Recent highlights of ATVB: macrophages.

Ziad Mallat, MD, PhD

From the Department of Medicine, Division of Cardiovascular Medicine, University of Cambridge, Cambridge, UK, and
Institut National de la Santé et de la Recherche Médicale, U970, Paris, France.

Correspondence to: Ziad Mallat, MD, PhD, at Department of Medicine, Division of Cardiovascular Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, CB2 2QQ, UK. E-Mail: zm255@medchl.cam.ac.uk.

Introduction:
Macrophages are the building blocks of intimal atherosclerotic lesions where they scavenge and accumulate modified lipoproteins, profoundly impacting lesion development and progression through their roles in lipid metabolism and as major sensors and effectors of the innate immune system [1]. Lesional macrophages originate from circulating monocytes, although more recent work indicates that a subpopulation may also derive from dedifferentiated vascular smooth muscle cells [2,3]. Resident macrophages are found in the heart at steady state and along with newly recruited monocyte-derived macrophages accumulate after myocardial infarction [4,5]. Macrophage turnover in atherosclerotic lesions and ischemic myocardium is under the control of several biological processes, including monocyte recruitment, macrophage proliferation and apoptosis as well as macrophage egress [6,7]. Moreover, local microenvironment whether in health or disease profoundly impacts macrophage phenotype [8,9] and consequently, both metabolic and immune-related responses [10]. Here, I summarize recent work published in *ATVB* on the roles of monocytes/macrophages in cardiovascular diseases (CVD) and review it in light of the most recent advances in this research area.

Monocytes, macrophages and the pathogenesis of atherosclerosis:
Monocyte activation/recruitment and macrophage trapping within the lesions: monocytes play central roles in atherogenesis and are rapidly attracted to sites of disturbed flow characterized by low-grade inflammation [11]. The use of advanced 10-fluorochrome, 13-prameter flow cytometry method recently allowed detailed characterization of the dynamics of immune cell accumulation during flow-induced (partial carotid artery ligation) atherogenesis in *Apoe*$^{-/-}$ mice. The study showed that monocytes/macrophages constitute the most abundant cell population in the intima where they rapidly accumulate as early as 4 days after induction of flow disturbance, peak at day 7 and remain relatively stable between day 14 and day 28 [12]. Disturbed flow promotes endothelial cell activation in part through repression of major anti-adhesive and anti-thrombotic transcription factors, Klf2 and Klf4 [13-16]. In fact, a single microRNA, mir-92a, coregulates Klf2 and Klf4 expression and contributes to the heterogeneity of endothelial cell phenotype in atheroprone versus atheroresistant arterial sites [17-19]. Hypercholesterlemia combined with low shear stress markedly upregulates endothelial expression of mir-92a in a Stat3-dependent manner. Blockade of mir-92a restores Klf2 and Klf4 expression, reduces endothelial inflammation and monocyte infiltration, thereby limiting atherosclerosis [19]. Disturbed flow as well as other pro-atherogenic factors also downregulate the endothelial expression of negative guidance cues, particularly Netrin-1 and Semaphorin3A, while upregulating EphrinB2, and facilitate chemokine-directed migration of monocytes and their infiltration within the intima [20]. Interestingly, Klf2, Klf4 and Netrin-1 are also expressed in monocytes/macrophages and regulate their phenotype/function during atherogenesis [21-24]. Klf2 and Klf4 limit monocyte/macrophage activation in part through the regulation of selective microRNAs (mir-124a, mir-150) [25] and promote an alternatively activated M2 phenotype associated with reduced susceptibility to atherosclerosis [22,23]. However, Netrin-1 expression in macrophages, which is highly upregulated by hypoxia-inducible transcription factor (HIF)-1α [26], promotes the progression of atherosclerosis through its profound impact on macrophage migration, limiting their emigration and retaining them within the developing atherosclerotic lesions [24]. The same group recently extended this concept to another guidance molecule, Semaphorin3E, and showed that it's upregulated in M1 macrophages where it blocks actin polymerization and migration in response to chemokine stimulation, thereby contributing to macrophage retention within the lesions [27]. Of note, low expression of Netrin-1 and Unc5b in macrophages and smooth muscle cells of human atherosclerotic lesions was associated with signs of plaque instability [28].

