Macrophage subsets in atherosclerosis as defined by single-cell technologies

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Abstract

Macrophages play a major role in the pathogenesis of atherosclerosis. Many studies have shone light on the different phenotypes and functions that macrophages can acquire upon exposure to local cues. The microenvironment of the atherosclerotic plaque contains a plethora of macrophage-controlling factors, such as cytokines, oxidised low-density lipoproteins and cell debris. Previous research has determined macrophage function within the plaque mainly by using immunohistochemistry and bulk analysis. The recent development and rapid progress of single-cell technologies, such as cytometry by time of flight and single-cell RNA sequencing, now enable comprehensive mapping of the wide range of cell types and their phenotypes present in atherosclerotic plaques. In this review we discuss recent advances applying these technologies in defining macrophage subsets residing in the atherosclerotic arterial wall of mice and men. Resulting from these studies, we describe three main macrophage subsets: resident-like, pro-inflammatory and anti-inflammatory foamy TREM2hi macrophages, which are found in both mouse and human atherosclerotic plaques. Furthermore, we discuss macrophage subset-specific markers and functions. More insights into the characteristics and phenotype of immune cells within the atherosclerotic plaque may guide future clinical approaches to treat disease.

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Introduction

Atherosclerosis is a lipid-driven inflammatory disease characterised by plaque formation in the large arteries. These fatty plaques contain lipid-laden macrophages that accumulate because of the recruitment of circulating monocytes and local differentiation and proliferation of macrophages [1–4]. Atherosclerosis is initiated by endothelial damage induced by classical risk factors such as high cholesterol, high blood pressure, obesity, diabetes, smoking and low shear stress [5–9], leading to local sub-endothelial accumulation of low-density lipoproteins (LDL), which are prone to oxidative modifications. Such modified lipoprotein particles trigger inflammatory responses inducing monocyte attraction. These monocytes subsequently differentiate into macrophages that scavenge oxidised LDL and eventually become foam cells. In atherogenesis, macrophages secrete numerous pro- but also anti-inflammatory mediators, pro-thrombotic tissue factor and enzymes such as matrix-degrading proteases, all of which influence plaque growth, cellular composition and stability. Although early lesions are dominated by plaque foam cells, plaque foam cell content decreases with atherosclerosis progression towards more advanced and fibrotic lesions or by induction of regression, such as mediated by cholesterol lowering [10–12]. Excessive LDL uptake induces macrophage apoptosis [13] and these apoptotic cells can be sensed by other macrophages via ‘find-me’ and ‘eat-me’ signals and are cleared through a process called efferocytosis [14]. When plaques become more advanced, the number of apoptotic cells increases and macrophage efferocytosis becomes limited, causing secondary necrosis, leading to the formation of a necrotic core [15,16]. Because of their complex and broad range of functions, macrophages are central in the formation, maintenance and rupture, and therefore clinical complications, of the atherosclerotic plaque.

Around the year 2000, the macrophage phenotype range was initially divided into two extremes of the
activation spectrum, namely pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages [17–19]. However, it has now become established by numerous more recent studies that the macrophage phenotype spectrum is much broader, containing a continuum of phenotypes induced by local tissue cues [20–22]. The atherosclerotic microenvironment is diverse and drives a range of macrophage phenotypes. Histological studies previously showed the presence of M1 and M2 macrophage subsets and their spatial localisation in atherosclerotic lesions [23–26]. Stöger et al [23] described that M1 macrophages are the predominant population in rupture-prone shoulder regions of the plaque. The plaque adventitia enclosed a pronounced M2 macrophage phenotype, whereas no significant differences in macrophage populations were seen in the fibrous cap. These studies highlighted the critical importance of macrophage polarisation regarding atherogenesis.

The discovery and rapid progress of single-cell RNA sequencing (scRNASEq) and tools exploiting large combinations of protein labelling of cells, such as cytometry by time of flight (CyTOF), have now enabled more detailed transcriptional and high-dimensional protein analysis of the cell types present in atherosclerotic plaques. Specific subsets of cells can now be distinguished based on overlapping or differing transcriptomes or protein marker sets. Using these tools, it has become possible to discriminate tissue macrophage subsets and characterise their functions. In recent years, scRNAseq and CyTOF were applied to mouse and human atherosclerosis, which has given novel insights regarding macrophage subsets and their functions. In recent years, scRNAseq and CyTOF were applied to mouse and human athero-

