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Who done it? Macrophage Mayhem in Atherosclerosis

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Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall in which apolipoprotein B containing lipoproteins and immune responses contribute to disease initiation, progression and clinical complications.[1] The accumulation of lipid-laden macrophages with “foamy” appearance is one hallmark feature of atherosclerotic lesions.[2] Lesional macrophages arise from recruitment of circulating monocytes and local proliferation.[2] A central question in the field is how lipid loading alters macrophage function, particularly their pro-inflammatory actions that may drive plaque instability and clinical atherosclerotic cardiovascular disease (CVD).

Non-foamy rather than foamy plaque macrophages are pro-Inflammatory in atherosclerosis

In the current issue, using bulk and single-cell RNA-seq (scRNA-seq), Kim et al.[3] report the important observation that non-foamy rather than foamy macrophages have pro-inflammatory characteristics in atherosclerosis of murine models.

The authors developed a flow cytometry-based approach to distinguish foam cells from other cells in the aortic wall using higher granularity (SSC^{hi}) and positivity for BODIPY493/503,

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Disclosures

None.

a fluorescent lipid probe that stains cytosolic neutral lipids. In *Ldlr*$^{-/-}$ mice fed a Western diet for 12 weeks, leukocyte-derived (CD45^{+}) foam cells accounted for ~81% of total foam cells, and ~97% of these CD45^{+} foam cells were CD11b^{+}CD64^{+} macrophages. This confirms established literature that macrophages are the major source of plaque foam cells[3] while also supporting more recent literature that non-leukocytes, particularly vascular smooth muscle cells, can form lesion foam cells.[4] The number of SSC^{hi}BODIPY^{hi} foam cells were correlated with the severity of atherosclerosis and were reduced in atherosclerosis regression models.[3]

Transcriptome analysis of foamy macrophages showed a striking lower expression of inflammatory genes and higher expression of genes related to cholesterol uptake, processing, and efflux compared with non-foamy macrophages, which expressed high levels of inflammatory genes, such as *Il1b*, *Nfkbia*, and *Tlf2*.[3] scRNA-seq of aortic CD45^{+} leukocytes in male *Ldlr*$^{-/-}$ mice fed a Western diet for 12 weeks identified 11 clusters with 8 macrophage clusters. The top differentially expressed genes enriched in foamy macrophages were mostly found in cluster 4, whereas many of those from non-foamy macrophages were specifically expressed in cluster 1. Pathway analysis supported that cluster 1 macrophages showed increased expression of genes in inflammation and toll-like receptor signaling pathways. In addition, clusters 0, 3 and 5 expressed high levels of *Lyve1*, a feature of resident-like macrophages. Cluster 8 showed highly enriched cell cycle-related genes, suggesting proliferating macrophages. The work confirms recent evidence[5, 6] of diverse leukocyte sub-populations in mouse atherosclerosis and highlighted non-foamy macrophages as abundant inflammatory cells in progressing atherosclerosis.

Do non-foamy macrophages drive atherosclerosis progression and clinical complications?

The success of CANTOS, a recent clinical trial of anti-IL-1β antibody in high-risk patients on optimal lipid lowering therapy,[7] as well as human genetic studies of inflammatory clonal hematopoiesis,[8, 9] reaffirm the inflammatory hypothesis of atherosclerotic CVD. The work of Kim et al.[3] challenge the field to have a more open view of lesion macrophage phenotypes and roles in atherosclerosis. Although challenging dogma, the finding of reduced inflammation in foamy macrophages is not entirely novel, and confirm recent work by the Glass group showing that generation of foamy macrophages by cholesterol loading *in vitro* and *in vivo* may suppress inflammatory status of peritoneal macrophages via activation of the liver X receptor.[10] Yet, much remains unknown from these single cell association data and extensive experimental and clinical follow-up is required.

First, why do non-foamy macrophages apparently remain not lipid-loaded in atherosclerosis progression? Do the lesion non-foamy and foamy macrophages have distinct spatial distribution with different degree of lipoprotein retention? Or do the non-foamy macrophages intrinsically possess lower lipid uptake capacity? Second, are non-foamy macrophages causal drivers of atherosclerosis initiation and progression independent of foam cell formation or are they in fact cells in transition to foam cells? Third and conversely, are foam cells less toxic than dogma suggests or indeed are they protective? Or does an

apparent less inflammatory gene expression profile mask their actions in complex lesion formation to drive plaque instability and clinical CVD complications?[11] Fourth, do these cells diminish during treatment and resolution of atherosclerosis or do distinct functional macrophage types emerge to promote regression? Fifth, how do these murine lesion macrophage subpopulations map to human plaques?