Recent studies have re-emphasized the subtle but crucial roles of post-translational glycosylation of endothelial cell or leukocyte ligands and receptors involved in cell-cell interactions, [29]. Disturbed flow and pro-inflammatory mediators increase hypoglycosylated, high-mannose and hybrid N-glycans on the endothelial cell surface at sites of early lesion development, which leads to increased monocyte adhesion [30]. On the other hand, sialyltransferase IV activity, which catalyzes the formation of a sialylated sLe^x, appears to promote monocyte recruitment into inflamed arteries and promotes atherogenesis through its essential role in the generation of a functional Ccr5 receptor [31], suggesting that selective targeting of these post-translational events may constitute an interesting therapeutic option. In addition to Ccr5, signaling events through Ccr2 and Cx3cr1 play non-redundant roles in atherosclerosis [32-34]. Interestingly, more recent studies on Cx3cr1 suggest upregulation of this chemokine receptor on platelets in response to hyperlipidemia, which promotes platelet-monocyte complex formation and supports monocyte arrest on inflamed vascular cells, thereby identifying a new mechanism leading to platelet accumulation and monocyte recruitment at sites of arterial injury [35]. Pharmacological targeting of Cx3cr1 through the administration of an amino terminus-modified Cx3cr1 ligand endowed with antagonist activity substantially limits monocyte trafficking into lesions and halts the development and progression of atherosclerosis [34].

Monocyte differentiation into macrophages is a pivotal process in atherogenesis and is regulated through a coordinated developmental program dependent on the balance between several transcription factors, including MafB, c-Maf, Icsbp/Irf8, Klf4, Pu.1 and the macrophage-specifying zinc finger transcription factors Egr-1 and Egr-2 [36,37]. A recent study published in *ATVB* proposed caveolin-1, the structure protein of caveolae, as a critical regulator of monocyte differentiation through Erk phosphorylation, leading to increased Egr-1 nuclear translocation and transcriptional activity [38]. A broader view of the molecular mechanisms controlling monocyte activation and priming towards the macrophage phenotype is emerging. Recent studies identified distinct dynamic epigenetic, transcriptional and metabolic programs controlling monocyte differentiation towards either tolerant or activated 'trained' phenotypes [39,40]. This is highly consistent with a more focused work showing that brief exposure of monocytes to oxLDL increases trimethylation of lysine 4 at histone 3 in promoter regions of several pro-inflammatory mediators and scavenger receptors (CD36, SR-A) leading to foam cell formation and long-term proinflammatory cytokine production [41] via epigenetic reprogramming. Such sustained changes in monocyte/macrophage phenotype may further promote and sustain immune cell activation. For example, CD36-dependent activation of monocytes generates an intracellular transduction pathway leading to Rac and Jnk2 activation downstream of the guanine nucleotide exchange factor Vav1, which contributes to enhanced monocyte adhesiveness to inflamed endothelium [42]. This is reminiscent of the role of CD36 in the trapping of macrophages within atherosclerotic lesions previously shown to be dependent on alteration of cytoskeletal dynamics through sustained activation of Fak and reduced migration [43]. Interestingly, AT1 receptor activation may upregulate CD36 expression on macrophages, sustain Fak activity and contribute to macrophage trapping in the intima, leading to heightened plaque inflammation and increased susceptibility to rupture [44]. In this regard, a more recent study indicates that ACE, the enzyme that converts angiotensin (Ang)I into AngII, is highly expressed in lesional macrophages where it contributes to lesion development [45], although the effect was modest and limited to the aortic arch. Superimposed metabolic stress factors, like high LDL and D-glucose concentrations may further prime monocytes and enhance their chemotaxis through increased S-glutathionylation and remodeling of actin downstream of NADPH oxidase 4. In contrast, regulators of lipid

metabolism such as Apoe may suppress atherosclerosis through reduced lipid accumulation in monocytes and downregulation of monocyte activation, independently of their lipid-lowering properties [46].