### Mouse atherosclerotic macrophages

Several studies have now investigated macrophages in murine atherosclerosis by either CyTOF or scRNASEq approaches (see supplementary material, Table S1) [27–32,34]. These investigated Ldlr<sup><-/-</sup> or apolipoprotein E (ApoE)<sup><-/-</sup> mouse models, either on chow or on a Western (high-fat) diet to accelerate atherogenesis. Most of the studies showed that macrophages are the most dominant immune cell type within the atherosclerotic plaque [28,30,35]. The total macrophage population proportion was further increased in mice with advanced atherosclerosis [28]. Generally speaking, three main subtypes of macrophage could be identified, involving resident-like macrophages, pro-inflammatory macrophages and foamy TREM2<sup>hi</sup> macrophages (Figure 1). Future additional differentiation of macrophage subsets can probably be made based on technical and numerical advances. However, currently available data suggest that plaque macrophages can be subdivided into at least three main macrophage populations. Several studies identified more than three populations These are probably further subdivisions of the three main clusters. We will discuss these three macrophage subsets in mouse and human atherosclerosis below.

### Atherosclerotic resident-like macrophages

Concerning their origin, macrophages found in tissues can be divided into two groups: infiltrating monocyte-derived macrophages and embryonically derived tissue-resident macrophages [36–38]. Whereas tissue-resident macrophages reside in a specific tissue and are mainly seeded during embryonic development, infiltrating monocyte-derived macrophages often accumulate in response to local inflammatory cues in the tissue [39,40]. Most of the tissues harbour resident macrophages. For example, Kupffer cells are tissue-resident macrophages of the liver and microglia are the tissue-resident macrophages of the brain. Tissue-resident macrophages not only have a role in tissue homeostasis but also form the first line of defence when a pathogen invades the tissue.

Ensan et al [41] identified mouse resident arterial macrophages that arose embryonically from C-X3-C motif chemokine receptor 1 (Cx3cr1)<sup>+</sup> precursors. These resident arterial macrophages specifically expressed lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1). Winkels et al [27] confirmed the expression of Cx3cr1 and Lyve by resident-like macrophages in healthy Apoe<sup><-/-</sup> mice. Depletion of Lyve1<sup>+</sup> macrophages in Lyve<sub>wt/cv</sub>Cx3cr1<sub>flox/flox</sub> mice resulted in increased arterial stiffness and collagen deposition, suggesting a key role in these processes [42]. Kim et al [29] identified three macrophage subsets expressing Lyve1 in atherosclerotic plaques of Ldlr<sup><-/-</sup> mice using scRNA-seq. The macrophage subset expressing the highest Lyve1 levels of all aortic CD45<sup>+</sup> cells also expressed increased levels of mRNA for anti-inflammatory markers such as mannose receptor Mr1 (also known as Cd206), transcription factor Mafb [43] and other genes mainly linked to the endocytosis pathway. Cochain et al [28] also identified a cluster of resident-like macrophages in atherosclerotic plaques expressing Lyve1 and other markers for resident-like macrophages, such as factor XIIIa (F13a1) and growth arrest-specific 6 (Gas6) [44]. The resident-like macrophages were present in both healthy and atherosclerotic aortas [27,28]. However, the resident-like macrophage subset of healthy aortas expressed higher levels of Lyve1, whereas the resident-like macrophages of the atherosclerotic aortas expressed higher levels of C-C motif chemokine receptor 2 (Ccr2), which is truly a marker for recruited macrophages. This suggests that atherosclerotic aortas contain resident macrophages originating from an embryonic pool, which upon atherosclerosis development are replaced by or accompanied by recruited monocyte-derived macrophages that adopt a resident-like macrophage phenotype. Resident-like macrophages
in lesions further express folate receptor β (Folr2), carbonyl reductase 2 (Cbr2), platelet factor 4 (Pf4), selenoprotein P (Sepp1) and (as in Kim et al [29]) Cd206. Folr2, Cbr2, Sepp1 and Cd206 are all associated with M2-like phenotype [24], thus suggesting anti-inflammatory characteristics of resident-like macrophages in atherosclerosis. Folr2 expression was increased in atherosclerotic plaques compared with normal artery walls [45]. Pf4 has previously been described as a platelet-specific molecule. However, more recent data also show Pf4 expression in macrophages [46,47]. Macrophage Pf4 (also known as CXCL4) was found to be positively correlated with clinical parameters such as lesion grade and the presence of symptomatic atherosclerotic disease in human carotid atherosclerotic plaques [46]. Pathway analysis revealed that resident-like plaque macrophages are involved in the receptor-mediated endocytosis [28], which is in line with the endocytosis pathway identified by Kim et al [29]. Likewise, Lin et al [31] identified a Cx3cr1<sup>+</sup> resident-like macrophage subset enriched for Mrcl, Gas6, F13a1, Sepp1, Cbr2, Pf4, Lyve1 and Folr2. There was no difference in the population proportion of resident-like macrophages between progressive and regressive plaques.

