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REVIEW

Macrophage heterogeneity in atherosclerosis: A matter of context

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During atherogenesis, plaque macrophages take up and process deposited lipids, trigger inflammation, and form necrotic cores. The traditional inflammatory/anti-inflammatory paradigm has proven insufficient in explaining their complex disease-driving mechanisms. Instead, we now appreciate that macrophages exhibit remarkable heterogeneity and functional specialization in various pathological contexts, including atherosclerosis. Technical advances for studying individual cells, especially single-cell RNA sequencing, indeed allowed to identify novel macrophage subsets in both murine and human atherosclerosis, highlighting the existence of diverse macrophage activation states throughout pathogenesis. In addition, recent studies highlighted the role of the local microenvironment in shaping the macrophages' phenotype and function. However, this remains largely undescribed in the context of atherosclerosis. In this review we explore the origins of macrophages and their functional specialization, shedding light on the diverse sources of macrophage accumulation in the atherosclerotic plaque. Next, we discuss the phenotypic diversity observed in both murine and human atherosclerosis, elucidating their distinct functions and spatial distribution within plaques. Finally, we highlight the importance of the local microenvironment in both phenotypic and functional specialization of macrophages in atherosclerosis and elaborate on the need for spatial multiomics approaches to provide a better understanding of the different macrophage subsets' roles in the pathogenesis of atherosclerosis.

Keywords: Atherosclerosis · Innate immunity · Macrophage heterogeneity · Macrophage niche · Multiomics

Introduction

Atherosclerosis is the most prevalent type of cardiovascular disease and remains the leading cause of premature death worldwide. It is characterized by excessive lipid deposition in the subendothelial layer of larger blood vessels over the course of a lifetime. Upon local oxidation or modification, these lipids are engulfed by macrophages. In response to this uptake, macrophages release proinflammatory signals leading to the attraction of leukocytes to the site of inflammation. Excessive uptake of lipids triggers the macrophages' apoptotic response and cell remnants accumulate

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to form a necrotic core, characteristic of an advanced plaque phenotype and further fuelling the inflammatory plaque environment [1].

Recent attempts to therapeutically target inflammation, such as the Cantos trial (anti-IL1β, canakinumab), have demonstrated the inflammatory character of atherogenesis but have proven limited success in reducing cardiovascular risk [2]. This points toward more complex disease mechanisms beyond the classical inflammatory/anti-inflammatory dichotomy and is in line with the paradigm shift from M1/M2 macrophages to a more multimodal view of the roles and phenotypes of macrophages [2–4]. The original M1/M2 macrophage paradigm suggested that macrophages polarize either into a pro- or an anti-inflammatory state [5]. This view has been nuanced through findings showing that macrophages in vivo display a multitude of phenotypes and change polarization throughout pathogenesis to adopt diverse, co-existing activation states [6]. As our understanding of macrophages evolved from population-based to single-cell biology, a whole panoply of macrophage subsets has been described. Importantly, it became clear that the identity and functional specialization of macrophages are highly dynamic and depend on their tissue and molecular context [7]. In parallel to the recent paradigm shift in macrophage biology in health and disease, the view on phenotypes and functions of macrophages in atherosclerosis has considerably changed as well [3, 8]. Especially single-cell RNA sequencing (scRNAseq) allowed the identification of new subsets in murine as well as in human atherosclerosis [9–12].

Whereas data on transcriptomic diversity of macrophages in atherosclerosis is multiplying, the influence of the molecular and cellular context on macrophage phenotypes and functions remains to be explored. However, the emergence of technological advances such as multiplex imaging and spatial transcriptomics will contribute to fill this gap by identifying macrophage phenotypes in their spatial context [13, 14].

The idea of the recently proposed "macrophage niche" states that macrophage phenotype and function are driven by cues originating from the immediate environment ("micro-environment") of the macrophages [15]. The dysregulation of this niche drives the development of inflammatory diseases such as atherosclerosis and, vice versa, inflammatory conditions derail the macrophage's niche [15]. This clearly emphasizes the necessity of studying the influence of the microenvironment on macrophages to understand atherogenesis. Here, we introduce the state-of-the-art research on the origin and specialization of macrophages, discuss macrophage heterogeneity in atherosclerosis, and consider how microenvironment(s) drive this phenotypic and functional diversity.