More on foam cell formation and macrophage activation/deactivation: Monocyte differentiation into macrophages is associated with upregulation of phagocytic activity leading to lipid accumulation and formation of typical foam cells. Recent findings suggest that cardiovascular risk factors other than elevated plasma cholesterol levels may significantly modulate macrophage foam cell formation. For example, the endogenous nucleoside adenosine, which is released extracellularly under stress conditions, profoundly impacts foam cell formation through G protein coupled receptor A(2A)-dependent regulation of reverse cholesterol transport (reviewed in [47]). Increased expression of xanthine oxidoreductase (XOR), a key enzyme in the uric acid production pathway, is upregulated in many cardiovascular disease settings and localizes to macrophages. Recent data indicate that overexpression of XOR promotes foam cell formation through upregulation of VLDL and scavenger receptors and downregulation of ATP-binding cassette transporters (ABCA1 and ABCG1) along with induction of a pro-inflammatory phenotype, all effects being prevented by treatment with allopurinol, which markedly limits lipid accumulation and lesion calcification in aortas of *Apoe*$^{-/-}$ mice [48]. Hypertriglyceridemia is an important cardiovascular risk factor and triglyceride-mediated pathways are causally related to coronary disease [49]. Bojic et al show that VLDL treatment of macrophages leads to triglyceride accumulation and induces AP1-dependent pro-inflammatory cytokine production, downstream of Erk1/2 and Akt/Foxo1. PPARδ agonists attenuated VLDL-stimulated triglycerides accumulation through reduction of lipoprotein lipase (LPL) activity, increased fatty acid uptake and enhanced β-oxidation, and inhibited VLDL-dependent inflammation through normalization of Erk and Akt/Foxo1 signaling [50], which led to an M2 macrophage phenotype and limited the progression of pre-established atherosclerosis in mice [51]. Angiopoietin-like 4 (ANGPTL4) is an endogenous inhibitor of LPL. Nonsynonymous variants in ANGPTL4 were initially associated with reduced triglycerides levels and increased HDL [52]. However, subsequent larger studies failed to establish a correlation between ANPTL4 variants or plasma levels and serum triglycerides, although plasma ANGPTL4 negatively associated with HDL-cholesterol [53]. Therefore, an uncertainty remained regarding the potential impact of such variants on cardiovascular disease. A recent work in *ATVB* clearly shows that transgenic overexpression of Angptl4 suppresses foam cell formation and significantly reduces atherosclerosis in mice despite no alteration of plasma cholesterol or triglycerides levels [54], supporting a protective effect of Angptl4 and a potential impact of ANGPTL4 variants on CVD beyond any role on serum lipid levels.

Macrophage foam cell formation and inflammation are prominent features of atherosclerotic lesions. However, recent work indicates that cholesterol accumulation does not invariably lead to inflammation. In fact, analysis of elicited peritoneal macrophages in WT and *Ldlr*$^{-/-}$ mice fed either a chow or a high fat high cholesterol diet revealed massive reprogramming of the lipidome in response to both diet and genotype, and an unexpected deactivation of the inflammatory response in the macrophage foam cells [55]. The underlying mechanisms involved regulated accumulation of desmosterol in foamy macrophages, which was responsible for downstream activation of LXR and inhibition of SREBP target genes, leading to selective reprogramming of fatty acid metabolism and the establishment of an anti-inflammatory homeostatic response [55]. In line with these observations, Suzuki M et al were unable to detect any evidence for increased expression of inflammatory proteins after in vitro loading of peritoneal macrophages with acetyl-LDL, and demonstrated that sterol loading blunted the macrophage response to LPS without altering the overall pattern of LPS-induced gene

expression [56]. However, their use of transcriptomics and proteomics to investigate molecular changes induced in cholesterol-loaded macrophages revealed a differential activation, mainly through post-transcriptional mechanisms, of 3 functional modules corresponding to lipid metabolism, lysosomal biology and, unexpectedly, complement activation, both the classic and the alternative pathways [56]. Thus, despite no direct induction of an inflammatory program in foam cell macrophages, complement regulation by sterol loading may have important consequences on the immune response and the development of atherosclerosis [57]. It should also be noted that enrichment of monocyte/macrophages with unesterified cholesterol generates biologically active PS-expressing microvesicles that may carry not only a thrombotic potential but also danger signals like peroxides, induced through activation of mitochondrial complex II and exported on the microvesicles, and capable of disseminating maladaptive immune responses through activation of pattern recognition receptors [58]. The true clinical in vivo relevance of these pathways is still unexplored.