Cochain et al [28] also identified a resident-like macrophage subset expressing Lyve1, Ccl9, F13a1, Folr2 and Sepp1 in atherosclerotic Apoe<sup>−/−</sup> mice. Cole et al [30] found a resident-like macrophage population expressing CD206 and CD169 in atherosclerotic Apoe<sup>−/−</sup> mice using CyTOF. This supports the notion that different atherosclerotic models contain resident-like macrophages. CD169 encodes the lectin-like adhesion molecule SIGLEC-1 that is expressed by certain resident-like macrophage subpopulations [48–50] and it can interact with scavenger receptor SR-B1 to mediate the uptake of oxidised LDL [51]. Furthermore, at least four studies described that resident-like macrophages can proliferate by showing either protein expression of proliferation marker Ki-67 or enrichment for cell cycle genes [29,31,39,52]. This confirms a previous study showing that the lesional microenvironment orchestrates macrophage proliferation in atherosclerosis [53].

McArdle et al [34] recently used atherosclerotic Apoe<sup>−/−</sup>Cx3cr1<sup>GFPCD11cYFP</sup> mice in which four macrophage subsets were identifiable by GFP, YFP, a double-positive and a double-negative signal. Bulk RNAseq data of these four flow-sorted macrophage subsets were compared with representative genes of each macrophage subset found by Cochain et al [28], Kim et al [29] and Lin et al [31] and revealed that three main macrophage populations: resident-like, inflammatory
and TREM2hi macrophages were overlapping with one of the subsets of the study of McArdle et al [34]. However, the fourth population (double-negative) of McArdle et al [34] did not overlap with a macrophage subset from the other studies. These resident-like macrophages again showed enrichment for Sepp1, Tonip and Pp4 and live-cell microscopy showed that these cells migrate less compared with other macrophage subsets and their shape resembles that of dendritic cells.

Immunostaining studies further showed that resident-like macrophages are predominantly present in the adventitia, both in the healthy blood vessel [41] and in the atherosclerotic aorta, where their number is increased compared with healthy settings [52].

In summary, various single-cell studies identified resident-like macrophages within the adventitia of the healthy and atherosclerotic aorta of Ldlr−/− and Apoe−/− mice. Different studies reported that resident-like macrophages proliferate, play a role in endocytosis and are enriched for genes such as Lyve1, Cx3cr1, Folr2, Cd206, F13a1, Cbr2, Sepp1 and Pp4 expression, which resembles an M2-like phenotype.

Atherosclerotic inflammatory macrophages

In atherosclerosis, the increased number of circulating monocytes infiltrate the arterial wall using different chemokine–chemokine receptor dyads, followed by support of the endothelial adhesion molecules [54–56]. In the intima, infiltrated monocytes can differentiate into macrophages. Inflammatory macrophages upon activation will express surface markers, including major histocompatibility complex class II, Fc receptor CD64 and costimulatory molecules CD80 and CD86, and will release IL-6, TNF and IL-23, and express nitric oxide synthase 2. Inflammatory macrophages are essential for the phagocytosis and intra- and extracellular killing of bacterial, fungal and viral infections [57–59]. However, chronic macrophage activation will lead to tissue injury, matrix degradation and impaired wound healing.

