

Macrophages heterogeneity in atherosclerosis – implications for therapy

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Abstract

Atherosclerosis is a chronic inflammatory disease occurring within the artery wall and is an underlying cause of cardiovascular complications, including myocardial infarction, stroke and peripheral vascular disease. Its pathogenesis involves many immune cell types with a well accepted role for monocyte/macrophages. Cholesterol-loaded macrophages are a characteristic feature of plaques and are major players in all stages of plaque development. As well as modulating lipid metabolism, macrophages secrete inflammatory cytokines, chemokines and reactive oxygen and nitrogen species that drive pathogenesis. They also produce proteases and tissue factor that contribute to plaque rupture and thrombosis. Macrophages are however heterogeneous cells and when appropriately activated, they phagocytose cytotoxic lipoproteins, clear apoptotic bodies, secrete anti-inflammatory cytokines and synthesize matrix repair proteins that stabilize vulnerable plaques. Pharmacological modulation of macrophage activity therefore represents a potential therapeutic strategy for atherosclerosis. The aim of this review is to provide an overview of the current understanding of the different macrophage subsets and their monocyte precursors, and, the implications of these subsets for atherosclerosis. This will present a foundation for highlighting novel opportunities to exploit the heterogeneity of macrophages as important diagnostic and therapeutic targets for atherosclerosis and its associated diseases.

Keywords: monocyte • macrophage • M1 • M2 • activation • atherosclerosis • inflammation • immunomodulation • imaging • plaque stability

Introduction

Atherosclerosis is now well recognized as a chronic inflammatory disorder involving many immune cell types [1]. In the 1960s macrophages were the first inflammatory cells to be identified within atherosclerotic plaques [2]. Macrophages are a key feature of all stages of atherogenesis where they have a very significant impact on lesion progression. As part of their innate immune role, macrophages infiltrate developing lesions and respond to and phagocytose oxidized low-density lipoprotein (oxLDL). This results in the secretion of cytokines, chemokines and toxic oxygen and nitrogen radicals that not only direct and amplify the local immune response but also leads to tissue injury. Moreover macrophages cause plaque destabilization, rupture and thrombosis,

highlighting their destructive role [3]. However, macrophages are heterogeneous cells and can also develop functions that facilitate tissue repair, remodelling and restoration of normal tissue homeostasis [4, 5]. Importantly, macrophages as professional phagocytes remove senescent and apoptotic cells as well as cytotoxic oxidized lipids from developing lesions. Macrophages also secrete anti-inflammatory mediators to down-regulate inflammatory responses within the lesion; the consequences of removing all macrophages are therefore likely to be significant. Indeed, the functions of macrophages in developing and advanced plaques are dictated by several factors including the subset of monocytes from which they derive, how they integrate signals from their local

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tissue environment and the exact stage in the disease process in which they infiltrate.

The marked infiltrate of macrophages within atherosclerotic plaques and the contradictory functions macrophages can develop have significant clinical implications. The development of therapies that switch off macrophage cytotoxic and plaque destabilizing functions and exploit their natural reparative properties would prove a powerful new approach to curtail macrophage-mediated injury and enhance plaque stability. Moreover, macrophage-rich areas are more frequently associated with unstable plaques. Detection of macrophage-rich areas using new imaging tools and metabolic labelling could therefore provide non-invasive methods for estimating plaque stability, permitting earlier detection of high-risk patients and more effective assessment of new therapies. The purpose of this review is firstly, to outline the different subsets of monocyte and macrophages and their activation and function; secondly, to discuss the role of macrophage subsets in atherogenesis and the heterogeneity of plaque macrophages and thirdly, to examine the potential of pharmacological modulation and non-invasive imaging of plaque macrophages to provide novel and better therapeutic, diagnostic and more importantly preventative regimes for cardiovascular disease.

