REVIEW

Diversity of macrophage phenotypes and responses in atherosclerosis

Hiroyuki Jinnouchi[1] · Liang Guo[1] · Atsushi Sakamoto[1] · Sho Torii[1] · Yu Sato[1] · Anne Cornelissen[1] · Salome Kuntz[1] ·
Ka Hyun Paek[1] · Raquel Fernandez[1] · Daniela Fuller[1] · Neel Gadhoke[1] · Dipti Surve[1] · Maria Romero[1] ·
Frank D. Kolodgie[1] · Renu Virmani[1] · Aloke V. Finn[1]

Abstract

The presence of macrophages within the plaque is a defining hallmark of atherosclerosis. Macrophages are exposed to various microenvironments such as oxidized lipids and cytokines which effect their phenotypic differentiation and activation. Classically, macrophages have been divided into two groups: M1 and M2 macrophages induced by T-helper 1 and T-helper 2 cytokines, respectively. However, for a decade, greater phenotypic heterogeneity and plasticity of these cells have since been reported in various models. In addition to M1 and M2 macrophage phenotypes, the concept of additional macrophage phenotypes such as M (Hb), Mox, and M4 has emerged. Understanding the mechanisms and functions of distinct phenotype of macrophages can lead to determination of their potential role in atherosclerotic plaque pathogenesis. However, there are still many unresolved controversies regarding their phenotype and function with respect to atherosclerosis. Here, we summarize and focus on the differential subtypes of macrophages in atherosclerotic plaques and their differing functional roles based upon microenvironments such as lipid, intraplaque hemorrhage, and plaque regression.

Keywords Macrophage · Phenotype · Lipid · Hemorrhage

Introduction

Activation of various cell types contributes to the advancement of atherosclerotic changes in arteries. These cells include endothelial cells, smooth muscle cells, lymphocytes, monocytes, and macrophages [1]. While all of these cell types contribute to the formation and progression of atherosclerosis, macrophage retention within the arterial wall is the sine qua non of atherosclerosis. Macrophages are the major inflammatory cells involved in its progression. Prevailing paradigms describe atherosclerosis as a T-helper type 1 (Th1)-driven disease in which the recruitment and retention of monocytes and lymphocytes in the arterial wall result from an increase in circulating low-density lipoprotein (LDL)-cholesterol in the blood and the accumulation of oxidized LDL in the subendothelial space. Macrophages scavenge lipoprotein particles, transforming into foam cells which secrete pro-inflammatory molecules and play an important role in collagen and matrix breakdown leading to plaque rupture [2–4].

Apoptosis of these resident macrophages in the lipid core of the lesion leads to progression of atherosclerosis. Phagocytes, mainly macrophages, play a role in the clearance of apoptotic cells. These apoptotic cells are recognized as dead cells in the process of programmed cell removal or 'efferocytosis' [5]. In early lesions, macrophages have a capability of clearing apoptotic cells to avoid progression of atherosclerosis. However, in progressive lesions, macrophages are no longer able to engulf dead cells and eventually the necrotic core is formed and expanded by the accumulation of apoptotic debris. Necrotic core expansion is strongly associated with inflammation, risk for plaque rupture, and thrombus formation [5]. Throughout the body, macrophages play an important role in maintaining efferocytosis, ensuring the rapid clearance of dead cells to prevent inflammatory consequence-associated apoptotic debris. Impairment of these mechanisms in atherosclerotic lesions appears to be critical in the progression of plaques [6].

Recently, it was reported that macrophages themselves can proliferate within atherosclerotic lesions. An increase in macrophages was observed in mouse lungs without any

✉ Aloke V. Finn
afinn@cvpath.org

1 CVPath Institute, 19 Firstfield Road, Gaithersburg,
   MD 20878, USA

interaction of circulating monocytes, an observation which challenged existing beliefs that tissue-resident macrophages were incapable of proliferating [7]. Both resident and recruited macrophages were thought to be mediated through the action of IL-4 which was sufficient to drive accumulation of macrophages through self-renewal [8]. Additionally, although macrophages in early lesions are derived from recruited monocytes, proliferation of macrophages occurs in advanced mouse atherosclerosis and microenvironmental signals drive their proliferation [9]. Therefore, the capability of local proliferation may be related to differentiation subtypes of resident macrophages [7, 10].

While the role of lipids and cell death dominated the field of atherosclerosis for many years, only more recently have we come to appreciate the heterogenous nature of macrophages. Microenvironment directs monocytes in functionally distinct cell types described within the classic binary paradigm described by Gordon [11]. Within this context, at least two different subtypes of macrophages so-called M1 and M2 macrophages were initially described. Activated (M1) macrophages are induced by a type 1 T-helper cell (Th1) cytokines such as interferon and lipopolysaccharide and are involved with pro-inflammatory activities. Alternative M2 macrophages are stimulated by Th2 cytokines such as cytokine interleukin (IL)-4 or IL-13 and produce anti-inflammatory cytokines such as IL-10 [12] and counterbalance inflammatory responses to M1 macrophages by promoting the resolution of inflammation and inducing tissue repair. Although M1/M2 dichotomy provides a conceptual framework for our understanding of the function of macrophages in the setting of injury, the mechanisms by which macrophages orchestrate inflammation and its resolution to promote tissue repair are incompletely understood.

Evidence for the existence of M2 macrophages in human atherosclerosis was shown by Chinetti-Gbaguidi et al. using histological analysis of human atherosclerotic plaques [8]. M2 macrophages were characterized as a subpopulation of CD68 cells which were positive for the mannose receptor, a surface marker for M2 macrophages. This and many other investigations have helped us go beyond the initial belief that atherosclerosis is primarily driven by Th1-type inflammatory responses [13, 14]. Because macrophages differentiate into morphologically and functionally distinct phenotypes depending upon their microenvironment, the differentiation of macrophages within atherosclerotic plaques is related to cellular events that take place there which may be dependent upon the stage of development of atherosclerotic lesions. Distinct macrophage subtypes can be identified based on their differential expression of surface markers and chemokine receptors [11, 15]. The prevailing view is that M1 macrophages are enriched in progressing lesions while M2 macrophages are present in regressing lesions but even this understanding is beginning to change.

Recent studies have demonstrated additional complexity to this paradigm. In general, the M2 phenotype has been divided into subgroups depending upon their activation stimuli with M2a macrophages induced by IL-4 or IL-13 and M2b induced by IL-1β or LPS. However, other subtypes have also been described such as M4 macrophages induced by CXCL4, Mox macrophages induced by oxidized LDL, and M (Hb) or Mhem macrophages induced by hemoglobin in the setting of intraplaque hemorrhage. As mentioned before, each has a distinctive role in plaque evolution and our understanding of their various roles with respect to atherosclerosis is still evolving and to this day is not completely understood. As an example, studies using single-cell RNA-sequencing methods revealed new clusters of macrophages by transcriptome signatures at single-cell resolution and suggested that the foamy macrophages are not related to inflammatory reactions [16–18].

In this review, we summarize and focus on the differential subtypes of macrophages in atherosclerotic plaques and their functional roles within different milieu such as lipid, intraplaque hemorrhage (IPH), and plaque regression.

Distinct types of macrophage in atherosclerosis

Monocytes enter into the plaque through processes such as capture, rolling, and transmigration with each step regulated by multiple factors. Lipoprotein, especially in its modified forms, accumulates in the proteoglycan rich layer of the intima where it is ingested by macrophages giving rise to foam cells.

Activation of these cells by toll-like receptors (TLR) as well as interferon-gamma induced by engulfed lipoproteins polarizes them into M1 macrophages. Pro-atherosclerotic cytokines such as IL-6, IL-1b, tumor necrosis factor (TNF), IL-23 and IL-12 are derived from M1 macrophages which sustain the inflammatory response [19]. M1 macrophage plays a role in killing intracellular pathogens and their polarization to this phenotype also has other consequences within the context of atherosclerosis. Reactive oxygen and nitrogen species are generated by M1 macrophages to eliminate bacterial, fungal, and viral infections, which also may worsen oxidative stress in the plaque [20]. Further M1 macrophages expressing different chemokine (C-X-C motif) receptor ligands (i.e., CXCL9, CXCL10, and CXCL5) induce the recruitment of Th1 and natural killer cells to kill intracellular pathogens [21]. These functions have important benefits especially in acute infection but they cause tissue damage and impair wound healing under the milieu of sterile inflammation generated within the context of lipid-driven atherosclerosis [22]. In atherosclerotic lesions of humans, M1 macrophages are located in lipid-rich areas away from

M2 macrophages [8]. These cells have decreased ability to migrate, and their accumulation and death are responsible for necrotic core formation and enlargement, a key feature of advancing plaques.

In in vitro and mouse studies, M1 macrophages were distinguished from M2 macrophages. The former induce plaque inflammation and the latter are thought to be involved with the resolution of inflammation and tissue repair. M2 macrophages are induced by IL-4 and IL-13 cytokines which are secreted by Th2 cells [12, 23]. Therefore, the term M2 macrophage is influenced by the TH2 nomenclature.

