



# New Classification of Macrophages in Plaques: a Revolution

Hao Deng<sup>1</sup> · Yingxin Sun<sup>2</sup> · Wenyun Zeng<sup>2</sup> · Huhu Li<sup>2</sup> · Maojuan Guo<sup>2</sup> · Lin Yang<sup>2</sup> · Bin Lu<sup>2</sup> · Bin Yu<sup>2</sup> · Guanwei Fan<sup>1</sup> · Qing Gao<sup>2</sup> · Xijuan Jiang<sup>2</sup>

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## Abstract

**Purpose** Macrophages play vital roles in the development of atherosclerosis in responding to lipid accumulation and inflammation. Macrophages were classified as inflammatory (M1) and alternatively activated (M2) macrophage types based on results of in vitro experiments. On the other hand, the composition of macrophages in vivo is more complex and remains unresolved. This review summarizes the transcriptional variations of macrophages in atherosclerosis plaques that were discovered by single-cell RNA sequencing (scRNA-seq) to better understand their contribution to atherosclerosis.

**Recent Findings** ScRNA-seq provides a more detailed transcriptional landscape of macrophages in atherosclerosis, which challenges the traditional view. By mining the data of GSE97310, we discovered the transcriptional variations of macrophages in LDLR<sup>-/-</sup> mice that were fed with high-fat diet (HFD) for 11 and 20 weeks. Cells were represented in a two-dimensional tSNE plane and clusters were identified and annotated via Seurat and SingleR respectively, which were R toolkits for single-cell genomics. The results showed that in healthy conditions, Trem2<sup>hi</sup> (high expression of triggering receptors expressed on myeloid cells 2)-positive, inflammatory, and resident-like macrophages make up 68%, 18%, and 6% of total macrophages respectively. When mice were fed with HFD for 11 weeks, Trem2<sup>hi</sup>, monocytes, and monocyte-derived dendritic cells take possession of 40%, 18%, and 17% of total macrophages respectively. After 20 weeks of HFD feeding, Trem2<sup>hi</sup>, inflammatory, and resident-like macrophages occupied 12%, 37%, and 35% of total macrophages respectively.

**Summary** The phenotypes of macrophages are very different from the previous studies. In general, Trem2<sup>hi</sup> macrophages are the most abundant population in healthy mice, while the proportion of monocytes increases after 11 weeks of HFD. Most importantly, inflammatory and resident-like macrophages make up 70% of the macrophage populations after 20 weeks of HFD. These strongly indicate that inflammatory and resident-like macrophages promote the progression of atherosclerosis plaques.

**Keywords** Macrophages · Atherosclerosis plaques · Classification · Single-cell RNA sequencing

Hao Deng, Yingxin Sun, and Wenyun Zeng contributed equally to this work and should be considered co-first authors.

Qing Gao and Xijuan Jiang contributed equally to this work and should be considered co-corresponding authors.

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✉ Qing Gao  
gaoq88@126.com

✉ Xijuan Jiang  
xijuanjiang@foxmail.com

<sup>1</sup> Tianjin Key Laboratory of Translational Research of TCM Prescription and Syndrome, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China

<sup>2</sup> School of Integrative Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China

## Introduction

Macrophage is an important initiator in the development of atherosclerosis plaques and its growth and rupture via mediating inflammation and lipid infiltration. It has been studied by many researchers and was shown to consist of several phenotypes in plaques that are interchangeable [1]. The inflammatory phenotype, termed as M1 macrophage, produces numerous pro-inflammatory cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin1 $\beta$  (IL-1 $\beta$ ), as well as chemokines such as monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-C motif) ligand 2 (Ccl2), to attract more monocytes to eliminate pathogens. M1 macrophage also produces reactive oxygen species (ROS) through activating NADPH oxidase to scavenge microorganisms [2]. On the other hand, a subset of alternatively activated macrophage, termed M2 macrophage, produces anti-inflammatory

molecules such as IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ). It is important to note that the classification of M1 and M2 phenotypes is based on *in vitro* experimental systems, in which M1 macrophages are induced by interferon- $\gamma$  (IFN- $\gamma$ ) and lipopolysaccharide (LPS), while M2 macrophages are induced by IL-4 and IL-13 [3]. However, neither M1 nor M2 macrophages present in atherosclerotic plaques alone where macrophages are exposed to a complex microenvironment and respond with expression of different cell-surface markers to exert by diverse functions [4]. Other macrophage populations which were identified *in vivo* include Mhem, Mox, M4, and M (Hb) variants, which are induced by haem, oxidized phospholipids, chemokine CXCL4, and hemoglobin-haptoglobin complexes respectively. Still, new macrophage phenotypes are discovered continuously with ongoing research [5].