Current concepts in monocyte heterogeneity

An early event in atherosclerosis is monocyte recruitment to the activated endothelium and subsequent diapedesis in response to chemoattractive stimuli. These events have been extensively reviewed elsewhere [6–8]. Circulating monocytes originate from a common monoblast precursor in the bone marrow and are rapidly released into the circulation, especially in response to inflammatory stimuli. Upon infiltrating tissue, monocytes differentiate into macrophages, dendritic cells or, in some cases, osteoclasts [9]. The mechanisms controlling monocyte emigration from bone marrow is an area of active investigation but the chemokine receptor CCR2 and its ligand CCL7 are thought to be important. Circulating monocytes are heterogeneous in that they express distinctive chemokine receptors and have different migratory and differentiation properties. At least two blood monocyte populations, based on expression of chemokine and other receptors, adhesion molecules and differences in size and granularity, have been classified in human beings and rodents [10]. Different subsets use different chemokine receptors for plaque entry [reviewed in 11]. In human beings, the majority of monocytes (about 90%) express high levels of CD14, low levels of the IgG receptor CD16 (CD14⁺⁺CD16⁻), high levels of CCR2 and low levels of the chemokine receptor CX3CR1 and CCR5 [12–14]. These cells are larger (18.4 μm) [15] and phagocytic, and demonstrate low pro-inflammatory cytokine production and high interleukin (IL)-10 when activated *in vitro* with lipopolysaccharide (LPS). A recent genomic study concluded that that in patients with coronary heart disease these monocytes express a more anti-inflammatory profile [16]. The remainder are CD14⁺CD16⁺ or CD14^{dim}CD16⁺. These CD16⁺ monocytes are smaller (13.8 μm), express high

levels of CX3CR1 and CCR5 and lower levels of CCR2 [14] and scavenger receptor type-A [17]. Their numbers are reported to be increased in patients with acute inflammation [18], obesity [19], hypercholesterolemia [20] and coronary heart disease [21]. CD16⁺ monocyte numbers are decreased in the blood after treatment with glucocorticoids [22], again suggesting that their abundance is related to inflammatory stimuli. Interestingly, however, in one study the percentage of CD16⁺ monocytes was reduced in high-risk coronary artery disease patients whereas the percentage of CD14⁺⁺CD16⁻ monocytes increased with the number of risk factors [23]. Information on the change in numbers or proportions of the circulating CD14⁺⁺, CD16⁻ subset in other human pathologies is lacking.

In mice, two equally represented subsets are divided into Ly6C^{high} CCR2⁺ CX3CR1^{low} and Ly6C^{low} CCR2⁻ CX3CR1^{high} and these probably represent different stages of a continuous maturation pathway [24]. Ly6C^{high} monocytes are short lived in the circulation and rapidly infiltrate inflamed sites (*e.g.* atherosclerotic plaques) where they differentiate into inflammatory macrophages [25–27]. They are phenotypic equivalents to human CD14⁺⁺CD16⁻, CCR2⁺ CX3CR1^{low} monocytes [28]. Although the Ly6C^{high} monocytes are phenotypic equivalents to human CD14⁺⁺, CD16⁻, CCR2^{high} CX3CR1^{low} monocytes there is a discrepancy in the functional properties of the respective subtypes of cells. This is important, leading to caution in directly extrapolating any of the findings from mouse to human beings. Numbers of Ly6C^{high} monocytes increase in response to inflammation or hypercholesterolemia [25, 26]. In contrast, the Ly6C^{low} monocytes persist longer in the circulation and can differentiate into tissue resident macrophages or dendritic cells. Under steady state conditions, Ly6C^{low} monocytes patrol the endothelium without extravasation. These monocytes play important functions in scavenging oxidized lipids, dead cells and potential pathogens. In response to endothelial injury, Ly6C^{low} monocytes rapidly extravasate and develop pro-inflammatory properties. Some monocyte subsets therefore heighten disease while others attenuate it. Consequently, studying the kinetics of monocyte recruitment into tissue would represent an effective prognostic tool. A recent study has devised methods using magnetic nano-sensors to profile and quantify peripheral monocyte subsets (*e.g.* phagocytic activity) and the fluctuations that occur in patients with atherosclerosis [29], highlighting a potential screening method for 'at risk' patients.

Monocytes migrating into inflamed sub-endothelial tissue differentiate into macrophages and become activated to exhibit distinct gene expression patterns and functions [30–32]. It is still debated whether a transition from a distinct monocyte subpopulation to a specific macrophage type exists or whether macrophage phenotypes within plaques are influenced by the heterogeneity of circulating monocytes [33]. Studies in inflamed murine kidney have demonstrated, however, that recruitment of a single lineage of Ly6C^{high} monocytes differentiate into three populations of kidney macrophages, including a pro-fibrotic Ly6C^{low} population. This suggests macrophage heterogeneity is complex and not due to a simple transition of specific monocyte subtypes [34].