Different single-cell studies have described the presence and phenotype of inflammatory macrophages in the atherosclerotic aorta [27–29,31,34]. Cochain et al [28] showed that these inflammatory macrophages are enriched for numerous classical pro-inflammatory transcripts such as Cxcl2, Ccl3, Ccl4, Il1a, Il1β, Tlr2, Tnf, nucleotide-binding oligomerisation domain, leucinerich repeat and pyrin domain containing 3 (Nlrp3) and specific transcription factors such as CCAAT enhancer-binding protein beta (Cebpβ) [60] and early growth response 1 (Egr1) [61]. Il1a, Il1β, and Nlrp3 are important in regulating atherosclerosis progression [62–64]. As a result of macrophage activation, negative feedback mechanisms are activated that partially compensate for the pro-inflammatory and pro-atherogenic effect and stimulate cell survival [65]. Cochain et al [28] found enrichment of negative feedback NF-κB inhibitors (Nfkbia, Nfkbid and Nfkbiz), nuclear receptor subfamily 4 group A member 1 (Nrl4a1), immediate early response 3 (Ier3) and the mRNA-binding protein Zpf36. Nrl4a1 and Zpf36 have both been shown to inhibit the inflammatory macrophage phenotype in atherosclerosis [66,67]. The inflammatory response was the most significantly enriched pathway among the inflammatory macrophages. Lin et al [31] showed that inflammatory plaque macrophages were enriched for the expression of different chemokines (Ccl2-5, Cxcl1, Cxcl2, Cxcl10), Nlrp3, Tnf and type I interferons signalling genes such as Ifitm3, Irf7, Isg15 and Mnda. Importantly, it has been shown that type I interferons are generally pro-atherogenic [68,69]. The relative frequency of the inflammatory macrophage subset increased with a Western-type diet compared with chow feeding, confirming the positive association with plaque progression.

Kim et al [29] investigated the association of macrophage foam cell phenotype with their inflammatory characteristics and showed that inflammatory plaque macrophages are particularly non-foamy. Many inflammatory transcripts, including Il1β, Nlrp3, Nfkbia, Tlr2 and Tnf, were upregulated in these non-foamy inflammatory macrophages. Inflammatory pathways (TLR and TNF signalling, cytokine–chemokine interaction and NF-κB signalling pathways) were confirmed to be dominant in non-foamy inflammatory macrophage function in plaques.

McArdle et al [34] also found that inflammatory plaque macrophages were enriched for the expression of Nlrp3, Tnf and Ccl3. Intravital imaging identified that inflammatory macrophages have elongations, are dendritic-shaped and migrated significantly more compared with resident-like macrophages.

Different single-cell studies have described that inflammatory macrophages are only present in the atherosclerotic aorta [27,28] where they represent the largest (about half) macrophage subset [28,29]. This was confirmed in both atherosclerotic Ldlr−/− and Apoe−/− mice. Stöger et al [23] observed in human patient material that inflammatory macrophages were the predominant population of the plaque shoulder regions, which is comparable with the finding of Kim et al [29], who found that inflammatory macrophages reside in the intima, which includes the plaque shoulder regions.

Overall, inflammatory macrophages form the major macrophage population within the intima of the plaque, are mainly non-foamy cells that are exclusively present in the atherosclerotic aorta and accordingly can be considered the main drivers of lesional inflammation. These inflammatory macrophages typically express inflammatory pathways and markers, including Tnf, Nlrp3, Il1β, Egr1, Zpf36, Ier3, Cephp, Cxcl2 and Ccl2-5, and are therefore associated with an M1-like phenotype.

Atherosclerotic TREM2hi macrophages

TREM2 is a myeloid-specific transmembrane glycoprotein that can interact with APOE, APOI, anionic ligands,
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TREM2 is expressed on anti-inflammatory macrophages and has been shown to limit macrophage activation [76]. Macrophage TREM2 protects against infections, adipocyte hypertrophy, systemic hypercholesterolemia, body fat accumulation and glucose intolerance in mice [77,78] and has been shown to negatively correlate with human plaque stability [79].

Cochain et al [28] detected TREM2 hi macrophages exclusively in the plaque and not in the healthy aorta. TREM2 hi macrophages expressed increased levels of Trem2, Cd9, secreted phosphoprotein 1 (Spp1), hydrogen voltage-gated channel 1 (Hven1) and several cathepsins (Ctsb, Ctsd and Ctsz). Cathepsins are lysosomal proteolytic enzymes of macrophages that promote atherosclerotic inflammation and plaque vulnerability [80–82]. For example, cathepsin Z stimulates the production of pro-inflammatory cytokines and cathepsin D controls multiple aspects of apoptosis and modifies LDL to promote LDL engulfment by macrophages and subsequent foam cell formation. Cathepsin B has been shown to mediate the degradation of the extracellular matrix. Pathway analysis of lesional TREM2 hi macrophages showed enrichment in lipid metabolism, regulation of cholesterol efflux, oxidative stress and catabolism, suggesting a link with intracellular lipid accumulation and foam cell formation.