Origin and functional specialization of macrophages

Like their phenotypic heterogeneity, the understanding of macrophage ontogeny has evolved over time. In 1968, van Furth and Cohn described the mononuclear phagocyte system concept, where in homeostasis tissue-resident macrophages rely on replacement by circulating BM-derived monocytic precursors over time, with peritoneal macrophages being replaced by 0.1% per hour [16]. Much later, it was shown that in Th2-related pathologies, tissue-resident macrophage accumulation through self-renewal is sufficient for pathogen control or wound repair, indicating monocyte recruitment is context-dependent [17]. Recent work showed that besides BM-derived monocytes, tissue-resident macrophages can derive from the yolk sac (YS) macrophages and fetal liver (FL) monocytes [18]. Fate mapping studies demonstrated that in some tissues these embryonic macrophages persist into adulthood while in other tissues (e.g. heart and intestine) they are progressively replaced by monocyte-derived macrophages lacking self-renewal capacities [19, 20]. This results in a variation in the proportion of monocyte-derived and tissue-resident macrophages between tissues [21, 22].

When assessing the ontogeny of arterial macrophages using lineage tracing models, such as pulse-labeled $Csf1r^{MerCreMer(MCM)}Rosa26^{YFP}$ and $Tie2^{MCM}Rosa26^{YFP}$ embryos with 4-hydroxytamoxifen at E8.5 and E7.5 or E10.5, it was shown that arterial resident macrophages arise from $Tie2^{+}Csf1r^{+}$ erythro-myeloid progenitors during YS hematopoiesis [23]. During aging in healthy mice, both erythro-myeloid progenitor and BM-derived macrophages contribute to an increased adventitial macrophage population, leading to a gradual replacement of the YS-derived population [23]. In parallel, another study showed that embryonic-derived macrophages from $CX3CR1^{+}$ precursors are maintained during homeostasis by the proliferation of monocyte-derived macrophages that seeded the arteries directly postnatal, explaining the mixed origin of arterial macrophages [24].

Macrophage ontogeny was shown to impact arterial macrophage functional specialization. These monocyte-derived macrophages are $CD115^{-}$ (*Csf1R*) $Lyve1^{-}$ and have a greater phagocytic capacity compared with $CD115^{+}Lyve1^{+}$ embryonic-derived arterial macrophages [24]. This is in line with Weinberger et al. [23] who show that steady-state $Lyve1^{+}$ YS-derived macrophages mainly have homeostatic and anti-inflammatory properties while monocyte-derived macrophages displayed an inflammatory phenotype.

Apart from recruited monocytes, mesenchymal cells can transdifferentiate and adopt a macrophage-like phenotype and functionality during pathogenesis and embryogenesis, like mesothelial cell–macrophage transition observed in mesentery [25, 26]. This underlines the importance of lineage tracing in determining the ontogeny of macrophages in arteries.

Macrophage ontogeny has been shown to impact arterial macrophage functional specialization. In addition to ontogeny-related differences, it has also been shown that the local microenvironment plays an important role in macrophage specialization [18, 27]. Upon in vivo adoptive transfer into a macrophage-depleted murine lung, macrophages from YS, FL, or BM origin could differentiate into alveolar macrophage-like cells, while FL monocytes had a competitive advantage of occupying the niche. However, the transfer of tissue-specific macrophages from one organ to another is not capable of efficiently repopulating the organ, indicating that epigenetic tissue imprinting

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cannot be reversed [18, 27]. This can be explained by the "macrophage niche" model that suggests self-renewing tissue-resident macrophages occupy a specific niche in steady-state, which is replenished by circulating monocytes [15, 28]. It is unclear if these differences in ontogeny underlie any phenotypic differences in arterial macrophages discussed below.