Pro-atherogenic role of plaque macrophages

Activated macrophages up-regulate their expression of both scavenger receptors and toll-like receptors (TLR) to enhance phagocytosis and remove harmful substances (*e.g.* dying cells or oxLDL) as part of the normal inflammatory response to sub-intimal pathogenic lipoproteins [35]. Usually, inflammation is self-limiting and homeostasis is restored. If engulfed cholesterol cannot exit the cell then macrophages become lipid-laden foam cells. Foam cells secrete additional extracellular matrix (ECM) components that further endorse lipoprotein retention within the sub-endothelium. CD36 signalling *via* oxLDL promotes adhesion and trapping of foam cell macrophages [36], provoking an inflammatory response at the local site. Foam cells within the developing fatty streak produce chemokines, cytokines, proteases, growth factors, bioactive lipids and angiogenic factors that perpetuate the inflammatory process. This also results in migration of smooth muscle cells from the media to the intima of the artery. Subsequent proliferation of smooth muscle cells in response to foam cell secreted mediators contributes to the enlarged fibro-fatty plaque and fibrous cap. Moreover, activated macrophages in developing plaques produce reactive oxygen species (ROS), which cause cell apoptosis and lipoprotein oxidation [37]. OxLDL engulfed by macrophages is digested, processed and peptide antigens presented to T lymphocytes, activating the adaptive immune system and initiating a cytotoxic T helper type 1 (Th1) immune response. The interferon- γ (IFN- γ) produced by Th1, as well as amplifying inflammation, causes induction of pro-coagulant tissue factor expression in macrophages.

More advanced plaques contain large numbers of macrophages and can develop into complex atherosclerotic lesions that are classified as stable or unstable [38]. Stable plaques are characterized by a thick fibrous cap overlying a plaque that does not contain a cholesterol-rich necrotic core. Unstable plaques are lipid-filled necrotic core, have a thin fibrous cap and a high ratio of macrophages to smooth muscle cells [39]. Here, macrophages secrete proteolytic enzymes that degrade matrix components, as well as the protective fibrous cap, leading to plaque destabilization and increased risk of plaque rupture and thrombosis [40]. Indeed, the number of macrophages present within plaques correlates with plaque stability and it is well known that plaques tend to rupture at sites of increased macrophage content [41]. Lesional macrophages also contribute to death of surrounding cells by release of toxic oxygen and nitrogen radicals and *via* Fas-Fas ligand interactions [42]. Large lipid-laden cells undergoing apoptosis present a difficult target for phagocytosis and failed apoptosis results in necrotic death. This leads to accumulation of insoluble lipids and other cellular components, causing increased pro-inflammatory responses. Pro-inflammatory mediators cause further apoptosis of smooth muscle cells, endothelial cells and leucocytes that account for much of the plaque-disrupting core of vulnerable plaques. Uncleared apoptotic cells shed plasma membrane microparticles that stimulate

thrombosis [43]. Advanced plaque macrophages also contain large quantities of tissue factor which are responsible for much of the pro-coagulant and pro-thrombotic activity following plaque rupture [44].

Anti-atherogenic role of plaque macrophages

The highly pathogenic role of macrophages in atherosclerosis suggests their removal from vulnerable, rupture prone plaques would be beneficial. Indeed, animal studies have demonstrated that macrophage depletion in developing plaques provides resistance to atherosclerosis [45, 46]. However, macrophages also have important anti-inflammatory, reparative effects and importantly are essential for the normal inflammatory response protecting the host from infection. Their depletion is therefore not a strong therapeutic option. In the early stages of atherogenesis, macrophages scavenge cytotoxic lipoproteins and senescent and apoptotic cells preventing accumulation and cytotoxicity within developing lesions. Efficient clearance of apoptotic cells is essential for preventing secondary necrosis and also triggers an anti-inflammatory response through induction of transforming growth factor (TGF)- β , IL-10 and other anti-inflammatory cytokines [47] to down-regulate ongoing inflammation. In advanced plaques, appropriately activated macrophages promote tissue repair by promoting ECM synthesis and smooth muscle cell proliferation that enhance plaque stability. Macrophages are also necessary for resolution of injury that occurs within the plaque or at sites of rupture. Here they secrete anti-inflammatory cytokines, matrix repair proteins and angiogenic factors, and clear tissue debris. Data from other tissues including lung [48], skin [49], heart [50] and kidney [51, 52] demonstrate the importance of macrophages for repair processes, and data are emerging that shows a similar reparative role in atherosclerosis. Intraplaque haemorrhage accelerates atherosclerosis and contributes to lesion development and destabilization. Normally, macrophages scavenge haemoglobin-haptoglobin complexes *via* CD163, and this process provokes the secretion of the anti-inflammatory atheroprotective mediators IL-10 and heme oxygenase-1 [53]. Finally, macrophages secrete u-PA and t-PA; these plasminogen activators activate the serine protease plasmin, which plays a key role in fibrinolysis of blood clots [54]. The role of monocytes/macrophages in atherosclerosis is therefore critically dependent on the exact stage of plaque development. A similar phenomenon is described in liver injury where functionally distinct subpopulations of macrophages were shown to exist in the same tissue favouring ECM accumulation during ongoing injury but enhancing matrix degradation during recovery [55]. Regulating macrophage function is therefore a fundamental approach for atherosclerosis treatment but each step in atherogenesis creates a new microenvironment which in turn influences apoptotic, cytotoxic and phagocytotic processes differently. Consequently, strategies targeting macrophage functions may need to be tailored to the stage in the disease process.

