDLr$^{-/-}$) mice *via* increased interleukin (IL)-6 production by macrophages. Previous studies showed that the increase in IL-6 might contribute to the development of atherosclerotic lesions. However, whether an anti-mouse IL-6 receptor antibody (MR16-1) can protect atherosclerotic lesions in atherogenic mice remains to be elucidated. We investigated atherosclerotic lesions in LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice after 16 weeks consumption of a high-fat diet. All mice received intraperitoneal injections of MR16-1 or phosphate-buffered saline (PBS) (control) once a week during a high-fat diet consumption. Treatment of MR16-1 yielded no adverse systemic effects, and we detected no significant differences in serum cholesterol levels in either group. The atherosclerotic lesions were significantly increased in IκBNS$^{-/-}$/LDLr$^{-/-}$ compared with LDLr$^{-/-}$ mice ($p < 0.01$) under treatment of PBS. However, MR16-1 treatment abolished the significant difference of atherosclerotic lesions between IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice. Interestingly, MR16-1 also significantly decreased atherosclerotic lesions in LDLr$^{-/-}$ mice compared with PBS treatment ($p < 0.05$). Immunostaining revealed percent phospho-STAT3-positive cell were significantly decreased in the atherosclerotic lesions of MR16-1 treated both IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice compared with PBS-treated mice, indicating MR16-1 could suppress atherosclerotic lesions *via* the inhibition of IL-6–STAT3 signaling pathway. This study highlights the potential therapeutic benefit of anti-IL-6 therapy in preventing atherogenesis induced by dyslipidemia and/or inflammation.

Keywords: interleukin-6, atherosclerosis, inflammation, IκBNS, dyslipidemia

OPEN ACCESS

Edited by:
Ichiro Manabe,
Chiba University, Japan

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

Received: *30 September 2017*
Accepted: *08 December 2017*
Published: *22 December 2017*

Citation:
Akita K, Isoda K, Sato-Okabayashi Y, Kadoguchi T, Kitamura K, Ohtomo F, Shimada K and Daida H (2017) An Interleukin-6 Receptor Antibody Suppresses Atherosclerosis in Atherogenic Mice. Front. Cardiovasc. Med. 4:84. doi: 10.3389/fcvm.2017.00084

INTRODUCTION

Nuclear factor-κB (NF-κB) plays essential roles in mediating immune systems. Cytoplasmic IκB family proteins regulate the transcriptional activity of NF-κB, because excessive activation is detrimental to the host (1). After translocation of NF-κB from the cytoplasm to the nucleus, nuclear proteins that are structurally similar to cytoplasmic IκBs take part in the mediating of NF-κB transcriptional activity, as activators or inhibitors, by associating with NF-κB subunits (2). Thus, the regulatory IκB-like nuclear molecules are described as nuclear IκB proteins. Although IκBNS is one of nuclear IκB

proteins, the role of IκBNS in the development of atherosclerosis is poorly understood. Our previous study demonstrated that deficiency of IκBNS induces atherogenesis in LDL receptor-deficient ($LDLr^{-/-}$) mice fed a high-fat diet and increases in interleukin (IL)-6 production by macrophages, indicating that IκBNS plays an important role in the suppression of atherosclerotic lesions *in vivo* (3). However, whether or not increased IL-6 production in IκBNS-deficient $LDLr^{-/-}$ mice accelerates atherogenesis remains undetermined.

Several previous reports indicate that IL-6 could accelerate atherosclerosis. Increased plasma levels of IL-6 were associated with cardiovascular mortality over 5-year independent of other risk factor of atherosclerosis (4, 5). Injection of IL-6 itself accelerated atherosclerosis in apolipoprotein E-null mice and C57Bl/6 mice either (4). The genetic polymorphism in the IL-6 signaling pathway concordantly associates with lifetime lower risks of coronary heart disease (6). However, whether inhibition of IL-6 might be effective for the suppression of atherogenesis remains to be elucidated.

We demonstrated here that treatment of an anti-mouse IL-6 receptor antibody (MR16-1) suppressed atherosclerosis lesion in atherogenic mice *via* the inhibition of IL-6–STAT3 signaling pathway.

MATERIALS AND METHODS

Mice

The generation of $LDLr^{-/-}$ mice that lacked IκBNS ($IκBNS^{-/-}$/$LDLr^{-/-}$) used in this study has been described previously (3). Details of IκBNS-deficient mice were described in the previous report (7). We investigated atherosclerotic lesions in $LDLr^{-/-}$ and $IκBNS^{-/-}$/$LDLr^{-/-}$ mice after 16 weeks consumption of a high-fat diet (MF diet containing 0.5% cholesterol, Oriental Yeast Co.). This study was performed according to the protocols approved by the Juntendo University Board for Studies in Experimental Animals.

Plasma Lipid Measurement

Plasma total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol levels were measured by high-performance liquid chromatography at Skylight Biotech Inc. (Akita, Japan).

Quantification of Atherosclerotic Lesions

After blood collection, the animals were euthanized by pentobarbital injection, and the heart and aorta were flushed with 0.9% NaCl followed by 4% paraformaldehyde. After perfusion procedure, the aorta was harvested and fixed with 10% neutral-buffered formalin for 48 h, embedded in paraffin, and sectioned from just above the aortic valve throughout the aortic sinus (each 6-μm thickness). We used equally spaced 10 cross sections (100-μm interval) to qualify arteriosclerotic lesions in the aortic sinus for each mouse. The samples were stained with Elastica van Gieson, and then photographed using a BX53 microscope (OLYMPUS, Tokyo, Japan). The luminal, arteriosclerotic lesions and medial areas were calculated using NIH Image J 1.42 (National Institutes of Health, public domain software). Quantification of the atherosclerotic lesions was performed by two blinded observers.

The whole aortas were also stained with Sudan IV. The surface atherosclerotic lesions were expressed as the percent of the lesion area extending from the ascending aorta to the iliac bifurcation.

Immunohistochemistry

Activation of STAT-3 was detected by phospho-Stat3 (Tyr705) (pSTAT3) staining (1:50; Cell Signaling Technology, #9145). Activation both of pSTAT3 was evaluated for percentage of positive nuclei to total nuclei in the arteries.

Inhibition of IL-6 by Anti-Mouse IL-6 Receptor Antibody (MR16-1) in Mice

All mice received intraperitoneal injections of phosphate-buffered saline (PBS) or MR16-1 (2 mg) once a week during a high-fat diet consumption. MR16-1 was kindly provided from Chugai Pharmaceutical (Japan).

Statistical Analysis

Results are shown as mean ± SEM. The two groups were compared using Student's *t*-test. Differences between groups were analyzed using one-way ANOVA test followed by Bonferroni's *t*-test to determine statistical differences after multiple comparisons. $p < 0.05$ was considered statistically significant.

RESULTS

Treatment of MR16-1 Yielded No Adverse in Mice

We investigated atherosclerotic lesions in $LDLr^{-/-}$ and $IκBNS^{-/-}$/$LDLr^{-/-}$ mice after 16 weeks consumption of a high-fat diet. Both $IκBNS^{-/-}$/$LDLr^{-/-}$ and $LDLr^{-/-}$ mice received injection of PBS (control) or MR16-1 (2 mg) once a week during a high-fat diet consumption of 16 weeks. MR16-1 treatment yielded no adverse systemic effects. Systolic blood pressure and body weight were similar among four groups (**Figures 1A,B**). Weight gains of mice after consumption of high-fat diet were similar among the four groups. Moreover, analysis of plasma lipid profiles revealed no statistically significant differences in total cholesterol (**Figure 2A**), LDL cholesterol (**Figure 2B**), triglyceride (**Figure 2C**), and HDL cholesterol (**Figure 2D**) among these groups.

MR16-1 Inhibited Atherosclerosis in both $LDLr^{-/-}$ and $IκBNS^{-/-}$/$LDLr^{-/-}$ Mice

We investigated the effect of MR16-1 treatment on atherogenesis in both $LDLr^{-/-}$ and $IκBNS^{-/-}$/$LDLr^{-/-}$ mice after 16 weeks consumption of a high-fat diet. As reported previously (3), the extent of atherosclerosis in the mice aortas (en face) was significantly increased in $IκBNS^{-/-}$/$LDLr^{-/-}$ compared with single $LDLr^{-/-}$ mice ($p < 0.001$) under treatment of PBS (**Figure 3**). However, MR16-1 treatment abolished the significant difference of atherosclerotic

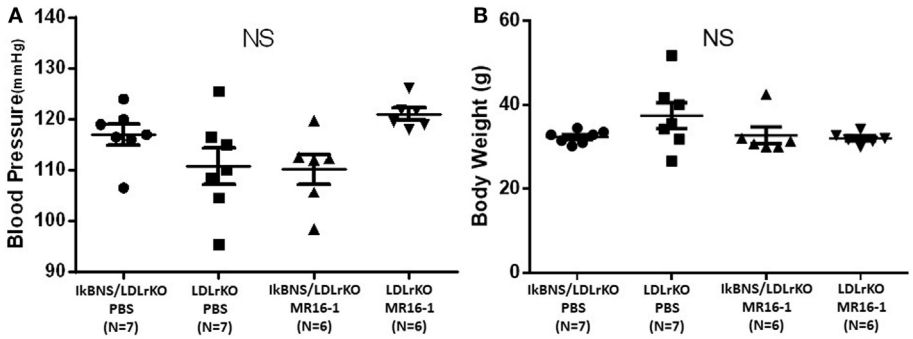

FIGURE 1 | Treatment with MR16-1 did not change blood pressure or body weight in both IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice. Both mice were received injection of phosphate-buffered saline (PBS) or MR16-1 (2 mg) once a week during a high-fat diet consumption of 16 weeks. Systolic blood pressure **(A)** and body weight **(B)** were similar among four groups. IκBNS/LDLr KO PBS: PBS-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO PBS: PBS-treated LDLr$^{-/-}$ mice. IκBNS/LDLr KO MR16-1: MR16-1-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO MR16-1: MR16-1-treated LDLr$^{-/-}$ mice. Data are expressed as mean ± SEM. NS, not significant.

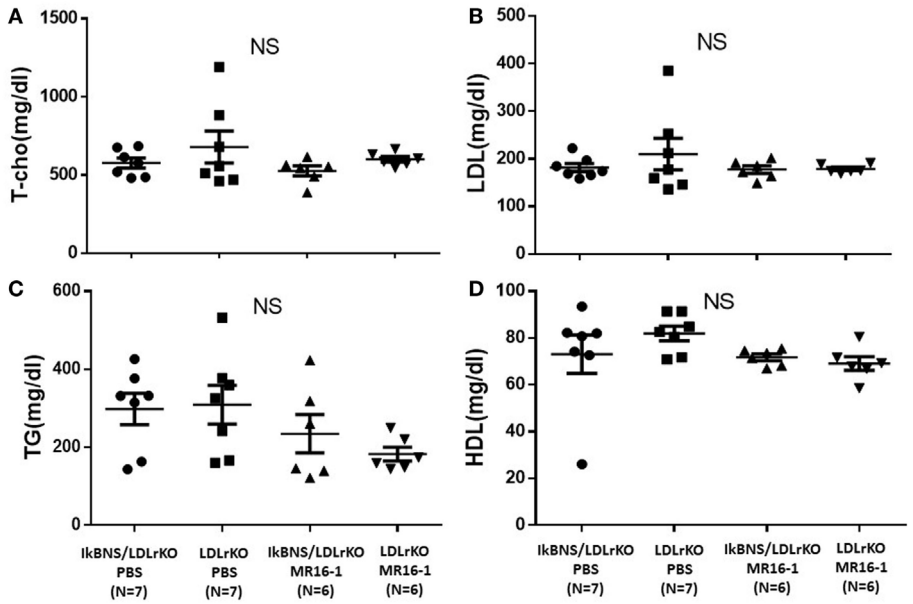

FIGURE 2 | Treatment with MR16-1 did not change cholesterol profiles in both IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice. The levels of total cholesterol (T-cho) **(A)**, low-density lipoprotein (LDL)-cholesterol **(B)**, triglyceride (TG) **(C)**, and high-density lipoprotein (HDL)-cholesterol **(D)** after treatment of phosphate-buffered saline (PBS) or MR16-1 (2 mg) once a week during a high-fat diet consumption of 16 weeks. IκBNS/LDLr KO PBS: PBS-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO PBS: PBS-treated LDLr$^{-/-}$ mice. IκBNS/LDLr KO MR16-1: MR16-1-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO MR16-1: MR16-1-treated LDLr$^{-/-}$ mice. Data are expressed as mean ± SEM. NS, not significant.

lesions between IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice (**Figure 3**). Interestingly, MR16-1 treatment also significantly decreased atherosclerotic lesions in LDLr$^{-/-}$ mice compared with PBS treatment (**Figure 3**).

Aortic root atherosclerotic lesions of IκBNS$^{-/-}$/LDLr$^{-/-}$ mice were also significantly larger than those in LDLr$^{-/-}$ mice ($p < 0.05$) under treatment of PBS (**Figure 4**). MR16-1 treatment significantly reduced atherosclerotic lesions in both LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice compared with PBS treatment (**Figure 4**).

These results show that an anti-mouse IL-6 receptor antibody significantly suppresses IκBNS in both LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice without major confounding effects on LDL-cholesterol or HDL-cholesterol, suggesting that inhibition of IL-6 might be significantly effective to prevent atherogenesis.

MR16-1 Suppressed STAT3 Activation in the Atherosclerotic Lesions of both LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ Mice

To examine STAT activation in the atherosclerotic lesions of both MR16-1 and PBS groups, we performed pSTAT3 staining in the atherosclerotic lesions of four groups after 16 weeks consumption of a high-fat diet. We detected much more pSTAT3-positive cells in the atherosclerotic lesions of PBS-treated IκBNS$^{-/-}$/LDLr$^{-/-}$

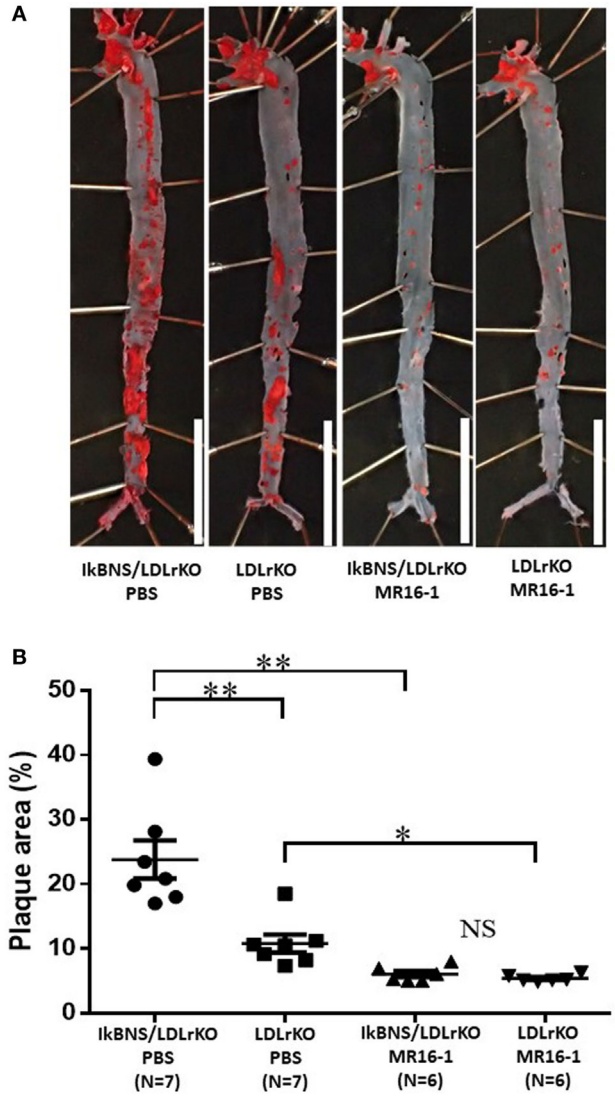

FIGURE 3 | Treatment with MR16-1 decreased in the extent of atherosclerotic lesions in the mice aortas (en face) of both IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice. **(A)** Sudan IV staining of aortas from four groups after treatment of phosphate-buffered saline (PBS) or MR16-1 (2 mg) once a week during a high-fat diet consumption of 16 weeks (bars = 10 mm). **(B)** Quantitative analysis of the relative surface area of the atherosclerotic lesion in aortas. IκBNS/LDLr KO PBS: PBS-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO PBS: PBS-treated LDLr$^{-/-}$ mice. IκBNS/LDLr KO MR16-1: MR16-1-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO MR16-1: MR16-1-treated LDLr$^{-/-}$ mice. Data are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$. NS, not significant.

mice compared with PBS-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice (**Figure 5A**). MR16-1 treatment significantly decreased pSTAT3-positive cells in the atherosclerotic lesions of both LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice compared with PBS treatment (**Figure 5A**).

To quantify STAT3 activity, we calculated the percentage of pSTAT3-positive nuclei to total nuclei in the arteries. The percentage of pSTAT3-positive nuclei in the aorta of MR16-1-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice was significantly lower than that in PBS treated group (47.3 ± 3.1 versus 8.4 ± 1.5%; $p < 0.01$; **Figure 5B**). Interestingly, MR16-1 treatment also decreased percentage of pSTAT3-positive nuclei in LDLr$^{-/-}$ mice compared with PBS treatment (27.7 ± 1.5 versus 7.2 ± 1.8%; $p < 0.01$; **Figure 5B**).

DISCUSSION

Persistent IL-6 production has been shown to play a critical role in chronic inflammatory disease (8). IL-6 signaling has also been linked to plaque initiation and destabilization (9) and to adverse outcomes in the setting of acute ischemia (10). IL-6 stimulates growth of promoters of macrophage and vascular smooth muscle cells, which are major components of plaque (11–13), and IL-6 expression is also detected in human atherosclerotic lesions (14). These findings indicate that IL-6 participates in the development of atherosclerosis. Thus, targeting IL-6 is a rational approach for atheroprotection. To block the effect of IL-6 on the development of atherosclerosis, we used anti-mouse IL-6 receptor antibody (MR16-1). MR16-1 has proved to be effective in experimental model of arthritis (15) and humanized anti-IL-6 receptor antibody (tocilizumab) has been approved by the Food and Drug Administration for the treatment in the patients with rheumatoid arthritis.

Our previous study demonstrates that IL-6 expression and STAT3 activation were increased in the foam cell rich-atherosclerotic lesions of IκBNS$^{-/-}$/LDLr$^{-/-}$ mice *in vivo* and IκBNS$^{-/-}$/LDLr$^{-/-}$ macrophages produced much higher level of IL-6 than LDLr$^{-/-}$ macrophages *in vitro*. These results indicate deficiency of IκBNS increases in the production of IL-6 in macrophage and an increase of IL-6 contributes to the susceptibility to atherogenesis in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice (3). Consequently, because we believe that the mice were suitable for evaluating the effect of IL-6-blocking therapy in atherogenesis, we used IκBNS$^{-/-}$/LDLr$^{-/-}$ mice in this study. As expected, MR16-1 treatment abolished the significant difference of atherosclerotic lesions between IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice. Interestingly, MR16-1 treatment also significantly decreased atherosclerotic lesions in LDLr$^{-/-}$ mice compared with PBS treatment.