TREM2 hi macrophages were present in mice with both early and advanced atherosclerotic lesions and found in both Ldr−/− and Apoe−/− atherosclerotic mice [28]. Moreover, TREM2 hi macrophages were detected during both the progression and the regression of atherosclerosis [31]. Lin et al [31] showed that TREM2 hi macrophages were enriched for the expression of Trem2, Cd9, Ctsb, galectin-3 (Lgals3), Spp1 and aldolase (Aldoa), of which CD9 expression was confirmed by flow cytometry. Tetraspanin CD9 is an exosome and anti-inflammatory marker of monocytes and macrophages and is associated with CD36 expression, which triggers foam cell formation in response to oxidised LDL exposure [83,84]. Kim et al [29] showed that TREM2 hi macrophages are indeed lipid-laden foam cells with increased expression of Trem2, Lgals3, ATP binding cassette subfamily G member 1 (Abcg1), Abca1, Ctsb, Fabp4, Meritk and Cd36. Galectin-3 expression drives the differentiation of monocytes to macrophages and is associated with CD36 expression, which triggers foam cell formation in response to oxidised LDL exposure [85,86]. Fabp4 has been previously described as a transcriptional marker of foamy macrophages in atherosclerotic Apoe−/− mice [29]. Pathways analysis by Kim et al [29] showed enrichment of oxidative phosphorylation, lysosome, cholesterol metabolism and peroxisome proliferator-activated receptors signalling pathways in these foamy TREM2 hi macrophages, again confirming the association of the foamy phenotype with metabolic pathways. Gene expression of foamy macrophages was also associated with plaque-resolving parameters such as efferocytosis and tissue repair compared with that of non-foamy inflammatory macrophages.

McArdle et al [34] showed that TREM2 hi macrophages were also enriched for Cd9, Trem2, Ctsd and Spp1. Live-cell imaging identified TREM2 hi macrophages to exist in a more rounded shape and to migrate significantly more compared with the resident-like macrophages.

Cochain et al [28] detected TREM2 hi macrophages exclusively in the plaque and not in the healthy aorta. Foamy macrophages predominantly reside in the intima where they have taken up atherogenic lipoproteins and subsequently form a lipid-rich core that can progress to become a necrotic core [1].

In summary, TREM2 hi macrophages are foamy lipid-laden macrophages accumulating in the plaque intima and its necrotic core and not in the healthy aorta. Pathway enrichment analysis confirmed the role of TREM2 hi macrophages in cholesterol metabolism and oxidative phosphorylation, which was already suggested by the foamy feature of the TREM2 hi macrophages. Trem2, Cd9, Spp1, Lgals3 and Ctsb are markers of TREM2 hi macrophages, as found by different studies. TREM2 hi macrophages seem to have an M2-like phenotype.

Smooth muscle cell transdifferentiation

Several studies have described that smooth muscle cells (SMCs) can also become foam cells after lipid loading [88–91]. Upon foam cell formation, SMCs express lower levels of typical SMC markers (α-SM actin and α-tropomyosin) and increased levels of macrophage markers such as CD68, galectin 3 and the macrophage foam cell marker ABCA1 [89]. It has been described that these transdifferentiated foam cells may make up a major component of advanced plaques. Co-staining with CD68 and α-SM actin of human coronary artery sections suggested that 40% of CD68+ cells originated from SMCs in advanced human coronary atherosclerosis [90]. Also, Kim et al [29] reported that a foam cell subset expressed SMC markers (α-SM actin and SM22α) and showed the absence of CD45 in murine atherosclerosis. However, Wirka et al [32] analysed the single-cell transcriptional profile of SMC-specific lineage-traced mouse plaque cells together with scRNAseq data of human atherosclerotic plaques, which raised the suggestion that both mouse and human SMCs transdifferentiate into fibroblast-like cells and do not acquire macrophage characteristics [31]. They confirmed this suggestion using multiple protein-based assays. Although experimental data clearly showed SMC conversion into cells with macrophage characteristics, the actual contribution of transdifferentiation from SMCs to macrophages (or potentially vice versa) to the human atherosclerotic process and subsequent clinical complications still need to be substantiated.