Origins and functional specialization of monocytes and macrophages in atherosclerosis

Throughout the initiation and progression of atherosclerosis, the origin of monocytes and macrophages in the atherosclerotic plaque is diverse. Williams et al. [29] showed that self-renewing aortic intima-resident macrophages (Mac^{AIR}), important for atherosclerosis initiation, arise from monocyte progenitors that seed the artery at birth. While these cells have been shown to differentiate into the first foam cells within the arterial intima, resident macrophage proliferation within the atherosclerotic plaque is insufficient for long-term maintenance of foam cells, resulting in them largely being overgrown by recruited monocytes that play a critical role in the macrophage population expansion during atherogenesis [29]. Another study using *Apoe*$^{-/-}$ high cholesterol diet-fed mice showed that macrophage replenishment in atherosclerosis mainly depended on local proliferation rather than monocyte recruitment [30]. Psaltis et al. [31] showed that there is a shift from circulating monocytes to local expansion in progression from early to established intimal lesions [30]. In line with previous studies, Härdtner et al. [32] showed that lipid-lowering strategies inhibited local macrophage proliferation in APOE*3-Leiden.CETP mice leading to a decrease in macrophage number and plaque regression. Altogether, this indicates the importance of local proliferating, resident macrophages in the plaque versus monocyte recruitment. In addition to monocytes and local macrophages, 40%–60% of macrophages in the plaque are shown to be of mesenchymal origin [33]. Smooth muscle cells attaining a proatherogenic macrophage phenotype, partly in response to cholesterol, have indeed been shown to play a role in plaque formation and remodeling [26]. It has been shown by scRNAseq that rather than attaining a macrophage phenotype, these cells transform into unique fibroblast-like cells, termed "fibromyocytes", that act protective in the lesion and the fibrous cap [11]. Increased levels of HDL could restore these cells' smooth muscle cells phenotype through cholesterol efflux, thereby leading to the thickening of the plaque-stabilizing fibrous cap [34].

Macrophage heterogeneity in atherosclerosis

Macrophage diversity and functions in murine atherosclerosis

Next to different origins, the microenvironment that macrophages reside in, drives their phenotype and functional specialization. Particularly in atherosclerotic conditions, this microenvironment is significantly altered, highly complex, and instrumental/decisive for a wide range of macrophage phenotypes and functions, ultimately driving plaque progression and determining plaque (de)stabilization.

A meta-analysis integrating 9 scRNAseq datasets of murine atherosclerotic lesions [9, 35–37] by Zernecke et al. [8] allowed to identify five major plaque macrophage phenotypes, based on their transcriptional profiles, despite inter-study differences in subset abundances. Noticeably, macrophage diversity increases with the progression of atherosclerosis [8].

"Inflammatory macrophages" were characterized by their expression of chemokines *Cxcl1, Cxcl2, Ccl2*, *Ccl3*, *Ccl4*, inflammatory cytokines *Il1b* and *Tnf*, alongside the expression of *Ccr2* that suggests they are monocyte-derived (Table 1). These inflammatory macrophages are transcriptionally distinct from foam cells [8]. However, previous work reported an inflammasome-dependent expression of *Il1b* that is activated by cholesterol crystals [38]. Thus, even if the inflammatory macrophages display a different transcriptional profile than foam cells which are not inherently proinflammatory, it cannot be excluded that both subsets partly share differentiation trajectories [8, 39]. Hence, one possibility is that these proinflammatory monocytes/macrophages represent an initial polarization state of foam cells [39].

Interferon-induced macrophages ("IFNIC") represent a small population that displays expression of genes characteristic for a type I IFN response, such as *Ifit3*, *Irf7*, and *Isg15*. Recent data indicate that the accumulation of lipids in monocytes/macrophages specifically dampened the type I IFN response in macrophages, providing a possible explanation for the low number of IFNIC macrophages in advanced atherosclerotic lesions [40].

In scRNAseq of heart macrophages at steady state, a cluster with macrophages showing a similar interferon-stimulated transcriptional profile was detected, and these cells shared a lineage relation with a *Ccr2*$^{+}$ and *Mhc-II*$^{+}$ macrophage cluster [41]. Thus, it is unclear whether these cells mainly play a role in homeostasis or are also involved in the induction of atherogenesis.

The "resident macrophage" subset expresses genes such as *Lyve1* and *Mrc1* (Table 1) [24, 42]. A fraction of these cells express *Ccr2* and low *Lyve1*, indicating that they may partly be monocyte-derived [8, 37]. This finding stipulates a certain phenotypic diversity of macrophages within the cluster that forms the resident macrophages. This subset resembles the recently described 'TLF' (Timd4 + Lyve1 + Folr2+) resident macrophages conserved across mouse organs. When assessing their transcriptional similarity, these macrophages seem to have important developmental and homeostatic functions through vesicle-mediated transport and receptor-mediated endocytosis [43].