Macrophage heterogeneity and *in vitro* classification

The extent of macrophage heterogeneity and cues that induce polarization in atherosclerotic plaques are still being unravelled. Activation studies *in vitro* have, however, phenotypically and functionally characterized several polarized macrophage subtypes. Classical or M1a macrophage activation requires priming by IFN- γ together with a second activating signal such as a microbial product (*e.g.* LPS, CpG-DNA) ligating TLR, or a pro-inflammatory cytokine such as TNF- α or IL-1 [5, 56, 57]. M1a macrophages produce copious amounts of reactive oxygen and nitrogen intermediates and release chemokines and pro-inflammatory cytokines including TNF- α and IL-6 that direct endothelial injury. M1a-activated macrophages mediate resistance against intracellular parasites and tumours but also elicit tissue damage. They express high levels of MHC class II and co-stimulatory molecules CD80/86, which increases their capacity as antigen presenting cells, and IL-12 that supports Th1-driven immune responses [51]. They also secrete a variety of tissue-degrading proteases. Functionally, M1a macrophages exhibit enhanced phagocytic capacity and bacterial (and cell) killing mechanisms [5]. Innate or M1b activated macrophages are consequences of pathogen-associated molecular patterns, *e.g.* LPS, CpG-DNA or flagellin engaging pattern recognition receptors [58], such as TLRs and nucleotide oligomerization domain receptors [59]. They display a phenotype similar to M1a activated cells but do not exhibit enhanced phagocytosis and they secrete low levels of IL-12.

Alternative or M2-activated macrophages, in contrast to M1 macrophages, kill intracellular pathogens poorly and are anti-inflammatory, having a critical role in resolution of injury [5, 57, 60]. The precise properties of M2 macrophages vary depending on the activating conditions and they have been divided into M2a, M2b and M2c subtypes [5]. M2a activation occurs when macrophages are stimulated by IL-4 or IL-13, typically associated with Th2 responses. They provoke increased deposition of ECM, *e.g.* by increased fibronectin and TGF- β production [57] and consequently have also been referred to as 'tissue reparative' or 'wound healing' macrophages [33]. M2a macrophages are strongly associated with extracellular parasite infections, allergy, humoral immunity and fibrosis. They show increased expression of C-type lectins including dectin-1 and macrophage mannose receptor, as well as both the IL-1 receptor antagonist (IL-1ra) and the decoy IL-1 β type II receptor, which inhibits inflammation elicited by IL-1. Arginase expression is enhanced in M2a activated mouse (not human) macrophages, and in contrast to M1 activated macrophages, the expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide is reduced [5]. M2b macrophages develop after exposure to LPS, CD40 ligand or IL-1 β in cells primed by IgG immune complexes that are recognized by Fc gamma receptors [60, 61]. They secrete enhanced IL-10 and decreased IL-12 but produce the pro-inflammatory cytokines TNF- α , IL-6 and IL-1. M2c macrophages similarly produce high levels of IL-10 and thus can down-regulate pro-inflammatory cytokines to limit inflammation. They are induced by IL-10, TGF- β or glucocorticoids [5] and have increased debris scavenging

activity and a pro-healing functional program. M2b and M2c have recently been described as 'regulatory macrophages' due to their high secretion of IL-10 [33]. Apoptotic cell uptake induces another type of anti-inflammatory macrophage outwith the M1/2 classification [62, 63] although these have been referred to as regulatory cells due to production of IL-10 [33]. These are characterized by transient expression of small amounts of pro-inflammatory cytokines and chemokines, followed by later and sustained TGF- β and PGE₂ synthesis and other anti-inflammatory lipid mediators that down-regulate inflammatory responses and dominate resolution of tissue injury [63].