M2 macrophages have been classified into three different subtypes including M2a, M2b, and M2c macrophages. IL-4 and IL-13 trigger M2a macrophages which express high levels of the mannose receptor [24–28]. M2a macrophages secrete pro-fibrotic factors (i.e., fibronectin, insulin-like growth factor (IGF), and transforming growth factor β (TGF-β) which contributes to the tissue repair [24–28]. M2b macrophages are triggered by exposure to immune complexes and TLR ligands or IL-1 receptor agonists [29]. These cells produce not only anti-inflammatory (IL-10) and pro-inflammatory (IL-1, IL-6, TNFα) cytokines but also TGF-β1. M2c macrophages are triggered by IL-10 and glucocorticoids. M2a macrophages are classically recognized as "wound healing macrophages", whereas M2b and M2c macrophages are called as "regulatory macrophages" [29]. All M2 macrophages have an anti-inflammatory cytokine profile such as low production of IL-12 and high production of both IL-10 and TGF-ß [21]. However, the phenotypic spectrum of macrophages in vivo is not as simple as this schema might suggest. Thus, it remains unclear how this above subclassification of M2 macrophages relates to the specific roles on different subtypes of M2 macrophages in atherosclerosis.

More recent data suggest a role for M2 macrophages in atherosclerosis regression in mouse models [30, 31]. The fact that M2 macrophages have been found in human plaques suggests there is potential for these cells to cause plaque regression but role they actually play in this process in humans is unknown. Common to experimental models of atherosclerosis (mostly mouse studies) is the findings that during regression plaque macrophages decrease and in some cases become enriched in M2 macrophages [30, 32]. But these results cannot directly be translated into humans because macrophage subtypes detected in mice and humans do not directly coincide, and mouse and human atherosclerotic plaque evolution are very different.

This classification can be further broadened into other types of macrophages that have been found in either mouse or human atherosclerosis or both [33] (Fig. 1). Mox macrophages have been found in mouse atherosclerosis where they are induced by oxidized LDL and have proatherogenic properties. M4 macrophages are induced by platelet chemokine CXCL4 and are identified by loss of the hemoglobin–haptoglobin scavenger receptor CD163 and express a combination of CD68, S100A8 and MMP7. They are predominated expressed in the adventitia and intima and have been associated with plaque instability [34]. Advanced plaques also may develop intraplaque hemorrhage (IPH) in which the release of free Hb and its subsequent uptake binding by haptoglobin via the CD163 receptor causes differentiation into M (Hb) or Mhem macrophages. As will be discussed below, emerging data both in human and mouse studies suggest these macrophages may be pathogenic in the setting of IPH.

M2 macrophages were first identified in human carotid endarterectomy lesions by Bouhlel et al. Two distinct types of cells were described: (1) M1 macrophages characterized by activation of MCP-1, IL-6, and TNF-alpha; and M2 cells with upregulation of MR (CD206) and CD163, CCL-18, and IL-10. Staining for MR and MCP-1 demonstrated distinct and separate tissue localizations. A correlation was shown between PPAR gamma mRNA levels and markers of M2 but not M1 macrophages [14]. In vitro priming of human monocytes increased expression of MR but not CD163. Although the authors stated these data showed the presence of M2 cells in advanced atherosclerotic lesions, the stimulus for M2 differentiation was not clearly resolved, as there is very little IL-4 expression that has been found in human atherosclerotic plaques [35]. Since the same cells that were described by Bouhlel were also found by the same group to contain very little in the way of lipid droplets, it remains uncertain to what extent these cells overlap with hemoglobin-induced macrophages described below [8].

Because different subtypes of M2 macrophages express some of the same markers this suggests the presence of the significant overlap which might make their phenotypic differentiation difficult. From this point of view, macrophage phenotypes should be categorized considering not only the expression of surface markers but also their specific function. Notably, it is believed that macrophages are not terminally differentiated but can switch from one phenotype to another through changes in microenvironment [36, 37]. The diversity of macrophage phenotypes within atherosclerosis has become a hot topic and still remains controversial.

What single-cell RNA-seq studies tell us about macrophage populations in atherosclerosis

The characterization of macrophages subtypes in atherosclerosis has been challenging and continues to evolve as our techniques to evaluate them continue to change. With the recent advent of single-cell RNA-seq (sc-RNA-seq) methods, new clusters of macrophages have been described in
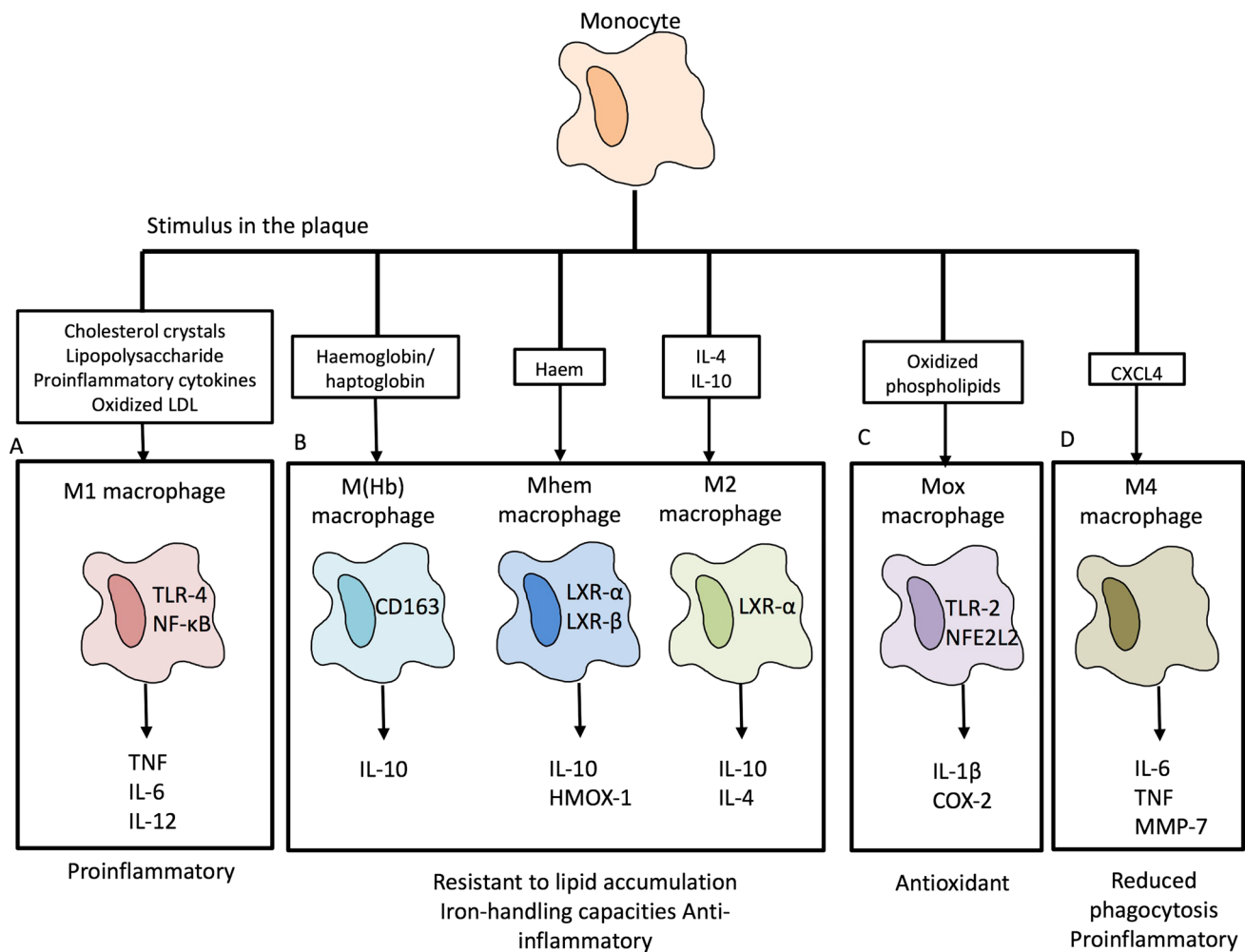
Fig. 1 Main macrophage subtypes found in atherosclerotic lesions. Stimuli present in atherosclerotic lesions drive the differentiation of monocytes towards different macrophage phenotypes. **a** M1 macrophages release pro-inflammatory cytokines. **b** M (Hb), Mhem, and M2 macrophages are resistant to lipid accumulation, possess iron-handling capacities, and have anti-inflammatory effects. **c** Mox macrophages display an antioxidant gene expression profile. **d** M4 macrophages, like M1 macrophages, are pro-inflammatory, but lack the capacity for phagocytosis. *COX-2* cyclooxygenase, *CXCL4* C-X-C motif chemokine 4, *HMOX-1* haem oxygenase (decycling) 1, *LDL* low-density lipoprotein, *LXR* liver X receptor, *MMP-7* matrix metalloproteinase-7, *NFE2L2* nuclear factor (erythroid-derived 2)-like 2, *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells, *TLR* toll-like receptor, *TNF* tumor necrosis factor (Ref. [33])

atherosclerosis by transcriptome signatures at single-cell resolution.