Human atherosclerotic plaque macrophage subsets

Recently, the first publications applying single-cell technologies on human atherosclerotic plaques were released
and NFKBIA. The foamy anti-inflammatory plaques expressed increased levels of activation of macrophages and foam cells [94,95]. PLIN2 expression is induced upon lipid uptake in macrophages and foam cells [93] and perilipin 2 (PLIN2). PLIN2 expression was enriched for the expression of LGALS3 and foam cell-related transcripts such as CD11b+HLA-DRmed and a CD11b+CD36+ population. The pro-inflammatory and foamy anti-inflammatory macrophage subset was found in CD206hiCD163hi and CD206hiCD163lo macrophages using mass cytometry. The CD206hiCD163hi macrophage subset may be similar to the phenotype of the resident-like macrophage subset described in murine atherosclerosis based on CD206 expression and the fact that haemoglobin–haptoglobin complex scavenger receptor CD163 and CD206 are not found to be expressed by foam cells [23,92]. Single-cell transcriptional analysis of plaque macrophages by Fernandez et al [33] further revealed the presence of pro-inflammatory and foamy anti-inflammatory macrophage subpopulations. The pro-inflammatory macrophages in human plaques expressed increased levels of activation markers such as HLA-DR, CD74, CYBA, LYZ2, AIF1, TLR4 agonist ST100A8/A9, MALAT1, JUNB and NFKBIA. The foamy anti-inflammatory macrophage subset was enriched for the expression of LGALS3 and foam cell-related transcripts such as APOC1, APOE, CTSB, FABP5 and perilipin 2 (PLIN2). PLIN2 expression is induced upon lipid uptake in macrophages and foam cells [94,95]. Moreover, Plin2-deficiency protects Apoe−/− mice from atherosclerosis. The phenotype of the foamy anti-inflammatory macrophage subset may be comparable with the TREM2hi macrophages found in mouse atherosclerotic plaques. Furthermore, Kim et al [29] showed that human foamy macrophages were more anti-inflammatory compared with non-foamy macrophages in human atherosclerotic plaques, as foamy macrophages expressed significantly less Il1b. Studies focusing on other diseases, such as multiple sclerosis and obesity, also provide supporting evidence regarding the translation of the mouse to human foamy macrophage subset phenotype. TREM2 is highly expressed on myelin-laden foamy macrophages in actively demyelinating multiple sclerosis lesions [96]. Moreover, foam cells in multiple sclerosis lesions have a more anti-inflammatory phenotype [97]. CD9hi adipose tissue macrophages of obese patients had higher intracellular lipid content compared with CD9lo adipose tissue macrophages [98]. Jaitin et al [77] identified a lipid-associated macrophage subset with an enriched transcriptional signature of Trem2, Lipa, Lpl, Ctsb, Ctsl, Fabp4, Fabp5, Lgals1, Lgals3, Cd9 and Cd36 in the adipose tissue of obese mice, which could be confirmed in humans. Moreover, protein expression of CD9 was also confirmed in these cells.

Thus, it seems that the murine and human plaques both enclose pro-inflammatory macrophages, foamy anti-inflammatory macrophages and resident-like macrophages. However, future studies are required to draw a more powerful and complete picture of the mouse-to-human translation. Such studies should involve direct computational comparisons of the single-cell macrophage transcriptomes between mice and men.

Limitations of single-cell studies

The high technical noise and variability of scRNAseq data are known issues that are larger compared with bulk RNAseq [99,100]. A major fraction of the transcripts is not detected in all cells, resulting in false-negative read counts, also referred to as dropouts. To improve sequencing depth, previous studies sorted the cells of interest using fluorescence-activated cell sorting (FACS) before scRNAseq library preparation [29,34]. For generating unbiased and reproducible scRNAseq results, dropouts and low-quality cells should be identified and excluded during quality control. Variability among different cells can be reduced by using either unique molecular identifiers or RNA spike-ins, which can be used to normalise the scRNAseq data [101–103]. Numerous methods are available that identify dropout events and impute these missing values, resulting in more accurate scRNAseq analysis [104–108]. The imputation of missing values is based on either gene expression of similar cells or gene-to-gene relationships. Tools that were originally designed for bulk RNAseq data may not be suitable for scRNAseq data. New tools have been developed to improve normalisation [109–111], subpopulation identification and comparison, including cross-species comparisons [112], differential expression analysis [113] and batch effect correction [114,115]. To handle the high dimensional scRNAseq data, dimensionality reduction and feature selection approaches have been created, such as t-distributed stochastic neighbour embedding [116], uniform manifold approximation and projection [117], and scvis [118]. Most of the scRNAseq studies to date sequenced only the 3′ transcriptome, although there are also techniques available that capture either the 5′ or full-length transcriptome and enable the detection of RNA splicing, RNA editing variants, long non-coding RNA and circular RNA [119,120].