The technology of single-cell RNA sequencing (scRNA-seq) provides a new and specific way to identify single-cell transcriptional features of the examined gene in tissue. Cochain et al. divided macrophages into three populations by applying scRNA-seq to total aortic CD45<sup>+</sup> cells, which were extracted from the non-diseased (chow-fed) and atherosclerotic aorta (11 weeks of high-fat diet to LDLR<sup>-/-</sup> mice). These populations are resident-like macrophages, inflammatory macrophages, and Trem2<sup>hi</sup> macrophages, which did not fit into classifications of macrophages using traditional methods. Cochain uploaded their original data to GEO dataset (GSE97310), and we analyzed macrophage transcriptional variations in atherosclerotic mice. R was used in mining data from total aortic CD45<sup>+</sup> cells from both healthy group and high-fat diet groups. T-SNE analysis showed 18 clusters, among which 7 are macrophages or monocytes, 8 are T cells, 1 is B cell, 1 is NK cell, and 1 is granulocytes (Figs. 1 and 2).

## Trem2<sup>hi</sup> Macrophages

Cluster 0 of macrophages is characterized by high expressions of Trem 2 (Triggering receptors expressed on myeloid cells 2), Spp1 (Secreted phosphoprotein1), CtSL (cathepsin L), and CD9. According to our analysis, Trem2<sup>hi</sup> macrophages occupy 68% of total macrophages in the healthy group, 43% in the HFD 11-week group, and 12% in HFD 20-week group. Obviously, the proportion of Trem2<sup>hi</sup> macrophages in plaques drops with longer time of high-fat diet.

Trem2 encodes a protein that is part of transmembrane signaling complex essential to the immune response of myeloid cells such as macrophages and microglia [6, 7]. Also, Trem2 drives gene expression associated with energy metabolism, phagocytosis, and lipid catabolism [8]. In Trem2-deficient mice, lipid uptake and storage within macrophages were abolished; as a result, the development of obesity and hypercholesterolemia accelerated [9•]. Other studies have

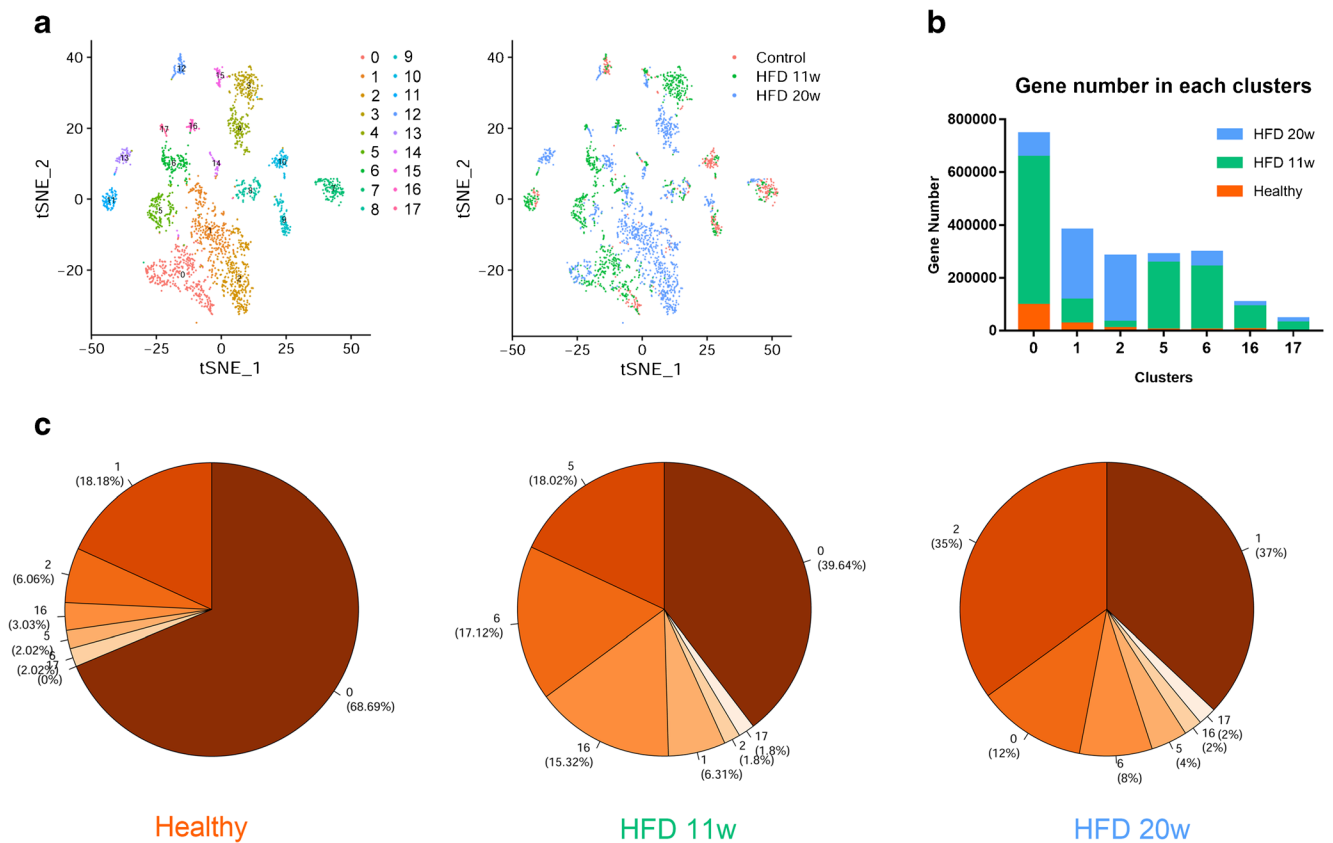
reported that Trem2 is required for clearance of apoptotic cells via phagocytosis in nonalcoholic steatohepatitis, Alzheimer's disease, and Parkinson's disease [10]. Therefore, Trem2-pathway represents a conserved and general macrophage response to scavenge extracellular pathogenic lipids across multiple tissues [11].