Interleukin-6 is an upstream inflammatory cytokine key player, propagating the downstream inflammatory response in atherosclerosis (16). Previous study demonstrated that inflammatory gene, such as IL-6 (17), which is known as an inducer of STAT3 was highly expressed in atherosclerotic plaques. Moreover, activation of STAT3 has been detected in the plaque (18), and its activation is involved in the progression of atherosclerotic lesions (19). In this study, we analyzed pSTAT3-positive nuclei to evaluate the inhibitory effect of MR16-1 in IL-6 signaling pathway. MR16-1 inhibited STAT3 activation and development of atherosclerotic lesions in both IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice, suggesting IL-6 is a main contributor to atherogenesis, and inhibition of IL-6 might be a new method to prevent the development of atherosclerosis.

Our data provide solid evidence for a causal role of the IL-6 pathway in the development of atherosclerosis, raising expectations for ongoing trials testing anti-inflammatory strategies for cardiovascular diseases (20).

Although we could not find any adverse systemic effects including signs of infection in MR16-1-treated mice, further

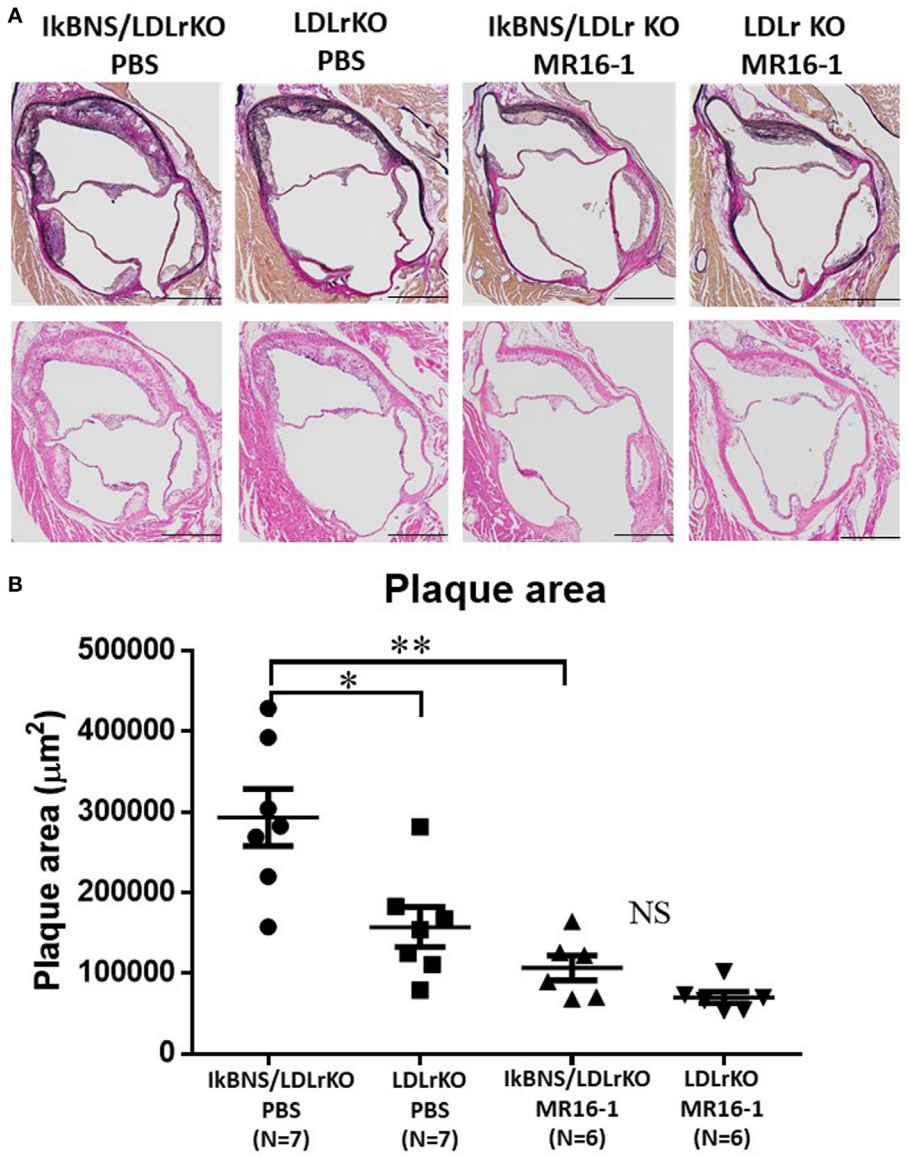

FIGURE 4 | Treatment with MR16-1 decreased in aortic root atherosclerotic lesions of both IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice. **(A)** Representative photomicrographs of sections of aortic sinus plaque from four groups after treatment of phosphate-buffered saline (PBS) or MR16-1 (2 mg) once a week during a high-fat diet consumption of 16 weeks. Adjacent sections were processed for Elastica van Gieson staining (upper panels) and H&E staining (lower panels) (bars = 500 μm). **(B)** Quantitative comparison of the atherosclerotic lesion in the aortic sinus. IκBNS/LDLr KO PBS: PBS-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO PBS: PBS-treated LDLr$^{-/-}$ mice. IκBNS/LDLr KO MR16-1: MR16-1-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO MR16-1: MR16-1-treated LDLr$^{-/-}$ mice. Data are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$. NS, not significant.

study must be needed for checking any adverse effects of MR16-1 in the immune systems.

In our experiments, levels of IL-6 in plasma and liver were not different between IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice after consumption of high-fat diet. On the other hand, Kuwata et al. (7) demonstrated intraperitoneally injection of LPS induced significantly high-serum levels of IL-6 in IκBNS$^{-/-}$ mice compared to wild-type (IκBNS$^{+/+}$) mice. These findings suggest that feeding a high-fat diet could induce local increase of IL-6 produced by IκBNS$^{-/-}$ macrophages around the vessels, but not serum levels of IL-6. In our previous study, IκBNS expression was observed in all cell types resident to the plaque microenvironment (i.e., smooth muscle cells, macrophages, and endothelial cells) in LDLr$^{-/-}$ mice, but not in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice. Because main IκBNS expression cells were macrophages, we previously examined the effects of the IκBNS deficiency in macrophages. We also investigated the effect of IκBNS deficiency in smooth muscle cells using small interference RNA, because they play important roles in atherogenesis. However, IκBNS deficiency in aortic smooth muscle cell did not

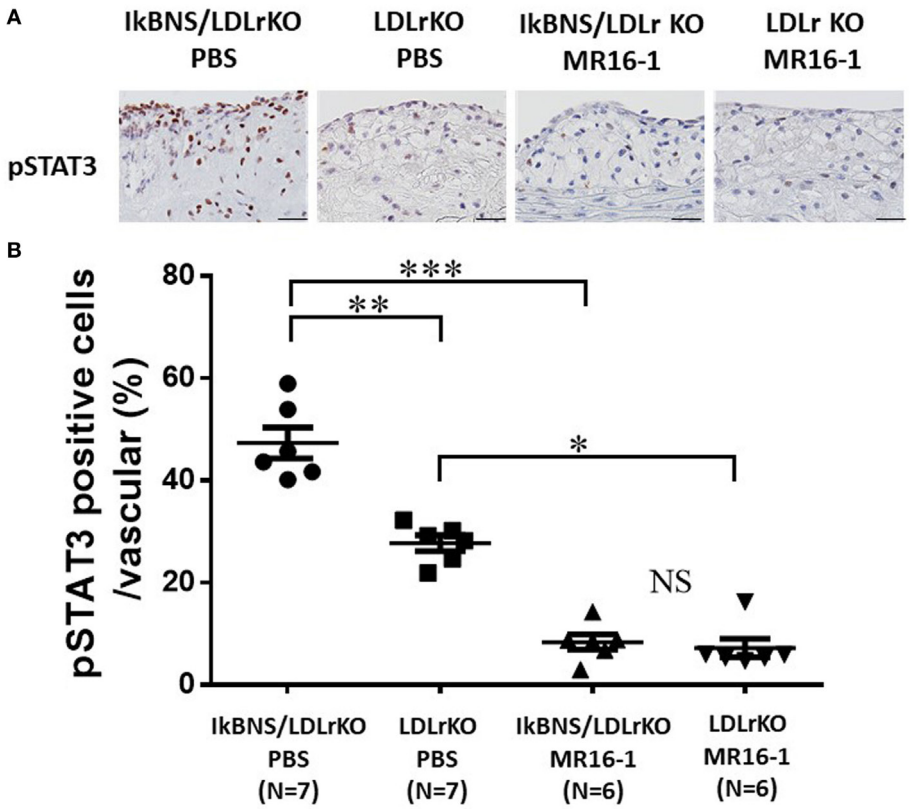

FIGURE 5 | MR16-1 treatment significantly inhibited STAT3 activation in the atherosclerotic lesions of both IκBNS$^{-/-}$/LDLr$^{-/-}$ and LDLr$^{-/-}$ mice. **(A)** Representative photomicrographs of sections of aortic sinus plaque from four groups after treatment of phosphate-buffered saline (PBS) or MR16-1 (2 mg) once a week during a high-fat diet consumption of 16 weeks. Sections were processed for pSTAT3 staining (bars = 25 μm). **(B)** Quantitative analysis of pSTAT3-positive cell in vascular wall in sections from four groups. IκBNS/LDLr KO PBS: PBS-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO PBS: PBS-treated LDLr$^{-/-}$ mice. IκBNS/LDLr KO MR16-1: MR16-1-treated IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, LDLr KO MR16-1: MR16-1-treated LDLr$^{-/-}$ mice. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. NS, not significant.

affect in IL-6 production *in vitro*. Further experiments might be needed to clarify which cells are main players in atherogenesis induced by IκBNS deficiency.

In conclusion, this study highlights the potential therapeutic benefit of anti-IL-6 therapy in preventing atherogenesis in the mice with dyslipidemia and/or inflammation.

ETHICS STATEMENT

The studies were performed according to the protocols approved by the Juntendo University Board for Studies in Experimental Animals.

AUTHOR CONTRIBUTIONS

KA, KI, YO-S, TK, and KK performed the research. KI, KS, and HD designed the research study. KA, YO-S, TK, KK, and FO contributed essential reagents or tools. KA, KI, YO-S, TK, KK, FO, KS, and HD analyzed the data. KA, KI, YO-S, TK, KK, and FO, KS, and HD wrote the paper.

FUNDING

This work was supported by Grant-in-Aid for Scientific Research (C) (JSPS KAKENHI Grant Number 16K09522).

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

frontiers in Cardiovascular Medicine

REVIEW
published: 22 December 2017
doi: 10.3389/fcvm.2017.00087

Anti-inflammatory Nanomedicine for Cardiovascular Disease

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Coronary artery disease, in the development of which inflammation mediated by innate immune cells plays a critical role, is one of the leading causes of death worldwide. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are a widely used lipid-lowering drug that has lipid-independent vasculoprotective effects, such as improvement of endothelial dysfunction, antioxidant properties, and inhibitory effects on inflammation. Despite recent advances in lipid-lowering therapy, clinical trials of statins suggest that anti-inflammatory therapy beyond lipid-lowering therapy is indispensible to further reduce cardiovascular events. One possible therapeutic option to the residual risk is to directly intervene in the inflammatory process by utilizing a nanotechnology-based drug delivery system (nano-DDS). Various nano-sized materials are currently developed as DDS, including micelles, liposomes, polymeric nanoparticles, dendrimers, carbon nanotubes, and metallic nanoparticles. The application of nano-DDS to coronary artery disease is a feasible strategy since the inflammatory milieu enhances incorporation of nano-sized materials into mononuclear phagocytic system and permeability of target lesions, which confers nano-DDS on "passive-targeting" property. Recently, we have developed a polymeric nanoparticle-incorporating statin to maximize its anti-inflammatory property. This statin nanoparticle has been tested in various disease models, including plaque destabilization and rupture, myocardial ischemia-reperfusion injury, and ventricular remodeling after acute myocardial infarction, and its clinical application is in progress. In this review, we present current development of DDS and future perspective on the application of anti-inflammatory nanomedicine to treat life-threatening cardiovascular diseases.

Keywords: coronary artery disease, inflammation, nanomedicine, monocytes, macrophages

OPEN ACCESS

Edited by:
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Reviewed by:
Vasilios Gabriel Athyros, Aristotle University of Thessaloniki, Greece
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *21 September 2017*
Accepted: *12 December 2017*
Published: *22 December 2017*

Citation:
Katsuki S, Matoba T, Koga J, Nakano K and Egashira K (2017) Anti-inflammatory Nanomedicine for Cardiovascular Disease. Front. Cardiovasc. Med. 4:87. doi: 10.3389/fcvm.2017.00087

ANTI-INFLAMMATORY THERAPEUTICS FOR CORONARY ARTERY DISEASE

Coronary artery disease is the leading cause of death worldwide and can be life threatening especially when it develops into acute myocardial infarction (AMI), the most severe type of atherosclerotic cardiovascular disease. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are potent inhibitors of cholesterol synthesis and established therapies for the

prevention of coronary artery disease. The first randomized trial to demonstrate that cholesterol-lowering therapy with statins improves prognosis in patients with high cholesterol and coronary artery disease, the Scandinavian Simvastatin Survival Study (4S), was reported in 1994 (1). Since then, lipid-lowering therapy with statins has become the mainstay for the prevention of coronary artery disease. The subsequent studies for the secondary prevention such as the CARE (Cholesterol and Recurrent Events) (2) and LIPID (Long Term Intervention with Pravastatin in Ischemic Disease) trials (3) further extended that the benefits of statins to the majority of patients whose cholesterol levels were in the normal range. The WOSCOPS (West of Scotland Coronary Prevention Study) (4) and the AFCAPS/TexCAPS (Air Force/Texas Coronary Atherosclerosis Prevention Study) (5) also extended the benefits for the primary prevention of atherosclerotic cardiovascular diseases. A decade after 4S, Ridker et al. demonstrated in the JUPITER (Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin) trial that high-intensity cholesterol-lowering therapy with statins reduced high-sensitivity C-reactive protein levels to lower than 2 mg/L and then achieved further risk reduction of cardiovascular events even among patients with normal cholesterol levels (6). Statins have a wide range of lipid-independent cardiovascular protective effects (so called "pleiotropic effects"), such as improvement of endothelial dysfunction, antioxidant properties, and inhibitory effects on inflammation (7) This landmark study suggested that anti-inflammatory therapy beyond lipid-lowering therapy is needed to further reduce cardiovascular events. At present, plaque erosion appears on the rise as a cause of acute coronary syndrome (ACS) in this statin era (8), but plaque rupture of unstable plaques with macrophage accumulation, fibrous cap thinning, and lipid deposition, followed by arterial occlusive thrombosis remains the primary mechanisms of ACS. Despite recent advances in lipid-lowering therapy by the emergence of Niemann-Pick C1-Like 1 inhibitor (ezetimibe) (9, 10) and proprotein convertase subtilisin/kexin type 9 inhibitors (11), there are still residual risks of cardiovascular events. One possible approach to the residual risks is to directly intervene in the inflammatory process during atherogenesis. Current medicinal therapy, including statins, has limitation of bioavailability in the diseased organs. Most small molecule drugs are absorbed from the intestines, metabolized in the liver, delivered to the bloodstream, and excreted from the kidneys, that is, drugs need to overcome the physiological barriers to achieve effective concentration in the blood and tissue. Adverse effects that any drugs possess may also limit their maximum dose in terms of safety. Recent innovation in nanomedicine can achieve effective drug delivery to targeted organs and cells, and spare undesirable adverse effects. In the first part of this review, we summarize recent advances in nanotechnology-based drug delivery system (nano-DDS). In the second part, we demonstrate the data of nano-DDS of statins in coronary artery disease for maximizing its anti-inflammatory effects.

VARIOUS NANO-SIZED MATERIALS AS DRUG DELIVERY SYSTEMS

Currently, various nano-sized materials (nanomaterials) have been tested and approved in clinical settings: lipid nanoparticles such as micelles or liposomes (12), polymeric nanoparticles (13), dendrimers (14), carbon nanotubes, and metallic nanoparticles. The characteristics of these materials and clinical applications are as follows (**Table 1**).

Micelles

Micelles consist of lipids and other amphiphilic artificial molecules, such as polymers. Micelles are self-assembling in aqueous solution to form a monolayer with a hydrophobic phase inside so that they can incorporate hydrophobic therapeutic agents. The diameter of micelles is usually 10–100 nm and the enclosed space is more confined than that of liposomes.

Liposomes

Since United States Food and Drug Administration (FDA) approved liposomal formulations of doxorubicin and amphotericin B in the mid-1990s (15), liposomes have been investigated most extensively in nanomedicine with approval due to less toxicity for *in vivo* application and capacity to deliver a variety of payloads. Liposomes mainly consist of phospholipids to form bilayers with an aqueous phase inside, which confer superior biocompatibility on liposomes. They can not only incorporate hydrophilic therapeutic agents inside but also hydrophobic agents in the liposomal membrane. Macromolecular drugs, including nucleic acid and crystalline metals, can be also incorporated in liposomes.

TABLE 1 | Characteristics of various nano-sized materials as drug delivery systems.

	Micelle	Liposome	Polymer nanoparticle	Dendrimer	Carbon nanotube	Metallic nanoparticle
Geometry	Spherical vesicles composed of a monolayer of lipids or synthetic amphiphiles	Spherical vesicles composed of phospholipids bilayers	An assembly of macromolecular polymers	Highly branched monodisperse macromolecules from a central core	Single or multi-walled tubular form of graphite sheets	A magnetic core coated with hydrophilic polymers
Size (nm)	10–100	40–1,000	20–1,000	3–20	0.5–3.0 × 20–1,000	60–150 (core 4–5)
Features	Encapsulation of hydrophobic agents inside	Encapsulation of hydrophilic agents inside, embedding hydrophobic agents in the membrane	Incorporation of hydrophilic and hydrophobic agents, controlled release of incorporated agents	Multivalent properties by exterior funciton groups	Encapsulation of agents into its inner space with chemically modified external surface	Contrast agents for biological imaging, photothermal properties

The diameter of liposomes is usually 40–1,000 nm. Liposomes obtain specific characteristics through modification of its surface with polymers, antibodies, and protein. PEGylated liposomal doxorubicin (Doxil®) is the first FDA-approved nanomedicine and enhanced the drug concentration in malignant effusions by 4-fold to 16-fold, while reducing cardiotoxic side effects (16).