Two sc-RNA-seq studies in Ldlr−/− [10] and Apoe−/− mice [18] used an unsupervised clustering method to map leukocyte heterogeneity. Both showed the macrophages and T cells are the predominant leukocytes in atherosclerotic plaques, and the proportion of macrophages increases as the plaques progress. Different clusters of macrophages have been described, the inflammatory cytokines (e.g., IL-1β, Cxcl2, Ccl2, and TNF were mostly expressed in non-foamy "inflammatory" macrophages [17, 18]. Foamy macrophages expressed high Trem2, Cd9, Ctsd, or Spp1 (osteopontin), but express lower levels of inflammatory cytokines [16]. Using single-cell RNA sequencing

and genetic fate mapping, a recent study showed profiling of CX3CR1+ precursors cells during both progression and regression of atherosclerosis revealed a spectrum of macrophage activation states. This includes "resident-like macrophages", "inflammatory macrophages", as well as "TREM2 hi macrophages" (Folr2 hi, chemokine hi, and Trem2 hi macrophage, respectively). Differentiated and activated macrophages were highly enriched in cells from progressing versus regressing plaques. Interestingly, a cluster of CX3CR1 high expressing macrophages, distinct from other macrophage populations, maintain a stem cell-like proliferative state, are enriched for cell cycle genes, and have been termed "stem-like macrophages". These results indicate that plaque microenvironment determines stimulating

factors that could drive the activation and differentiation of macrophages as plaques progress. Reversal of hyperlipidemia improves the microenvironment in the plaque during regression, reducing the heterogeneity and complexity of macrophages [38]. These single-cell RNA-seq studies provided important transcriptome data in lesional cell populations, and by combining the lineage tracing models as well as proteomic profiling, more studies will further reveal the diversity of macrophage phenotypes and their functions in disease pathogenesis. However, still missing in such experimental models is the link to human atherosclerosis where such dynamics are much less understood. More recently, Fernandez et al. conducted single-cell proteomic and transcriptomic analyses of human carotid plaques of patients with symptomatic and asymptomatic disease. Plaques from symptomatic patients contained alternatively activated macrophages, including CD163 positive subsets. Distinct macrophage subsets displayed activated and pro-inflammatory functional states, genes expressed in lipid metabolism, and unique anti-inflammatory signatures, suggesting that subsets of macrophages within the human plaque environment are not accurately defined by M1 and M2 phenotypical descriptions characterized by certain surface markers [39].

Efferocytosis

Efferocytosis is one of the most basic functions of macrophages. Phagocytic clearance of apoptotic macrophages in early lesions is usually efficient, giving physiological benefit by tempering inflammatory responses. That is because areas with early lesional apoptotic macrophages are cleared before they become necrotic, with release of anti-inflammatory cytokines and pro-resolving lipids. On the other hand, apoptotic macrophages are more frequently observed within advanced lesion relative to early lesions. This fact suggests that phagocytic clearance in advanced lesions is not effective. In human carotid arteries, it has been shown that a fair amount of apoptotic macrophages were not engulfed by phagocytes. On the other hand, in human tonsillar tissue, apoptotic macrophages were mostly found inside phagocytes. Many studies demonstrated that areas close to the necrotic core have larger concentrations of lesional apoptotic macrophages [40–43]. Defective clearance of these cells leads to macrophage necrosis and enhanced inflammatory reactions as well as release of immunogenic antigens [44–46]. In fact, in the Apoe−/− mouse model of atherosclerosis defective clearance of apoptotic cells correlated with an increase in the level of inflammatory markers including TNF-α [47]. Eventually, defective phagocytic clearance of apoptotic macrophages provokes plaque progression through several important mechanisms. First, non-cleared apoptotic cells provide a source for pro-coagulant molecules, leading to thrombus formation [48]. Second, the necrotic core consisting of apoptotic and necrotic cells leads to increases in lysophosphatidic acid derivatives which are associated with platelet aggregation and prevention of emigration of macrophages out of the lesion [49, 50]. Third, macrophages secrete matrix-degrading proteases via inflammatory factors in the necrotic core [51]. Lastly, antigenic factors such as thymidine phosphorylate are released from necrotic macrophages, accelerating atherosclerotic changes such as intraplaque angiogenesis and inflammation [52, 53].

Although it is not fully understood why the inefficiency of phagocytic clearance of apoptotic cells is observed within advanced lesions, previous studies have introduced important mechanisms [54]. One such mechanism is the role of oxidized molecules which inhibit the uptake of apoptotic cells by phagocytes [54]. Competitive inhibition of receptors occurs since the oxidized molecules are recognized by certain phagocyte receptors expressed on apoptotic cells. Also, oxidized lipid may affect the affinity of phagocytes for detecting and taking up apoptotic cells [55, 56]. Recognition and ingestion by phagocytes are inhibited because oxidized LDL competes with apoptotic cells for macrophage binding, suggesting they both share oxidatively modified moieties on their surface than serve as ligands to macrophage recognition [57]. However, clinical trials in humans have been able to show that antioxidants were able to prevent progression of atherosclerosis. Perhaps the most powerful evidence for the role of efferocytosis in plaque progression was found by demonstrating that an antibody to CD47, a key 'don't eat me' molecule reversed defective efferocytosis, normalized clearance of diseased vascular tissue and reduced atherosclerosis in multiple mouse models [58].

Relationship between macrophages and lipid

Oxidative stress-modified LDL generates signals that are recognized by pattern recognition receptors (PRR) on macrophages and other immune cells [4]. Scavenger receptors are a type of PRR expressed by macrophages and numerous ones have been identified such as scavenger receptor A1, scavenger receptor B1, CD36, scavenger receptor B1, etc., all of which can bind oxidized LDL and promote foam cell formation. In the late endolysosomal compartment, cholesterol esters from lipoprotein are hydrolyzed to free cholesterol and fatty acids. In vitro SRA1 and CD36 mediate > 75% of the degradation of modified LDL with combined deficiency of both receptors reducing but not totally preventing foam cell formation in mice, suggesting other mechanisms of LDL uptake exist in vivo [59, 60]. Other studies suggest native LDL may also be taken into

macrophages by pinocytosis which also delivers it to the endolysosomal compartment where it undergoes esterification [61].

The ability of macrophages to store cholesterol can be overwhelmed in the setting of abundant cholesterol uptake and results in pathological changes to the function of these cells. Cholesterol esters are mostly biologically stable whereas free cholesterol can cause significant toxicity to cells. As free cholesterol builds up in cells, the ability to esterify it via acetyl-coenzyme A: cholesterol acetyltransferase 1 (ACAT1) starts to decline which further promotes its accumulation. This enhances inflammatory signaling via TLR signaling and NF-kB activation. Because of impaired trafficking of free cholesterol out of lysosomes cholesterol efflux may also become impaired. As a result of these changes in cholesterol metabolism, endoplasmic reticulum stress results in cell death.

Phagocytic function of macrophages is extremely important in the clearance of dead cells. The process of programmed cell removal (or efferocytosis) is dysregulated in atherosclerosis. Defects in lipid metabolism and upregulation of CD47, an important "don't eat me" molecule that has been implicated in defective efferocytosis. The combination of lipid toxicity and defective dead cell clearance results in necrosis and the release of cell components and lipids that contribute to the growth of the necrotic core, a key feature of advanced atherosclerotic plaques.

Cholesterol crystals are also present at the early stage of atherosclerotic lesions and stimulate inflammatory activation of macrophages [62]. In lipopolysaccharide (LPS)-primed human peripheral blood mononuclear cells and in vivo in mice, cholesterol crystals are responsible for the stimulation of the caspase-1-activating-NLRP3 inflammasome and which causes the cleavage and the release of IL-1 family cytokines (including IL-1β and IL-18) [62]. Cholesterol crystals accumulate in atherosclerotic lesions and play a role in the differentiation of M1 macrophages. A causal role in the activation of the NRLP3 inflammasome in atherosclerosis progression remains controversial with some mouse models with deficiency in components of the inflammasome showing decreased atherosclerosis while others failing to show a protective effect of its deletion [62, 63].

Most aspects of lipid and its by-products drive the differentiation of macrophages into M1 cells. Oxidized lipoproteins inhibit Kruppel-like factor 2 (KLF2) while minimally modified LDL (mmLDL) acts via activated TLR-4 and TLR-2 pathways [64]. Cholesteryl linoleate, the major cholesteryl ester in atherosclerotic plaques, stimulates M1 polarization via a TLR4/NF-kB dependent mechanism while its oxidation products such as 7-keto-cholesteryl-9 carboxinonanoate promote an anti-inflammatory phenotype dependent TGF-β signaling pathway [65–67].

Responses to hemorrhage

More than 75 years ago, Wartman et al. reported that IPH might be responsible for the progression of coronary artery lesions [68]. Neovascularization often occurs in atherosclerotic plaques and can result in IPH after vessel rupture [69]. Barger et al. have shown a rich microvascular network extending from the adventitia through the media and into the thickened intima after the injection of silicone polymer into atherosclerotic human coronary arteries [70]. IPH is the essential trigger for alternative macrophage conversion in the atherosclerotic lesions. IPH is caused by plaque neovascularization, increased microvessel permeability, and leakage of blood [71, 72] (Fig. 2). These findings are recognized as one of features of advanced atherosclerotic plaques, are related to rapid progression of atherosclerotic lesions with accumulation of erythrocyte membranes, and promote necrotic core enlargement [69]. In a large series of human plaques from victims of sudden coronary death, there was a greater frequency of previous IPH (as identified by immunostaining for glycophorin A, an anion exchange protein specific to erythrocytes) in high-risk plaques prone to rupture compared to early lesion morphologies and stable plaques. IPH is thought to result in expansion of the necrotic core through deposition of free cholesterol-rich erythrocyte membranes. As the amount of glycophorin A increased so did macrophage infiltration, suggesting hemorrhage is an inflammatory stimulus.