"Foamy macrophages" were identified expressing several genes important in lipid processing (*Abcg1*, *Trem2*, *Fabp4) but also Cd11c, Cd9*, and *Spp1* among others (Table 1) [8]. Expression of *Cd14* in part of these cells indicates that foam cells can be monocyte-derived [8]. Evidence from yolk sac-derived microglia that acquire a similar transcriptional profile reinforces the hypothesis that foamy macrophages are a heterogeneous subset

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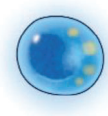
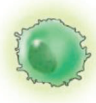
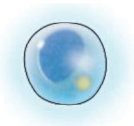
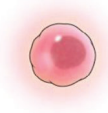
Table 1. Macrophage subsets and their marker expression in advanced murine atherosclerosis.

Subset		Protein markers	Transcriptional profiles
Advanced foam cells		Perilipin2^{+++}, F4/80^{+++}, CD68^{+++}, Dectin1^{++}, CD44^{+}, MHCII^{+}, CD206^{+}	*Abcg1, Trem2, Fabp4, Cd9,Lgals3 Ctsd, Ctsl, Spp1,Mmp12, Mmp14, Itgax*
Resident macrophages		CD206^{+++}, MHCII^{++}, F4/80^{+}, Dectin1^{+}	*Lyve1, Mrc1, Folr2, F13a1, Ccl8*
Early foam cells		Perilipin2^{+++}, Dectin1^{+++}, F4/80^{++}, MHCII^{+}, CD68^{+}, CD44^{+}	*Abcg1, Trem2, Fabp4, Cd9,Lgals3 Ctsd, Ctsl, Spp1,Mmp12, Mmp14, Itgax*
Smooth muscle cell-derived foam cells		Perilipin2^{+++}, CD44^{+++} Dectin1^{++}, F4/80^{+}, CD68^{+},	*Abcg1, Trem2, Fabp4, Cd9,Lgals3 Ctsd, Ctsl, Spp1,Mmp12, Mmp14, Itgax*
Inflammatory macrophages		MHCII^{+++}, F4/80^{++}, Dectin1^{+}	*Ccl2, Cxcl1, Il1b, Tnf, Cd14, Ccl3, Cxcl2, Ccl4*

including macrophages of different ontogenies [44]. The existence of a foamy macrophage signature among many (metabolic) diseases underlines the central role of this subset in disease mechanisms and progression [45]. These macrophages seem to play a role in lipid metabolism and catabolism while having a low expression of inflammatory genes [35]. Furthermore, their gene signature is similar to osteoclasts, indicating a potential role in plaque calcification [37].

"Cavity macrophages" were previously uncharacterized in atherosclerosis and can be defined as *Cd226*, *Cd11c, Ccr2*, *Retnla*, and *Mhc-II* expressing cells [8]. They strongly resemble a monocyte-derived macrophage subset identified as IRF-4-dependent peritoneal/pleural resident macrophages [46].

The introduction of scRNAseq to the field of cardiovascular research contributed substantially to the discovery and definition of macrophage subsets in atherosclerotic plaque. However, the scRNAseq datasets that have played a major role in identifying plaque macrophage subsets may miss certain subsets and lack information about their spatial context. Therefore, to understand how the biochemical and cellular neighborhood influences macrophage phenotype and function, the use of techniques that maintain the tissue's spatial organization is crucial.

In recent work from our lab, we compared the identified transcriptomic profiles from the above-mentioned meta-analysis of Zernecke et al. [8] to the macrophage subsets we identified in situ [13]. Our data show strong compartmentalization of the macrophage subsets, with high homogeneity within cell neighborhoods rather than intermingled macrophage subsets (Fig. 1). Parallel mass spectrometry imaging on the same cohort showed distinct lipid profiles in these respective plaque regions. These findings underpin the existence of macrophage phenotype-driving niches in the murine atherosclerotic plaque [13, 15].

The proinflammatory macrophages identified by Zernecke et al. [8] correspond with MHCII-expressing monocyte macrophages that line the luminal border and are freshly infiltrated (Fig. 1). Another subset of inflammatory macrophages lines the plaques' adventitial border (Fig. 1). These two subsets show a strong correlation with the above-described IFNIC-macrophages as well, in line with the lineage-relation of these cells with a *Ccr2* and *MhcII* expressing macrophage cluster that was previously reported [41].