Polymeric Nanoparticle

Food and Drug Administration-approved polymers, polylactic acid (PLA), polyglycolic acid (PGA), and poly lactic-co-glycolic acid (PLGA), are widely used for the synthesis of polymeric biodegradable nano-DDS, because they are eliminated from the body in the form of water and carbon dioxide. PLGA is a copolymer of PLA and PGA, the most frequently used constitution among these polymers, and is being tested for a DDS for intractable diseases, including cardiovascular disease. PLA is more hydrophobic, whereas PGA is more hydrophilic; the degradation speed of PLGA can be adjusted by the PLA:PGA ratio and their molecular weights, which achieves controlled release of incorporated drugs. PLGA polymers incorporate hydrophilic and hydrophobic therapeutic agents, including chemicals and nucleotides by emulsion solvent diffusion methods. The diameter of polymeric nanoparticles widely ranges from 20 to 1,000 nm. FDA-approved PLGA nanoparticle utilizing these advantages is leuprolide acetate (a testosterone inhibitor)-incorporated nanoparticle for prostate cancer (Eligard®). PLGA achieves slow and sustained leuprolide acetate release after subcutaneous injection.

Dendrimer

Dendrimers are dendritically expanded macromolecules with monodisperse structure that consist of a central core, branching interior, and exterior functional groups (14). Dendrimers can incorporate therapeutic agents in their three-dimensional branching interior voids and work as a drug delivery carrier. The number of repeating branching cycles is called generations. Exterior function groups increase exponentially as generation increases, which confer multivalent properties on dendrimers. This multivalent effect has the advantage of enhancing the binding capacity when its exterior surface is modified with some ligands or antibodies as an active targeting (17).

Carbon Nanotube

Carbon nanotubes are a subfamily of fullerenes and consist of graphite sheets rolled up into tubular form. As drug carriers, they can incorporate drugs into its inner space and have a chemically modified external surface with biomolecules, such as proteins and nucleotides, for selective targeting. Carbon nanotube-based anticancer therapy such as cisplatin-incorporated carbon nanohorn is currently being investigated (18).

Metallic Nanoparticle

Metallic nanoparticles include iron oxide and gold nanoparticles. Iron oxide nanoparticles consist of a magnetic core (4–5 nm) and hydrophilic polymers such as dextran or poly(ethyleneglycol)s. Superparamagnetic iron oxide (SPIO) (60–150 nm) has been investigated as a contrast agent for magnetic resonance imaging (MRI). Ferumoxytol (Feraheme®), caraboxymethydextran-coated iron oxide, is the only FDA-approved SPIO. The clinical use of ferumoxytol is limited to iron replacement therapy for patients with chronic kidney disease and is under investigation as an imaging agent. Resovist®, carboxydextran-coated iron oxide, has been withdrawn from FDA-approved drugs due to lack of clinical users, and is currently available in limited countries including Japan (19). Gold nanoparticles has unique photothermal properties, tunable size and shape, and easily modified surface. No gold nanoparticles have been clinically approved to date, but they are actively investigated especially in research fields targeting cancer (20).

Although micro-sized particles are considered to be biologically inert, nano-sized materials could activate innate immune sensors. In fact, nano-sized inorganic metal oxides, such as silica dioxide (SiO_2) and titanium dioxide (TiO_2) (21), and silver nanoparticle (22) have been reported to activate the NLR pyrin domain containing 3 (NLRP3) inflammasome in human macrophages. Since carbon nanotubes are fiber-shaped material, the morphology of which is similar to that of asbestos, the concern for the toxicity of carbon nanotubes has been raised (23). In animal models, intratracheal exposure with the above-mentioned nanomaterials demonstrated airway inflammation (21, 24). The activation of inflammasome is mediated by potassium efflux (25), lysosome degradation that results in cathepsin B leakage, generation of reactive oxygen species (ROS) (26), and production of adenosine (27). These data suggested that greater caution might be needed to these widely produced nanomaterials.

IN VIVO KINETICS OF NANOPARTICLE-MEDIATED DRUG DELIVERY SYSTEM

Although there are numerous determinants to affect *in vivo* kinetics of nano-DDS other than geometry as previously discussed, the size and surface modification of nanoparticles are the most important in the physiological behaviors of nano-DDS. Large-sized nanomaterials (>1,000 nm in diameter) tend to accumulate in the liver and lungs, and sometimes can be the cause of microemboli in capillaries, while small-sized nanomaterials (<10 nm) tend to be excreted from the kidneys. Middle-sized nanomaterials (10–1,000 nm) remain in circulation for longer time avoiding renal excretion. These circulating nanomaterials are generally incorporated by the mononuclear phagocytic system (MPS) in the liver, spleen, lymph nodes, and bone marrow (28). A surface modification with polyethylene glycol (PEG) serves as a hydrophilic shield that reduces protein absorption and undesirable non-specific interaction with MPS, which is preferable for nano-DDS in cancer. However, incorporation of nanomaterials into MPS itself is one of the intended mechanisms of drug delivery, especially when targeting inflammatory diseases including atherosclerosis. On the other hand, accumulation of nanomaterials depends on the permeability of target lesions. In tumor blood vessels, inflammatory atherosclerotic lesions, and ischemic myocardium, nanomaterials extravasate from blood vessels due to enhanced permeability. Tumors lack functional lymphatic vessels in their tumor microenvironment, which

enhances the accumulation of nanomaterials. These phenomena are referred to as "enhanced permeability and retention effects" (29) or "passive-targeting" (**Figure 1**).

By contrast, "active-targeting" strategy employs target-specific structures, such as antibodies and proteins on nanomaterials, which bind to the target molecule that is specific for a certain disease process. "Active-targeting" strategy is being developed in cancer therapeutics targeting molecules associated with angiogenesis and cell proliferation (30). Adhesion molecules are one of the candidates for cardiovascular imaging as described later in this review, and innovative cardiovascular imaging using "active-targeting" strategy is being investigated. Regardless of the benefits of "active targeting," only a few clinically validated nanomaterials utilize this strategy. Engineered protein combining IL-2 and diphtheria toxin (Ontak®) is the only FDA-approved "active-targeting" nanomaterial to date (15).

THE ROLE OF MONOCYTES/ MACROPHAGES IN CORONARY ARTERY DISEASE

Monocytes/macrophages play a key role in the inflammatory hypothesis of atherothrombosis (31). Atherogenesis starts with endothelial dysfunction caused by oxidative, hemodynamic, or biochemical stimuli (from smoking, hypertension, or

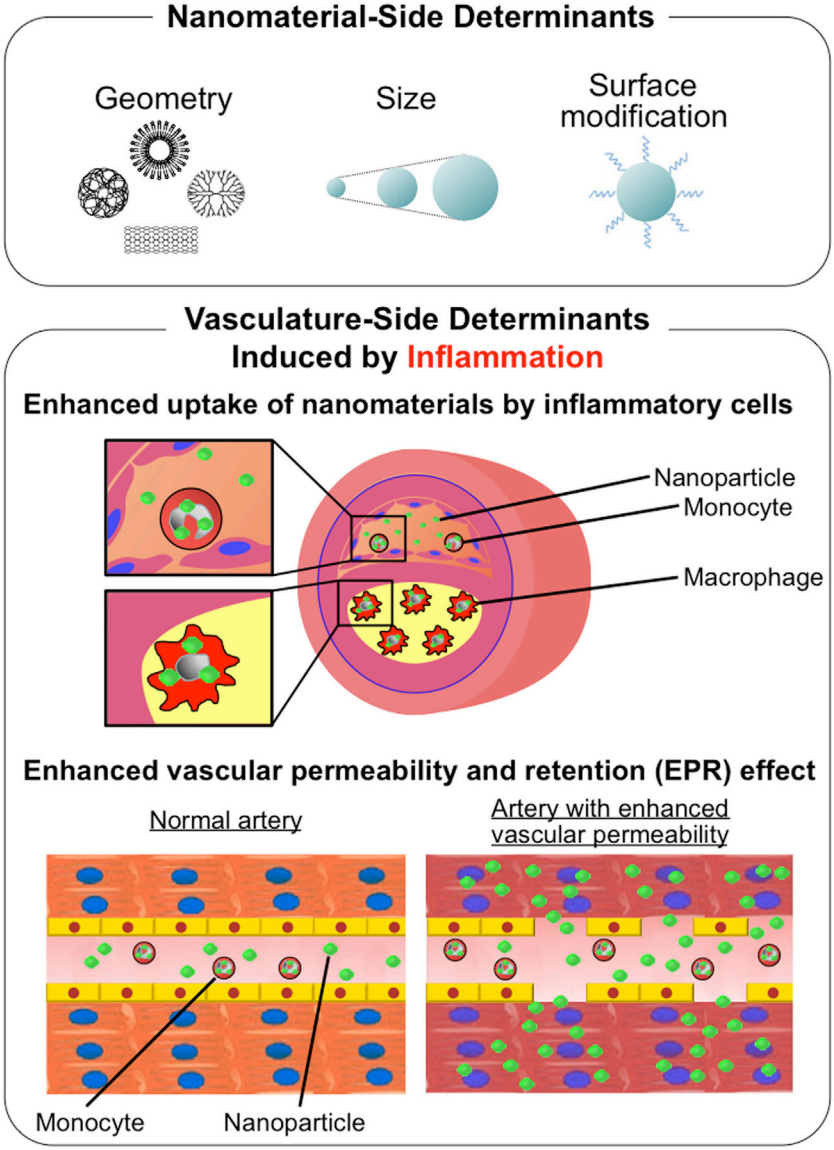

FIGURE 1 | Schematic description of determinants of physiological behavior of nanomaterials. (Upper panel) Nanomaterial-side determinants: geometry, size, and surface modification. (Lower panel) Vasculature-side determinants induced by inflammation: enhanced uptake of nanomaterials by inflammatory cells and enhanced vascular permeability and retention (EPR) effect.

dyslipidemia) and inflammatory factors. Endothelial dysfunction leads to enhanced permeability for cholesterol-containing low-density lipoprotein (LDL) particles and the expression of adhesion molecules to promote adhesion of circulating monocytes. Adhesive monocytes migrate into subendothelial space by chemoattractant proteins including monocyte chemoattractant protein-1 (MCP-1), where monocytes are differentiated into macrophages by macrophage-colony stimulating factor and activated through phagocytosis of oxidized lipid components to become foam cells. Activated macrophages secrete inflammatory cytokines to trigger positive feedback loop between cytokines and immune cells and matrix metalloproteinases (MMPs) leading to fibrous cap thinning. Rupture-prone plaques are characterized by the abundant accumulation of innate immune cells (mainly monocytes/macrophages), lipid core formation, and induction of several proteinases that catabolize the extracellular matrix (32).

Monocytes are functionally heterogeneous and are classified into at least two major subsets in mice: inflammatory monocytes (Ly-6C^{high}CCR2^{+}CX3CR1^{low}) and non-inflammatory monocytes (Ly-6C^{low}CCR2^{-}CX3CR1^{high}) (33). Previously, we developed a mouse model of "plaque rupture" utilizing a high-fat diet and angiotensin II infusion in apolipoprotein E (ApoE)-deficient mice and reported that adoptive transfer of Ly-6C^{high}CCR2^{+} inflammatory monocytes increases buried fibrous caps in the brachiocephalic arteries (34), suggesting a critical role for inflammatory monocytes in plaque destabilization. Although their functional similarities have not been fully determined, CD14^{++}CD16^{-} classical monocytes and CD14^{+}CD16^{++} non-classical monocytes are described as their respective counterparts of Ly-6C^{high} and Ly-6C^{low} monocytes according to their chemokine receptor expression in humans, and intermediate monocytes (CD14^{++}CD16^{+}) have been recently identified as not only a transitory state but also likely to possess unique features (35). There were not any significant differences in the peak levels of circulating CD14^{++}CD16^{-} monocytes among the patients with AMI, unstable angina pectoris (UAP), and stable angina pectoris (SAP), but that of circulating CD14^{+}CD16^{+} monocytes (defined as CD14^{++}CD16^{+} plus CD14^{+}CD16^{++} monocytes) were significantly decreased in AMI patients compared with those in patients with UAP or SAP (36). A conventional paradigm proposed two subpopulations in macrophages corresponding to the monocyte heterogeneity: "classically activated or inflammatory M1 macrophages" and "alternatively activated or anti-inflammatory M2 macrophages." Although the balance between "M1" and "M2" subpopulations may contribute to the development of cardiovascular disease, emerging evidence suggests that heterogeneity of macrophage subpopulation seems much more complex than "M1" and "M2" dichotomy (37). "M2" macrophages are divided into at least three subpopulations according to the stimulus they are activated; "M2a," "M2b," and "M2c." All "M2" macrophages have anti-inflammatory properties to secrete IL-10 and TGF-β. "M2b" is an exception because they additionally secrete pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα. The tissue microenvironment in atherosclerotic plaques can affect macrophage population (38). Oxidized phospholipids induce "Mox" and intraplaque hemorrhage induces "Mhem (HA-mac)" or "M(Hb)." Although localization of "Mox" in human atherosclerotic lesions remains to be elucidated, the population was detected in approximately 30% of macrophages in advanced atherosclerotic plaque of LDL receptor-deficient mice. "Mox" not only shows an pro-inflammatory phenotype by producing cyclooxygenase-2 and IL-1β but also control redox status by inducing redox-regulating genes such as heme oxygenase-1 (HO-1), sufiredoxin-1 (Srnx1), and thioredoxin reductase 1 *via* nuclear factor erythroid 2-like 2 (NFE2L2). They also display reduced chemotactic and phagocytic capacities, which might contribute to perpetuation of inflammation and tissue damage (39). "Mhem" (previously named as "HA-mac" by the same investigators), originally defined as CD163^{high} human leukocyte antigen-DR^{low}, was detected in human hemorrhaged atherosclerotic plaque. "Mhem" is an atheroprotective subpopulation that drives adaptation to intraplaque hemorrhage through activation transcription factor 1-mediated induction of HO-1 by heme (40). Another investigators named this population "M(Hb)" and further demonstrated that "M(Hb)" has high expression of "M2 markers" mannose receptor and CD163, and reduces ROS production and promotes LXRα-mediated reverse cholesterol transport (41). Platelet chemokine CXCL4 induces "M4" macrophages. They lack CD163 and are unable to increase HO-1 production in response to hemoglobin or hemoglobin–haptoglobin complexes. Co-expression of MMP7 and S100 calcium-binding protein A8 (S100A8) in "M4" in human atherosclerotic plaques might lead to plaque destabilization by increased expression of MMP7 and affect lesion progression through decreased macrophage proliferation (42). In this way, the description of macrophage activation has been expanded and confusing, and then a group of scientists proposed the updated nomenclature to define the activator, i.e., M(IFNγ), M(LPS), M(IL-4), and M(IL-10), instead of the current complex nomenclature in *in vitro* experiments. This helps researchers to avoid using different definitions of activated macrophages. They also provide the framework within which researchers place a given population for *in vivo* experiments (37).

Macrophage is also a protagonist in myocardial ischemia-reperfusion (IR) injury and wound healing process of ischemic heart disease following AMI. In patients with ST-segment elevation acute MI (STEMI), early reperfusion is a standard therapy to salvage viable myocardium and limit MI size. Regardless of significant reductions in door-to-balloon time in the last decade, the mortality of patients with MI has not improved as shown in recent cohort studies. It is well recognized that the reperfusion of coronary arteries can paradoxically induce cardiomyocyte death, called "myocardial IR injury." The major contributing factors in myocardial IR include ROS, calcium overload, and the rapid restoration of physiological PH at reperfusion, which induces the opening of the mitochondrial permeability transition pore that leads to the necrosis and apoptosis of cardiomyocytes in the first several minutes of reperfusion. Over several hours after reperfusion, myocardial inflammation contributes to cardiomyocyte apoptosis and the healing of infarcted myocardium (43, 44). The recruitment of neutrophils and inflammatory monocytes is an established phenomenon after myocardial injury. Uncontrolled macrophage infiltration following MI results in ischemic heart failure. We and another group demonstrated that infarcted myocardium sequentially recruits Ly-6C^{hi} monocytes and Ly-6C^{lo}

monocytes in murine MI model (45) and IR injury model (46); Ly-6C^{hi} monocyte dominates in early phase to exhibit inflammatory functions including phagocytic and proteolytic activity, while Ly-6C^{low} monocyte dominates in later phase to resolute inflammation and promote angiogenesis. In consistent with this preclinical data, circulating CD14^{++}CD16^{-} and CD14^{+}CD16^{+} monocytes sequentially increase in patients with AMI. The peak levels of circulating CD14^{++}CD16^{-} monocytes were negatively correlated with the degree of myocardial salvage, suggesting that manipulating classical inflammatory monocyte could be a therapeutic target for salvaging for ischemic myocardial damage after MI (36).

Monocyte chemoattractant protein-1 belongs to the CC chemokine subfamily and its primary receptor CCR2 is highly expressed on a subpopulation of monocytes (Ly-6C^{high} monocytes in mice and CD14^{++}CD16^{-} monocytes in humans). These inflammatory monocytes depend on CCR2 to migrate into the injured tissue. We previously demonstrated that systemic gene therapy with plasmids encoding 7ND, a deletion mutant of MCP-1, limits atherogenesis associated with increased lesional extracellular matrix content, one of characteristics of stable plaques, without any effects on serum lipid concentration (47). Furthermore, this gene therapy not only limits progression of established preexisting atheroma but also leads to plaque stabilization (48). We then utilized the 7ND-incorporated PLGA nanoparticle for interfering with MCP-1/CCR2 signaling and demonstrated inhibition of macrophage accumulation in the atherosclerotic plaque, followed by amelioration of morphological characteristics similar to human destabilized/ruptured plaque in the brachiocephalic artery of ApoE-deficient mice (34). Leuschner et al. demonstrated that inhibition of monocyte CCR2 with siCCR2-incorporated lipid nanoparticle inhibits macrophage accumulation in sites of inflammation and reduces atherosclerotic formation and infarct size in myocardial IR injury (49). Another group reported that blockade of CCR2 markedly reduced macrophage infiltration in ischemic lesions that results in attenuation of myocardial IR injury in mice *via* inhibition of macrophage-related oxidative stress and MMPs (50) They also demonstrated that macrophage infiltration into infarcted tissue was impaired in CCR2$^{-/-}$ mice after coronary ligation and the CCR2 deficiency ameliorates post-MI left ventricular (LV) remodeling *via* inhibition of macrophage-related MMPs (51). These data suggested that inflammatory subpopulations of monocytes/macrophages are feasible therapeutic targets for coronary artery disease.