IPH results in erythrocyte lysis through oxidative stress and afterward free hemoglobin is released (Hb) [73]. Hb is immediately complexed with the plasma protein haptoglobin and macrophages internalize hemoglobin:haptoglobin (HH) complexes via CD 163 receptor which is exclusively expressed on macrophages [74, 75]. CD163 is involved in clearance and endocytosis of HH, and is necessary for effective hemoglobin clearance after IPH [76]. The heme subunit of hemoglobin is degraded by the heme oxygenase enzymes (HO) which produces antioxidants carbon monoxide and biliverdin and also releases free Fe^{2+}. Once iron has been released, it is either used by the cell, sequestered in a non-toxic form as ferritin, or exported from the cell via the iron exporter ferroportin (FPN) which also converts it to the less redox-active form Fe^{3+} by ceruloplasmin. Although elements of this process such as HO have been studied within the context of atherosclerosis, our understanding of the impact of how IPH affects macrophage function and the process of atherogenesis remains incomplete. Ingestion of HH by CD163 results in differentiation of macrophages into a subtype we have termed M (Hb) and others Mhem. M (Hb) macrophage is mainly located in areas of neoangiogenesis and hemorrhage, and characterized by high surface expression of CD163 and CD206 (mannose receptor).
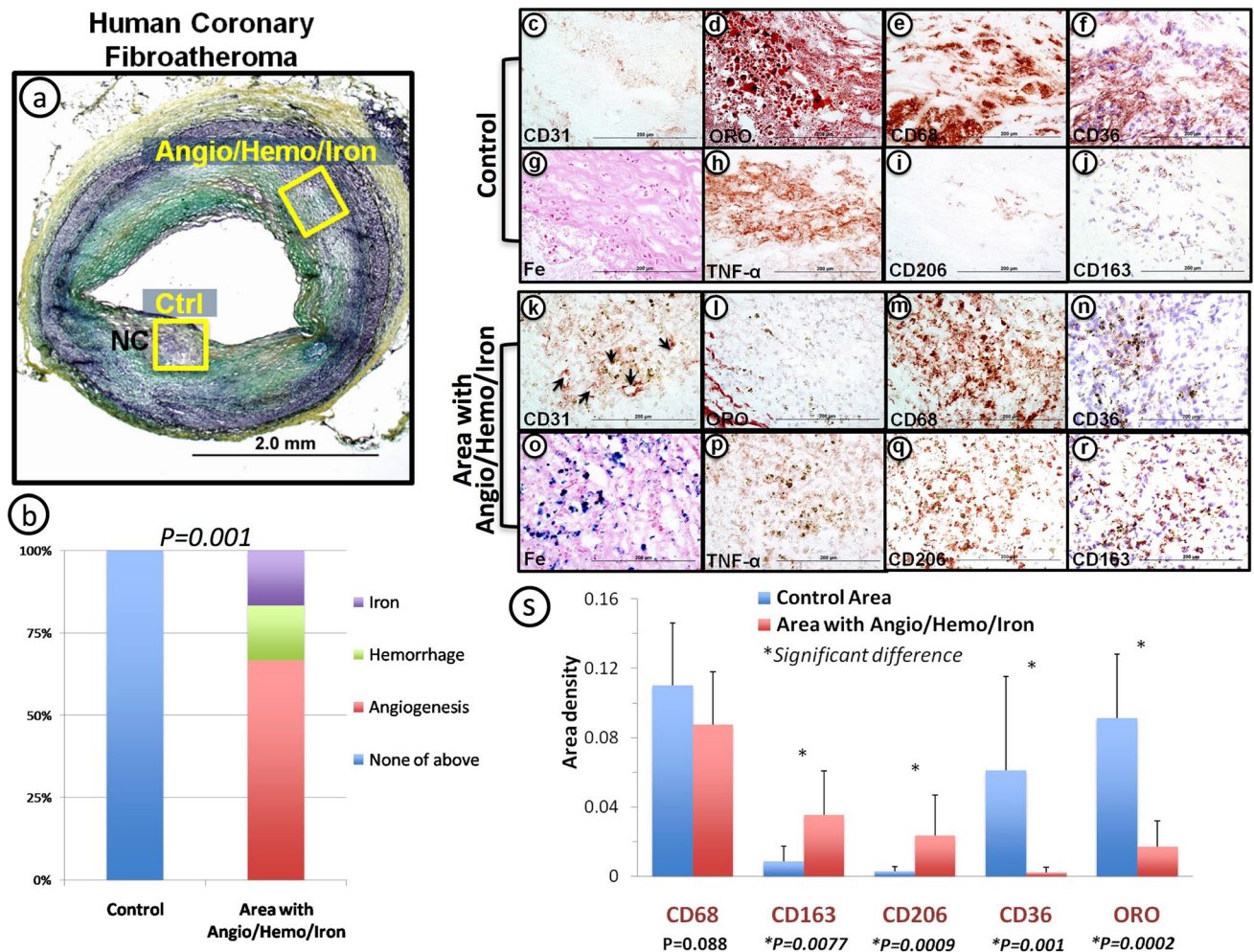
Fig. 2 M2 markers CD206/CD163 are present in human atherosclerotic lesions at sites of prior hemorrhage and are distinct from foam cells. **a** Representative frozen section of human coronary fibroatheroma from a 48-year-old man who died suddenly. High-power image from ctrl (red box) shows foamy macrophages in the perinecrotic core (NC) region. The blue box shows an area rich in angiogenesis and iron (low-power image, Movat stain; high-power images, hematoxylin and eosin [H&E] stain). Photomicrographs of the boxed areas from ctrl (red boxes) (**c–j**) and area of angio/hemo/iron (blue boxes) (**k–r**). Note that the ctrl area shows a lack of CD31 staining (brown). **b** Plaque regions were identified by the presence of angiogenesis/hemorrhage/iron (angio/hemo/iron) and compared with control (ctrl) pericore regions of macrophages devoid of angio/hemo/iron (**c**), abundant oil red O (ORO) positivity (red) (**d**), macrophage infiltration (CD68, brown) (**e**), CD36 staining (brown) (**f**) and no iron

staining (blue, Perls' Prussian blue) (**g**). It also demonstrates abundant tumor necrosis factor (TNF-) positivity (brown) (**h**), but there is minimal MR (CD206, brown) (**i**) and CD163 (brown) (**j**) immunostaining. **k–r** From an area of angio/hemo/iron showing abundant CD31 staining (**k**, black arrows point to angiogenesis), rare positive cells for oil red O (**l**), but abundant C68 staining (**m**). This area also demonstrates minimal CD36 staining (**n**), but abundance of iron (**o**), minimal TNF staining (**p**), but positive staining for CD206 and CD163 (**q**, **r**). Quantitative analysis (**s**) from ctrl and angio/hemo/iron areas from 14 plaques demonstrated equivalent macrophage area density (CD68) but higher expression of CD163 and CD206 in regions of angio/hemo/iron than in ctrl regions. Scale bars: low-power, 2 mm; high-power: 200 m (non-normal distribution CD68 and CD163). Fe iron (Ref. [71])

The M (Hb) macrophage phenotype is induced in vitro by HH complexes, and is characterized by the production both anti-inflammatory (IL-10, IL-1Ra) and pro-inflammatory (VEGF, IL-1β) factors. As compared to foam cells, M (Hb) macrophage lacks lipid retention which distinguishes M (Hb) macrophage from foamy macrophages [71, 77]. Owing to increased activity of the transcription factor oxysterols receptor LXR-α (also known as liver X receptor-α),

induction of cholesterol efflux, and downregulation of scavenger receptors involved in lipid uptake these macrophages are protected against lipid accumulation [71]. As eluded to above, free iron is exported through upregulation of the iron exporter FPN in M (Hb) macrophage, which leads to a decrease of intracellular free iron in M (Hb) macrophage [71, 78]. M (Hb) macrophages also produce less reactive oxygen species (ROS) than foam cells [71]. In response to

HH complex ligation via CD163, these macrophages produce IL-10 by a mechanism involving the phosphoinositide 3-kinase (PI3K)–Akt pathway [79, 80]. On the basis of their lower ROS, elaboration of anti-inflammatory cytokines and non-foam cell appearance, M (Hb) macrophage has thought to be atheroprotective based on these findings [77, 81]. However, the association of M (Hb) macrophage with areas of intraplaque angiogenesis and permeability raises the important question of whether M (Hb) macrophage causes these processes or is merely associated with them.

Vascular endothelial growth factor (VEGF) is a critical signal protein produced by a variety of cells that stimulates the formation of blood vessels by binding to tyrosine kinase receptors (VEGFR) in the cell surface [82]. The production of VEGF is stimulated by hypoxia-inducible factors (HIFs). A relationship between iron and HIF1α can be found in its interactions with the prolyl hydroxylase proteins (PHDs). In the milieu with normoxia or hyperoxia, HIF1α becomes hydroxylated by PHDs on proline residues 402 and 564, within its oxygen-dependent domain. This allows it to be recognized by the von Hippel–Lindau tumor suppressor protein (pVHL), which targets it for ubiquitin-mediated degradation [83]. Because