The resident macrophage subset identified by Zernecke et al. [8] links to CD206-expressing macrophages identified by Goossens et al. [13]. This subset is mainly localized at the media-adventitia border and present at both initial and advanced atherosclerotic lesion sites (Fig. 1) [8, 47].

Three different foam cell subsets were identified based on the expression of Perilipin2, a marker of intracellular lipid accumulation (Table 1, Fig. 1). Two of these correspond to the *Trem2*+ foamy macrophages, the first one likely representing an early foam cell subset, based on limited lipid uptake (as determined by histopathology) and their relative proximity to the aortic lumen, while the second is plausibly an advanced foam cell subset with higher lipid content and a localization deeper in the plaque. The latter also displayed signs of disintegrating membrane as a sign of early necrosis [13]. Finally, a third Perilipin2-positive subset presumably represents smooth muscle cell-derived foam cells with low expression of myeloid cell markers [26, 35, 48, 49]. They

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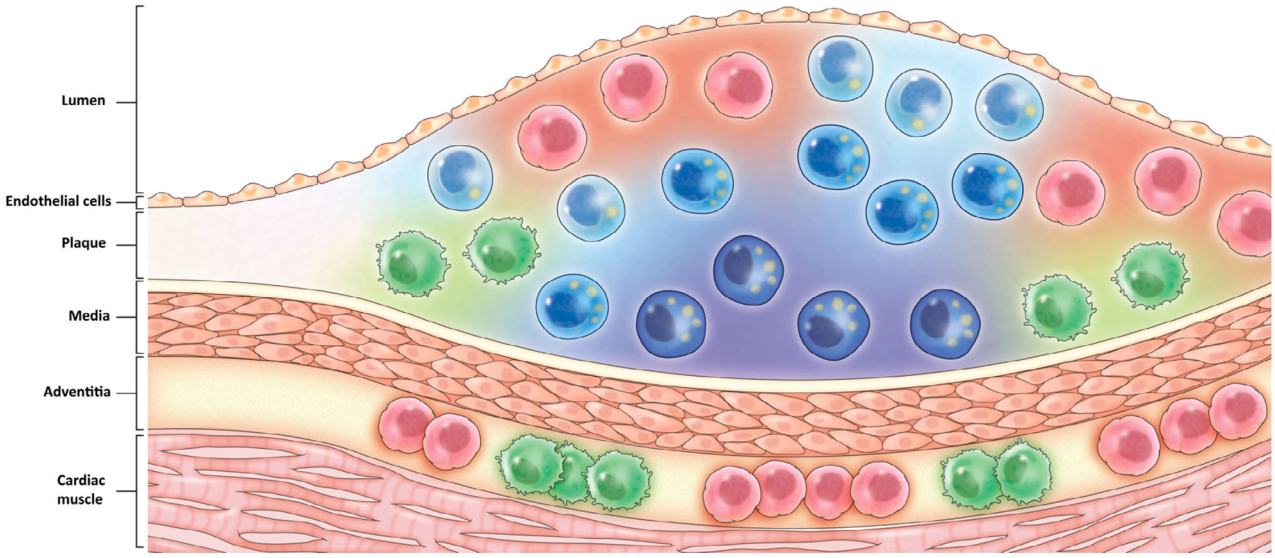

Inflammatory macrophages	Early foam cells	Advanced foam cells	SMC-derived foam cells	Resident macrophages
• Close to the lumen • Adventitia • Plaque-media border	• Close to the lumen	• Deeper in the plaque compared to early foam cells	• Plaque-media border • Necrotic core regions	• Adventitia • Close to the media in the plaque
• Progression of inflammation	• Low lipid content	• High lipid content • Signs of early necrosis	• High lipid content • Proliferative	• Homeostasis
• Monocyte-derived with a fraction of IFN-induced cells	• Partly monocyte derived • Subset comprises various ontogenies	• Likely to be derived from early foam cells	• Potentially derived from medial smooth muscle cells	• Mainly derived from embryonic macrophages

Figure 1. Macrophage subsets and their distribution in the advanced murine atherosclerotic plaque.

display a proliferative phenotype with no visible signs of cell death, despite residing deep in the plaque, in and near the necrotic core. The comparison of non- vs. atherosclerotic aortas confirmed that the presence of foam cells is linked to atherosclerotic disease progression [37, 50]. These findings are supported by their intra-plaque restricted localization [13].