Other immune cells, including dendritic cells (DCs), T cells, and B cells, also play important roles in atherosclerosis. A predominant subpopulation of T cells in atherosclerotic lesion is CD4^{+} T cells. CD8^{+} T cells and natural killer T cells are minor T cell subpopulations. Antigen-presenting cells such as macrophages and DCs process the disease-related antigens including oxidized LDL, heat-shock proteins and microbes, and present them to CD4^{+} T cells as fragments loaded onto major-histocompatibility-complex class II molecules, which translates innate to adaptive immunity and is one of the therapeutic targets in adaptive immunity. In fact, vaccination targeting oxidized LDL-immuned DCs ameliorates atherosclerosis in LDL receptor-deficient mice (52). Activated T cells subsequently produce the type 1 helper T (Th1) cytokines (e.g., interferon γ) to initiate pro-inflammatory responses. Atherosclerotic lesions usually contain Th1 cytokines rather than the type 2 helper T (Th2) cytokines. In contrast, regulatory T cells modulate the process by producing anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β (TGF-β) (53). The role of B cells in the progression of atherosclerosis still remains poorly understood, but it has recently gained more attention. B cells have two main subsets in mice based on their origin; B1 and B2. The role of the majority subset B2 cells in atherosclerotic lesion is controversial, while B1 cells seem to be atheroprotective (54). The corresponding B cell subsets in humans have not been clearly demonstrated. Nanomedicine can be applied to those atherosclerosis-regulating immune cells. However, the application of nanomedicine to atherosclerotic disease is extremely limited so far. A previous study demonstrated that intranasal immunization with chitosan/pCETP nanoparticles inhibits atherosclerosis in rabbit, in which the nanoparticulation enabled an efficient delivery of the antigen peptide to antigen presenting cells while sparing mucociliary clearance (55). Further studies are needed for the development of nanomedicine targeting adaptive immunity by utilizing nanoparticles as antigen delivery carriers of vaccination.

NANOMATERIALS FOR IMAGING OF MACROPHAGES

We have described that macrophage-mediated inflammation plays a crucial role in coronary artery disease. Hence, imaging of macrophages provides insight for future therapeutic options for cardiovascular diseases, in which several nanomaterials are being investigated as new imaging modalities. Developing those modalities for imaging macrophages is also indispensable in terms of quantitative analysis of therapeutic effects of anti-inflammatory drugs. Computed tomography (CT) is widely used for imaging of coronary artery in clinical setting. Nanomaterials for CT are limited because it requires high concentrations of absorbent nanomaterials to detect macrophages with X-ray. N1177, a crystalline iodined aroyloxy ester covered with a polymer, can detect macrophage-rich arterial walls in rabbits by CT (56). Macrophage-targeted gold high-density lipoprotein (HDL) nanoparticle can be localized in the macrophages of atherosclerotic plaque in the aorta of ApoE-deficient mice (57). MRI has high soft tissue contrast resolution and can detect plaque morphology non-invasively without radiation exposure. SPIO nanoparticle and ultrasmall superparamagnetic iron oxide (USPIO) are nanomaterial developed as the negative contrast for MRI. SPIO- and USPIO-based contrast agents consist of iron oxide core with hydrophilic polymeric coating, such as dextran-coated monocrystalline iron oxide nanoparticle-47 (MION-47) and dextran-crosslinked iron oxides. High-resolution MRI after administration of MION-47 can assess macrophage burden in atheromata induced by balloon injury of cholesterol-fed New Zealand White rabbits (58). In clinical setting, ATHEROMA trial has been conducted to examine USPIO-related signal change in

patients with carotid stenosis >40%, but USPIO-enhanced MRI did not predict cardiovascular events significantly (59). On the other hand, USPIO-based contrast, ferumoxytol, has succeeded in characterizing infarcted myocardium mainly by detecting infiltrating macrophages (60). There are some reagents that adopt "active-targeting" strategy to image lesional macrophages. One good example is that iron oxide nanoparticle conjugated with ligand of vascular cell adhesion molecule 1 (VCAM-1) visualized VCAM-1-expressing endothelial cells and macrophages in ApoE-deficient mice (61). Nano-sized probes for near infrared fluorescence (NIRF) are used in animal studies. The excitation and emission wavelengths of NIRF probes range from 600 to 900 nm. In that range, the absorbance and scattering of biological tissues are relatively low. These probes are designed to be activated when target protease cleaves protease-specific peptide substrates linked to quenched fluorescent dyes. MMP (62, 63) and cathepsin are used as the substrates to image macrophage burden (64).

ANTI-INFLAMMATORY THERAPEUTICS WITH NANOMATERIALS FOR CORONARY ARTERY DISEASE

Atherosclerosis

We have recently developed an innovative nano-DDS utilizing polymeric PLGA nanoparticle-incorporating pitavastatin (Pitava-NP) without PEGylation to enhance the anti-inflammatory effects of statin on monocyte/macrophage-mediated inflammation of coronary artery disease. The average diameter of polymeric nanoparticles is 200 nm. Fluorescence-labeled nanoparticle (FITC-NP) was incorporated mainly Lineage (CD90/B220/CD49b/NK1.1/Ly-6G)$^-$CD11b$^+$ monocytes in blood and Lineage$^-$CD11b$^+$ monocytes/macrophages in aorta by intravenous injection (**Figure 2A**). Fluorescence microscopic images demonstrated that FITC signal was observed in atherosclerotic plaque of brachiocephalic artery 24 h after intravenous injection of FITC-NP, suggesting that FITC-NP was passively delivered to atherosclerotic lesion with enhanced permeability (**Figure 2B**). Time course analysis of FITC signal in peripheral and aortic leukocytes by flow cytometry revealed that the delivery of FITC-NP to peripheral monocytes was followed by its delivery to aortic macrophages over 2–7 days after injection, suggesting a direct delivery of intravenous PLGA nanoparticles to blood monocytes, which gradually migrate to the atherosclerotic aorta. Weekly intravenous treatment with Pitava-NPs reduced circulating inflammatory Ly-6C^{high} monocytes, macrophage accumulation in the atherosclerotic lesions of the aortic root, and ameliorated morphological characteristics similar to human destabilized/ruptured plaque in the brachiocephalic arteries of ApoE-deficient mice (**Figures 2C,D**) (34). In consistent with these data, a preclinical study from other investigators reported that a statin-loaded reconstituted HDL (rHDL) nanoparticle inhibits atherosclerotic formation. Using dual gadolinium and fluorescent dye-labeled rHDL nanoparticle, they demonstrated that intravenously administered rHDL was incorporated into lesional monocytes and macrophages, and inhibits plaque formation with reduced macrophage content in the aortic root (65).

Myocardial IR Injury

Several pharmacological agents, including statins and erythropoietin analogs, have been shown to reduce MI size in preclinical studies (44). However, several clinical trials on pharmacological cardioprotection for myocardial IR injury have failed to demonstrate a positive impact on clinical outcome, and there is no effective therapy for preventing myocardial reperfusion injury in STEMI patients (44, 67). One possible explanation for the failure of current clinical trials is an insufficient drug delivery during a limited interventional time window, while administered at the time of reperfusion. Therefore, from a clinical perspective, it is feasible to apply an effective DDS that facilitates delivery to the sites of IR injury during reperfusion, a clinically feasible time point. In addition to macrophage-mediated inflammation, the activation of pro-survival kinases including PI3K/Akt and Erk1/2 that are known as reperfusion injury salvage kinases (RISK) in ischemic myocardium is another potential therapeutic target to reduce reperfusion-induced necrosis and apoptosis, and then limits MI size. Statins are known to afford cardioprotection from IR injury in animals (68). The cardioprotection of statins on infarct size is mediated partly by activating the RISK pathway. Intravenously injected nanoparticles accumulate not only in MPS but also in injured tissues, including IR myocardium, where vascular permeability is enhanced (13). Thus, nano-DDS may be a feasible for myocardial IR injury targeting both inflammatory monocytes/macrophages and ischemic myocardium. Therefore, we examined the efficacy of Pitava-NP as a DDS for myocardial IR injury. In a rat model of myocardial 30-min IR, PLGA nanoparticles were found exclusively in the ischemic myocardium (**Figure 3A**). Flow cytometric analysis showed the incorporation of FITC-NPs in CD11b$^+$ leukocytes in blood and IR heart. Intravenous treatment with Pitava-NPs at the time of myocardial reperfusion significantly reduced infarct size 24 h after reperfusion (**Figure 3B**). The therapeutic effect of Pitava-NP derived from the inhibition of MCP-1/CCR2 pathway by inactivation of NF-κB, which resulted in reduced macrophage-mediated inflammation (**Figure 3C**) and the activation of RISK pathway in ischemic myocardium (69). To establish preclinical proof-of-concept, we also demonstrated the therapeutic efficiency of Pitava-NPs in a preclinical conscious pig IR injury model (70). These data suggested that nano-DDS of statin to inflammatory cells and ischemic myocardium is a feasible strategy for the treatment of myocardial IR injury. Other nanomaterials can be applied as a nano-DDS for myocardial IR injury. PEGylated liposomes also showed the prolonged circulating time in blood and the specific accumulation in ischemic/reperfused myocardium. The liposomes encapsulating adenosine demonstrated the enhanced cardioprotection effects of adenosine against myocardial IR injury in rats (71). Dendrimer nanoparticles might be another candidate for the nano-DDS due to selective localization in ischemic myocardium (72). Further studies are needed to assess the therapeutic effects of a dendrimer–drug conjugate in myocardial IR injury.

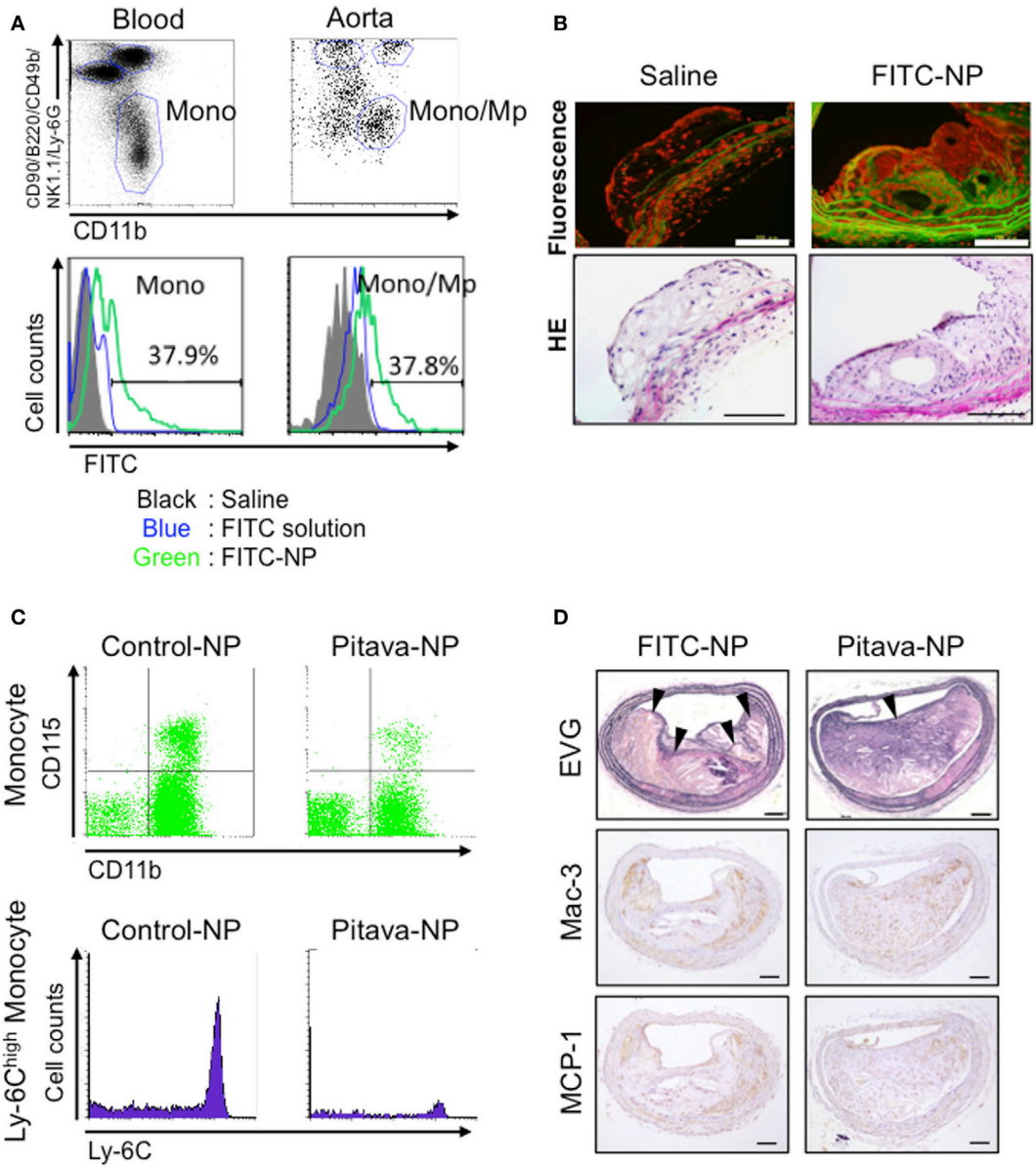

FIGURE 2 | Efficacy of pitavastatin-nanoparticle in atherosclerotic mice. **(A)** Flow cytometry of circulating leukocytes 2 days after intravenous injection of poly lactic-co-glycolic acid nanoparticles encapsulated with FITC (FITC-NP). The histograms demonstrate FITC uptake by monocytes in the blood and the aorta. **(B)** Fluorescent and light micrographs of brachiocephalic arteries 24 h after intravenous injection of saline or FITC-NP. **(C)** Flow cytometric dot plots and histograms of leukocytes from mice injected intravenously with control (empty)-NPs or pitavastatin-NPs (Pitava-NP). **(D)** Weekly intravenous injection of Pitava-NP increased fibrous cap thickness and decreased the number of buried fibrous caps in the atherosclerotic plaques with reduced macrophage accumulation (Mac-3) and monocyte chemoattractant protein-1 (MCP-1) expression. Arrows indicate disrupted/buried fibrous caps. We reused these data according to the Copyright Transfer Agreement with the publisher (34, 66).

Post-MI LV Remodeling

Recent advances in therapeutic strategy including coronary intervention and optimal medication decreased the acute phase mortality of MI, but the prevalence of chronic heart failure in MI survivors is increasing (73). Intractable heart failure due to LV remodeling (dilatation and remodeling) associated with increased fibrosis and wall thinning after MI remains a major clinical concern. Therefore, there is also an unmet need for cardioprotective modalities to ameliorate post-infarct LV remodeling. The infarcts show high inflammatory response with the recruitment of innate immune cells including macrophages and monocytes derived from extramedullary hematopoiesis such as spleen (74). We have examined the efficacy of PLGA nanoparticles as a DDS for post-infarct LV remodeling. After permanent left anterior descending coronary artery (LAD) ligation, Pitava-NP was intravenously administered for three consecutive days. Intravenously injected

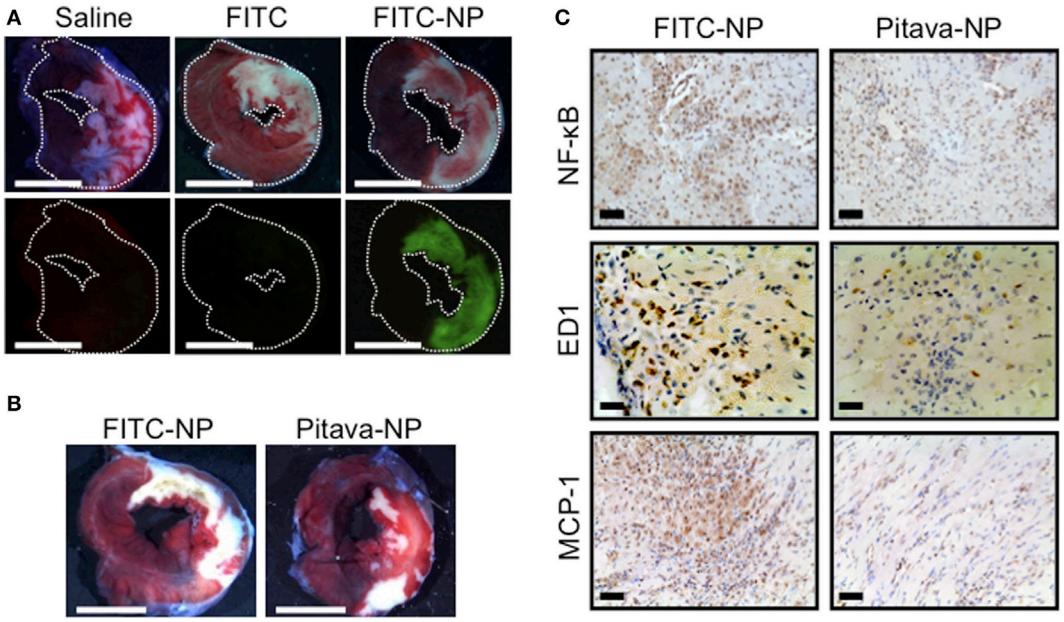

FIGURE 3 | Efficacy of pitavastatin-nanoparticle in myocardial ischemia-reperfusion injury. **(A)** Representative light (upper) and fluorescence (lower) stereomicrographs of cross-section of the ischemia-reperfusion (IR) hearts 3 h after intravenous injection of saline, FITC alone, or FITC-NP. **(B)** Intravenous treatment with Pitava-NP at the time of reperfusion reduced infarct size 24 h after reperfusion. **(C)** Cross-sections from IR myocardium stained with NF-κB, ED-1, and monocyte chemoattractant protein-1 (MCP-1). We reused these data according to the Copyright Transfer Agreement with the publisher (69).

FITC-NPs were delivered to Lineage$^-$CD11b$^+$ monocytes in the blood, spleen, and heart, but not in non-infarcted myocardium within the area at risk due to coronary ligation (**Figure 4A**), suggesting that the beneficial effects of Pitava-NPs on post-infarct LV remodeling can be predominantly derived from those on inflammatory cells. Actually, Pitava-NP decreased macrophage accumulation in the heart by inhibiting not only angiotensin II-mediated monocyte mobilization from the spleen (**Figure 4B**) but also monocyte mobilization from the bone marrow that is supposed to be mediated by CCR2 (75). Intravenous treatment with Pitava-NPs after MI induced by permanent LAD ligation attenuated post-infarct LV remodeling associated with reduced monocyte/macrophage accumulation in the heart (**Figure 4C**) (76). A delayed transition from inflammatory to anti-inflammatory macrophages by prolonged recruitment of classical inflammatory monocytes to myocardium may also interfere with post-infarct LV remodeling (77). Other investigators have intervened a transcription factor that shifts macrophages to inflammatory subsets by utilizing siRNA lipid nanoparticles targeting interferon regulatory factor 5 (IRF5). They demonstrated that siIRF5 improves post-infarct LV remodeling by reprogramming macrophages toward anti-inflammatory subsets (78).