it is an essential cofactor for the activity of PHDs, iron also indirectly controls the activation of HIFIα [84]. The hydroxylation of proline residues on HIF1α is controlled by 3 HIF prolyl hydroxylases (PHD1, PHD2, PHD3) [85]. PHD2 plays a primary role in cultured cells [85]. The addition of iron in vitro promotes PHD2 hydroxylase activity whereas its chelation is associated with PHD inhibition. Thus, the angiogenic potential of a cell can be affected by iron metabolism. We recently showed that lowering of intracellular iron within M (Hb) macrophages inhibits PHD2 function which increases HIF1 and VEGF levels. In vitro work suggested M (Hb) supernatants increased endothelial permeability and endothelial inflammation [i.e., upregulation of vascular cell adhesion molecule (VCAM)] which was also shown by comparing plaque progression in the brachiocephalic artery of $CD163^{+/+}$ and $CD163^{-/-}$ mice both on $ApoE^{-/-}$ background (Fig. 3) [82]. Blockade of VEGF both in vitro and in vivo inhibited these effects suggesting VEGF as the main stimulus. In human plaque the presence of CD163+ macrophages was correlated with vascular permeability (as measured by Evans Blue Dye staining), angiogenesis and inflammation. Finally, in 1-year-old $CD163^{-/-}$ $ApoE^{-/-}$ brachiocephalic
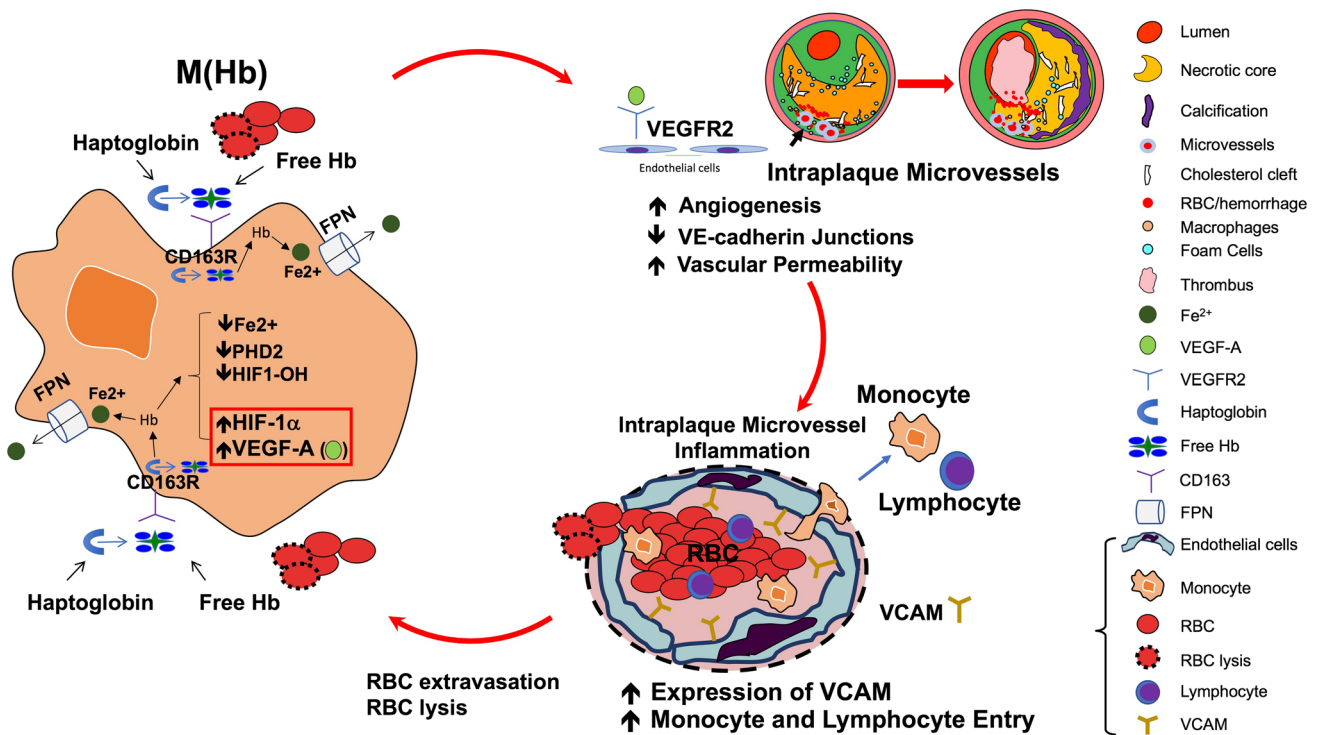

Fig. 3 Summary of the role of M (Hb) macrophages in plaque angiogenesis, permeability, vascular inflammation, and plaque progression. In areas of IPH, HH complex ingestion by macrophages induces angiogenesis via activation of HIF1α, which is a consequence of intracellular Fe^{2+} deprivation and PHD2 inhibition. VEGF-A, which is secreted by macrophages via HIF1α activation, promotes angiogenesis, endothelial expression of VCAM, inflammatory cell recruitment, and vascular permeability via VEGF-A/VEGFR2 signaling. This may cause further IPH, RBC lysis, and more Hb ingestion by CD163+ macrophages. This vicious cycle causes plaque progression, which eventually leads to plaque rupture (Ref. [82])

artery plaque progression was inhibited as compared to $CD163^{+/+}$ $ApoE^{-/-}$ mice, suggesting CD163 is a proatherogenic molecule.

These results challenge the concept that M (Hb) is atheroprotective and that hypoxia resulting from intimal thickening is the major cause of plaque angiogenesis. The relationship between hypoxia in human carotid artery and the bioreductive agent pimonidazole was reported by Sluimer et al. [86]. Hypoxia was strongly linked to regions of advanced atheroma with the macrophage marker CD68, angiogenesis, and thrombus, though pathological intimal thickening lesions are not related to hypoxia. At the sites of inflammation, HIF1α immunoreactivity and mRNA were detected even in areas within a close distance (20–30 µm) from the vessel lumen though the diffusion limit of oxygen is 100–250 µm. While hypoxia may be an additional and important factor, our data clearly demonstrate that Hb intake by human macrophages under conditions of normoxia drives their differentiation into proangiogenic cells.

These data suggest that the proangiogenic changes that occur within M (Hb) cells are almost certainly proatherogenic within the context of IPH. Small amounts of intimal angiogenesis and bleeding could be exacerbated by the inflammatory response which sets in place a vicious cycle by which bleeding begets more bleeding. Clinical studies using carotid MRI to detect IPH also confirms this event has longstanding deleterious effects on plaque growth that remain detectable for years after the initial bleeding event [87].

Role of macrophages in plaque stability

As the lesion becomes advanced, the number of macrophages increases. Symptomatic plaque has more macrophages than asymptomatic plaques [88–90]. Macrophages in shoulder regions of fibrous cap mainly express M1 polarization markers and these are recognized as unstable areas prone to cap rupture [91]. Macrophages found in fibrous plaque express both M1 and M2 polarized markers [88]. Ruptured human plaques express transcriptional markers of both M1 and M2 cells without preferential polarization. Ruptured versus stable plaques showed upregulation of both M1 markers such as CD68, HLA-DP/Q/R and MARCO and M2 markers DC-SIGN and PPARγ. Markers of IPH such as CD163 and HO-1 were very upregulated in ruptured plaques. Immunostaining revealed both types of markers accumulate as plaques increased as lesion severity increased. Lesional foam cells were characterized by some markers of both M1 (HLA-DP/Q/R and iNOS as well as dectin-1).

According to some experimental data, macrophage phenotype can switch to differential subtypes in response to the microenvironment. These might explain the reason why macrophage phenotypes expressing different types of markers exist in same regions or the fact that foam cells demonstrate mixed of surface markers. Mouse studies have reported phenotype switching of macrophages [92]. Arginase II (M1 markers) has been found next to arginase I (M2 markers) in macrophages within advance atherosclerotic plaques. Moreover, immunohistological staining showed equally distributed arginase I and II within advanced plaques [92]. Switching phenotypes of macrophages can be influenced by changes of cytokines in microenvironment with plaque progression and severity.

Atherosclerosis development in coronary arteries can be also be affected by macrophages in epicardial adipose tissue. There are more macrophage infiltration and cytokine expression in the epicardial adipose tissue of patients with coronary artery disease than those without coronary artery disease [93, 94]. The M1:M2 macrophage ratio in epicardial adipose tissue is positively correlated with the severity of coronary artery disease, indicating that epicardial adipose tissue as well as the plaque are related to macrophage polarization in atherosclerotic lesion of humans [94].

M1 macrophages are dominant within the carotid plaque of symptomatic patients during acute ischemic attack, whereas asymptomatic patients are likely to display M2 phenotype in the plaque [89]. CD11c + M1 macrophages were found within the advanced plaques related to acute myocardial infarction. On the other hand, there were no differences in the mannose receptor + M2 macrophages between acute myocardial infarction lesions and stable angina lesions [95]. Moreover, the different types of arteries are also associated with proportion of M1 and M2 phenotypes [90]. M1 macrophages are more frequently observed in carotid arteries than femoral arteries, whereas M2 markers were detected more common in femoral than carotid arteries [90]. The results of correlation studies indicate that M1 macrophages predominate in plaques with an unstable phenotype, suggesting that plaque instability might be the consequence of an imbalance between M1 and M2 phenotypes. However, whether the abundance of M1 macrophages in unstable plaques is the cause or consequence of plaque rupture remains unclear and further work is needed to understand how the ratio of macrophages phenotypes affects plaque progression.

Atherosclerosis regression

Historically, the progression of atherosclerosis in both human and animal studies has been the focus of most research. It was a long-held belief that atherosclerosis was essentially an irreversible process, though plaque regression remained poorly understood. The most convincing evidence supporting regression in advanced lesions has come from animal studies. These experiments date back to the 1920s

when Anichkov showed that switching cholesterol-fed rabbits to a low-fat chow over 2–3 years resulted in arterial lesions becoming more fibrous with reduced lipid content [96]. Subsequent studies in monkeys showed shrinkage and remodeling during longer follow-up as their diet was low-fat diet [97]. To further understand the mechanisms of plaque regression, murine transplantation models have been used. In these experiments, a segment of plaque containing aorta from a hyperlipidemic proatherogenic (e.g., ApoE$^{-/-}$) is transplanted into a wild-type recipient. Using this approach the degree of plaque complexity can also be examined. Transplanting early or advanced lesions into wild-type mice reduces the number of foam cells and increases the number of smooth muscle cells, particularly in the fibrous cap [98, 99]. Recent studies showed that continued recruitment of Ly6Chi inflammatory monocytes and their STAT6-dependent polarization to the M2 state are required for resolution of atherosclerotic inflammation and plaque regression, and single-cell RNA-seq analyses showed less complex macrophage populations in the regression than progression [38, 100].