As a member of tetraspanin superfamily, CD9 participates in a wide range of biological activities including cell growth, motility, adhesion, metastasis, differentiation, and signal transduction. In macrophages, CD9 is associated with CD36 on the surface and involved in foam-cell formation in response to oxidized low-density lipoproteins [12]. Although CD9 is previously known as a specific exosome marker, it is now regarded as an anti-inflammation marker of macrophages and monocytes (Table 1) [13]. Hence, CD9 is responsible for phagocytosis and inflammation responses in macrophages.

CtSL encodes Cathepsin L (CtL), which is a major cysteine protease that belongs to the papain-like family, which is responsible for degrading a large variety of lysosomal proteins [14]. It is overexpressed in several types of carcinomas arising from the lung, ovary, cervix, breast, and colon [15]. CtL also expressed at very high level in CD68-positive macrophages within in atherosclerotic lesions, which is significantly associated with apoptosis, necrotic core formation, and plaque destabilization of the lesion [16]. Moreover, CtL regulated expression of cytokines, especially TNF- $\alpha$ , Niemann-Pick Type C (NPC) proteins, and other genes involved in cholesterol metabolic pathways treated with LPS. In a word, CtL is responsible for inflammation, cholesterol metabolism, and apoptosis in macrophages.

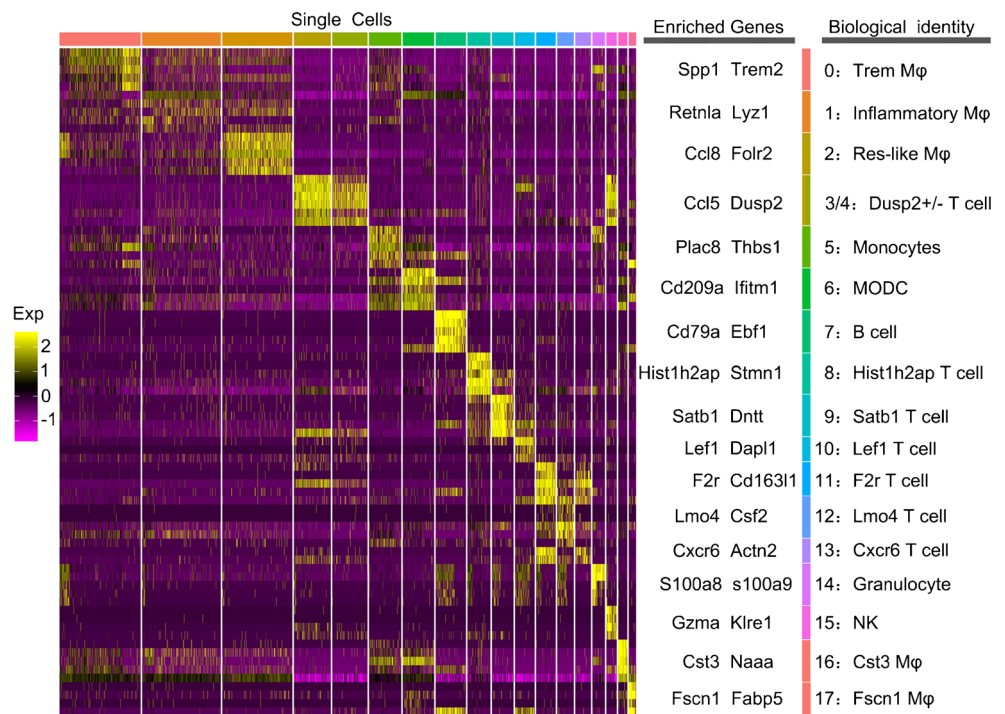
Spp1, also known as osteopontin (OPN), regulates diverse cellular processes including cell survival, cell adhesion, cell motility, and bone remodeling [17]. However, its function differs between variants due to structural heterogeneity and the timing of its expression. It was reported that OPN ablation resulted in reduced muscle fibrosis and enhanced regeneration. OPN also regulates TGF- $\beta$  and several other immune cell populations such as natural killer T cells [18]. Besides, macrophage polarization is altered in the absence of OPN. One study showed that OPN deficiency promoted macrophages polarization towards a pro-regenerative phenotype via reducing M1 and M2 subset like IL-4 but increasing M2 subsets like IL10, as well as increasing the expression of pro-regenerative factors such as insulin-like growth factor 1, leukemia inhibitory factor, and urokinase-type plasminogen activator [19]. By contrary, another study indicated that the macrophages with increased OPN transcriptional activity were M2 (IL10)-like phenotype [20]. Thus, the exact role of OPN in macrophages is still needed to be clarified.

Apart from the genes mentioned above, hexosaminidase B (Hexb), CD72, CD63, cathepsin B (ctsb), and cathepsin D (ctsd) are all highly expressed in Trem2<sup>hi</sup> macrophages. Gene Ontology (GO) enrichment showed that the functions



**Fig. 1** **a** Left: t-SNE representation of aligned gene expression in single cells extracted from atherosclerotic aortae of *Ldlr*<sup>-/-</sup> mice which shows 18 distinct clusters. Right: Groups (healthy, high-fat diet 11, and 20 weeks) distribution in each cluster. **b** Gene number distribution of each group in clusters 0, 1, 2, 5, 6, 16, 17. **c** The percentage of clusters 0, 1, 2, 5, 6, 16, and 17 in each group

**Fig. 2** Heatmap shows the upregulated genes (ordered by decreasing *p* value) in each cluster defined in Fig. 1a, and selected enriched genes used for biological identification of each cluster (scale: Log<sub>2</sub> fold change)