SUMMARY AND CLINICAL PERSPECTIVE

In this review, we have summarized a series of evidence including (1) the anti-inflammatory therapeutics for coronary artery disease, (2) a variety of nanomaterials for drug delivery systems, (3) the role and imaging of monocytes/macrophages in coronary artery disease, and (4) application of nano-DDS based on PLGA nanoparticle to coronary artery disease, such as plaque destabilization, IR injury, and post-MI LV remodeling. Applications of nano-DDS for coronary artery disease is a feasible strategy by utilizing increased incorporation of nanomaterials by MPS and enhanced permeability of inflammatory vasculature (**Figure 1**). In addition to the therapeutic effects of nano-DDS on coronary artery disease, there are a wide variety of opportunities to combine nano-DDS and various therapeutic agents, including chemical, nucleotide, peptide, and others, which may extend the possibility of current pharmacotherapy for several cardiovascular diseases. Possible application of nano-DDS in other cardiovascular diseases may include, pulmonary hypertension (79, 80), vein graft disease (81), and coronary artery stents (82). The clinical application of nano-DDS utilizing Good Manufacturing Practice-compliant Pitava-NPs in this review is in progress. We have already completed phase I/IIa investigators' initiated clinical trial testing the efficacy of Pitava-NP in patients with critical limb ischemia in the Kyushu University Hospital (UMIN000008011). We have also completed a phase I clinical trial testing the safety of an intravenous administration of Pitava-NP in healthy volunteers (UMIN000014940). Recently, phase III CANTOS trial targeting interleukin-1β with human anti-IL-1β monoclonal antibody, canakinumab, has demonstrated that the inhibition of systemic inflammation may reduce cardiovascular risk even in the absence of concomitant lipid lowering (83). This trial has proved the concept that anti-inflammatory therapeutics targeting innate immunity pathway can be applicable in humans, and our novel DDS utilizing PLGA polymer-based nanotechnology might be one of promising therapeutic strategies for various cardiovascular diseases in the near future.

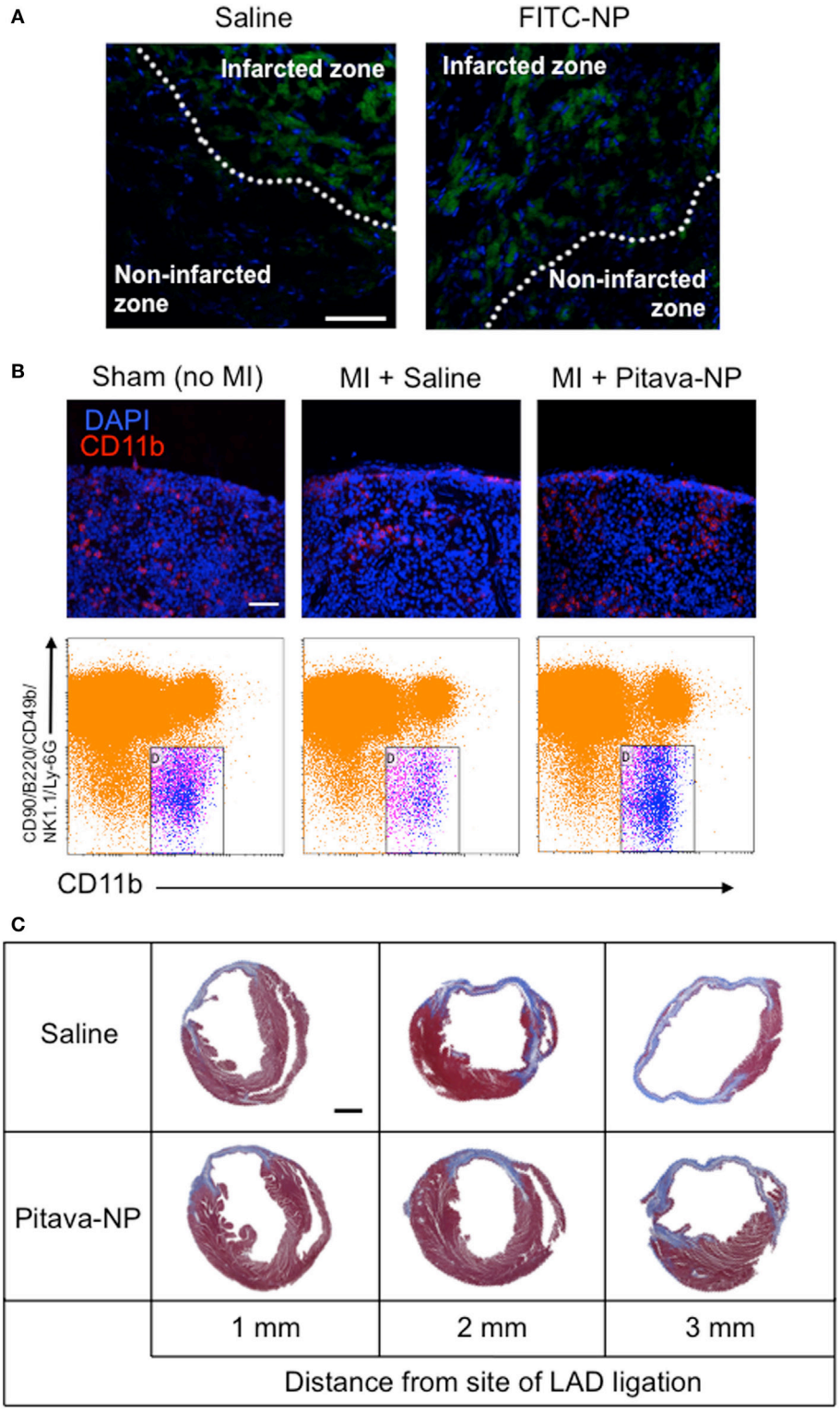

FIGURE 4 | Efficacy of pitavastatin-nanoparticle in post-myocardial infarction left ventricular (LV) remodeling. **(A)** Cross sectional pictures of the infarcted myocardium observed under fluorescent microscope 3 days after LAD ligation. Saline or FITC-NP were injected for three consecutive days after ligation. Weak fluorescent signals observed in infarcted zones are auto-fluorescence from dead cardiomyocytes. **(B)** (Upper panel) Splenic sections stained with anti-CD11b antibody (red) and DAPI (blue) showing the subcapsular red pulp. (Lower panel) Dot plots of flow cytometric analysis of spleen samples. Treatment with Pitava-NPs inhibited MI-induced Lineage (CD90/B220/CD49b/NK1.1/Ly-6G)$^-$CD11b$^+$ monocyte reduction in the spleen. **(C)** Intravenous treatment with Pitava-NPs for three consecutive days ameliorated LV remodeling 4 weeks after myocardial infarction. We reused these data according to the Copyright Transfer Agreement with the publisher (76).

AUTHOR CONTRIBUTIONS

SK, TM, J-iK, KN, and KE make substantial contributions to conception and design and acquisition, analysis, and interpretation of data.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan (25860607 to SK, 17K09590 to TM, 22390160 and 25293185 to KE), the Translational Research Network Program from the Japan Agency for Medical Research and Development, Tokyo, Japan (to KE) and Health Science Research Grants (Translational Research, Research on Intractable Diseases, and Research on Nanomedicine) from the Ministry of Health, Labour and Welfare, Tokyo, Japan (to KE). The authors thank Kazuhiro Nagaoka, Yajing Mao, Soichi Nakashiro, and Yasuhiro Nakano for their excellent works demonstrated in this review.

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Conflict of Interest Statement: KE is the inventor of an issued patent on part of the results reported in the present study (Pharmaceutical composition containing statin-encapsulated nanoparticle, WO 2008/026702). Applicants for this patent include Kyushu University (https://airimaq.kyushu-u.ac.jp/), KOWA Inc. (http://www.kowa.co.jp), and Sentan Medical Inc. (http://sentaniryou.co.jp). Sentan Medical Inc. is a drug discovery venture company from Kyushu University. KE is a founder of Sentan Medical Inc., possesses stocks, serves as one of Directors of the company, and reports personal fees from the company outside the submitted work. The intellectual property division of Kyushu University is reviewing that Sentan Medical Inc. did not play a direct role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript in KE's Laboratory.

frontiers in Cardiovascular Medicine

REVIEW
published: 11 May 2018
doi: 10.3389/fcvm.2018.00032

Insights From Pre-Clinical and Clinical Studies on the Role of Innate Inflammation in Atherosclerosis Regression

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Atherosclerosis, the underlying cause of coronary artery (CAD) and other cardiovascular diseases, is initiated by macrophage-mediated immune responses to lipoprotein and cholesterol accumulation in artery walls, which result in the formation of plaques. Unlike at other sites of inflammation, the immune response becomes maladaptive and inflammation fails to resolve. The most common treatment for reducing the risk from atherosclerosis is low density lipoprotein cholesterol (LDL-C) lowering. Studies have shown, however, that while significant lowering of LDL-C reduces the risk of heart attacks to some degree, there is still residual risk for the majority of the population. We and others have observed "residual inflammatory risk" of atherosclerosis after plasma cholesterol lowering in pre-clinical studies, and that this phenomenon is clinically relevant has been dramatically reinforced by the recent Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial. This review will summarize the role of the innate immune system, specifically macrophages, in atherosclerosis progression and regression, as well as the pre-clinical and clinical models that have provided significant insights into molecular pathways involved in the resolution of plaque inflammation and plaque regression. Partnered with clinical studies that can be envisioned in the post-CANTOS period, including progress in developing targeted plaque therapies, we expect that pre-clinical studies advancing on the path summarized in this review, already revealing key mechanisms, will continue to be essential contributors to achieve the goals of dampening plaque inflammation and inducing its resolution in order to maximize the therapeutic benefits of conventional risk factor modifications, such as LDL-C lowering.

Keywords: innate immunity, macrophages, atherosclerosis progression, atherosclerosis regression, pre-clinical models, clinical trials as topic

OPEN ACCESS

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

Received: *16 December 2017*
Accepted: *20 March 2018*
Published: *11 May 2018*

Citation:
Rahman K and Fisher EA (2018) Insights From Pre-Clinical and Clinical Studies on the Role of Innate Inflammation in Atherosclerosis Regression. Front. Cardiovasc. Med. 5:32. doi: 10.3389/fcvm.2018.00032

INTRODUCTION

Atherosclerosis, which underlies coronary artery disease (CAD), is characterized by a maladaptive immune response to lipoprotein and cholesterol accumulation in artery walls that results in the formation of plaques (also called lesions). The Pathobiological Determinants of Atherosclerosis in Youth study demonstrated that established coronary artery plaques begin their progression in childhood (1). Furthermore, a majority of patients likely have formation of advanced plaques even before physical symptoms of CAD, such as angina, manifest (2). Since plaques are established so early, efforts to reduce the morbidity and mortality of CAD requires inducing favorable changes to

pre-existing clinical disease (3–6). Unfortunately, there is ample evidence that the conventional risk reduction therapies, heavily weighted towards reducing low density lipoprotein cholesterol (LDL-C) levels, leave a large amount of residual risk (7). This motivated our group and others to develop pre-clinical models of atherosclerosis regression and study them in molecular detail in order to identify potential clinical approaches and therapeutic targets to reduce the residual risk. In this review, we will summarize the evidence for the role of the innate immune system, focusing on plaque macrophages, in both atherosclerosis progression and regression, as the large body of work in the former has informed the design and interpretations of the latter. We should first note that we are aware of the participation of the adaptive immune system in atherosclerosis. As plaques advance, T and B-cells, in particular, make increasingly important contributions to the inflammatory state of plaques and to the local and systemic responses to antigenic material generated from modification of apolipoprotein B (APOB)-containing lipoproteins (LPs) and from tissue damage and cell necrosis. For this topic, we refer the reader to other expert reviews, such as (8–17).

MACROPHAGES IN ATHEROSCLEROSIS PROGRESSION

Atherosclerotic lesion development begins with the accumulation of cholesterol-rich APOB-LPs, which include very low density lipoprotein (VLDL) and LDL particles, in the subendothelial space (18, 19). Circulating monocytes derived from the bone marrow and spleen enter the subendothelial space of the arteries, with some of them differentiating to macrophages, which in turn, ingest retained lipoproteins in probably both their native and modified forms [e.g., oxidized LDL (OxLDL)], and become activated. Activation of macrophages (and endothelial cells) also leads to the secretion of chemoattractant molecules such as CCL2 (MCP-1) and CCL5 (RANTES) that lead to further recruitment of circulating monocytes.

Humans have two main subsets of circulating monocytes, Cluster of Differentiation 14^+ ($CD14^+$) $CD16^-$ and $CD14^{low}CD16^+$, which correspond, respectively, to the lymphocyte antigen 6C ($Ly6C)^{high}$ and $Ly6C^{low}$ monocyte subsets in mice. These circulating monocytes, via CC-chemokine ligand 5 (CCL5) and CXC-chemokine ligand 1 (CXCL1), bind to endothelial cell glycosaminoglycans and P-selectin, as well as to vascular adhesion molecule 1 (VCAM1) and intracellular adhesion molecule 1 (ICAM1) binding via integrins very late antigen 4 (VLA4) and lymphocyte function-associated antigen 1 (LFA1), respectively. Together, these factors allow for monocyte adhesion to the activated endothelial cell layer (3, 20, 21).

The monocytes then transmigrate into the subendothelial space using surface receptors CC-chemokine receptor 2 (CCR2), CX3- chemokine receptor 1(CX3CR1), and CCR5, which bind to chemoattractant proteins released from the endothelial cells, as well as from existing macrophages in the plaque, such as CCL2, CX3CRL1, and CCL5, respectively (3, 20, 21). Importantly, it has been found that $Ly6C^{high}$ and $Ly6C^{low}$ monocytes differentially use these chemokine receptors for migration into plaques. $Ly6C^{high}$ monocytes preferentially use CCR2 and CX3CR1, while $Ly6C^{low}$ monocytes preferentially use CCR5 to enter plaques during plaque progression (22). Furthermore, the combined deficiency of these 3 receptor-ligand interactions led to a ~ 90% decrease in atherosclerosis burden (23), with the major fraction due to the loss of CCR2-CCL2 mediated monocyte migration into plaques (24, 25). Additionally, hypercholesteremia induces increased CCR2 expression in monocytes, leading to increased migration to CCL2, as well as $Ly6C^{high}$ monocytosis (26–28). This suggests that $Ly6C^{high}$ monocyte recruitment, mediated by CCR2 and CCL2, to the plaque is essential for atherosclerosis progression. Furthermore, loss of macrophage colony stimulating factor (M-CSF), which stimulates differentiation of monocytes to macrophages, led to almost complete loss of plaque development (29, 30).

Monocyte-derived macrophages ingest APOB-LPs, particularly LDL, through the LDL receptor (LDLR), beginning the formation of foam cells, a key step in the initiation and progression of atherosclerotic lesions (3, 20, 21). However, LDLR expression quickly decreases due to increased intracellular cholesterol levels (3, 21). Some of the remaining LDL becomes modified in a number of ways, including by oxidation (OxLDL) in the artery wall (31). After LDLR downregulation, macrophages then take up normal and modified LDL particles via pinocytosis and binding to scavenger receptors, most notably scavenger receptor A1 (SR-A1) and CD36, eventually becoming overwhelmed by the ingested lipids (3, 21, 32–34). As macrophages accumulate intracellular cholesterol, they upregulate molecules involved in cholesterol efflux pathways, such as ATP-binding cassette subfamily A member 1 (ABCA1) and ABCG1, via cholesterol precursors or derivatives that induce Liver X Receptor (LXR) activation, to induce the removal of intracellular cholesterol (3, 21). ABCA1 promotes efflux to lipid-poor apolipoprotein A1 (APOA1), which is a major component of high density lipoprotein (HDL) particles, while ABCG1 promotes efflux to mature lipid-rich HDL particles (35, 36). Despite this attempt at restoring homeostasis in cellular lipid content, plaques macrophages continue to accumulate cholesterol, which has many adverse effects, as will be described in a number of places in this review.

Macrophages have multiple roles in inflammation, for example, by promoting it during infection or by resolving it during wound and tissue repair (37–39). One influential classification of macrophages that recognizes the wide range of macrophage inflammatory phenotypes- from causative to protective- is based on the work of Siamon Gordon and Alberto Mantovani and their colleagues, who broadly described M1 (classically activated) and M2 (alternatively activated) states (40, 41). Since the initial descriptions of the M1 and M2 states, there has been much research- and controversy- about the numbers of sub-types of macrophages not only within each category, but also along the spectrum between the categories [e.g., (39)]. Indeed, we have participated in a recent perspective on macrophage activation and polarization (42). For the purposes of this review, as summarized in **Figure 1** of that perspective, our referring to M1 or M2 macrophages in mice corresponds

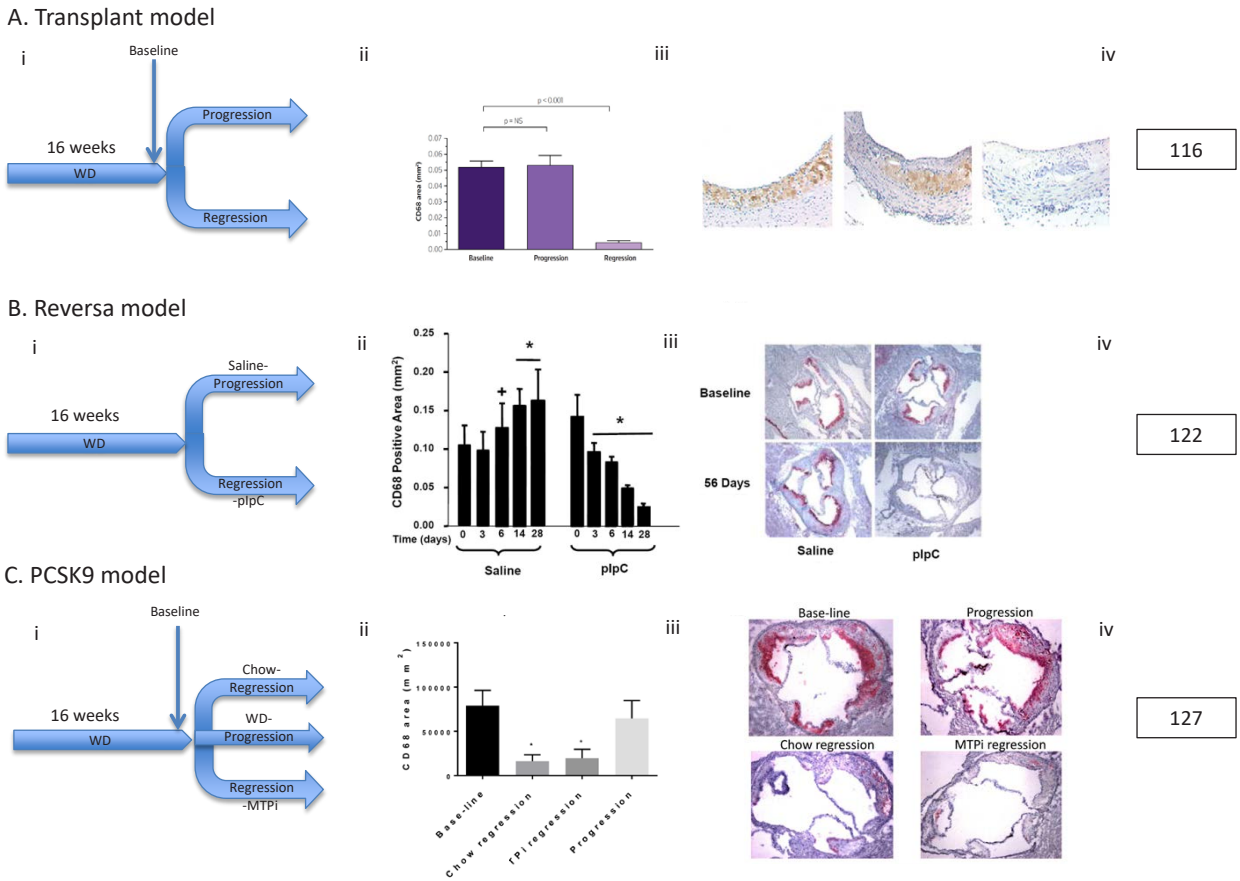

FIGURE 1 | Representative Murine Plaque Regression Models. Selected models taken from those described in the text: **(A)** Transplant Model, **(B)** Reversa Model,and **(C)** PCSK9 model. For each model: (i) Schematic of experiment, (ii) Quantification of $CD68^+$ (macrophage) area, *<0.05, (iii) representative $CD68^+$ stained immunohistochemical sections, and (iv) original reference from which the images are modified from. Despite different methods to drastically lower circulating lipids,all of these models show a significant (*<0.05) reduction in $CD68^+$ macrophage content in regression groups compared to respective baseline and/or progression groups.

to M[lipopolysaccharide (LPS)] or M[Interleukin-4 (IL-4)], respectively, based on the markers we have documented in macrophages in progressing and regressing plaques (43, 44). Looking ahead, as advanced sequencing techniques, including but not limited to single cell RNA sequencing and CEL-Seq2 (45–47), become incorporated into atherosclerosis studies, the phenotyping of plaque macrophages will undoubtedly continue to be refined.