Common to these mouse models of atherosclerosis regression is a decline in the number of macrophages and some changes in their phenotypic characterization with enrichment of M2 characteristics, suggesting these cells may play an active role in this process. As more data accumulated for the existence of multiple macrophage phenotypes in atherosclerosis, the emigration of macrophages and the presence of tissue-remodeling M2 macrophages in human and animal plaques were reported. They raise the hope that atherosclerosis regression can be achieved at the clinical level. In progressing and regressing plaques of an aortic transplantation mouse model, transcriptomic profiling of macrophages isolated by laser capture microdissection showed > 700 differentially regulated genes [31]. Downregulated genes included adhesion molecules, such as members of the cadherin family [31] with the upregulation of cellular motility factors. Transcriptome analyses from other models of regression will be needed to determine how conserved these changes are.

Because it remains impossible to detect macrophages phenotypes in humans, the association of M2 macrophages with plaque regression in living patients remains uncertain. Clinical trials in which patients with acute coronary syndromes as well as stable angina were treated with high-dose statins and evaluated by intravascular ultrasound (IVUS) have shown changes in plaque composition [101, 102]. However, meta-analysis from eight pooled clinical trials evaluating the effects of intensive statin therapy on plaque volume by IVUS showed that high-intensity statin therapy significantly increases the volume of calcification within plaque without a decrease of atheroma volume [103]. Additional clinical studies also support these results [104, 105]. Taken together, statin therapy may provide plaque stabilization

with volume reduction of atheroma with some incremental increase in calcification volume. Yet it remains unknown how such treatments affect plaque macrophage phenotypes in humans.

Conclusions

During the development and progression of atherosclerotic lesions, macrophages respond to various environmental signals such as lipid and their derivatives, pro- and anti-inflammatory cytokines and heme from senescent erythrocytes. They can regulate distinct phenotypes of macrophage in atherosclerotic lesions. The spectrum of macrophage phenotypes probably changes over the lifetime of a plaque since these signals may vary as plaques progress or regress depending upon the cellular events taking place. Therefore, macrophages adapt their phenotype both over time and in response to what is taking place around them. Although it is well recognized that foam cell type macrophages play a critical role in atherosclerosis progression, the role of function of alternative macrophage phenotypes is still being explored. In fact, the recent single-cell-RNA-seq studies revealed that the foamy macrophages express lesser inflammatory cytokines discrepant with the commonly held perception that foamy macrophages are related to the inflammatory burden. While IPH is a well-recognized event in the life of advanced plaques, the macrophage inflammatory response to such an event may also be an important predictor of plaque evolution. The role of M2 macrophages as associated with plaque regression in humans is still not fully understood. The concept of therapeutic targeting of macrophages within atherosclerotic lesions is attractive as a method to prevent the progression or even hasten regression of plaques. However, modulation of macrophage phenotypes as a therapeutic approach remains an attractive concept but is not yet feasible yet since several questions about the role of alternative macrophages in plaque evolution remain unanswered.

Compliance with ethical standards

References

1. Moore KJ, Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. Cell 145:341–355
2. Lusis AJ (2000) Atherosclerosis. Nature 407:233–241
3. Libby P (2002) Inflammation in atherosclerosis. Nature 420:868–874

4. Libby P, Aikawa M, Schonbeck U (2000) Cholesterol and atherosclerosis. Biochem Biophys Acta 1529:299–309

5. Tabas I (2005) Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arterioscler Thromb Vasc Biol 25:2255–2264

6. Tabas I (2010) Macrophage death and defective inflammation resolution in atherosclerosis. Nat Rev Immunol 10:36–46

7. Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, MacDonald AS, Allen JE (2011) Local macrophage proliferation, rather than recruitment from the blood, is a signature of th2 inflammation. Science 332:1284–1288

8. Chinetti-Gbaguidi G, Baron M, Bouhlel MA, Vanhoutte J, Copin C, Sebti Y, Derudas B, Mayi T, Bories G, Tailleux A, Haulon S, Zawadzki C, Jude B, Staels B (2011) Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the ppar-gamma and lxralpha pathways. Circ Res 108:985–995

9. Robbins CS, Hilgendorf I, Weber GF, Theurl I, Iwamoto Y, Figueiredo JL, Gorbatov R, Sukhova GK, Gerhardt LM, Smyth D, Zavitz CC, Shikatani EA, Parsons M, van Rooijen N, Lin HY, Husain M, Libby P, Nahrendorf M, Weissleder R, Swirski FK (2013) Local proliferation dominates lesional macrophage accumulation in atherosclerosis. Nat Med 19:1166–1172

10. Jenkins SJ, Ruckerl D, Thomas GD, Hewitson JP, Duncan S, Brombacher F, Maizels RM, Hume DA, Allen JE (2013) Il-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by csf-1. J Exp Med 210:2477–2491

11. Gordon S, Taylor PR (2005) Monocyte and macrophage heterogeneity. Nat Rev Immunol 5:953–964

12. Gordon S (2003) Alternative activation of macrophages. Nat Rev Immunol 3:23–35

13. Waldo SW, Li Y, Buono C, Zhao B, Billings EM, Chang J, Kruth HS (2008) Heterogeneity of human macrophages in culture and in atherosclerotic plaques. Am J Pathol 172:1112–1126

14. Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G (2007) Ppargamma activation primes human monocytes into alternative m2 macrophages with anti-inflammatory properties. Cell Metab 6:137–143

15. Geissmann F, Jung S, Littman DR (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 19:71–82

16. Winkels H, Ehinger E, Vassallo M, Buscher K, Dinh HQ, Kobiyama K, Hamers AAJ, Cochain C, Vafadarnejad E, Saliba AE, Zernecke A, Pramod AB, Ghosh AK, Anto Michel N, Hoppe N, Hilgendorf I, Zirlik A, Hedrick CC, Ley K, Wolf D (2018) Atlas of the immune cell repertoire in mouse atherosclerosis defined by single-cell rna-sequencing and mass cytometry. Circ Res 122:1675–1688

17. Cochain C, Vafadarnejad E, Arampatzi P, Pelisek J, Winkels H, Ley K, Wolf D, Saliba AE, Zernecke A (2018) Single-cell rna-seq reveals the transcriptional landscape and heterogeneity of aortic macrophages in murine atherosclerosis. Circ Res 122:1661–1674

18. Kim K, Shim D, Lee JS, Zaitsev K, Williams JW, Kim KW, Jang MY, Seok Jang H, Yun TJ, Lee SH, Yoon WK, Prat A, Seidah NG, Choi J, Lee SP, Yoon SH, Nam JW, Seong JK, Oh GT, Randolph GJ, Artyomov MN, Cheong C, Choi JH (2018) Transcriptome analysis reveals nonfoamy rather than foamy plaque macrophages are proinflammatory in atherosclerotic murine models. Circ Res 123:1127–1142

19. Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, Kastelein R, Kolk A, de Waal-Malefyt R, Ottenhoff TH (2004) Human il-23-producing type 1 macrophages promote

20. but il-10-producing type 2 macrophages subvert immunity to (myco) bacteria. Proc Natl Acad Sci USA 101:4560–4565

20. Adamson S, Leitinger N (2011) Phenotypic modulation of macrophages in response to plaque lipids. Curr Opin Lipidol 22:335–342

21. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25:677–686

22. Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, Rayner KJ, Boyer L, Zhong R, Frazier WA, Lacy-Hulbert A, El Khoury J, Golenbock DT, Moore KJ (2010) Cd36 ligands promote sterile inflammation through assembly of a toll-like receptor 4 and 6 heterodimer. Nat Immunol 11:155–161

23. Stein M, Keshav S, Harris N, Gordon S (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 176:287–292

24. Jetten N, Verbruggen S, Gijbels MJ, Post MJ, De Winther MP, Donners MM (2014) Anti-inflammatory m2, but not pro-inflammatory m1 macrophages promote angiogenesis in vivo. Angiogenesis 17:109–118

25. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA (2001) Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta (1). J Exp Med 194:809–821

26. Spencer M, Yao-Borengasser A, Unal R, Rasouli N, Gurley CM, Zhu B, Peterson CA, Kern PA (2010) Adipose tissue macrophages in insulin-resistant subjects are associated with collagen vi and fibrosis and demonstrate alternative activation. Am J Physiol Endocrinol Metab 299:E1016–E1027

27. Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB, Beelen RH (2011) Macrophages in skin injury and repair. Immunobiology 216:753–762

28. Sierra-Filardi E, Vega MA, Sanchez-Mateos P, Corbi AL, Puig-Kroger A (2010) Heme oxygenase-1 expression in m-csf-polarized m2 macrophages contributes to lps-induced il-10 release. Immunobiology 215:788–795

29. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958–969

30. Feig JE, Rong JX, Shamir R, Sanson M, Vengrenyuk Y, Liu J, Rayner K, Moore K, Garabedian M, Fisher EA (2011) Hdl promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. Proc Natl Acad Sci USA 108:7166–7171