**Table 1** The markers and functions of each macrophage subset

Cluster name	Markers	Common functions	Unique functions
Trem2 <sup>hi</sup> macrophages	Trem2	Neutrophil degranulation	Lipid catabolic process
	Spp1	Myeloid leukocyte activation	Osteoclast differentiation
	Ctsl		Vasculature development
	CD9	Response to lipoprotein particle	Response to reactive oxygen species Positive regulation and maintenance of locomotion
Inflammatory macrophages	Retnla	Inflammation response	Cell chemotaxis
	LYZ1	<i>Staphylococcus aureus</i> infection	NF-kappa B signaling pathway
	FN1		Regulation of smooth muscle cell migration
	CCL12	Antigen processing and presentation	
Resident-like macrophages	CCL8	Inflammation response	Endocytosis
	Folr2	Leukocyte migration	Proteolysis
	Pf4	Neutrophil degranulation	Lipid storage
	Cbr2	Myeloid leukocyte activation <i>Staphylococcus aureus</i> infection	Respiratory burst Platelet activation Cellular catabolic process Apoptotic signaling pathway
Plac8 <sup>hi</sup> monocytes	PLAC8	Inflammation response	Lysosome
	Thbs1	Neutrophil degranulation	Phagosome
	Lgals3	Myeloid leukocyte activation	Phagocytosis
	OSM		Regulation of proteolysis Apoptotic cell clearance Glycoside metabolic process Dendritic cell differentiation Reactive oxygen species metabolism
Monocyte-derived dendritic cells	CD209a	<i>Staphylococcus aureus</i> infection	Dendritic cell differentiation
	IFITM1		MHC class II antigen presentation
	NAPSA	Myeloid leukocyte activation	
	IFI30	Inflammation response	
Cst3 <sup>hi</sup> macrophages	Cst3	Antigen processing and presentation	Endocytosis
	NAAA		Proteasome
	IRF8	Neutrophil degranulation	Muscle cell migration
	IFI205		Lymphocyte activation Carbohydrate catabolism Activate glycosphingolipid catabolism
Fscn1 <sup>hi</sup> macrophages	Fascin1	Antigen processing and presentation	Apoptosis
	Fabp5		NF-kappa B signaling
	Tbcl4	Neutrophil degranulation	Actin cytoskeleton organization
	CCR7		Positive regulation of hydrolase activity

of Trem2<sup>hi</sup> macrophages are myeloid leukocyte activation, positive regulation and maintenance of locomotion, osteoclast differentiation, vasculature development, lipid catabolic process, responding to lipoprotein particle, reactive oxygen species, etc. Therefore, Trem2<sup>hi</sup> macrophages specialized in macrophage activation, adhesion, migration, and lipid metabolism.

## Inflammatory Macrophages

Cluster 1 macrophages express resistin-like alpha (Retnla), lysozyme (LYZ1), fibronectin 1 (FN1), chemokine (C-C motif) ligand 12 (CCL12), etc. in high level. Retnla, also known as hypoxia-induced mitogenic factor (HIMF) or FIZZ1, acts as a marker of M2 macrophages that are

associated with tissue remodeling and vascular growth in responding to chronic inflammatory conditions [21]. However, other M2 macrophage markers, such as v-abl Abelson murine leukemia viral oncogene 2 (Arg) and chitinase-like 3 (Ym1) [22, 23], were not highly expressed in cluster 1. LYZ1 is an antimicrobial enzyme that lyses the cell walls of certain Gram-positive bacteria by cleaving the glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid of the peptidoglycan [24]. Also, it enhances phagocytosis of macrophages [25]. As an extracellular matrix glycoprotein, Fn1 participates in cell differentiation, growth, and migration. It is also involved in tissue remodeling and wound healing [26]. Besides CCL12 (also known as MCP-5), other chemokine (C-C motif) ligand family members, including CCL2 (MCP-1), CCL3 (MIP-1 $\alpha$ ), CCL4 (MIP-1 $\beta$ ), and CCL7 (MCP-3) were significantly increased in this cluster. That is why cluster 1 was named inflammatory macrophages. Moreover, other pro-inflammation cytokines such as IL-1 $\beta$ , NFKB $\zeta$ , and CXCL12 are also enriched. GO analysis showed that the function of cell chemotaxis, NF-kappa B signaling pathway, regulation of smooth muscle cell migration stands out in inflammatory macrophages. In sum, this subtype of macrophages mainly takes part in promoting migration of macrophages, recruiting smooth muscle cells into atherosclerosis plaques and producing pro-inflammation cytokines. In the plaques of HFD 20-week group, cluster 1 and cluster 2 expand to almost 34% of total macrophages respectively.

## Resident-Like Macrophages

Cluster 2 was classified as resident-like (res-like) macrophages by Cochain et al. [27••], because the resident macrophage markers which are coagulation factor XIII (F13a1) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVEL) were both highly expressed in this cluster [28, 29]. Tissue-resident macrophages derived from monocytes or embryonic progenitors can be self-renewal in adult [30]. Smooth muscle cell-derived macrophages are not taken into consideration, since the smooth muscle-derived foam cells do not express the leukocyte marker CD45 [31]. Other markers in this analysis are CCL8, folate receptor 2 (Folr2), platelet factor 4 (Pff4), and carbonyl reductase (Cbr2). Interestingly, myocardial tissue-resident macrophages also expressed high levels of Lyve1 and Folr2 [32]. CCL8, also known as MCP-2, is responsible for the recruitment of leukocytes [33]. Folate receptor 2 (Folr2) is a family member of glycosylphosphatidylinositol-anchored glycoproteins with a high affinity to folic acid and 5-methyltetrahydrofolate. Folr2 is present on activated macrophages rather than on quiescent macrophages or other immune cells. However, whether it was expressed on M1 or M2 macrophages was controversial: some literatures reported that it is solely expressed on the M1 subset