Macrophage heterogeneity exists in tissue *in vivo* although the phenotype observed may not directly correspond to simplistic phenotypes generated *in vitro*. *In vivo*, macrophages are exposed to a complex microenvironment generated from several cell types. The way infiltrating macrophages are activated is dictated by a multitude of signals impinging on their receptors (see Fig. 1). These signals change during the evolution of the underlying disease process and have the capacity to influence the outcome of the disease. Other less-defined macrophage subtypes induced by metabolic factors are liable to be present in atherosclerotic plaques. For example, uptake of oxidized or acetylated LDL, as a model of foam cell formation increases the expression of commonly used M1 markers and transcription factors such as iNOS, metalloproteinase-1 and NF- κ B [64]. Fatty acids present in developing plaques activate an inflammatory programme; saturated fatty acids are robustly pro-inflammatory, polyunsaturated fatty acids are weakly inflammatory or neutral while omega-3 unsaturated fatty acids induce more anti-inflammatory functions [65]. Moreover, under appropriate conditions, infiltrating monocytes differentiate into osteoclast-like cells within plaques and promote lesion calcification [9], whereas macrophages exposed to particulate calcium mineral have been reported to undergo osteoclastic differentiation [66].

Macrophage heterogeneity within atherosclerotic plaques

Studies in several rodent models have shown evidence of macrophage heterogeneity within the atherosclerotic plaque [26, 27, 67]. Heterogeneity amongst macrophages infiltrating human atherosclerotic lesions has also been recognized for many years [68] although it has been difficult to distinguish the specific activation phenotypes at the single cell level. This is due to lack of markers that clearly delineate different activation states in plaque tissue although MCP-1 has been used as an M1 marker and macrophage mannose receptor and CD163 as M2 markers. The differences in phenotype of plaque macrophages are reported to be due to the time of residence within the plaque [68], the initial phenotype of infiltrating monocytes, or site of location within the plaque (inflamed/non-inflamed) [69]. Waldo *et al.* have characterized CD14⁺ macrophages (CD68⁺) within active plaques whereas CD14⁻ macrophages were abundant in disease-free regions [69]. They propose this could be due to dissimilar activating