31. Feig JE, Vengrenyuk Y, Reiser V, Wu C, Statnikov A, Aliferis CF, Garabedian MJ, Fisher EA, Puig O (2012) Regression of atherosclerosis is characterized by broad changes in the plaque macrophage transcriptome. PLoS One 7:e39790

32. Feig JE, Shang Y, Rotllan N, Vengrenyuk Y, Wu C, Shamir R, Torra IP, Fernandez-Hernando C, Fisher EA, Garabedian MJ (2011) Statins promote the regression of atherosclerosis via activation of the ccr7-dependent emigration pathway in macrophages. PLoS One 6:e28534

33. Chinetti-Gbaguidi G, Colin S, Staels B (2015) Macrophage subsets in atherosclerosis. Nat Rev Cardiol 12:10–17

34. Chistiakov DA, Bobryshev YV, Nikiforov NG, Elizova NV, Sobenin IA, Orekhov AN (2015) Macrophage phenotypic plasticity in atherosclerosis: the associated features and the peculiarities of the expression of inflammatory genes. Int J Cardiol 184:436–445

35. Uyemura K, Demer LL, Castle SC, Jullien D, Berliner JA, Gately MK, Warrier RR, Pham N, Fogelman AM, Modlin RL (1996) Cross-regulatory roles of interleukin (il)-12 and il-10 in atherosclerosis. J Clin Investig 97:2130–2138

36. Porcheray F, Viaud S, Rimaniol AC, Leone C, Samah B, Dereuddre-Bosquet N, Dormont D, Gras G (2005) Macrophage

activation switching: an asset for the resolution of inflammation. Clin Exp Immunol 142:481–489

37. Lee S, Huen S, Nishio H, Nishio S, Lee HK, Choi BS, Ruhrberg C, Cantley LG (2011) Distinct macrophage phenotypes contribute to kidney injury and repair. J Am Soc Nephrol 22:317–326

38. Lin JD, Nishi H, Poles J, Niu X, McCauley C, Rahman K, Brown EJ, Yeung ST, Vozhilla N, Weinstock A, Ramsey SA, Fisher EA, Loke P (2019) Single-cell analysis of fate-mapped macrophages reveals heterogeneity, including stem-like properties, during atherosclerosis progression and regression. JCI Insight 4(4):e124574

39. Fernandez DM, Rahman AH, Fernandez NF, Chudnovskiy A, Amir ED, Amadori L, Khan NS, Wong CK, Shamailova R, Hill CA, Wang Z, Remark R, Li JR, Pina C, Faries C, Awad AJ, Moss N, Bjorkegren JLM, Kim-Schulze S, Gnjatic S, Ma'ayan A, Mocco J, Faries P, Merad M, Giannarelli C (2019) Single-cell immune landscape of human atherosclerotic plaques. Nat Med 25:1576–1588

40. Kolodgie FD, Narula J, Burke AP, Haider N, Farb A, Hui-Liang Y, Smialek J, Virmani R (2000) Localization of apoptotic macrophages at the site of plaque rupture in sudden coronary death. Am J Pathol 157:1259–1268

41. Akishima Y, Akasaka Y, Ishikawa Y, Lijun Z, Kiguchi H, Ito K, Itabe H, Ishii T (2005) Role of macrophage and smooth muscle cell apoptosis in association with oxidized low-density lipoprotein in the atherosclerotic development. Mod Pathol 18:365–373

42. Hegyi L, Skepper JN, Cary NR, Mitchinson MJ (1996) Foam cell apoptosis and the development of the lipid core of human atherosclerosis. J Pathol 180:423–429

43. Geng YJ, Libby P (1995) Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme. Am J Pathol 147:251–266

44. Libby P, Sukhova G, Lee RT, Galis ZS (1995) Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. J Cardiovasc Pharmacol 25(Suppl 2):S9–S12

45. Ball RY, Stowers EC, Burton JH, Cary NR, Skepper JN, Mitchinson MJ (1995) Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. Atherosclerosis 114:45–54

46. Schaefer HE (1981) The role of macrophages in atherosclerosis. Haematol Blood Transfus 27:137–142

47. Grainger DJ, Reckless J, McKilligin E (2004) Apolipoprotein e modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein e-deficient mice. J Immunol 173:6366–6375

48. Toschi V, Gallo R, Lettino M, Fallon JT, Gertz SD, Fernandez-Ortiz A, Chesebro JH, Badimon L, Nemerson Y, Fuster V, Badimon JJ (1997) Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. Circulation 95:594–599

49. Siess W, Tigyi G (2004) Thrombogenic and atherogenic activities of lysophosphatidic acid. J Cell Biochem 92:1086–1094

50. Llodra J, Angeli V, Liu J, Trogan E, Fisher EA, Randolph GJ (2004) Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. Proc Natl Acad Sci USA 101:11779–11784

51. Galis ZS, Sukhova GK, Lark MW, Libby P (1994) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Investig 94:2493–2503

52. Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E, Lo KM, Gillies S, Javaherian K, Folkman J (2003) Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. Proc Natl Acad Sci USA 100:4736–4741

53. Boyle JJ, Wilson B, Bicknell R, Harrower S, Weissberg PL, Fan TP (2000) Expression of angiogenic factor thymidine phosphorylase and angiogenesis in human atherosclerosis. J Pathol 192:234–242

54. Sambrano GR, Steinberg D (1995) Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. Proc Natl Acad Sci USA 92:1396–1400

55. Miller YI, Viriyakosol S, Binder CJ, Feramisco JR, Kirkland TN, Witztum JL (2003) Minimally modified ldl binds to cd14, induces macrophage spreading via tlr4/md-2, and inhibits phagocytosis of apoptotic cells. J Biol Chem 278:1561–1568

56. Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, Chen PP, Witztum JL (2001) Human-derived anti-oxidized ldl autoantibody blocks uptake of oxidized ldl by macrophages and localizes to atherosclerotic lesions in vivo. Arterioscler Thromb Vasc Biol 21:1333–1339

57. Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, Dennis EA, Witztum JL (1999) Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc Natl Acad Sci USA 96:6353–6358

58. Kojima Y, Volkmer JP, McKenna K, Civelek M, Lusis AJ, Miller CL, Direnzo D, Nanda V, Ye J, Connolly AJ, Schadt EE, Quertermous T, Betancur P, Maegdefessel L, Matic LP, Hedin U, Weissman IL, Leeper NJ (2016) Cd47-blocking antibodies restore phagocytosis and prevent atherosclerosis. Nature 536:86–90

59. Manning-Tobin JJ, Moore KJ, Seimon TA, Bell SA, Sharuk M, Alvarez-Leite JI, de Winther MP, Tabas I, Freeman MW (2009) Loss of sr-a and cd36 activity reduces atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice. Arterioscler Thromb Vasc Biol 29:19–26

60. Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF, Freeman MW (2002) Scavenger receptors class a-i/ii and cd36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 277:49982–49988

61. Kruth HS (2011) Receptor-independent fluid-phase pinocytosis mechanisms for induction of foam cell formation with native low-density lipoprotein particles. Curr Opin Lipidol 22:386–393

62. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E (2010) Nlrp3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 464:1357–1361

63. Menu P, Pellegrin M, Aubert JF, Bouzourene K, Tardivel A, Mazzolai L, Tschopp J (2011) Atherosclerosis in apoe-deficient mice progresses independently of the nlrp3 inflammasome. Cell Death Dis 2:e137

64. Bae YS, Lee JH, Choi SH, Kim S, Almazan F, Witztum JL, Miller YI (2009) Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of nadph oxidase 2. Circ Res 104:210–218 **(221p following 218)**

65. Huber J, Boechzelt H, Karten B, Surboeck M, Bochkov VN, Binder BR, Sattler W, Leitinger N (2002) Oxidized cholesteryl linoleates stimulate endothelial cells to bind monocytes via the extracellular signal-regulated kinase 1/2 pathway. Arterioscler Thromb Vasc Biol 22:581–586

66. Fang L, Harkewicz R, Hartvigsen K, Wiesner P, Choi SH, Almazan F, Pattison J, Deer E, Sayaphupha T, Dennis EA, Witztum JL, Tsimikas S, Miller YI (2010) Oxidized cholesteryl esters and phospholipids in zebrafish larvae fed a high

cholesterol diet: macrophage binding and activation. J Biol Chem 285:32343–32351

67. Huang Z, Li W, Wang R, Zhang F, Chi Y, Wang D, Liu Z, Zhang Y, Matsuura E, Liu Q (2010) 7-Ketocholesteryl-9-carboxynona-noate induced nuclear factor-kappa b activation in j774a1 mac-rophages. Life Sci 87:651–657

68. Wartman WB (1938) Occulusion of the coronary arteries by hemorrhage into their walls. Am Heart J 15:459–470

69. Kolodgie FD, Gold HK, Burke AP, Fowler DR, Kruth HS, Weber DK, Farb A, Guerrero LJ, Hayase M, Kutys R, Narula J, Finn AV, Virmani R (2003) Intraplaque hemorrhage and progression of coronary atheroma. N Engl J Med 349:2316–2325

70. Barger AC, Beeuwkes R 3rd, Lainey LL, Silverman KJ (1984) Hypothesis: vasa vasorum and neovascularization of human coronary arteries. A possible role in the pathophysiology of ath-erosclerosis. N Engl J Med 310:175–177

71. Finn AV, Nakano M, Polavarapu R, Karmali V, Saeed O, Zhao X, Yazdani S, Otsuka F, Davis T, Habib A, Narula J, Kolodgie FD, Virmani R (2012) Hemoglobin directs macrophage differentia-tion and prevents foam cell formation in human atherosclerotic plaques. J Am Coll Cardiol 59:166–177