[34], while others showed it was a marker of M2 population too [35]. In atherosclerosis plaques, high Folr2 expressed area is of increased number of activated macrophages and high degree of hypoxia, suggesting that it can serve as an indicator of plaque vulnerability [36]. What's more, folate-targeted liposomes have been reported to be utilized as a fluorescent molecule deliver or an anti-inflammatory drug (betamethasone) target for diagnostic or therapeutic applications respectively [34]. Pff4 encodes CXCL4 protein, which has been identified as a monocyte survival factor that induces the differentiation of monocytes towards "M4" macrophages with expression of markers of CD68, MMP7, and S100A8. Pff4 promotes macrophage differentiating into the so-called "M4" phenotype [37]. Pff4 also promotes monocyte phagocytosis and oxygen radical formation which contributes to the evolution of atherosclerosis lesions and its knockout reduces plaque size [38]. Cbr2 encodes AP27 protein, which is required for adipocyte differentiation. However, the role of Cbr2 in macrophages is still to be elucidated. Selenoprotein P1 (SEPP1) is also highly expressed in this type of macrophages. As a major selenoprotein, it serves two main roles: supplying tissues with selenium and acting as part of antioxidant defense system [39].

GO analysis showed that resident-like macrophages contribute to endocytosis, apoptotic signaling, platelet activation, proteolysis, lipid storage, cellular catabolic process, and respiratory burst. Thus, res-like macrophages mainly exert antioxidant and metabolism effect. In the present analysis' results, res-like macrophage populations only thrived at HFD 20-week group.

## Plac8<sup>hi</sup> Monocytes

Cluster 5 was identified as monocytes with high expression of placenta-specific 8 (PLAC8). Other expressed markers include thrombospondin1 (Thbs1), galectin-3 (Lgals3), and oncostatin M (OSM). PLAC8 is a small protein known to be highly expressed in giant trophoblasts and the spongiotrophoblast layer of the placenta. Also, it is a highly reversed, negative-regulated target of the c-Myc-gene [40]. Stimulating monocytes with LPS results in PLAC8 upregulation. PLAC8-overexpressed human monocytic cell line (THP-1) was associated with significant decrease of IL-1 $\beta$ , pro-IL-1 $\beta$ , pro-IL-18, and IL-18 through the enhancement of autophagy pathways [41]. Thbs1, a large (~450 kD) glycoprotein, is a key angiogenic checkpoint [42]. It plays a major role in both physiological and pathological tissue repair. Moreover, TSP1 binds and activates TGF- $\beta$ , then inhibits osteoblast differentiation [43]. TGF- $\beta$  is also highly expressed in Plac8<sup>hi</sup> macrophages (supplementary material). However, TSP1 deficiency had no effect on formation of obesity-induced aortic atherosclerotic lesion [44]. Galectin-3 is a 32 kDa protein secreted by macrophages, which is involved in processes such as cell activation, chemotaxis, and phagocytosis [45]. Recent studies

reported that Lgals3 enhances monocyte-derived macrophage efferocytosis of apoptotic granulocytes in asthma [46]. Also, it plays an important pro-inflammatory role in the induction phase of acute colitis by activating NLRP3 inflammasome and producing of IL-1 $\beta$  in macrophages [47]. OSM, which belongs to the interleukin 6 cytokine families, is secreted by activated T lymphocytes and monocytes. It plays vital roles in various processes including regulation of inflammation, fibrosis, and wound repair. As a HIF-1 $\alpha$  target gene, OSM directly inhibits the macrophage hypoxia signaling and relieves excessive fibrosis in the heart through inhibiting TGF- $\beta$ /smad pathway [48, 49]. However, in hepatic macrophages, OSM upregulated the expression of fibrogenic factors such as TGF- $\beta$  and PDGF [50]. Besides, OSM suppressed the expression of genes related to fatty acid synthesis and increased the expression of genes related to fatty acid oxidation. Furthermore, OSM decreased lipid absorption and increased the expression of active glucagon-like peptide-1 in the intestine [51].