In vitro, macrophages are typically polarized towards M1 by incubation with LPS alone or LPS combined with interferon γ (IFNγ), which activates Toll-like receptor-4 (TLR-4) and its downstream effector, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). M2 polarization can be induced by interleukin-13 (IL-13) or, more commonly, IL-4, via activation of IL-4/IL-13 receptor and its downstream effector, signal transducer and activator of transcription 6 (STAT6) (40, 41, 48). M1 macrophages promote inflammation by highly expressing pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), IL-6, and IL-12. M2 macrophages resolve inflammation by expressing anti-inflammatory mediators, such as transforming growth factor β (TGF-β), IL-1 receptor antagonist, and IL-10, which also induces M2 macrophage polarization and collagen production (49). In reality, macrophages span a range of phenotypes, but the M1/M2 scheme has nevertheless served as a convenient classification system. As advanced sequencing techniques (45–47) become incorporated into atherosclerosis studies, we will better be able to identify and understand the roles of the macrophage subsets during plaque progression and regression. For this review, we will focus on the M1/M2 paradigm as many of the studies we cite assess macrophage polarization within the simplified model.

There are multiple inflammatory stimuli that influence the macrophage polarization state in progressing plaques. One comes

from the formation of cholesterol crystals (50, 51), which induces inflammasome activation and consequently IL-1β secretion. IL-1β is a potent inflammatory cytokine that promotes atherosclerosis progression as well as M1 polarization (15, 51–53). Cholesterol accumulation also increases plasma membrane cholesterol content, which sensitizes TLRs to their ligands, amplifying the inflammatory response (54, 55). As noted above, intracellular cholesterol accumulation activates LXR, which in addition to promoting cholesterol efflux, is also a repressor of NF-κB, leading to dampening of inflammation (3, 21, 49, 56, 57). However, the pro-inflammatory response and the associated M1 macrophage polarization predominates during plaque progression due to reinforcement by additional signals, such as TLR activation by pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS; see below) (3, 21, 49). One PAMP is thought to be a lipid component of OxLDL that is recognized by TLR4 and leads to the production of TNF-α and IL-6 (58). OxLDL can also activate the TLR4-TLR6 heterodimer via CD36, leading to NF-κB activation and chemokine secretion that induces monocyte recruitment into plaques (3, 21, 59–61). Another inflammation-related mechanism involves T-cell infiltration into the plaque during progression. The infiltrating T-cells secrete inflammatory type 1 helper T cell (Th1) cytokines (IL-1, IL-6, TNF-α) that promote inflammation and M1 macrophage polarization (62–64), and in advanced plaques, inhibit smooth muscle cell collagen synthesis (65). Coupled with robust secretion by M1 macrophages of matrix metalloproteinases (MMPs) that degrade collagen, this results in thinning of the sub-endothelial fibrous cap, a protective and desirable component in human plaques, thought to prevent their rupture.

Furthermore, advanced atherosclerosis lesions are characterized by increased accumulation of dying/dead cells. There are a number of likely stimuli in progressing plaques that promote cell death by apoptosis, and by a related process, pyroptosis. These include the activation of ER stress by cholesterol, accumulation of reactive oxygen species, oxysterols and other modified lipids, and the activation of the inflammasome by multiple TLR ligands, TNF-α, and cholesterol crystals (3, 21, 50, 66–76). These deteriorating cells are normally cleared via efferocytosis, but as plaques advance, macrophages lose their efferocytosis capability. M2 macrophages are thought to have more efferocytosis capability than M1 macrophages, thus the predominance of M1 macrophages during plaque progression may also contribute to efferocytosis not being able to keep pace (49). This combination of increased accumulation of apoptotic cells and defective efferocytosis leads to secondary necrosis, which contributes to large lipid-filled necrotic cores and the release of more DAMPS (15, 77), which as noted above, are TLR ligands and stimulate inflammatory pathways. While secondary necrosis after inefficient efferocytosis of apoptotic or pyroptotic cells contributes to further inflammation and the formation of the necrotic core of plaques, recent studies have also highlighted the contributions to cell death of primary macrophage necrosis as a result of activation of the necroptosis pathway by TNF and OxLDL (78–81).

The combination of large necrotic cores and thinning of the fibrous cap destabilizes the plaque and increases risk of rupture and thrombus formation that precede heart attacks and strokes (2, 3, 15, 20, 21, 49, 82). In human plaques, M1 macrophages are localized to rupture prone regions, such as the necrotic core and plaque shoulder, while M2 macrophages are localized in the adventitia and farther away from the necrotic core (83, 84). This further solidifies the role of M1 macrophages in not only plaque progression, but also in the events that directly precede plaque rupture. This progression from the response to retained APOB-LPs to plaque rupture represents a maladaptive innate immune response. Normally, after the recruitment of monocytes to damaged tissues and the enrichment in M1 macrophages, there is eventual resolution of the inflammation and beneficial tissue remodeling by M2 macrophages (3, 15, 21, 28, 85). As will be presented below, the value of atherosclerosis regression models is to first illustrate that the failure to resolve inflammation during plaque progression can be overcome, and then to provide discovery platforms with which to accomplish this.

ATHEROSCLEROSIS REGRESSION IN PRE-CLINICAL MODELS

Rabbits, Non-Human Primates, and Pigs

The history of plaque regression in rabbit, non-human primates, and pigs has been detailed extensively in our previous review (4). More briefly, evidence of plaque regression was observed as early as the 1920s, when it was observed that arterial lesions from cholesterol-fed rabbits that were switched to low-fat chow had increased fibrous content and reduced lipid content (86). In 1957, Friedman and colleagues performed one of the first prospective, interventional studies that showed plaque regression in cholesterol-fed rabbits that were injected with phosphatidylcholine (PC). Over the next two decades, similar plaque regression was observed in response to injections of dispersed phospholipids by multiple groups in experimental atherosclerosis models in rabbits (87–90) and baboons (91).

These findings were attributed to the ability of dispersed phospholipids to spontaneously form liposomes in aqueous solutions that can extract un-esterified cholesterol from membranes and cells, including plaque macrophages (90). These findings were further bolstered when Badimon and colleagues found that infusions of HDL, a known acceptor of cholesterol from macrophages, led to atherosclerosis regression in cholesterol fed-rabbits (92). Aikawa and Libby found that dietary lipid lowering led to plaque composition changes in rabbit atherosclerotic lesions that signaled increased plaque stability and reduced thrombotic potential. This included reduced tissue factor (TF) expression and activity (93, 94) along with decreased macrophage content, reduced expression and activity of MMPs, increased collagen accumulation, and increased smooth muscle cell area in the fibrous cap (94). Taken together, these studies provided strong evidence that dietary lipid lowering along with treatments to remove cholesterol from membranes and cells led to plaque regression in rabbit models of atherosclerosis (4, 90).

Further evidence of plaque regression was found in squirrel monkeys in 1968. A switch from an atherogenic to chow diet led to

significant loss of lipid content compared to baseline lesions (95). This was further confirmed in rhesus monkeys (4, 96–100). A common theme in these studies was that lesions at varying stages of plaque progression (fatty streaks to more advanced lesions) all regressed due to dietary lipid depletion (96), consistent with findings in rabbit models of plaque regression.

Pigs have also been used for atherosclerosis regression research. It was found that after atherosclerosis was induced by a combination of arterial injury and a high cholesterol diet, there were favorable plaque composition changes including decreases in lipid content and necrotic core area after the animals were switched to a regular chow diet (101). In 1981, the same group published extensive histological analysis of plaques at different stages of regression. Interestingly, they reported that the advanced lesions showed changes compatible with a "healing process" characterized by the disappearance of foam cells, a significant decrease in necrotic areas, and increased replacement of necrotic debris by fibrous tissue and calcified areas (102). Furthermore, they reported that early in the regression process, the decrease in foam cells and necrotic areas was accompanied by an increase in non-foam cell macrophages (102). This led them to hypothesize that the disappearance of the necrotic core areas occurred because the necrotic debris was removed by newly recruited, functioning, healthy macrophages (102).

Mice

Mice have naturally low LDL-C levels, with most of their plasma cholesterol carried by HDL, and it is thought that this plasma lipoprotein profile underlies their natural resistance to atherosclerosis (4). The use of murine models in atherosclerosis was catalyzed by the development of the apolipoprotein E (APOE) knockout mouse by the Breslow (103) and the Maeda (104) laboratories, and the LDL receptor (LDLR) knockout mouse by the Brown and Goldstein laboratory (105) in the early 1990s. In mice, APOE is the major ligand for the LDL receptor, even for APOB-containing lipoproteins. Thus, both models eliminate pathways for efficient lipoprotein cholesterol clearance by removing either a ligand ($Apoe^{-/-}$) or a receptor ($Ldlr^{-/-}$), and result in above normal plasma levels of cholesterol carried by APOB-lipoproteins. The circulating plasma levels of cholesterol can be further increased by feeding the mice a high fat and high cholesterol diet ("western diet", WD), which accelerates the development of atherosclerotic plaques (4).

With mouse atherosclerosis progression models in place, we and others focused on adapting them for studies of regression. Similar to the rabbit, non-human primate, and pig studies, plaque regression was induced by significant lowering of circulating lipid levels, especially LDL-C, or by elevating HDL particles either genetically or by infusion (4, 49, 106). Significant LDL-C lowering could not be accomplished, however, by statin treatment, given the absence of either the ligand for the LDL receptor or the receptor itself. One approach to lipid lowering involved the hepatic overexpression of *Apoe* in $Apoe^{-/-}$ mice (107–111) and *Ldlr* in $Ldlr^{-/-}$ mice (105, 112–115) using adenoviral-mediated gene transfer. Both approaches led to the normalization of atherogenic lipid profiles, and favorable changes in plaque size, composition, or macrophage content (4, 105, 107–115). However, in many of these models, the regression of early fatty streaks was much more pronounced than in advanced lesions. While fatty streaks reduced in lesion size by a factor of 10 compared to baseline, advanced lesions usually had smaller reductions in size by <20% (110, 116). This difference in plaque regression may have been due to the transient nature of the adenoviral-mediated lipid lowering with the early vectors, in part because of an immune response against virally transduced cells. Thus, models were needed in which lower lipid levels could be sustained indefinitely so that the regression of lesions of any complexity could be studied (116).

Towards this goal, the Fisher lab developed a plaque regression model that involved transplanting an atherosclerotic thoracic arch (116) or aortic arch segment (117) from a hyperlipidemic donor ($Apoe^{-/-}$ fed a WD) into a normolipidemic recipient (wild type (WT) fed a chow diet). This rapid environmental change in circulating lipoprotein/lipid levels induced plaque regression over a surprisingly short time (starting at 3 days) (43, 116–120). The regression group plaques showed significantly decreased lesion, macrophage, and lipid areas compared to their baseline counterparts.

Another regression model developed by our lab (in collaboration with Dr. Stephen Young, UCLA) is the Reversa mouse ($Ldlr^{-/-}$ $Apob^{100/100}$ $Mttp^{fl/fl}Mx1Cre^{+/+}$) (121, 122), where after plaque progression occurs while the mice are on WD, polyinosinic-polycytidylic (pIpC) injection induces the Mx1-Cre gene leading to the inactivation of the microsomal triglyceride transfer protein (MTTP) gene. MTTP inactivation inhibits APOB-lipoprotein particle secretion from the liver. The resulting decrease of circulating cholesterol-rich VLDL and LDL induced plaque regression characterized by reduced macrophage content, reduced lipid area, and increased collagen content (122–124). Similar plaque regression was seen with treatment with a MTTP inhibitor in WD fed $Ldlr^{-/-}$ mice (125).

A common denominator in the above models of plaque regression was the requirement of an intervention to reduce lipid levels considerably below what could be achieved by switching from WD to chow alone. However, Nagareddy and colleagues have shown that with longer periods of moderate lipid lowering induced by switching the WD to chow after plaques have been established in $Ldlr^{-/-}$ mice, regression can occur (126).

Space does not permit their full descriptions, but a rapid expansion of the number of non-surgical models to study atherosclerosis regression is expected based on the combined use of PCSK9-adenovectors to make mice LDLR-deficient, followed by antisense oligonucleotides (ASOs) against APOB or MTTP to lower APOB-lipoproteins (127, 128). In addition, there are still other regression models that are based on non-LDL lowering strategies, such as raising HDL particles either transgenetically, by infusion of HDL, and treatment with anti-miR-33 (43, 106, 123, 129, 130). As will be presented later, there has been remarkable consistency in the changes in the macrophage properties in the plaques, independent of the mode of regression.

In summary, plaque regression has been observed in multiple pre-clinical models (see **Figure 1** for representative results from

key models described above). We will next review the evidence of plaque regression in humans.

ATHEROSCLEROSIS REGRESSION IN HUMANS

One of the first reports of possible regression of established human atherosclerotic lesions was seen in autopsy studies by Aschoff in 1924 when he observed that there was reduced atherosclerosis severity during the famine Germany suffered after World War I (131). These findings were confirmed in 1946 by Vartianen and Kanerva (132), in 1947 by Wilens (133), and in 1951 by Wanscher and colleagues (134) in diverse populations that had suffered from severe food restrictions, chronic wasting diseases, and cancer-induced cachexia (135), suggesting that atherosclerosis severity could decrease after lesions were established.

In 1966, Ost and Stenson performed one of the first prospective, interventional studies that demonstrated plaque regression in humans. They found that 10% of patients treated with niacin, which lowers triglycerides, raises HDL-C, and lowers LDL-C, showed improved femoral angiograms as measured by reduction in flow-limiting stenosis (4, 136). Brown and colleagues summarized larger trials from 1984 to 1993 that examined the relationship between lipid therapies and plaque regression, as measured by angiographic evidence of improved arterial flow or changes in plaque size compared to their baseline angiograms (137). They found that while the absolute decreases in arterial narrowing after lipid therapies were statistically significant, they were remarkably small, especially compared to the reduction in adverse clinical events (137). This discrepancy between absolute changes in angiographic arterial narrowing and reduction in coronary events was termed the "angiographic paradox" (4). The paradox was at least in part clarified when it was found that vulnerable plaques prone to the thrombotic events preceding heart attack and stroke cause less than 50% stenosis compared to stable, but more occlusive, lesions (4, 137, 138). An interpretation we favor, based on the pre-clinical models, for example a few reviewed in (122, 124, 125), is that lipid lowering most likely led to remodeling and stabilization of the smaller, vulnerable plaques, some perhaps not even readily visible on the angiograms (137). This suggests that lipid lowering interventions can induce beneficial changes in plaques (via compositional changes that lead to stabilization of vulnerable plaques and significant reduction in coronary events), that imaging methods then (and, for that matter, now) are inadequate to easily detect.

Later trials switched from angiographic analysis to intravascular ultrasonography (IVUS) because of being able to measure arterial wall thickness (assumed to be equivalent to plaque volume) in addition to vascular lumen size (4). For example, Nissen and colleagues reported two major prospective trials that observed patients with CAD who were treated with high dose statins using IVUS: (1) the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial (139), and, (2) A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden (ASTEROID) (140). In the REVERSAL study, they found that LDL-C reduction greater than 50 percent in patients treated with atorvastatin for 18 months was associated with decreases in plaque volume of 0.4 percent (139). In the ASTEROID trial, LDL-C dropped from 130.4 mg/dl to an average of 60.8 mg/dl with rosuvastatin treatment for 24 months, and this was associated with a 0.98 percent decrease in plaque volume (140). The Study of Coronary Atheroma by Intravascular Ultrasound: Effect of Rosuvastatin versus Atorvastatin (SATURN) showed similar findings using IVUS, where plaque volume decreased by 0.99 percent in the atorvastatin treatment group and by 1.22 percent in the rosuvastatin treatment group after 24 months of LDL-C lowering treatment (141).

More recently, Nicholls and colleagues assessed the efficacy of proprotein convertase subtilisin kexin type 9 (PCSK9) antibodies to lower circulating LDL-C in the Global Assessment of Plaque Regression With a PCSK9 Antibody as Measured by Intravascular Ultrasound (GLAGOV) trial (142). They found that the group treated with PCSK9 antibody had significantly lower LDL-C levels compared to controls (36.6 mg/dL compared to 93.0 mg/dL after 76 weeks of treatment) and a 0.95 percent decrease in plaque volume compared to baseline (142). These studies provide consistent evidence that extensive lipid lowering can lead to plaque regression as measured by decreases in plaque volume. The modest quantitative changes in plaque volume, however, recall the surprising results of Brown and colleagues, which suggested that changes in plaque composition might pre-dominate over changes in plaque size after lipid-based interventions. As implied above, confirmation of this hypothesis will require more sensitive, preferably non-invasive, imaging methods as reviewed in (143).

Another therapeutic intervention that has been recently studied using IVUS was the infusion of HDL-like complexes into patients with acute coronary syndromes (ACS). Nissen and colleagues used APOA1-Milano. This form was originally identified in 40 carriers in northern Italy who had very low levels of HDL-C, but less CAD risk than expected (144, 145), suggesting increased HDL functionality, for example by removal of more cholesterol from plaque foam cells. They found that the group treated with APOA1-Milano infusions for 5 weeks had a 1.06 percent decrease in plaque volume compared with baseline volume (144). Interestingly, they found that increasing the dose from 15 to 45 mg/kg did not improve the results (144). In a subsequent study, Tardif and colleagues used reconstituted HDL using wild-type APOA1, and found that patients treated for 4 weeks had a 3.4 percent decrease in plaque volume compared to baseline (146).