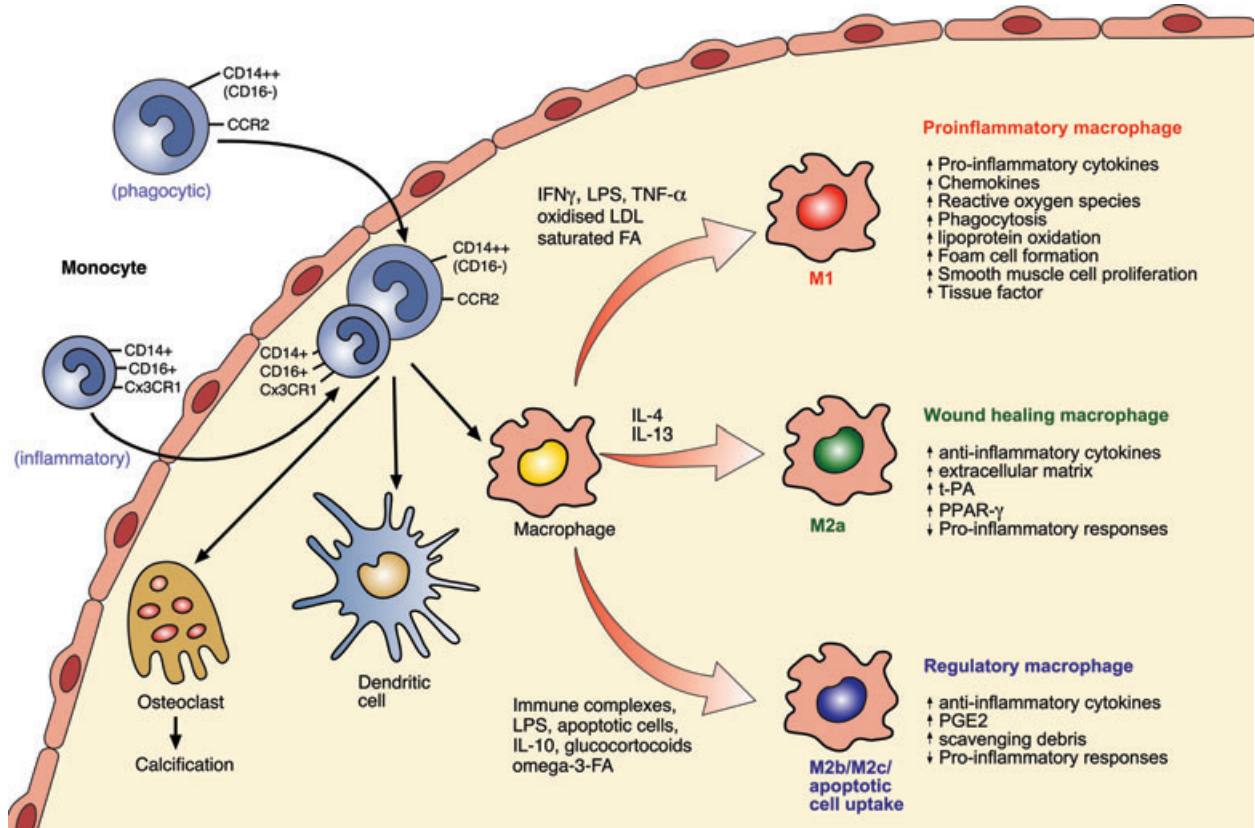


Fig. 1 Activation of macrophages in developing and advanced human atherosclerotic plaques. Monocytes (CD14⁺⁺CD16⁻ or CD16⁺ subsets) enter the developing atheroma guided by adhesion molecules and chemokines and once infiltrated can differentiate into macrophages, dendritic cells or osteoclasts. Differences in monocyte subsets also exist for mice (see text for details). In response to microenvironmental stimuli, macrophages become activated to develop either atherogenic or atheroprotective functions. The factors controlling entry of the different monocyte subsets into plaque and whether specific monocyte subsets differentiate into distinct functional macrophage subsets requires further investigation. M1 macrophages are microbicidal and involved in host defence. They are pro-inflammatory and cause tissue injury and promote lesion development as well as enhancing plaque vulnerability. M2a macrophages are involved in tissue repair. They are anti-inflammatory and can stabilize vulnerable plaques. M2b (e.g. immune complex/LPS activated) and M2c macrophages, as well as macrophages that take up apoptotic cells in the presence of pro-inflammatory stimuli, are immunoregulatory and anti-inflammatory and stabilize or even regress atherosclerotic plaques, see text for further details. Abbreviations: IFN- γ , interferon- γ ; LPS, lipopolysaccharide; LDL, low-density lipoprotein; FA fatty acid; t-PA, tissue plasminogen activator; PPAR- γ , peroxisome proliferator activated receptor- γ ; PGE2, prostaglandin-2.

environments but different CD14 expressions could also be due to time of residence within plaques given CD14 is lost on maturation of monocytes. Interestingly, macrophages expressing low levels of CD14 express five times more peroxisome proliferator activated receptor- γ (PPAR- γ), a nuclear hormone receptor, than macrophages expressing high levels of CD14, and PPAR- γ is associated with an M2-anti-inflammatory/wound healing phenotype [70]. The haptoglobin/haemoglobin scavenger receptor CD163 has also been associated with an anti-inflammatory macrophage. In a separate study, scattered macrophages within diffuse intimal lesions showed strong positivity for CD163 whereas foamy plaque macrophages were weakly positive [71]. Distinct populations of CD163⁺ macrophages have also been identified in haemorrhaged atherosclerotic plaques and importantly these had low levels of human leucocyte antigen-DR and

were unlike classical lipid core macrophages [53]. These were thought to suppress the impact of haemorrhage on atherosclerotic progression.

Bouhrel *et al.* also provide evidence for macrophage heterogeneity in atherosclerosis since both M1 and M2 macrophage markers (MCP-1 and macrophage mannose receptor, respectively) were detectable in human atherosclerotic lesions, and these markers were present in distinct locations [70]. Interestingly, they showed that PPAR- γ expression correlated with the expression of M2 activation markers and PPAR- γ activation skewed human monocytes towards an anti-inflammatory phenotype [70] (see also section 'Pharmacological modulation of macrophage function'). Taken together, these observations provide evidence for the presence of several macrophage phenotypes within developing and advanced plaques and their complexity in promoting, as well as inhibiting,

plaque progression. Phenotyping isolated plaque macrophages through several 'omics' approaches will undoubtedly provide a more detailed picture of the complex functional role of individual subtypes in the progression and stabilization of the plaque.