Innate Inflammatory signaling pathways: An intriguing role for TLR7/9 signaling in foam cell formation has been reported recently [59] beyond its role in macrophage activation. However, the molecular mechanisms behind this observation remain to be elucidated. Other work has expanded on the role of inflammatory signaling in macrophages as a major driver of atherogenesis. Some studies proposed a role for NOD2-mediated activation of macrophages within the lesions, leading to p38-dependent activation of COX-2 through upregulation of pro-inflammatory cytokines IL1β and TNFα, and culminating in increased eicosanoid production (mostly prostaglandin E2) [60]. Macrophages can also be primed for increased eicosanoid (including PGE2) secretion following LXR-mediated induction of lysophosphatidylcholine acyltransferase 3 [61]. In this regard, suppression of myeloid-derived, but not vascular cell-derived, PGE2 substantially reduces atherosclerosis [62]. Other studies confirmed a pivotal role for NF-κB pathway, and more particularly myeloid-specific IκB kinase activity, in sustaining macrophage inflammation and promoting lesion development [63]. A number of interesting inflammatory pathways that converge on NF-κB activation have been reported. Sphingomyelin generation in macrophages through sphyngomyelin synthase 1 promotes atherosclerosis through accentuation of TLR4-dependent NF-κB signaling [64]. TWEAK-mediated activation of monocytes/macrophages through fibroblast growth factor inducible 14 increases the release of the pro-inflammatory and pro-atherogenic HMGB1 [65,66], and upregulation of resistin-like molecule (RELM)β on foam cell macrophages by saturated fatty acids potentiates the classical NF-κB pathway and accelerates lesion development [67]. An additional intriguing mechanism of macrophage activation involves interference with extracellular matrix remodeling through activation of heparanase activity, which cleaves heparin sulfate side chains. Blich et al nicely showed that heparanase potently activated macrophages to upregulate TNFα, IL-1, MCP-1 and MMP-9, likely through TLR2/4, PI3K, MAPK, and NF-κB signaling pathway [68]. How heparanase activates TLRs is till unknown but may involve conformational changes in cell membrane HS proteoglycans, generation of HS-cleavage products, or other HS-independent function. Of note, vulnerable coronary plaques showed increased heparanase activity compared with stable lesions, and elevated plasma heparanase levels were found in patients with acute myocardial infarction compared with stable patients, highly suggesting a clinically-relevant pathway [68].

Metabolic disturbances associated with obesity and insulin resistance drive cardiovascular complications [69,70]. Free fatty acids (FFA) are elevated in patients with the metabolic syndrome and are proposed to promote inflammatory responses through TLR and NF-κB-dependent signaling. Recent studies provided additional mechanisms for FFA-dependent regulation of the inflammatory response. Saturated FFA palmitate induces the activation of NLRP3-ASC

inflammasome leading to increased production of IL-1β (and IL-18) [71], whereas unsaturated oleate selectively induces inflammasome-independent IL-1α production through activation of mitochondrial uncoupling [72]. Recent work published in *ATVB* indicates that stearic acid, a major saturated FFA in atherosclerotic lesions, activates TLR2/4-independent inflammation in macrophages, with induction ER stress and macrophage apoptosis [73], suggesting potentially important consequences with regard to lesion progression, given the well-documented roles of ER stress, macrophage apoptosis and defective efferocytosis in promoting plaque inflammation and necrotic core formation [74-76]. Conversely, n-3 fatty acids are endowed with anti-inflammatory properties. For example, eicosapentaenoic acid has recently been shown to limit the induction of macrophage endothelial lipase in response to a variety of stimuli, including LPS, palmitic acid or PPARγ agonists. The effect was associated with reduced production of pro-inflammatory mediators but upregulation of anti-inflammatory IL-10 [77]. Moreover, administration of omega-3 fatty acids to *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice led to significant reduction of Ly6C^{hi} monocytosis and monocyte recruitment into developing lesions, independently from effects on plasma cholesterol [78]. More generally, omega-3 fatty acids were recently shown to uncouple NF-κB binding and histone acetylation at enhancers and promoters from subsequent steps required for induction of inflammatory genes, particularly histone methylation and eRNA production [79].
Intraplaque hemorrhage (IPH) is an important risk factor for plaque progression and vulnerability where erythrophagocytosis by macrophages appears to play a determinant role [80, 81]. However, the mechanisms behind this increased susceptibility to atherosclerosis are poorly understood. Two studies published in *ATVB* suggest a critical role for hepcidin, a key regulator of iron homeostasis, in driving foam cell formation (in part through modulation of ABCA1 and ABCG1 expression), oxidative stress and production of pro-inflammatory cytokines by macrophages, particularly in association with erythrophagocytosis [82, 83], highlighting a mechanistic link between iron retention in macrophages, inflammatory activation and lesion progression.