Other highly expressed genes in cluster 5 include fatty acid-binding protein 5 (Fabp5), glycoprotein, and serum/glucocorticoid-regulated kinase1. GO analysis showed that the main functions of plac8<sup>hi</sup> macrophages were phagosome, phagocytosis, reactive oxygen species metabolism, apoptotic cell clearance, dendritic cell differentiation, regulation of proteolysis, receptor catabolic process and glycoside metabolic process. Therefore, plac8<sup>hi</sup> macrophages exert phagocytosis and lipid metabolism function. Plac8<sup>hi</sup> macrophages occupy 18% in HFD 11-week group, but only 2% and 3% in healthy and HFD 20-week group respectively (Fig. 1c).

## Monocyte-Derived Dendritic Cells

Another monocyte subtype is cluster 5, which take up 16% of total macrophages in both HFD 11-week and HFD 20-week groups. Cluster 6 is identified as monocyte-derived dendritic cells (DCs) because it highly expresses CD209a, a marker of dendritic cells [52]. Other markers expressed at cluster 6 are interferon-induced transmembrane protein 1 (IFITM1), napsin A aspartic peptidase (NAPSA), and interferon-gamma-induced protein 30 (IFI30). IFITM1, a 17 kDa membrane protein, is part of membrane complexes that deliver homotypic adhesion signal in lymphocytes. It plays a central role in many cellular functions such as adhesion, proliferation, and angiogenesis [53]. What's more, it is essential for the formation of functional blood vessels, and it stabilizes endothelial cells by regulating tight junction assembly [54]. NAPSA, a human aspartic proteinase found primarily in type II pneumocytes and alveolar macrophages, is used as an immunohistochemical marker in probing the origin of lung neoplasms. But there are not many studies to examine IFITM1, NAPSA, and IFI30 in macrophages. Interestingly, sepp1, pf4, and ApoE are downregulated greatly in DCs. Numerous

studies have reported the important role of dendritic cells in atherosclerosis [55, 56]. GO analysis showed that this cluster of macrophages played roles in mediating *Staphylococcus aureus* infection, dendritic cell differentiation, and MHC class II antigen presentation.

## Cst3<sup>hi</sup> Macrophages

Cluster 6 make up to only 6% of all macrophages in HFD 11-week group, and almost absent in healthy and HFD 20-week groups. The markers of cluster 6 are cystatin C (Cst3), *N*-acylethanolamine acid amidase (NAAA), interferon regulatory factor 8 (IRF8), and interferon-activated gene 205 (IFI205). Cystatin C is the best studied type II cystatins, a family of secreted small proteins that inhibit cysteine proteases of the papain family and legumain. DC and macrophages expressed the highest levels of the CST3 gene among all cell types. Low level of extracellular cystatin C associates with pathologic protease activity in atherosclerosis, emphysema, aortic aneurism, cancer, and arthritis. Cst3 deficiency enhances atherosclerosis in mice due to autophagy dysfunction and macrophage apoptosis [57]. NAAA is a cysteine enzyme that hydrolyzes of palmitoylethanolamide (PEA). Pharmacological blockage of NAAA elevates PEA levels and exerts powerful anti-inflammatory activities [58]. Another study reported that inhibition of NAAA suppressed LPS-induced macrophage activation [59]. IRF8, a transcription factor, controls genes that are induced by both developmental and inflammatory stimulus in macrophages. Meantime, it is required for the expression of autophagy-related genes and can be induced by IFN- $\gamma$ , Toll-like receptor, and bacterial infection [60]. IRF8 is also required for optimal activation of the NLRC4 inflammasome [61]. The role of IFI205 in macrophages is not elucidated yet.

GO analysis showed the main functions of this type of macrophages are negative regulation of immune system, lymphocyte activation, activate glycosphingolipid catabolism, endocytosis, proteasome, muscle cell migration, and carbohydrate catabolism.