Macrophage heterogeneity in obesity and obesity-associated disorders

Obesity is well known to contribute strongly to the risk of atherosclerosis as well as insulin resistance, type II diabetes and the metabolic syndrome, all of which are associated with atherothrombotic vascular disease [72, 73]. Macrophages are also important players in the development of obesity and are coupled with low grade inflammation in adipose tissue [74]. High numbers of circulating CD16⁺ monocytes are associated with obesity [19] and macrophages are present in much higher numbers in adipose tissue of obese patients than in that of lean patients [75]. Both M1 and M2 populations of macrophages have been identified in rodent and human adipose tissue with M2-type macrophages as the main population in tissue from lean rodents [76] and human beings [77]. Importantly, Lumberg *et al.* reported that high fat diet-induced obesity caused a switch in adipose tissue macrophages from an M2 anti-inflammatory state to M1 pro-inflammatory cells [76] favouring inflammation. Further studies by Fujisaka *et al.* confirmed that insulin resistance is associated with both increased numbers of M1 macrophages and an increased M1/M2 ratio in adipose tissue [78]. On the other hand, weight reduction promotes the occurrence of M2-like macrophages in adipose tissue of obese patients [79, 80]. The nuclear receptor PPAR- γ is required for maturation of M2 macrophages and deletion of the PPAR- γ gene in myeloid cells results in a shift of macrophage differentiation towards M1, predisposing mice to diet-induced obesity, insulin resistance and glucose intolerance [81, 82]. Agonizing PPAR- γ could therefore promote M2 polarization in adipose tissue protecting from insulin resistance [83]. Together, these studies suggest, as is the case for atherosclerosis, M1 macrophages are pathogenic and polarization of macrophages towards an M2-status has potential as a new strategy for treatment of obesity-induced metabolic disorders.

Macrophage modulation by pathogens and tumours

Several pathogens have developed systems to dampen the innate response and survive the hostile environment produced by M1 macrophages. This is achieved by inhibiting pro-inflammatory intracellular pathways and/or enhancing anti-inflammatory responses (M1- to M2-macrophage switch). Moreover, to avoid macrophage-mediated destruction, pathogens can interfere with receptor-mediated recognition, phagocytosis and trafficking of bacteria to degradative lysosomes (reviewed in [84, 85]). Tumours, like pathogens, can skew macrophage functions by reducing their ability to produce IL-12 while enhancing autocrine

IL-10 production [86] and by decreasing their antigen presenting ability. Tumours also cause enhanced production of matrix-degrading proteases by macrophages that favour metastasis, and they induce enhanced angiogenic factors that promote vascular growth [87]. The capacity of tumours and pathogens to subvert macrophage function from M1 to M2 to avoid immunosurveillance and promote their own survival provides a clear precedent for altering macrophage activation to favour resolution of plaque-induced tissue injury and enhance plaque stability. A key challenge is to devise methods to therapeutically switch M1 to M2 macrophages within developing or unstable plaques (or adipose tissue of obese patients) as effectively as pathogens and tumours.

Pharmacological modulation of macrophage function

Many available standard therapies for atherosclerotic vascular disease (*e.g.* angiotensin converting enzyme inhibitors, β -blockers, aspirin, corticosteroids) influence general immune responses but these lack specific macrophage targeting, and are usually only mild modifiers of macrophage activity [88–91]. General immunosuppressive therapies also leave patients vulnerable to infection and cancer and highly effective therapies targeting macrophage activation are thus more desirable. Several common pharmacological agents have already been proposed to modulate macrophage activity for prevention and treatment of inflammatory-related diseases, including atherosclerosis.

PPAR- γ is a key factor in regulating macrophage lipid metabolism and inflammatory responses. It is induced by natural ligands such as prostaglandins and some pharmacologicals including anti-diabetic thiazolidinediones (TZD), which slow progression of atherosclerosis. As discussed previously, PPAR- γ is highly up-regulated in M2 macrophages and PPAR- γ agonists have been shown directly to induce M2-like differentiation of monocytes *in vivo* and *in vitro* [70]. Conversely, in mice, selective inactivation of macrophage PPAR- γ impairs M2 activation and exacerbates diet-induced obesity [82], suggesting that PPAR- γ agonists have therapeutic potential. However PPAR- γ activation did not switch the function of M1 macrophages to M2 in atherosclerotic lesions [70]. PPAR- γ activation by TZD also increased transcription of many genes that cause weight gain and increased LDL cholesterol [92] and modifications of this compound are required should its therapeutic potential be developed for atherosclerosis.

Liver X receptors (LXRs) are up-regulated in M2 macrophages and like PPAR- γ , exert important atheroprotective effects by regulating cholesterol metabolism and M1 macrophage-induced inflammatory gene responses [93]. In experimental models macrophage-specific loss of LXRs resulted in a marked increase in lesion size [94] while LXR agonists reduced the size of pre-existing plaques and this reduction was dependent on macrophage LXR activity [95]. The exact mechanisms controlling LXR-induced macrophage responses are unknown but they are thought to up-regulate PPAR- γ , thus a switch from M1 to M2 is likely. However, LXR activation induces lipogenesis and

hypertriglyceridemia and new LXR ligands need to be designed without these undesirable side effects [96].