Anti-inflammatory signaling pathways: IPH has also been shown to generate a distinct adaptive macrophage state in lesions [84] dependent on a key transcription factor ATF1 induced by heme [85], and the associated induction of LXR and HO-1 target genes [86, 87]. Heme-induced HO-1 in macrophages requires NRF-2 [88], recently shown to prevent oxidative stress in these cells, leading to limitation of plaque inflammation and progression [89]. Moreover, activation of 5'-AMP-activated protein kinase (AMPK) is instrumental in heme-induced ATF1 and downstream suppression of macrophage oxidative stress and protection from foam cell formation [87]. This may provide potential explanation for the vasculoprotective effects of the AMPK activator metformin and its unique ability to reduce the macrovascular complications of diabetes [90] amongst many other anti-diabetic treatments. Remarkably, AMPK activation by metformin was recently shown to suppress trained immunity in macrophages through inhibition of mTOR [40], revealing a potentially broader role for this pathway in the regulation of macrophage immune activation versus tolerance.

Macrophage polarization is proposed to play determinant roles in cardiovascular diseases and the topic has been the subject of many recent reviews in *ATVB* [91-98]. Additional original work further highlighted the mechanisms and the impact of macrophage polarization on atherosclerosis. For example, deficiency of Klf4 in macrophages limits M2 and promotes a pro-atherogenic M1 phenotype [23], whereas control of oxidative stress by thioredoxin is reported to favor the development of an athero-protective M2 phenotype [99]. M2 polarization is dependent on fatty acid oxidation. Interestingly, cell-intrinsic lysosomal lipolysis by lysosomal acid lipase (LAL) appears to be essential for the induction of a coordinated

program leading to alternative activation of macrophages [100]. Autophagy has previously been shown to regulate cholesterol efflux in macrophages through targeting of LAL to lipid droplets with subsequent hydrolysis of cholesteryl esters and generation of free cholesterol for ABCA1-dependent efflux [101-103]. However, no evidence is available yet to directly implicate autophagy in LAL-dependent induction of an M2 phenotype, although it plays pivotal roles in macrophage homeostasis, limiting oxidative stress, inflammasome activation and promoting efferocytosis and anti-atherogenic pathways [104,105]. Additional studies expanded on the role of TIMP3 in macrophage biology based on previous work that highlighted the invasive potential of TIMP3(-)MMP14(+) foam cell macrophages [106]. The new results show that TIMP3 is upregulated by classic but downregulated by alternative macrophage activation [107], and that overexpression of TIMP3 in macrophages leads to reduction of oxidative stress, decreased inflammation, and a stable plaque phenotype [108]. The exact mechanisms behind this observation remain to be elucidated. Additional work on the consequences of PPARγ activation in macrophages revealed selective activation of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in M2 versus M1 macrophages [109]. Upregulation of 11β-HSD1 would increase conversion of cortisone into active cortisol. The authors suggested that this might promote improved resolution of the inflammatory response. However, direct inhibition of 11β-HSD1 or its deletion in bone marrow-derived cells do not promote but rather limit atherogenesis through reduction of inflammatory macrophage phenotype and improved cholesterol ester export [110]. Thus, additional work is needed to better clarify the relevance of PPARγ-induced 11β-HSD1 in M2 macrophages. Finally, a new anti-inflammatory mechanism for apoA-I mimetic peptide 4F was reported, involving disruption of lipid rafts, reduced recycling of TLR4 after LPS stimulation and inhibition of NF-κB-dependent pro-inflammatory gene expression [111].

Macrophages in aortic aneurysm

Abdominal aortic aneurysm is characterized by extensive remodeling of the arterial wall, leading to wall thinning, weakening, dilatation and rupture [112]. Despite extensive studies implicating the inflammatory response in AAA [113], little is known about the role of monocyte/macrophages in disease pathogenesis. Monocyte depletion reduces AAA formation in mice [114]. However, the distinct contributions of the various monocyte/macrophage subsets to AAA are still relatively unexplored. A few recent studies published in *ATVB* started to address some of these issues. Two studies highlighted the important role of chemokine-dependent monocyte recruitment through Ccr2 or Cxcl4-Ccl5 in the development of AAA [115,116] using AngII-dependent [115,116] or elastase-induced models [115]. The studies suggested new therapeutic medical strategies for this disease using peptide-mediated inhibition of Cxcl4-Ccl5 interactions [115] or treatment with everolimus to inhibit AngII-induced Ifnγ production and Ifnγ-induced Ccr2 expression on monocytes [116]. A note of caution however should be added for everolimus, recently shown to trigger cytokine release by macrophages through mTOR inhibition and activation of p38 Mapk [117]. Additional studies addressed the impact of modulation of macrophage functions on the development of AAA. Haploinsufficiency of *Notch1* in bone marrow-derived cells reduced the incidence of AngII-induced AAA in *Apoe*$^{-/-}$ mice, which was associated with reduced aortic influx and accumulation of macrophages, reduced macrophage migration and proliferation in vitro and a switch towards an M2 phenotype [118]. The results extend previous work that implicated Notch1 signaling in the pathogenesis of aortic valve calcification/bicuspidy and thoracic aortic aneurysm. Another study addressed the role of macrophage-derived Angptl2 in the pathogenesis of AAA [119]. Angptl2 expression is known to be induced in several inflammatory settings, including obesity-related insulin resistance [120], and is particularly associated with smoking, the dominant