Different macrophage subsets, their localization, functional aspects, and origin as defined by the comparison of scRNAseq- and multispectral imaging data of the advanced murine atherosclerotic plaque in the aortic root [8, 13]. The colored background behind the subsets symbolizes the context-driven compartmentalization of macrophages in murine atherosclerosis. Below, the localization, potential role, and ontogeny of the depicted macrophage subsets are summarized.

The different macrophage subsets are shown in Fig. 1 and their protein marker expression and transcriptional profiles, as reported in Goossens et al. and Zernecke et al. [8, 13].

Macrophage diversity and functions in human atherosclerosis

Analogous to murine atherosclerosis, several single-cell and mass cytometry datasets have permitted the identification of a new macrophage subset in human atherosclerosis [9–12]. Zernecke et al. [8] performed an integration of the scRNAseq data from two of the aforementioned studies [10, 11] as well as a cross-species comparison of human and murine plaque scRNAseq [50].

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Broadly, three main macrophage subsets were defined: Resident, inflammatory, and foamy macrophages [50, 51]. Additionally, two minor macrophage subsets and one monocyte subsets were identified [50].

The resident macrophage subset is characterized by the expression of genes like *LYVE1*, *CD206*, *CD163*, and *FOLR2* [50], an expression profile that was also found in murine resident-like macrophages [8]. Mass cytometry further subdivided them into two resident subsets, $CD206^{hi}$ $CD163^{hi}$ and $CD206^{lo}$ $CD163^{lo}$, both present in plaques from symptomatic and asymptomatic plaques [10]. Previous studies associated $CD163^{+}$ macrophages with a more advanced plaque phenotype, colocalizing with intraplaque hemorrhage, a sign of plaque rupture and thrombotic events [52, 53]. The CD163-expressing macrophages are present in the shoulder regions of the plaque [53] and were found to be the major CD68-expressing macrophages in the adventitia [52]. Interestingly, the reported roles of $CD163^{+}$ macrophages in atherosclerosis diverge from serving proinflammatory [53, 54] to anti-inflammatory functions [53, 55]. The different locations, association with distinct plaque components [52, 53] and subsets [10] of the CD163-expressing macrophages could be a possible explanation for the reported functional divergence in atherosclerosis.

The proinflammatory macrophages identified in the scRNAseq data showed expression of *CD74*, CXCL2, CCL3, CCL4, and IL1B among others, in line with the proinflammatory macrophages characterized in murine atherosclerosis [50]. Analogous to the progression of inflammation, the presence of macrophages with a proinflammatory signature increased with plaque progression. Proinflammatory macrophages colocalized strongly with the necrotic core and were found to a lower extent in the adventitia [52].

The foam cell subset characterized by Zernecke et al. [50] is enriched in *TREM2*, CD9, GPNMB, SPP1, CTSL, LIPA, ACP5, and PLIN2, like the foam cell signature reported in murine atherosclerosis. While localization, heterogeneity, and functions in human atherosclerosis are not well studied, lipid-associated macrophages with a similar gene expression profile have been characterized in mice and humans in other disease and homeostatic contexts [56–59]. Future studies will require the use of in situ techniques such as multiplex imaging and spatial transcriptomics to get deeper insights into foam cell subsets and their respective abundance and functions throughout human atherogenesis.

The macrophage niche in the plaque as a key driver of macrophage specialization and function

Recently, the niche concept has been refined [15]. The macrophage niche is regulated by a variety of factors, including cell–cell interactions, oxygen levels, nutrients, metabolites, or other soluble signals as well as (composition or modifications of) the extracellular matrix. These signals can influence the recruitment and survival of macrophages, as well as their differentiation and polarization [15, 60]. The homeostatic arterial niche is composed by resident macrophages of embryonic origin and is replenished by circulating monocytes that acquire the identity of the niche-residing macrophages [18]. In atherosclerosis, plaque macrophage niches are regarded to be significantly altered (i.e. hyperlipidaemic, oxidative, and inflamed conditions), yet remain poorly characterized/largely uncharacterized. Guilliams *et al.* hypothesized that a macrophage niche must provide a physical structure or framework to the macrophage and provide nutrients or growth factors that allow the macrophage to sustain itself, driving the activation of transcription factors that confer a unique "niche-specific" identity [15].