Statins are effective cholesterol-lowering agents that also dampen immune responses through inhibition of macrophage inflammatory activity [97], again possibly *via* PPARs [98]. In one study, statin treatment slightly increased the number of plaque macrophages yet other markers of inflammation were reduced with the authors concluding that it is not the presence of macrophages but activation and subsequent protease and cytokine release that is attenuated by statin use [98]. Interestingly, the orally available drug FTY720, a mimetic of sphingosine-1-phosphate (a biologically active sphingolipid) has been shown to increase the proportion of M2 macrophages in atherosclerotic lesions and slow lesion progression in mice [99], indicating significant potential for therapy.

Pharmacological agents that selectively target macrophages are not widely available. Small interfering RNA (siRNA) therapeutics offers a potential new class of pharmaceutical drug to target inflammatory genes and signalling pathways that skew macrophage function [100]. Our group, for example, has shown that modulating expression of the suppressor of cytokine signalling proteins by siRNA efficiently switches macrophage function from M1 to M2 [32]. New methods have been devised for delivering siRNA specifically to macrophages [101] and an exciting study by Aouadi et al. [102] has shown that targeting macrophage mitogen-activated protein kinase-4 (using siRNA) *via* an oral delivery system suppresses systemic inflammation. It will be fascinating to see whether this approach could open up a novel avenue to manipulate macrophage function to treat patients suffering from atherosclerosis and related diseases.

Macrophages as a target to non-invasively image vulnerable plaques

The abundance of macrophages in unstable plaques makes their visualization very relevant for both diagnostic purposes and for the evaluation of therapeutic interventions [103]. This could present an important adjunct to morphological markers for the detection of high-risk plaques using methods such as computed tomography. Several non-invasive imaging techniques have been described for macrophage detection in tissue, including atherosclerotic plaques, and advances in these suggest they could soon provide an excellent new diagnostic option.

A promising approach for imaging vulnerable plaques involves superparamagnetic iron oxide particles (SPIOs) and ultra SPIOs that are rapidly taken up by macrophages *via* phagocytosis. When clumped within the phagolysosomes, these nanoparticles produce a strong MRI signal. Their use permits the direct visualization of plaque macrophage in models of atherosclerosis and in patients with severe carotid disease [104, 105]. It is thought that uptake only occurs in recently migrated cells and accumulation is indicative of acute ongoing inflammation that is present in rupture-prone or already ruptured plaques, thus providing a good means to assess plaque activity [106]. Inflammatory cytokines within the inflamed

lesion enhance phagocytic uptake of USPIOs [107] and after uptake there is the added advantage in that production of TNF- α is decreased and anti-inflammatory IL-10 is enhanced. [108]. New MRI probes for imaging plaque macrophages include macrophage scavenger receptor-targeted immunomicelles loaded with gadolinium [109]. Iodine-containing contrast agents have also labelled lesional macrophages for detection with computed tomography [110].

Nuclear agents, *e.g.* ^{64}Cu -labelled nanoparticles, are efficiently taken up by macrophages and detected by positron emission tomography (PET) [111]. Radioactive fluorodeoxyglucose (^{18}F -FDG) competes with glucose for uptake into activated macrophages and is proportional to macrophage density when imaged by PET [112, 113]. Furthermore, increased ^{18}F -FDG uptake occurs in symptomatic carotid plaques compared to the asymptomatic contra-lateral carotid artery [95]. However, not all atherosclerotic plaques showed high ^{18}F -FDG uptake [114] reflecting the fact that some patients were receiving statin therapy which can alter macrophage metabolic activity and ^{18}F -FDG uptake in human aortic and carotid arteries [115]. Another PET-imaged biomarker successfully used to detect plaque macrophage activity is the peripheral benzodiazepine receptor ligand, a mitochondrial protein that is highly expressed in activated macrophages [116]. New technologies are being developed to selectively image macrophage subsets, *e.g.* M2, [117, 118] and whether this is clinically translatable for more efficiently monitoring plaque vulnerability will be an exciting area of future research.

Summary

It is now clear that monocytes and macrophages are heterogeneous and that their subsets have either harmful or beneficial functions in atherogenesis. Clinical trials for anti-atherogenic drugs should not only determine the effect of therapy on macrophage numbers but also their activation status and whether macrophages can be skewed to a more reparative phenotype. Elucidating the exact roles of macrophages localizing within lesions and the molecular cues that drive differential activation will undoubtedly aid in the development of novel strategies for early diagnosis, stabilization or even regression of vulnerable atherosclerotic plaques, which in the long term will effectively help reduce the burden of cardiovascular disease on modern societies.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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