Perhaps the most intriguing questions relate to the origin, drivers, dynamics, and human translation of distinct macrophage subpopulations in lesions. Plaque microenvironment factors, such as lipids and cytokines, hypoxia, apoptotic and necrotic cells, and matrix can shape macrophage identities.[12] It is plausible also that circulating and recruited monocyte subsets, and the macrophages derived from them, have intrinsic properties with distinct roles in atherogenesis. Indeed, hypercholesterolemic mice demonstrate monocytosis primarily attributable to an increase in the more inflammatory Ly6C^{hi} monocyte subset, and these make up the majority of cells recruited to atherosclerotic plaques.[13, 14] These questions can be probed in rodent models but critically require both independent discovery within the human risk context and validation of mouse findings in humans. Key questions include the relationship of subpopulations to CVD-related inflammatory myeloid cells in human lesions that are driven by clonal hematopoiesis and age-related somatic mutations in *TET2* and other genes?[8, 9] From a therapeutic perspective, understanding which of these macrophages subpopulations are modulated by targeting IL-1β will facilitate clinical translation of the CANTOS trial findings.[7] Kinetic profiling to map the temporal and spatial trajectories of all human circulating and lesion monocyte and macrophage subsets and understanding how known CVD risk factors, including genetic predisposition, affect their plasticity and survival will provide new insights into mechanisms of human atherosclerotic CVD.

Strengths and limitations of scRNA-seq in understanding atherosclerosis

Three independent studies[3, 5, 6] published recently in *Circulation Research* use scRNA-seq to examine CD45^{+} aortic leukocytes subpopulations and their transcriptome signatures in mouse model of atherosclerosis (Table 1). Winkels et al.[5] and Cochain et al.[6] profiled leukocytes in healthy and atherosclerotic aortas from chow-fed and Western diet/high fat diet-fed mice, while Kim et al.[3] focused on plaque leukocytes in Western diet-fed mice. Although all three studies agree in major leukocyte populations identified and have successfully discovered and validated novel subpopulations,[15] the cell type clusters reported have important differences (Table 1). Winkels et al.[5] and Cochain et al.[6] have identified multiple T cell subpopulations. Kim et al.[3] showed macrophages with the largest cell number and the most diverse subpopulations. These differences may be attributable to the mouse strain, disease model and timing, type of diet, tissue sampling and digestion as well as analytic framework. Foremost however, this may simply reflect the nascent state of single cell profiling in atherosclerosis, in particular a lack of sensitivity to detect lower frequency populations.

In summary, Kim et al.[3] and others[5, 6] are driving a conceptual shift towards defining the roles of diverse plaque leukocytes, which have previously underappreciated heterogeneity. Coupled to quickly evolving experimental and computational protocols, and applications to human lesions, single-cell profiling has the potential to transform our understanding of

plaque biology, reveal causal cell types, their key master regulators and effectors, and thus novel therapeutic targets for human atherosclerosis and its clinical complications.[15]

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Table 1.

Methods and findings of scRNA-seq analysis of $CD45^+$ aortic leukocytes in atherosclerotic mice model in three independent studies.

	Kim et al.[3]	**Winkels et al.[5]**	**Cochain et al.[6]**
Mice	*Ldlr*$^{-/-}$ (8 wks male)	*Apoe*$^{-/-}$ (8 wks female)	*Ldlr*$^{-/-}$ (6-8 wks male)
Diet	Western Diet (Test Diet, AIN-76A)	Chow or Western diet (Envigo, TD.88137)	Chow or high-fat diet (Altromin, 15% milk fat, 1.25% cholesterol)
Duration	12 wks	12 wks	11 wks
Enzyme Mix	Ca^{2+} Mg^{2+} PBS + Collagenase I (675 U/mL) + Col XI (187.5 U/mL) + Dnase I (90 U/mL) + Hyaluronidase (90 U/mL)	HBSS + Collagenase I (450 U/mL) + Collagenase XI (250 U/mL) + Dnase I (120 U/mL)	RPMI + Collagenase I (450 U/mL) + Collagenase XI (125 U/mL) + Hyaluronidase (60 U/mL)
Incubation Time	37 °C, 70 min	37 °C, 60 min	37 °C, 40 min
Platform	10X Genomics	10X Genomics	Drop-seq
Analysis	Cell Ranger, SEURAT		
# of $CD45^+$ Cells Analyzed After QC Filtering	3,781 (Western diet)	909 (chow) 2,077 (Western diet)	372 (chow) 854 (Western diet)
Clusters	11	11	13
	Macrophages: 8 DCs: 2 T cells: 1	T cells: 5 B cells: 2 Monocytes: 2 Macrophages: 1 NK cells: 1	T cells: 4 Macrophages: 3 B cells: 1 DCs: 1 Granulocytes: 1 Monocytes: 1 NK cells: 1 Mixed cells/mast cells: 1
scRNA-seqReplication	Total foam cells ($SSC^{hi}BODIPY^{hi}$) from *Apoe*$^{-/-}$ mice fed a high-fat diet for 27 wks	$CD45^+$ leukocytes from *Ldlr*$^{-/-}$ (8 wks male) fed a high cholesterol diet for 12 wks	$CD45^+$ leukocytes from *Apoe*$^{-/-}$ (8 wks female) fed Western diet (Envigo, TD.88137) for 12 wks
Validation	FACS, histology, and RNA-seq for foamy and non-foamy macrophages	Mass Cytometry and FACS for 3 B-cell subsets	Immunohistochemistry for 3 macrophage subsets (inflammatory, resident-like, and $TREM2^{hi}$)
Human Translation	*In situ* hybridization of *Il1b* mRNA and KI-67 staining in human lesional macrophages	Enumerate leukocyte frequencies in 126 human plaques by a genetic deconvolution strategy	Immunohistochemistry of human lesions