risk factor for AAA [121]. Deletion of Angptl2 in bone marrow-derived cells led to protection from CaCl2-induced AAA, which was associated with reduced macrophage activation and blunted MMP9 production [119], suggesting a major impact on vascular inflammation, probably through inhibition of IκB degradation [120] and matrix remodeling. The relevance of macrophage phenotype to AAA pathogenesis in humans is exemplified in one study that used advanced laser capture microdissection to characterize macrophage subsets in aortic tissue, and showed that different macrophage subsets localized to distinct regions in the aneurysmal tissue, with preferential localization of an inflammatory CD68(+)mannose receptor(-) subset to adventitia, whereas the intraluminal thrombus predominantly harbored an anti-inflammatory CD68(+)MR(+) macrophages [122]. Interestingly, the inflammatory subset was enriched in peroxiredoxin-1, suggesting a predominant contribution to oxidative stress in AAA, and a potential role in AAA development and progression, given that serum peroxiredoxin-1 levels positively correlate with AAA size and growth rate [123].

Macrophages in heart diseases

The last few years witnessed important progress in our understanding of the immune response to ischemic injury, and more particularly the role played by the various subsets of monocytes/macrophages in this process [124]. Selective macrophage subsets (mainly of yolk-sac origin) populate the heart at steady state, are capable of self-renewal and probably play a role in immune surveillance [4,5]. Following injury, the contribution of monocytes and monocyte-derived macrophages become predominant with distinct pathogenic and/or protective roles for $Ly6C^{hi}$ and $Ly6C^{lo}$ monocyte subsets [76,125], respectively, but still poorly defined roles for the newly accumulated macrophage subsets in coordinating inflammation versus reparative processes [4]. A few recent studies published in *ATVB* examined the regulation and function of macrophages in response to heart injury. Deletion of the chemokine decoy receptor D6, which binds and selectively inactivates inflammatory CC-chemokines, led to larger infarcts and increased incidence of cardiac rupture in mice subjected to coronary artery ligation [126]. The results were attributed to heightened Ccl2/3 activity, increased accumulation of neutrophils and $Ly6C^{hi}$ monocytes in the ischemic heart, along with upregulation of inflammatory signaling and Mmp2/9 activities. The whole phenotype was reversed after reconstitution of $D6^{-/-}$ animals with Ccr2-deficient bone marrow, clearly implicating leukocyte-selective Ccr2 signaling in the myocardial response to ischemic injury. Deletion of Irak-M, a functional decoy that lacks endogenous kinase activity and inhibit Tlr and I1-1 responses, resulted in enhanced post-ischemic inflammation and adverse myocardial remodeling [127]. The effects were attributed to direct regulation of MMPs and inflammatory signaling in cardiac fibroblast, which led to secondary accumulation of $Ly6C^{hi}$ monocytes. Two studies addressed the role of macrophage phenotype in mediating the cardiac fibrogenic effects of AngII [128,129]. Both studies concluded to an important role of M2 induction in this process, which stimulated cardiac fibroblasts to differentiate into α-smooth muscle actin- and collagen I-positive myofibroblasts. One study identified a requirement for serum glucocorticoid kinase 1 in promoting optimal Stat3 phosphorylation downstream of AngII, favoring the differentiation towards M2. In the other study, the authors unraveled an interaction between $CD4^{+}$ T cells and macrophages that coordinated the induction of the M2 phenotype. Deletion of Il-12p35 in macrophages favored T cell differentiation towards Th2, which in turn, was required for an optimal switch towards the M2 phenotype.