Further, a constant provision of growth factors such as M-CSF (CSF-1) or GM-CSF (CSF-2) is essential for macrophages to maintain or develop within a tissue [15]. CSF-1 was found to enhance the differentiation of macrophages in vitro toward an anti-inflammatory phenotype whereas CSF-2 was shown to promote the differentiation into proinflammatory macrophages [61]. In atherosclerotic conditions, oxidized LDL was reported to induce the secretion of CSF-1 and CSF-2 by endothelial cells and of CSF-1 in human aortic smooth muscle cells [62, 63]. More recently, it was shown that locally-produced CSF-1 derived from endothelial cells and smooth muscle cells drives atherosclerotic lesion growth. Further, in the absence of CSF-1, proliferation of macrophages was reduced while apoptosis was increased [64, 65]. While several studies exist on CSF-1 in the atherosclerotic plaque, barely any data is available on CSF-2 and its effects on macrophages in the plaque. Nonetheless, it was reported that CSF-2 deficiency in $ApoE^{-/-}$ mice leads to a reduction in atherosclerotic lesion size [66]. This leaves the opposing or complementary roles of CSF-1/-2 in the plaque and more specifically their effects on macrophage functional specialization open for further studies. Other atherogenic modulators such as the IFN cytokine family have been shown to shape both macrophage phenotype and functional specialization in atherosclerosis. IFN-γ, mainly produced by local Th1 cells, can induce endothelial cell adhesion molecules leading to leukocyte recruitment to the plaque [67, 68]. Although the role of IFNs in the plaque is not yet fully elucidated, IFN type I and II seem to impair lipid handling of macrophages, promoting foam cell formation [69]. Furthermore, data shows that IFN families play a proapoptotic role, partly through their overlapping STAT-1 pathway [68, 70–72].

Finally, the macrophages should return some kind of advantage or benefit to their niche. In atherosclerosis, niche benefits could comprise functions of macrophages such as lipid- or cellular debris clearing, stimulation of angiogenesis, enhancement of fibrosis, and stabilization of the fibrotic cap [15].

Though the concept of macrophage niches in atherosclerosis is yet to be explored, a high diversity of distinct biochemical and mechanical micromilieus co-exist within the plaque [73]. Among these, we appreciate lipid-rich parts, neovascularization, hypoxia, inflammatory regions and necrotic cores, smooth muscle cell-rich areas, fibrosis, micro- and macrocalcification. Certain subsets could already be associated with a specific trait or location in murine and human plaques [13, 42, 52, 53].

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Conclusion

Advances in the field of single-cell studies have generated vast data on transcriptional and phenotypic diversity of macrophages in the plaque. On the other hand, the niche concept describes that this macrophage diversity is driven (or restricted) by local cues, available in the macrophages' immediate environment [15, 28].

However, macrophage subsets can express different levels of their subset-defining transcriptional and cell surface markers and it remains to be elucidated how these diverging expression profiles reflect differences in niche composition localization and function.

Therefore, a more holistic characterization of macrophage subsets in their respective plaque neighborhoods is needed. Such a characterization should be based on a comprehensive transcriptional and/or surface marker profile in combination with information on the subsets' histological location, the cellular and molecular context, and association to plaque traits. An in-depth characterization of macrophage niches in atherosclerosis and their relation to beneficial vs. detrimental macrophage subsets will be beneficial for the development of therapeutical approaches that aim to specifically target macrophage subsets or functions by altering microenvironmental characteristics.

In line with this, it will be important to use advanced histological approaches to complement the wave of scRNAseq data on atherosclerotic plaques in mice and humans. Recently, new promising single-cell-based spatial techniques have emerged and been applied to different disease contexts [13, 14, 74]. The application of multi-omics (e.g. spatial transcriptomics and proteomics) approaches to the field of atherosclerosis research will further help to refine the current view of macrophage heterogeneity and function.

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Abbreviations: **FL**: fetal liver · **scRNAseq**: single-cell RNA sequencing · **YS**: yolk sac

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