Previous work has shown that macrophages are more susceptible than other cell types to becoming damaged during the enzymatic and mechanical dissociation procedures required for scRNAseq, often resulting in an under-representation of macrophages [121]. Moreover, different digestion protocols may favour different cell subsets to survive, resulting in over-representation of certain populations. To overcome this problem, CIBERSORT has been developed, which estimates the abundance of cell populations based on gene expression data from bulk RNAseq [122]. Alternatively, techniques without tissue dissociation, such as spatial transcriptomics, which visualises and determines the transcriptome of tissue sections [123], and the combination of laser capture microscopy and Smart-seq2 may be an outcome in the confirmation of the current findings [124].

In summary, the application of numerous wet-laboratory protocols and many different algorithms makes it currently challenging to directly compare different...
studies with each other. This may also explain differences in identified macrophage subsets in atherosclerosis. Cochain et al [28] showed that a higher resolution analysis may result in an increased number of macrophage subsets by extracting and re-analysing the single-cell transcriptional data from the macrophage clusters. Fortunately, the previous limitations of scRNAsseq, such as batch effects, are minimised by the rapid development of new bioinformatics software.

**Future perspectives**

Here we have discussed three main macrophage populations that have been identified within atherosclerotic plaques. A better understanding of the cell phenotype and function of these populations and their association with disease, clinical features of patients and future events may aid in steering future approaches to limit atherosclerosis complications. Current mouse data may support such progress. For instance, Kim et al [29] showed that foam cell activity correlates with cell profiles of resolution and plaque regression, whereas inflammatory macrophage activity associates with factors involved in atherosclerosis progression. Linking current mouse studies with upcoming and available human data will be essential for extrapolating model observations to clinical settings. Moreover, expanding datasets in future studies on human atherosclerosis should involve association with clinical data including plaque features (e.g. cellular content, necrotic core, collagen deposition) to better associate specific transcriptomes to clinically relevant parameters. We suggest that ongoing research should further characterise these macrophage subsets. For instance, future CyTOF experiments applying series of macrophage markers described in this review may confirm and further detail the existence of macrophage populations in atherosclerosis of mice and men. In addition, subpopulation-specific markers can also be applied to isolate these populations by flow-sorting and subsequent detailed characterisation of cellular phenotypes. Furthermore, single-cell assay for transposase-accessible chromatin sequencing (ATAC-seq) and single-cell chromatin immunoprecipitation (ChIP-seq) studies may be used to characterise the epigenomic landscape of macrophage subsets to further unravel their regulatory pathways, such as the epigenetic processes and transcription factors that control them.

Based on these studies, detailed identification of cell populations that are detrimental in disease may be defined, as well as pathways, cytokines or other local inflammatory cues that promote them. Alternatively, reparative pathways dampening plaque worsening may also be identified. These insights will define new therapeutic interventions to control relevant macrophage populations in atherosclerotic disease. To limit off-target effects of potential therapeutics aimed at such macrophage populations, cell-specific targeting may be required. Nanomedicine has been applied as therapeutic agents that accurately target macrophages [125]. High-density lipoproteins are natural nanoparticles with an affinity for atherosclerotic plaque macrophages [126] that can be applied for the treatment of atherosclerosis [127,128], for example by loading with relevant drugs to re-educate lesional macrophages, as has been applied in the cancer field [129,130].

In conclusion, the development of single-cell technologies, such as scRNAsseq and CyTOF, has made it possible to characterise macrophage and other immune cell subsets in the atherosclerotic plaque in great detail and to compare atherosclerotic settings with those in the healthy aorta. So far, three key macrophage populations (resident-like, pro-inflammatory and anti-inflammatory foamy TREM2hi) have been described that exist in the atherosclerotic plaque. Future studies should particularly focus on the translation of mouse atherosclerotic immune cell populations to human pathophysiology and linkage to human disease.

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**Author contributions statement**

LW and MPJW researched data for the review, discussed its content, wrote the manuscript, and reviewed and revised it before submission.

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**SUPPLEMENTARY MATERIAL ONLINE**

Table S1. Overview of mouse and human aortic macrophage populations identified by single-cell technologies