Macrophages as diagnostic, prognostic or therapeutic targets in CVD

Macrophages as imaging targets: Non-invasive in vivo visualization of inflammatory processes within vascular lesions or injured tissues may allow better classification of patients at high risk of cardiovascular events. Non-conjugated ultrasmall superparamagnetic iron

oxide (USPIO) particles allow the detection of phagocytic cell activity and have shown promise in identification of inflammatory vascular lesions. In a recent study, Hasan et al have extended the methodology to the detection of inflammatory cerebral aneurysms in humans using ferumoxytol-enhanced magnetic resonance imaging (MRI) and showed selective uptake by CD68(+) macrophages [130]. Three other groups developed MRI protocols and used functionalized USPIO or micron-size range iron oxide (MPIO) particles to target cells expressing vascular cell adhesion molecule (Vcam)-1 within atherosclerotic lesions of *Apoe*$^{-/-}$ mice [131-133]. Significant plaque enhancement was detected with the functionalized compared with the uncoupled particles. Moreover, histological analyses revealed that iron localized not only to endothelial cells but accumulated mostly in cap and necrotic core macrophages when using USPIO to target Vcam-1, suggesting a high potential for this technique to identify vulnerable lesions. The Choudhury group in fact produced dual-ligand MPIO targeted to both Vcam-1 and P-selectin and imaged mice using a 9.4-T magnet [133]. The investigators showed selective targeting of atherosclerotic lesions using the functionalized MPIO and histological analysis revealed selective localization of MPIO to endothelium overlying atherosclerotic lesions, with no MPIO detected within lesions, indicating no or minimal uptake by macrophages. Nevertheless, dual labeling with Vcam-1 and P-selectin directly correlated with the extent of macrophage accumulation within the lesions over a time course of 30 weeks (with a peak of macrophage accumulation at 20 weeks of age), suggesting that imaging of activated endothelium can be used as a good surrogate of lesional macrophage content. The results nicely support the concept of macrophage accumulation being highly dependent on continuous monocyte recruitment but do not really fit with the idea that macrophage proliferation within the lesions plays a pivotal role in lesion progression [7]. To directly image macrophages within lesions, Biessen's group designed and validated a strategy to detect cells expressing SR-AI [134,135], a scavenger receptor abundantly expressed by macrophages. Injection of USPIO conjugated with a specific peptidic SR-AI ligand into humanized *Ldlr*$^{-/-}$ mice led to a 3-fold improvement in contrast-to noise ratio of atherosclerotic lesions in comparison with mice injected with nontargeted USPIO or mice with leukocyte SR-AI deficiency, suggesting an interesting strategy to target phagocytic activity in atherosclerotic lesions. In another work by Burtea et al, the authors used USPIO particles conjugated with a linear hexapeptide R826 to target phosphatidylserine, a biomarker of apoptosis [132]. Remarkably, injection into *Apoe*$^{-/-}$ produced specific negative enhancement of plaques rich in macrophages and neutral fat after visualization using a 4.7-T Burker MRI, again suggesting a potentially useful tool to track vulnerable plaques.

Additional imaging modalities are being tested, including intravascular optical coherence tomography using near-infrared light which provides images within 10- to 20-μm axial resolution, and recently shown to discriminate macrophage rich areas within *Apoe*$^{-/-}$ lesions [136]. Others have used multispectral and multimodal intravital fluorescence molecular imaging to detect and quantify the inflammatory process elicited in response to (FeCl3-induced) deep vein thrombosis (DVT) in mice and assess the impact of DVT on subsequent DVT resolution [137]. Their work demonstrates the feasibility of integrated multitarget imaging modality using macrophage and MMP activity fluorescence imaging agents in this setting to detect and quantify the intensity of thrombus inflammation, and predicts the magnitude of subsequent DVT resolution.

Monocytes/macrophages as prognostic targets: There's growing interest in the study of the relationship between murine and human monocyte/macrophage subsets [138,139], and more generally whether knowledge accumulated from animal studies can be extrapolated to the human setting [140,141]. Regarding monocytes and CVD, it is interesting to note that the few human translational studies conducted to date have so far provided support to the experimental data.

Circulating levels of classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes, but not $CD14^{+}CD16^{++}$ nonclassical monocytes, have been independently associated with cardiovascular events (death, MI, stroke) at follow-up in 2 relatively large cohorts of coronary patients [142] or individuals with no prevalent CVD [143]. In further support of the use of monocytes/macrophages as biomarkers of CVD in humans, Reiner et al convincingly reported an independent association between circulating levels of soluble CD14 and incident cardiovascular events or all-cause mortality in a cohort of >5000 European-American and black older adults, and identified new genetic determinants of sCD14 [144]. However, there was no evidence of association between CD14 genotype and CVD risk, suggesting no causal relationship.