The impact of these HDL infusion results on clinical thinking, however, has been limited not only by their small size, but also by a number of recent studies in which elevations in HDL-C are not necessarily linked to reductions in major adverse cardiovascular events. These studies include mendelian-randomization genetic (147–149), as well as HDL-C raising interventions with niacin (150, 151) or cholesteryl ester transfer protein (CETP) inhibitors (152–154). These results have highlighted the differentiation of the level of HDL-C from the function of HDL particles, with the latter being considered to be more strongly associated with risk

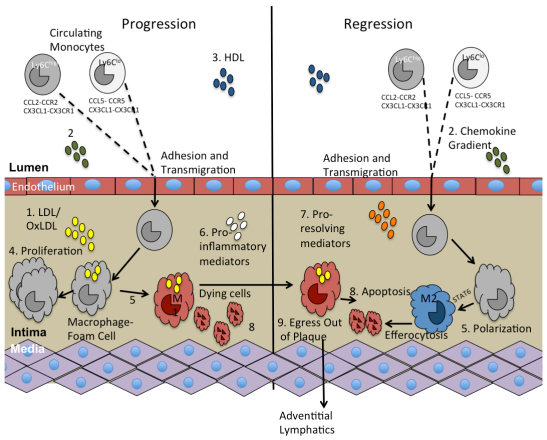

FIGURE 2 | Key molecules involved in innate immunity and atherosclerosis pathogenesis. There are multiple molecules and pathways involved in the progression of atherosclerosis that have also been implicated in atherosclerosis regression. They include: 1. LDL/oxLDL, 2. Chemokines that induce monocyte recruitment into the plaque, 3. HDL, 4. Proliferation, 5. Polarization, 6. Pro-inflammatory mediators, 7. Pro-resolving mediators, 8. Apoptosis and efferocytosis, and 9. Macrophage egress out of the plaque.

protection (155). Thus, the promise of the HDL infusion and the disappointment in the above cited mendelian randomization and intervention studies may reflect the different functional capabilities of the HDL particles depending on the method of increasing their levels in circulation (4, 155, 156). In particular, the results from the HDL infusion studies may reflect the ability of increased numbers of *functional* HDL particles to favorably remodel plaques, as we and others have observed in mouse (43, 106, 157) and rabbit models (92, 158).

In summary, observational studies going back to 1924 and in more recent intervention trials support the regressibility of atherosclerotic plaques in humans. In the intervention studies, plaque regression is typically small (<1%) with aggressive lipid reductions, yet where outcome data are available, this is associated with significantly fewer events, suggesting that plaque compositional changes, not detectable by IVUS or angiography (which predominately measure wall thickness or luminal stenosis, respectively), may be a major contributing factor. Imaging modalities to detect changes in plaque composition is a very active field (e.g., 131, 147–150). While there have been advances (such as the use of optical coherence tomography, near IR spectroscopy, and NMR) there are still limitations because of either invasiveness or limited applicability to coronary sites. Undoubtedly, further progress will be made so that coronary artery compositional changes will become more common outcomes of clinical studies. Even then, given the limited mechanistic information possible to glean from clinical studies, for the understanding of how extensive lipid lowering or other interventions can lead to plaque regression and compositional changes, as well as the identification of the molecular pathways within the plaques that may be therapeutic targets, the pre-clinical models described earlier are invaluable. These studies have led to a number of insights, particularly in regard to plaque macrophage biology, which will be reviewed next.

MACROPHAGES IN ATHEROSCLEROSIS REGRESSION

In regressing atherosclerotic plaques, we have observed that there is reduced expression of classical inflammatory genes characteristic of M1 macrophages, such as CCL2, TNFα, and iNOS. This reduction coincides with increased expression of genes encoding markers of alternatively activated, anti-inflammatory, tissue-remodeling M2 macrophages, such as Arg1, mannose receptor (MR/CD206), CD163, C lectin receptor, and IL-10 in $CD68^{+}$ cells (43, 118, 122, 124, 125). This intriguing finding is consistent with the studies in mouse models that have shown that loss of nuclear hormone receptor 77 (NUR77) or Krüppel-like factor 4 (KLF4), two factors associated with lower macrophage inflammation (i.e., a more "M2-like" phenotype) leads to more plaque inflammation and atherosclerosis progression (159–163). Additionally, the induction of IL-4 or IL-13 mediated M2 macrophage polarization also favorably changes plaque composition to a less inflammatory state and reduces atherosclerosis progression (3, 21, 164, 165). Thus, the enrichment in M2 macrophages is a signature of regressing plaques suggesting that the healthier microenvironment not only reduces the signals for macrophage activation, but also provides signals for M2 polarization. This results in reduced inflammation and favorable tissue remodeling (3), consistent with the results from altering the M1/M2 balance in progression.

We were interested in the source of the M2 macrophages in regressing plaques. We had previously observed that despite the rapid and significant lipid lowering we often used to induce regression, there was still ongoing recruitment of monocytes to the plaques (166), suggesting these new cells may be the precursors. Another possibility is that these M2 macrophages resulted from proliferation and polarization of existing macrophages already in the plaque (167, 168) that originated from circulating monocytes recruited during progression or from tissue resident macrophages of embryonic origin (yolk sac-derived) and maintained through self-renewal (169, 170). We did not find evidence of much proliferation of macrophages in regressing plaques [e.g., (44)], which focused our attention on the role of newly recruited monocytes.

Conventionally, it is thought that $Ly6C^{high}$ monocytes are the precursors of M1 macrophages, while $Ly6C^{low}$ monocytes are the precursors of M2 macrophage (22, 23, 27, 37). Using normolipidemic mice deficient in either CCR2 or CCR5, which are selectively used to recruit $Ly6C^{high}$ or $Ly6C^{low}$ monocytes, respectively into atherosclerotic plaques (22), we conclusively showed that M2 macrophages in regressing plaques were surprisingly derived from cells recruited from the $Ly6C^{high}$ circulating subset after lipid lowering (44). That the enrichment in M2 macrophages was not just associated with, but was required for, regression was demonstrated in experiments using normolipidemic mice deficient in STAT6, which as noted above, is a required factor for canonical (i.e., IL-4 or IL13-induced) M2 polarization. As will also be noted

TABLE 1 | Key molecules and therapeutic potential for resolving innate immune inflammation during atherosclerosis progression and regression.

	Examples of Molecules	Examples of Therapeutics or Potential Approaches	Effect on Plaque Progression	Effect on Plaque Regression
LDL/oxLDL		Statins, PCSK9 Inhibitors, antibodies to oxidized lipids, vaccination to oxLDL	Retarded progression in humans	Lowering LDL leads to some regression in humans and more in animal models. Antibodies to oxLDL retard progression in mice; not tested in regression
Chemokine Gradient	CCL2/MCP-1, CCL5, CX3CRL1	CCR2 Inhibitors	Inhibiting chemokine function impairs progression in mice	Inhibiting chemokine function (CCR2) impairs regression in mice
HDL	ApoA1, ABCA1, CETP	HDL Infusions (ApoA1-Milano, ApoA1- WT), Niacin, CETP Inhibitors; anti-miR33	Increasing number of functional HDL particles (HDL-P) impairs progression in mice; limited human studies agree; raising HDL-C has not been effective	Increasing HDL-P via infusions leads to some regression in humans and significant regression in mice; anti-miR33 treatment leads to significant regression in mice; being tested in non-human primates
Pro-inflammatory mediators	Cytokines, e.g., IL-1β, IL-6, IL-12, TNF-α	Canakinumab (IL-1β Inhibitor)	Lowering inflammatory cytokines impairs progression in humans and mice	Not tested
	Leuokotrienes	5-lipoxygenase activating protein inhibitor	Reducing leukotrienes impairs progression in mice; polymorphisms in the gene associated with atherosclerosis in human genetic studies	Not tested
Pro-resolving mediators	Cytokines includiing IL-4, IL-10, IL-13	The cytokines themselves delivered systemically or as part of targeted nanoparticles	Timing and context matter but in general decreasing anti-inflammatory cytokines promotes, and increasing them retards, progression in mice	STAT6 (downstream of IL-4 and IL-13) required for regression in mice
	Lipoxins, resolvins, maresins, protectins	Treatment with annexin 1, resolvin D1, resolvin D2, maresin 1, and resolvin E1 systemically or as part of targeted nanoparticles	Increasing pro-resolving mediators impairs progression in mice	Not tested
Apoptosis/efferocytosis		Stimulators of efferocytosis, such as LXR agonists delivered as targeted nanomedicines given hepatic toxicity when given systemically	Early apoptosis and active efferocytosis can retard plaque progression but apoptosis and failed efferocytosis at advanced stages promote plaque progression by contributing to necrotic core formation in mice	Not tested
Emigration/Egress Out of Plaque	Netrin 1, CCR7	Delivery of siRNA or anti-sense oligonucleotides to netrin 1 in targeted nanoparticles	Netrins inhibit emigration of macrophages and promote progression in mice	Blocking CCR7 leads to impaired emigration of macrophages and impairs regression in mice

in the next section, these results point to the limitations of lipid lowering alone to achieve atherosclerosis regression, as when this was achieved without being able to enrich in M2 macrophages, regression was impaired. The results also stimulated us to seek the nature of the signals that were responsible for the polarization of the newly recruited monocytes.

INFLAMMATION RESOLUTION AS A THERAPEUTIC TARGET AND ITS CLINICAL RELEVANCE

Besides the pre-clinical findings just reviewed, there is also ample evidence from the clinical literature to argue that new directions are clearly needed to augment the traditional approach of lowering LDL-C by statins in order to achieve atherosclerosis regression and cardiovascular risk reduction. This point was driven home by a summary of the results of 6 "mega-trials" of statins- the Scandinavian Simvastatin Survival Study (4S), the Cholesterol And Recurrent Events (CARE) study, the Air Force Coronary Atherosclerosis Prevention Study/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS), the Long-term Intervention with Pravastatin in Ischemic Disease study (LIPID) study, and the Heart Protection Study (HPS). While all showed reductions in coronary heart disease events, two thirds of patients still experienced events while on statin treatment, clearly demonstrating that LDL-C lowering alone does not lead to optimal therapeutic benefit (7).

The recent Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial (171) has brought to clinical focus what our pre-clinical studies have shown, namely that the benefits of LDL-C lowering are enhanced when inflammation is reduced. In this 2 year study, patients at high risk of CAD were treated with statins to lower LDL-C to 82 mg/dL, with half also receiving an antibody to the potent inflammatory mediator IL-1β. Indeed, there were decreased events in those in the antibody group, which demonstrated the clinical importance of the "residual risk of inflammation" that persisted after LDL-C lowering in some patients, particularly those whose levels of CRP, considered to be a biomarker of inflammation, were ≥2 mg/dL (171, 172). The CANTOS results have antecedents in the Pravastatin or Atorvastatin Evaluation and Infection Therapy–Thrombolysis in Myocardial Infarction 22 (PROVE IT–TIMI 22) study (172). After intensive statin therapy, Ridker and colleagues found there were 4 groups of patients with differential risk of having

a recurrent heart attack – (1) LDL ≥70 mg/dL, CRP ≥2 mg/L (highest risk), (2) LDL ≥70 mg/dL, CRP <2 mg/L, (3) LDL <70 mg/dL, CRP ≥2 mg/L, and (4) LDL <70 mg/dL, CRP <2 mg/L (lowest risk). Intriguingly, they found that patients with LDL ≥70 mg/dL, CRP <2 mg/L or LDL <70 mg/dL, CRP ≥2 mg/L had similar risk of recurrent risk, which suggested that heightened inflammation impeded the benefits of LDL-C lowering (172), consistent with the CANTOS results and our pre-clinical findings (44, 171).

Given the direct evidence it provides in humans, the CANTOS trial has catapulted the interest in clinical approaches to reducing plaque inflammation and promoting its resolution, whose benefits have heretofore been heavily based in pre-clinical settings. Potential therapies, in addition to enhancing STAT6-induced M2 macrophage polarization, could include using plaque targeted therapies to increase lipid-derived (e.g., resolvins) and protein mediators (e.g., cytokines) to promote resolution, by blocking inflammatory cell influx, promoting their egress, clearing pathogens and cellular debris, reducing inflammatory cytokines, increasing the clearance of dying macrophages by efferocytosis, and repairing tissue damage by creating an environment that promotes tissue remodeling M2 macrophage polarization (173, 174). In parallel developments, nano-vehicles to direct some of these therapies directly to plaques are being intensely pursued. These will allow not only more potent attacks on inflammation, but have the potential to avoid systemic adverse effects, such as the increased fatal infections observed with systemic IL-1β antagonism in the CANTOS trial.

CONCLUDING REMARKS

It has been considered for some time [reviewed in, e.g., (15, 18, 175)] that atherosclerosis plaque progression, like many inflammatory processes, begins as a typical defense mechanism against a threat, in this case retained APOB-lipoproteins. For the reasons discussed above, there are a number of unfortunate events, such as the relentless entry of lipoproteins (which in addition to the lipids they bring, give rise to PAMPS), and the ensuing tissue damage, which produces DAMPS. These phenomena both amplify the innate immune responses and extend the mayhem to involve adaptive immunity. Thus, the normally homeostatic resolution of inflammation does not occur. As we have reviewed, progress in understanding the immunology of atherosclerosis based on pre-clinical models of progression and regression, coupled with advances in clinical investigations, puts the field well on its way towards the goal of achieving this homeostasis, which will result in improved primary and secondary prevention strategies and a reduction in atherosclerotic cardiovascular disease. In **Figure 2** and **Table 1**, we have summarized some of the pathways and factors implicated in potentially limiting, or even better, resolving plaque inflammation.

AUTHOR CONTRIBUTIONS

KR and EF wrote and edited the manuscript, and also created all figures.

FUNDING

The research from the authors' laboratory that is described in this review was supported by NIH grants R01 HL084312, R01 HL129433, P01 HL098055, T32 GM007308, T32 AI100853, and F30 HL131183.

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

frontiers in Cardiovascular Medicine

MINI REVIEW
published: 05 June 2018
doi: 10.3389/fcvm.2018.00062

Antiinflammatory Therapy in Clinical Care: The CANTOS Trial and Beyond

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Inflammation is a critical pathway in the pathogenesis of atherosclerosis. Previous studies have shown that plasma levels of high-sensitivity C-reactive protein (hsCRP), a marker of inflammation, are associated with cardiovascular disease independent of traditional risk factors. Randomized trial data have also shown that statins reduce not only hsCRP but also cardiovascular event rates independent of their effect on low-density lipoprotein cholesterol (LDL-C) level. More recently, the CANTOS trial showed that directly reducing inflammation with canakinumab, an interleukin (IL)-1β neutralizing monoclonal antibody, could also reduce cardiovascular event rates. These mark the first phase 3 trial results validating inflammation as a viable target for preventing cardiovascular disease. In this review, we recap the role of inflammation in cardiovascular disease and highlight previous trial data showing its modulation with statins and other agents. We also detail the CANTOS trial results and discuss its implications for clinicians as well as future directions for anti-inflammatory therapy in the prevention of cardiovascular disease.

Keywords: vascular inflammation, atherosclerosis, canakinumab, prevention, randomized trials

OPEN ACCESS

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

Received: *21 March 2018*
Accepted: *16 May 2018*
Published: *05 June 2018*

Citation:
Aday AW and Ridker PM (2018) Antiinflammatory Therapy in Clinical Care: The CANTOS Trial and Beyond. Front. Cardiovasc. Med. 5:62. doi: 10.3389/fcvm.2018.00062

INTRODUCTION

Atherosclerosis is the central disease process underlying most instances of myocardial infarction (MI), ischemic stroke, and peripheral artery disease. Fortunately, cardiovascular medicine has witnessed the development of numerous therapies for the primary and secondary prevention of atherosclerotic disease. Beyond diet, exercise, and smoking cessation, intensive lowering of low-density lipoprotein cholesterol (LDL-C) remains the fundamental preventive tool for individuals at high risk. Accordingly, current professional guidelines recommend aggressive statin therapy in such patients (1, 2).

Despite the success of LDL-C lowering, researchers have long known that atherosclerosis is not solely a disease of cholesterol deposition and that inflammation plays a key role in its pathogenesis. Leukocytes are recruited to early atherosclerotic lesions by proinflammatory cytokines and, through endothelial adhesion and transmigration, help initiate plaque formation (3). As plaques evolve, activated macrophages and T cell lymphocytes colocalize within atherosclerotic plaques to sustain a local inflammatory response (4). Additionally, activated leukocytes drive smooth muscle cell proliferation and extracellular matrix deposition as plaques continue to mature (5). Inflammation is instrumental not just in plaque development but also plaque rupture. A greater accumulation of macrophages and lymphocytes within plaques is associated with an increased risk of plaque rupture (6). This is mediated in part by macrophage release of matrix metalloproteinases capable of degrading the fibrous cap of plaques as well as signals that impair collagen synthesis and, thus, plaque repair and reinforcement (6).

In addition to histologic and immunologic evidence documenting the role of inflammation in atherosclerosis, numerous clinical studies have also examined this association. Data have shown that elevated levels of high sensitivity C-reactive protein (hsCRP), a marker of systemic inflammation, are associated with traditional cardiovascular risk factors, including hypertension (7), type 2 diabetes (8), and obesity (9). However, studies also show this risk association for hsCRP persists independent of such risk factors. For instance, among 1,086 healthy men followed prospectively as part of the Physician's Health Study, individuals with levels of hsCRP in the highest quartile had a 2.9-fold greater risk of MI than those in the lowest quartile; this risk was independent of traditional lipid and non-lipid risk factors (10). Other prospective cohorts have found similar risk associations between elevations in hsCRP and incident cardiovascular disease (CVD) in both men and women (11, 12).

Clinical trial data have shown that statins have a beneficial effect in terms of inflammation reduction. The CARE study was a randomized, placebo-controlled trial of pravastatin 40 mg daily among 4,159 individuals with a history of MI and elevated levels of cholesterol (LDL-C 115–175 mg/dL) (13). In a cohort of 472 randomly selected individuals from the trial, those treated with pravastatin experienced a mean decrease in hsCRP of 21.6% over 5 years of follow up compared to placebo (14). Similarly, among 1,702 patients with no history of cardiovascular disease in the PRINCE study, those randomized to pravastatin 40 mg daily experienced a 16.9% reduction in hsCRP at 24 weeks compared to no reduction in the placebo group (14). These effects were independent of LDL-C reduction.

Therapy with more potent statins has a more pronounced effect on hsCRP. In the JUPITER study, 17,802 men and women free of CVD with low levels of LDL-C (<130 mg/dL) but elevated levels of hsCRP ($\geq$ 2.0 mg/L) were randomized to either rosuvastatin 20 mg daily or placebo (15). Overall, rosuvastatin led to a 37% median hsCRP reduction ($p < 0.0001$) compared to placebo (16). Similarly, PROVE-IT TIMI 22 randomized 3,745 individuals with an acute coronary syndrome (ACS) to either atorvastatin 80 mg or pravastatin 40 mg daily with a primary outcome of recurrent MI or coronary-related death (17). Following 30 days of therapy, 57.5% of individuals treated with atorvastatin achieved an hsCRP < 2 mg/L compared to 44.9% of those treated with pravastatin (18).