72. Jain RK, Finn AV, Kolodgie FD, Gold HK, Virmani R (2007) Antiangiogenic therapy for normalization of atherosclerotic plaque vasculature: a potential strategy for plaque stabilization. Nat Clin Pract Cardiovasc Med 4:491–502

73. Nagy E, Eaton JW, Jeney V, Soares MP, Varga Z, Galajda Z, Szentmiklosi J, Mehes G, Csonka T, Smith A, Vercellotti GM, Balla G, Balla J (2010) Red cells, hemoglobin, heme, iron, and atherogenesis. Arterioscler Thromb Vasc Biol 30:1347–1353

74. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK (2001) Identification of the haemoglobin scavenger receptor. Nature 409:198–201

75. Pulford K, Micklem K, McCarthy S, Cordell J, Jones M, Mason DY (1992) A monocyte/macrophage antigen recognized by the four antibodies ghi/61, ber-mac3, ki-m8 and sm4. Immunology 75:588–595

76. Nielsen MJ, Moller HJ, Moestrup SK (2010) Hemoglobin and heme scavenger receptors. Antioxid Redox Signal 12:261–273

77. Boyle JJ, Johns M, Kampfer T, Nguyen AT, Game L, Schaer DJ, Mason JC, Haskard DO (2012) Activating transcription factor 1 directs mhem atheroprotective macrophages through coordinated iron handling and foam cell protection. Circ Res 110:20–33

78. Habib A, Polavarapu R, Karmali V, Guo L, Van Dam R, Cheng Q, Akahori H, Saeed O, Nakano M, Pachura K, Hong CC, Shin E, Kolodgie F, Virmani R, Finn AV (2015) Hepcidin-ferroportin axis controls toll-like receptor 4 dependent macrophage inflam-matory responses in human atherosclerotic plaques. Atheroscle-rosis 241:692–700

79. Philippidis P, Mason JC, Evans BJ, Nadra I, Taylor KM, Haskard DO, Landis RC (2004) Hemoglobin scavenger receptor cd163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. Circ Res 94:119–126

80. Landis RC, Philippidis P, Domin J, Boyle JJ, Haskard DO (2013) Haptoglobin genotype-dependent anti-inflammatory signaling in cd163(+) macrophages. Int J Inflamm 2013:980327

81. Boyle JJ, Harrington HA, Piper E, Elderfield K, Stark J, Lan-dis RC, Haskard DO (2009) Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. Am J Pathol 174:1097–1108

82. Guo L, Akahori H, Harari E, Smith SL, Polavarapu R, Karmali V, Otsuka F, Gannon RL, Braumann RE, Dickinson MH, Gupta A, Jenkins AL, Lipinski MJ, Kim J, Chhour P, de Vries PS, Jin-nouchi H, Kutys R, Mori H, Kutyna MD, Torii S, Sakamoto A, Choi CU, Cheng Q, Grove ML, Sawan MA, Zhang Y, Cao Y,

Kolodgie FD, Cormode DP, Arking DE, Boerwinkle E, Morrison AC, Erdmann J, Sotoodehnia N, Virmani R, Finn AV (2018) Cd163+ macrophages promote angiogenesis and vascular perme-ability accompanied by inflammation in atherosclerosis. J Clin Investig 128:1106–1124

83. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr (2001) Hifalpha targeted for vhl-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 292:464–468

84. Nandal A, Ruiz JC, Subramanian P, Ghimire-Rijal S, Sinnamon RA, Stemmler TL, Bruick RK, Philpott CC (2011) Activation of the hif prolyl hydroxylase by the iron chaperones pcbp1 and pcbp2. Cell Metab 14:647–657

85. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J (2003) Hif prolyl-hydroxylase 2 is the key oxygen sensor set-ting low steady-state levels of hif-1alpha in normoxia. EMBO J 22:4082–4090

86. Sluimer JC, Gasc JM, van Wanroij JL, Kisters N, Groeneweg M, Sollewijn Gelpke MD, Cleutjens JP, van den Akker LH, Cor-vol P, Wouters BG, Daemen MJ, Bijnens AP (2008) Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. J Am Coll Cardiol 51:1258–1265

87. Sun J, Underhill HR, Hippe DS, Xue Y, Yuan C, Hatsukami TS (2012) Sustained acceleration in carotid atherosclerotic plaque progression with intraplaque hemorrhage: a long-term time course study. JACC Cardiovasc Imaging 5:798–804

88. Stoger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, Biessen EA, Daemen MJ, Lutgens E, de Winther MP (2012) Distribution of macrophage polarization markers in human ath-erosclerosis. Atherosclerosis 225:461–468

89. Cho KY, Miyoshi H, Kuroda S, Yasuda H, Kamiyama K, Naka-gawara J, Takigami M, Kondo T, Atsumi T (2013) The phe-notype of infiltrating macrophages influences arteriosclerotic plaque vulnerability in the carotid artery. J Stroke Cerebrovasc Dis 22:910–918

90. Shaikh S, Brittenden J, Lahiri R, Brown PA, Thies F, Wilson HM (2012) Macrophage subtypes in symptomatic carotid artery and femoral artery plaques. Eur J Vasc Endovasc Surg 44:491–497

91. Barlis P, Serruys PW, Devries A, Regar E (2008) Optical coher-ence tomography assessment of vulnerable plaque rupture: pre-dilection for the plaque 'shoulder'. Eur Heart J 29:2023

92. Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nico-letti A, Caligiuri G (2010) Macrophage plasticity in experimental atherosclerosis. PLoS One 5:e8852

93. Hirata Y, Kurobe H, Akaike M, Chikugo F, Hori T, Bando Y, Nishio C, Higashida M, Nakaya Y, Kitagawa T, Sata M (2011) Enhanced inflammation in epicardial fat in patients with coro-nary artery disease. Int Heart J 52:139–142

94. Hirata Y, Tabata M, Kurobe H, Motoki T, Akaike M, Nishio C, Higashida M, Mikasa H, Nakaya Y, Takanashi S, Igarashi T, Kitagawa T, Sata M (2011) Coronary atherosclerosis is associ-ated with macrophage polarization in epicardial adipose tissue. J Am Coll Cardiol 58:248–255

95. Lee CW, Hwang I, Park CS, Lee H, Park DW, Kang SJ, Lee SW, Kim YH, Park SW, Park SJ (2013) Macrophage heterogeneity of culprit coronary plaques in patients with acute myocardial infarction or stable angina. Am J Clin Pathol 139:317–322

96. Wissler RW, Vesselinovitch D (1976) Studies of regression of advanced atherosclerosis in experimental animals and man. Ann N Y Acad Sci 275:363–378

97. Armstrong ML (1976) Evidence of regression of atherosclerosis in primates and man. Postgrad Med J 52:456–461

98. Reis ED, Li J, Fayad ZA, Rong JX, Hansoty D, Aguinaldo JG, Fallon JT, Fisher EA (2001) Dramatic remodeling of advanced

atherosclerotic plaques of the apolipoprotein e-deficient mouse in a novel transplantation model. J Vasc Surg 34:541–547

99. Trogan E, Fayad ZA, Itskovich VV, Aguinaldo JG, Mani V, Fallon JT, Chereshnev I, Fisher EA (2004) Serial studies of mouse atherosclerosis by in vivo magnetic resonance imaging detect lesion regression after correction of dyslipidemia. Arterioscler Thromb Vasc Biol 24:1714–1719

100. Rahman K, Vengrenyuk Y, Ramsey SA, Vila NR, Girgis NM, Liu J, Gusarova V, Gromada J, Weinstock A, Moore KJ, Loke P, Fisher EA (2017) Inflammatory ly6chi monocytes and their conversion to m2 macrophages drive atherosclerosis regression. J Clin Investig 127:2904–2915

101. Nissen SE, Nicholls SJ, Sipahi I, Libby P, Raichlen JS, Ballantyne CM, Davignon J, Erbel R, Fruchart JC, Tardif JC, Schoenhagen P, Crowe T, Cain V, Wolski K, Goormastic M, Tuzcu EM (2006) Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the asteroid trial. JAMA 295:1556–1565

102. Nicholls SJ, Ballantyne CM, Barter PJ, Chapman MJ, Erbel RM, Libby P, Raichlen JS, Uno K, Borgman M, Wolski K, Nissen SE (2011) Effect of two intensive statin regimens on progression of coronary disease. N Engl J Med 365:2078–2087

103. Puri R, Nicholls SJ, Shao M, Kataoka Y, Uno K, Kapadia SR, Tuzcu EM, Nissen SE (2015) Impact of statins on serial coronary calcification during atheroma progression and regression. J Am Coll Cardiol 65:1273–1282

104. Raber L, Taniwaki M, Zaugg S, Kelbaek H, Roffi M, Holmvang L, Noble S, Pedrazzini G, Moschovitis A, Luscher TF, Matter CM, Serruys PW, Juni P, Garcia-Garcia HM, Windecker S (2015) Effect of high-intensity statin therapy on atherosclerosis in non-infarct-related coronary arteries (ibis-4): a serial intravascular ultrasonography study. Eur Heart J 36:490–500

105. Banach M, Serban C, Sahebkar A, Mikhailidis DP, Ursoniu S, Ray KK, Rysz J, Toth PP, Muntner P, Mosteoru S, Garcia-Garcia HM, Hovingh GK, Kastelein JJ, Serruys PW (2015) Impact of statin therapy on coronary plaque composition: a systematic review and meta-analysis of virtual histology intravascular ultrasound studies. BMC Med 13:229

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.