Monocytes/macrophages as therapeutic targets: Preliminary data suggest that inhibition of PAI-I/LRP1 interactions using a small molecule inhibitor impairs macrophage migration and limits macrophage accumulation in a model of renal injury [145]. The relevance of the finding to atherosclerosis is still uncertain as macrophage LRP1 regulates efferocytosis [146] and plays a major homeostatic role in vessels, preserving vascular integrity [147,148]. Two initially lipid-targeting strategies have now been shown to limit monocyte/macrophage-related inflammatory processes, independently from their effects on lipid metabolism. Treatment with omega-3 fatty acids is shown to limit atherosclerosis through reduction of $Ly6C^{hi}$ monocyte recruitment [78] and nicotinic acid (NA) may activate GPR109A to limit NF-κB-dependent monocyte activation, chemotaxis and adhesion on inflamed endothelium in vitro [149]. This is of high relevance to atherosclerosis, as GPR109A is expressed in lesional macrophages (although it may be downregulated in foam cells) and NA has been shown recently to limit lesion progression in a GPR109A-dependent manner using *Ldlr*$^{-/-}$ mice [150]. In further support of the anti-inflammatory properties of NA/GPR109A axis, GPR109A signaling promotes anti-inflammatory properties in colonic macrophages and dendritic cells (high production of IL-10) in vivo, and favors the differentiation of Tr1-like IL-10-producing T cells endowed with homeostatic properties [151]. Thus, targeting GPR109A signaling in monocytes, macrophages and dendritic cells may be useful in limiting maladaptive innate and acquired immune responses in CVD.

Macrophages in AAA can readily be imaged with the modified glucose 18 fluoro-deoxyglucose by positron emission tomography imaging [152], which is associated with enhanced expression of glucose transporters 1 and 3 [153]. Interestingly, administration of glycolysis inhibitor 2-deoxyglucose significantly attenuated AAA formation in 2 mouse models of the disease [153], which was associated with reduced monocyte/macrophage inflammation, MMP production and survival/proliferation. This agrees in general with the role of glycolysis in supporting trained immunity in macrophages [40] and dendritic cells [154] and could be extended to adaptive effector immune cells [155]. Thus, glycolysis inhibition might prove to be a viable therapeutic option to limit pathogenic immune responses in CVD.

Diagnostic and imaging modalities are being combined with therapeutic agents leading to the development of a new research area employing theranostics. A few examples have recently been published in *ATVB*. Maiseyeu et al showed that gadolinium nanoparticles conjugated with a neutralizing antibody against myeloid-related protein (Mrp)-8/14 (a secreted protein previously shown to promote leukocyte recruitment) limit Mrp-8/14-dependent inflammatory activation of bone marrow-derived macrophages in vitro and selectively image inflammatory cells in lesions of *Apoe*$^{-/-}$ mice [156], suggesting a potential theranostic approach for atherosclerosis. Others have developed photodynamic therapy to image and kill activated and detrimental macrophages within atherosclerotic lesions [157], using a combination of photosensitizers and light illumination. In particular, the authors developed a cathepsin B-activatable theranostic agent, which becomes fluorescent and generates singlet oxygen on

protease conversion followed by light therapy. They showed selective imaging of macrophage-dependent cathepsin B activity in lesions of *Apoe*$^{-/-}$ mice, followed by significant reduction of macrophage content after application of light therapy, mostly due to macrophage apoptosis [157]. Future work should define the utility and potential undesirable effects of this attractive 'see-and-treat' approach. Induction of massive macrophage apoptosis in advanced lesions might promote necrotic core formation in the absence of very effective efferocytosis pathways.

Conclusion

Inflammatory processes are central to the pathogenesis and complications of CVD. Yet, no anti-inflammatory strategy has been approved for the treatment of patients with CVD, and a few have even failed [158,159], although the inefficacy of non-selective sPLA2 inhibitors could have been predicted from both animal [76] and human mendelian randomization studies [160]. This highlights the urgent need for a better understanding of the cellular and molecular determinants of the inflammatory processes related to CVD and the assessment of their clinical relevance. The last few years have witnessed an immense advance in the characterization of the origins and distinct functions of monocyte/macrophage subsets as well as their differential roles in health and disease, and investigators have proposed ingenious strategies to track these cells and influence their behavior in vivo. The day is getting closer when this novel understanding will be translated into selective and effective diagnostic and therapeutic approaches for the benefit of patients.

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