## Fscn1<sup>hi</sup> Macrophages

Fscn1<sup>hi</sup> consists of only 2% of all macrophages in animals of HFD 11-week and HFD 20-week group. Markers of Fscn1<sup>hi</sup> include fascin actin-binding protein 1 (Fascin1, also named fascin-1), fatty acid-binding protein 5 (Fabp5), TBC1 domain family (Tbcl4), and chemokine (C-C motif) receptor 7 (CCR7). Fascin-1 is an actin-bound protein, suppression of which results in impaired cellular migration and invasion through extracellular matrix [62]. FABPs is a small protein with 14–15 kDa, which is abundantly expressed in cytoplasm

and is responsible for transporting hydrophobic ligands such as long-chain fatty acids, eicosanoids, and other lipids to specific cellular compartments. FABP4 and FABP5 are the only known isoforms co-expressed in adipocytes and macrophages. In leptin-deficient (*ob/ob*) mice, FABP4 and FABP5 deficiency contributes to severe obesity [63]. FABP5 is expressed in macrophages and plays significant roles in the development of diabetes and atherosclerosis [64]. FABP5 inhibits macrophages differentiate towards M2 phenotype in the liver [65]. Also, FABP5-deficient mice produced lower circulating inflammatory cytokines [66]. TBC1D4 is a Rab-GTPase-activating protein involved in insulin signaling [67]. It is critical for translocation of glucose transporter 4, from an inactive, intracellular, vesicle-bound site to the plasma membrane, where it promotes glucose entry into cells [68]. However, the role of TBC1D4 in macrophages is not yet well-understood. As the receptor of CCL19 and CCL21, CCR7 is positively correlated with macrophage migration and atherosclerosis progression [69]. GO analysis showed that the functions of *Fscn1<sup>hi</sup>* macrophages include apoptosis induction, positive regulation of hydrolase activity, NF-kappa B signaling, actin cytoskeleton organization, etc.

## Conclusions

With the development and prevalence of scRNA-seq technology, previous macrophage classification faces great challenge. The traditional macrophage phenotypes in atherosclerosis include M1, M2a, M2b, M2c, and M2d. However, the markers of those subtypes remain unresolved. For example, the reported markers of M1 including CD86, MHCII TNF $\alpha$ , IL-1b, IL-6, IL-12, IL-23, TNF $\alpha$ , CXCL9, CXCL10, CXCL11, CD80, and MARCO [1]. Analyzing GSE97310, markers are sorted in different clusters. As mentioned above, SPP1 and *FiZZ1*, which are the markers of *Trem2<sup>hi</sup>* and inflammatory macrophages respectively, are both recognized as markers of M2 phenotype in vitro. Therefore, a new method to clarify the subsets of macrophages is established based on scRNA-seq technology, which is totally different from the traditional one. We identified 7 clusters of macrophages, among which *Trem2<sup>hi</sup>* macrophages mainly appeared in healthy condition. In HFD 11-week group, the population of *Trem2<sup>hi</sup>* macrophages reduced while monocyte and monocyte-derived macrophages increased. Inflammatory and res-like macrophages are the two main clusters present in the HFD 20-week group. *Cst3<sup>hi</sup>* macrophages are the only subtype that can exert anti-inflammation effect in plaque, but its number was very low. However, none of those phenotypes is activated in vitro based on current reports.

Because the original data we used derived from GEO DataSets, several concerns should be clarified. Firstly, LDLR and ApoE knockout mice combined with high-fat diet are the

most extensively used atherosclerosis models [70]. The authors of GSE97310 data have proved that LDLR-negative mutants successfully developed atherosclerosis on high-fat diet [27••]. Secondly, based on our data analysis and the work of Cochain et al., the cluster phenotypes are the same between inflammatory, res-like, and *Trem2<sup>hi</sup>* macrophages in LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> atherosclerosis mice model. However, we were not sure for other populations due to lack of additional information of ApoE<sup>-/-</sup> mice in the reported experiments. Thirdly, since the authors focused on macrophage subsets in atherosclerosis plaques, other information about the mice model was not mentioned in the original article [27], such as the blood glucose levels, weight gain from the high-fat diet, and whether the environment of the site from which macrophages was isolated was highly inflamed with infiltration of cytokines and chemokines.

To sum up, there are many questions that needed to be further clarified such as why the number of res-like macrophages expanded only in HFD 20-week group. With more and more scRNA-seq experiments, a better understanding of macrophage classification in atherosclerosis plaques could be reached.

**Authors' Contributions** Hao Deng, Yingxin Sun and Wenyun Zeng contributed equally to this work.

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## Compliance with Ethical Standards

**Conflict of Interest** Hao Deng, Yingxin Sun, Wenyun Zeng, Huhu Li, Maojuan Guo, Lin Yang, Bin Lu, Bin Yu, Guanwei Fan, Qing Gao, and Xijuan Jiang declare no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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- Of major importance

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