Other classes of drugs have also been studied in terms of their impact on hsCRP reduction. In the FOURIER trial, 27,564 individuals with stable atherosclerotic disease and an LDL-C $\geq$ 70 mg/dL on statin therapy were randomized to evolocumab, a PCSK9 monoclonal antibody, or placebo (19). After a median of 2.2 years, therapy with evolocumab had no impact on hsCRP (median reduction 0.2 mg/L) (20). Similar results were seen with bococizumab, another PCSK9 monoclonal antibody (21). Randomized controlled trial data on fibrates and hsCRP are limited, but a meta-analysis of 16 trials and 1,635 patients found a 0.47 mg/L reduction in hsCRP with fibrates compared to placebo ($p = 0.046$) (22).

Beyond the impact of statins on hsCRP, additional trial data indicate a clinical benefit in terms of reducing cardiovascular events. Within PROVE-IT TIMI 22, individuals who achieved hsCRP levels < 2 mg/L had lower event rates regardless of the degree of achieved LDL-C lowering (23). Indeed, the reduction in event rates was nearly identical whether individuals achieved an LDL-C < 70 mg/dL or hsCRP < 2 mg/L. Similar results were seen with a combination of simvastatin and ezetimibe in IMPROVE-IT (24). The JUPITER study further tested the association between inflammation and cardiovascular disease in terms of primary prevention (15). After a median follow-up of 1.9 years, rosuvastatin led to a 54% reduction in MI, a 48% reduction in stroke, and a 20% reduction in all-cause mortality.

JUPITER did not address whether inflammation reduction in the absence of cholesterol lowering might reduce vascular event rates. To answer this question, a randomized trial was needed in which therapy targeting the IL-1 to IL-6 to CRP signaling pathway (25) with no effects on atherogenic lipids could be administered. This was the fundamental design issue that resulted in the Canakinuamb Anti-inflammatory Thrombosis Outcomes Study (CANTOS).

Among cytokines that mediate inflammation, the interleukin-1 (IL-1) family of proteins, which includes both IL-1α and IL-1β isoforms, emerged as a leading candidate for CANTOS. Inflammatory signals involved in atherosclerosis, including cholesterol crystals, hypoxia, and turbulent flow, activate the NLRP3 inflammasome, which is a multi-protein assembly that integrates these signals and specifically activates the IL-1β isoform (26). Once activated, IL-1 has several important roles in the pathogenesis of atherosclerosis. IL-1 stimulates vascular endothelial cells to express cell surface proteins that increase inflammatory cell adhesion (27). Additionally, IL-1 triggers increased vascular smooth muscle cell proliferation (28). IL-1 also upregulates IL-6, another pro-inflammatory cytokine that induces hepatocytes to synthesize and release different acute phase reactants, including CRP, fibrinogen, and plasminogen activator inhibitor (26). Mutations in inflammasome proteins are known to cause hereditary periodic-fever syndromes, including Muckle-Wells syndrome and familial cold urticarial (29). These mutations lead to dysregulated activation of IL-1β and cause severe episodes of inflammation and fever in affected individuals. Thus, IL-1β lies far enough upstream in the inflammation pathway that modulation of these proteins could dramatically impact numerous inflammatory components of atherosclerosis.

Several drugs have been developed to target IL-1 signaling. Anakinra, an IL-1 receptor antagonist, down regulates signaling through both IL-1α and IL-1β isoforms and is used to treat rheumatoid arthritis (30). Canakinumab is a fully human IL-1β neutralizing monoclonal antibody that was previously granted orphan drug status for treatment of Cryopyrin-Associated Periodic Syndromes, including Muckle-Wells Syndrome (30). In terms of CVD prevention, canakinumab was particularly attractive since known atherosclerosis risk factors upregulate IL-1β via the NLRP3 inflammasome. Additionally, canakinumab may be less likely to impair host immune function since signaling via IL-1α remains intact. In a pilot study performed in preparation for CANTOS among 556 diabetic individuals at high risk for CVD, canakinumab led to a significant decrease in

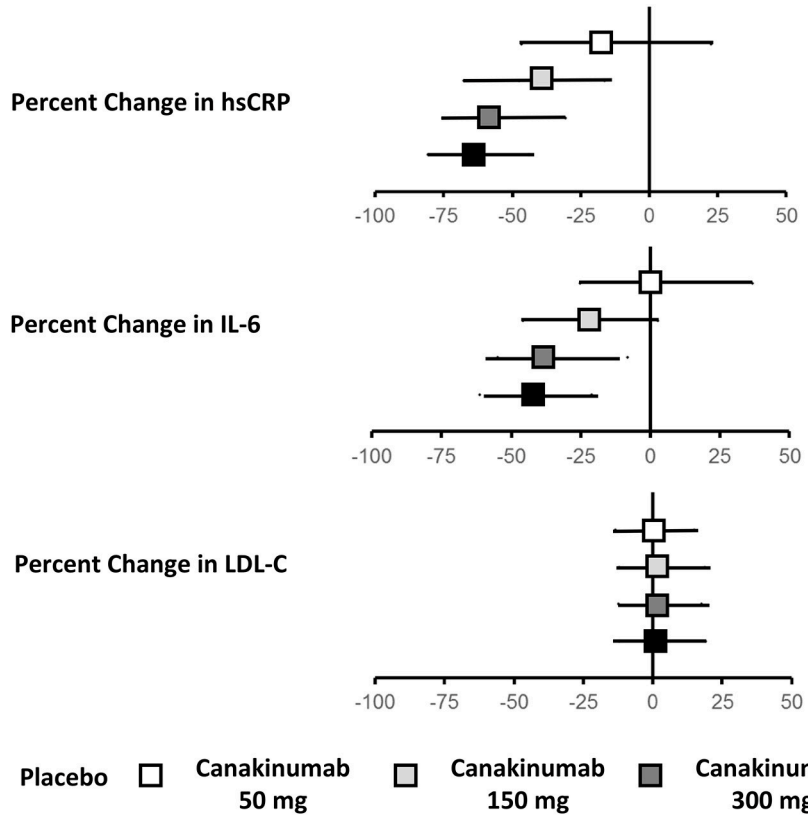

FIGURE 1 | Effects of canakinumab on hsCRP, IL-6, and LDL-C in CANTOS.Ridker (34).

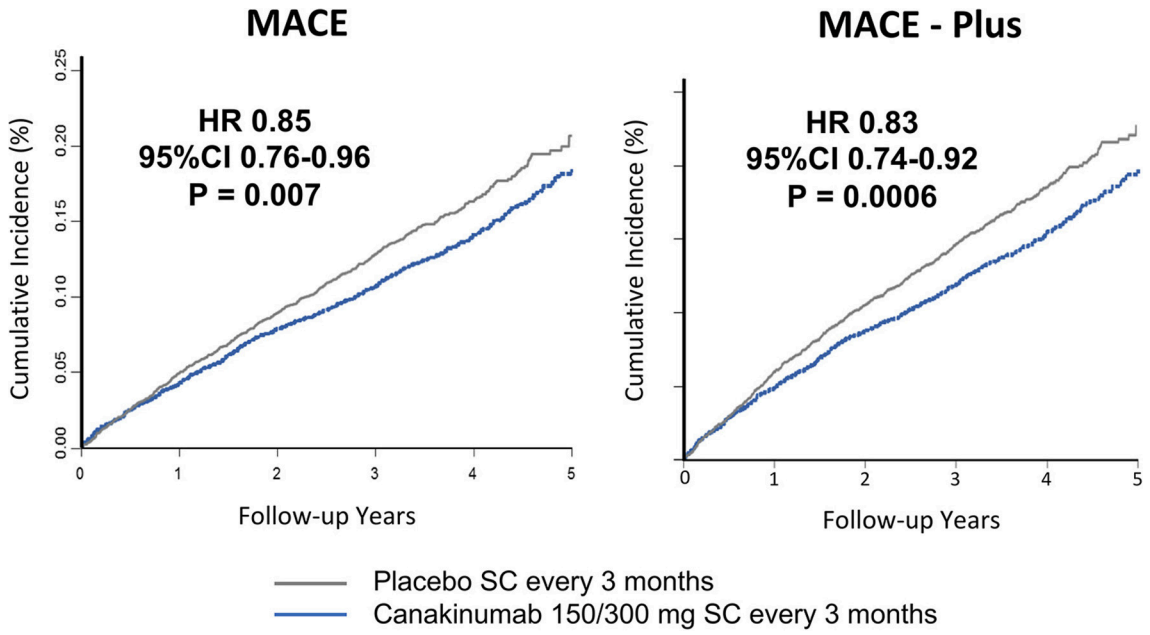

FIGURE 2 | Cumulative endpoints for individuals receiving 150 or 300 mg subcutaneous canakinumab every 3 months vs. placebo in CANTOS. **(Left)** Primary endpoint of myocardial infarction, stroke, or cardiovascular death (MACE). **(Right)** Secondary endpoint additional including hospitalization for unstable angina requiring urgent revascularization (MACE-plus). HR, hazard ratio; CI, confidence interval; SC, subcutaneous. Ridker et al. (35).

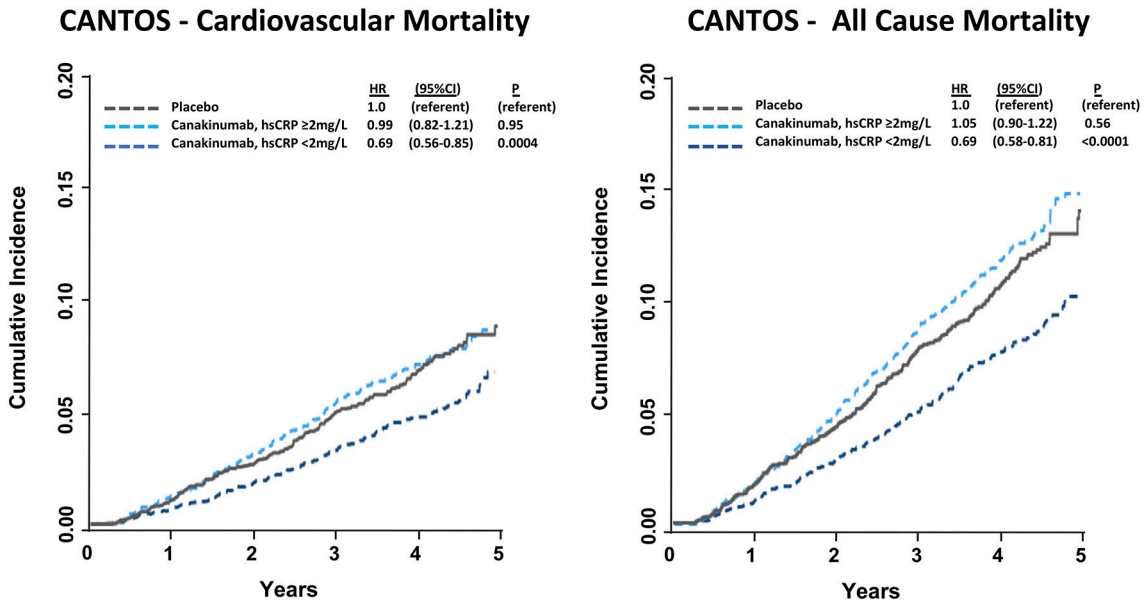

FIGURE 3 | Cumulative incidence and hazard ratios of cardiovascular mortality (**Left**) and all-cause mortality (**Right**) among CANTOS participants allocated to either placebo or canakinumab according to whether post-randomization on-treatment hsCRP levels were above or below 2 mg/L. Hazard ratios are adjusted for age, sex, smoking status, hypertension, diabetes, body mass index, baseline concentration of hsCRP, and baseline concentration of LDL-C. HR, hazard ratio; CI, confidence interval. Ridker (37).

hsCRP, fibrinogen, and IL-6 with no impact on LDL-C or other lipid measures (31).

CANTOS was a randomized, double-blind, placebo-controlled trial of canakinumab in 10,061 patients with a history of MI and hsCRP $\geq$ 2 mg/L; such patients with "residual inflammatory risk" rather than "residual cholesterol risk" are a common and very high risk group (32, 33). Individuals with a history of chronic or recurrent infections, cancer other than basal-cell skin carcinoma, an immunocompromised state, history of tuberculosis or human immunodeficiency virus, or current use of additional anti-inflammatory medications were excluded from participating in the trial. Three different doses of subcutaneous canakinumab were used: 50, 150, and 300 mg given every 3 months. The primary endpoint was a composite of non-fatal MI, non-fatal stroke, or cardiovascular death (MACE). There was an additional pre-specified secondary endpoint of MACE along with hospitalization for unstable angina requiring urgent revascularization (MACE+).

Among study participants, the mean age was 61 years, and nearly 75% were male. Approximately 40% had a history of diabetes, and 81% had undergone percutaneous or surgical coronary revascularization. More than 93% were receiving lipid lowering therapy at baseline with a mean entry LDL-C of 82 mg/dL, and 95% of individuals were taking either an antiplatelet agent or an anticoagulant. The median hsCRP at baseline was 4.2 mg/L.

Treatment with canakinumab led to a significant decrease in both hsCRP and IL-6. At 48 months, median hsCRP was reduced by 26–41% in a dose-dependent manner. Similarly, at 12 months, median IL-6 was reduced by 19–38%. Drug treatment had no impact on LDL-C or high-density lipoprotein cholesterol (HDL-C) and a modest 4–5% increase in median triglyceride levels (**Figure 1**).

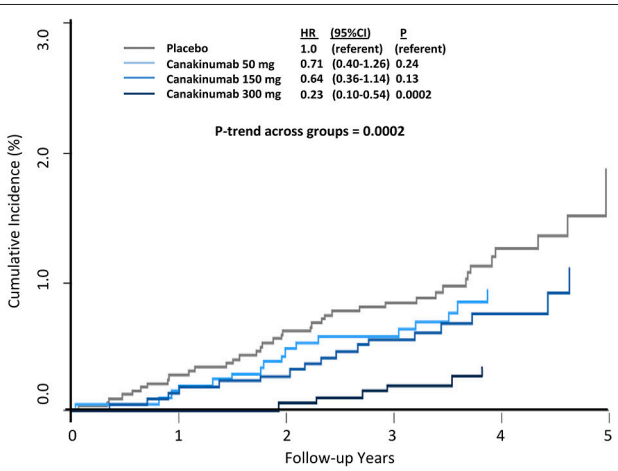

FIGURE 4 | Cumulative incidence of fatal lung cancer among CANTOS participants. HR, hazard ratio; CI, confidence interval. Ridker et al. (38).

Study participants were followed for a median of 3.7 years. Individuals receiving either the 150 or 300 mg doses of canakinumab experienced a 15% reduction in MACE ($P = 0.007$); there was a non-significant 7% reduction in the primary endpoint for those receiving the 50 mg dose. Similar significant reductions in MACE+ were seen in the higher dose groups

(17%, $P = 0.0006$) with a non-significant 10% reduction in the 50 mg group (**Figure 2**). Subgroup analyses showed no evidence of effect modification by sex, age, history of diabetes, smoking history, body-mass index, or baseline levels of lipids or hsCRP (36).

In a pre-specified analysis, canakinumab efficacy was found to differ considerably based upon the magnitude of inflammation reduction achieved by individual trial participants. Individuals with hsCRP concentrations < 2 mg/L after the first dose of canakinumab experienced a 25% reduction in MACE ($P < 0.0001$) compared to a non-significant 5% reduction in those with on-treatment hsCRP levels ≥ 2 mg/L. These differences persisted even after adjusting for potential confounders, including baseline hsCRP and LDL-C as well as clinical risk factors including age, sex, smoking history, hypertension, diabetes, and body-mass index, and were consistent in causal inference analyses. Additionally, individuals with a reduction in hsCRP < 2 mg/L also experienced a significant 31% reduction in cardiovascular death ($P = 0.0004$) and all-cause mortality ($P < 0.0001$); those with on-treatment hsCRP level ≥ 2 mg/L did not have a significant reduction in these endpoints (**Figure 3**). Within the overall trial, the number needed to treat for the 5-year composite endpoint of MI, stroke, coronary revascularization, or all-cause mortality was 24 (36). This number was 16 for those with on-treatment hsCRP levels < 2 mg/L and 57 for individuals with on-treatment hsCRP levels ≥ 2 mg/L.

Overall, canakinumab was tolerated well with essentially identical discontinuation rates compared to placebo. Mild neutropenia and thrombocytopenia were slightly more common in those treated with canakinumab. Rates of death due to infection or sepsis were low but more likely in the canakinumab group compared to placebo (incidence rate 0.31 vs. 0.18 per 100 person-years, $P = 0.02$). In terms of the types of infections that occurred during follow up, only pseudomembranous colitis was more common in the canakinumab group; no evidence of opportunistic infection was observed, data emphasizing that canakinumab is not a clinically immunosuppressive intervention. Further demonstrating this issue, random allocation to canakinumab as compared to placebo in CANTOS resulted in large and highly significant dose-dependent reductions in cancer fatality, incident lung cancer, and fatal lung cancer (**Figure 4**) (38).

With the publication of the CANTOS results, clinicians have definitive evidence that directly targeting inflammation is beneficial for the secondary prevention of cardiovascular disease, and this benefit is independent of cholesterol. This serves as a reminder that there are emerging therapeutic options for individuals with residual inflammatory risk, as defined by persistently elevated levels of hsCRP despite adequate LDL-C lowering (32). Successfully addressing traditional CVD risk factors should not falsely reassure clinicians and patients that nothing more can be done to further reduce risk, and this is particularly relevant given that CVD remains the leading global cause of mortality despite significant advances in terms of pharmacologic and lifestyle interventions for both primary and secondary prevention.

Beyond CANTOS, there are several ongoing trials of other anti-inflammatory agents, including low-dose methotrexate in the Cardiovascular Inflammation Reduction Trial (39), colchicine in the LoDoCo2 and COLCOT trials, as well as proposed trials involving other modulators of IL-1, IL-6, and the NLRP3 inflammasome. Although the vast majority of individuals in CANTOS were receiving lipid lowering therapy with low baseline levels of LDL-C, it remains to be seen whether anti-inflammatory therapy would be beneficial among patients on PCSK9 inhibitors with even lower LDL-C levels.

AUTHOR CONTRIBUTIONS

AA was responsible for conceptualization and drafting of the manuscript. PR was responsible for conceptualization and editing of the manuscript.

FUNDING

This work was supported by NIH T32 HL007575 (AA).

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Conflict of Interest Statement: PR served as the Principal Investigator of CANTOS and received research grant support to conduct CANTOS from its sponsor, Novartis. PR also served as the Trial Co-Chair for the SPIRE studies of bococizumab and received research grant support to conduct these trials. PR has also served as a consultant to Novartis, Pfizer, and Sanofi, and is listed as a co-inventor on patents held by the Brigham and Women's Hospital that relate to the use of inflammatory biomarkers in cardiovascular disease and diabetes that have been licensed to AstraZeneca and Seimens.

The other author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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