HHS Public Access

Author manuscript

Curr Opin Lipidol. Author manuscript; available in PMC 2019 October 01.

Published in final edited form as:

Curr Opin Lipidol. 2018 October ; 29(5): 389–396. doi:10.1097/MOL.0000000000000537.

Atherosclerosis in the single-cell era

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Abstract

Purpose of review—The immune system plays a critical role in the development and modulation of atherosclerosis. New high-parameter technologies, including mass cytometry (CyTOF) and single-cell RNA sequencing (scRNAseq), allow for an encompassing analysis of immune cells. Unexplored marker combinations and transcriptomes can define new immune cell subsets and suggest their functions. Here, we review recent advances describing the immune cells in the artery wall of mice with and without atherosclerosis. We compare technologies and discuss limitations and advantages.

Recent findings—Both CyTOF and scRNAseq on leukocytes from digested aortae show 10–30 immune cell subsets. Myeloid, T, B and natural killer cells were confirmed. Although cellular functions can be inferred from RNA-Seq data, some subsets cannot be identified based on current knowledge, suggesting they may be new cell types. CyTOF and scRNAseq each identified four B-cell subsets and three macrophage subsets in the atherosclerotic aorta. Limitations include cell death caused by enzymatic digestion and the limited depth of the scRNAseq transcriptomes.

Summary—High-parameter methods are powerful tools for uncovering leukocyte diversity. CyTOF is currently more powerful at discerning leukocyte subsets in the atherosclerotic aorta, whereas scRNAseq provides more insight into their likely functions.

Keywords

atherosclerosis; immune system; leukocytes; mass cytometry; single-cell RNA sequencing

INTRODUCTION

Heart attacks and strokes account for the majority of deaths worldwide. Most are caused by arterial thrombosis, a complication of ruptured or eroded atherosclerotic plaque [1]. The normal arterial wall contains endothelial cells, smooth muscle cells (SMCs), adventitial

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Conflicts of interest

There are no conflicts of interest.

fibroblasts and leukocytes including vascular macrophages [2], T cells and B cells [3■■,4]. Several arterial leukocyte lineages were discovered by immunostaining and studied by flow cytometry (FACS) of digested aortae of experimental animals. After onset of atherosclerosis, endothelial cells are activated and SMC phenotype changes [5,6], the number of leukocytes increases in the adventitia and a eointima forms that also contains many leukocytes. Vascular macrophages proliferate locally [7■], and new leukocyte subsets are recruited to the arterial wall from circulation, either across the luminal endothelium or from vasa vasorum. Stem cells have been observed in the adventitia that may give rise to both SMC and macrophages [8].

Immunohistochemistry uncovered the presence of myeloid, T, B and natural killer (NK) cells in atherosclerotic lesions. FACS allows the simultaneous use of 18 fluorochrome-tagged antibodies and forward scatter (cell size) and side scatter (granularity), thus revealing more leukocyte subsets in the atherosclerotic mouse aorta. FACS-defined myeloid cells include three subsets of macrophages [9], neutrophils [10] and monocytes [11]. Furthermore, dendritic cells [3■■,12] and small numbers of plasmacytoid dendritic cells [13,14] can be found. Among αβ T cells, CD4 and CD8 [15] were found to be present, and a small number of $\gamma\delta$ T cells [16]. FACS allows for intracellular staining of transcription factors, which identified Th1 [17], Th17 [18], follicular helper [19] and regulatory (Treg) [20] cells among CD4 T cells. Th1 cells are the predominant CD4 T-cell subset residing in the plaque [21]. Although Th2 cells are scarce in Apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice on the C57BL6/J background, possibly due to the known Th1 bias of this strain [22], single-cell RNA sequencing (scRNAseq) of murine aortae clearly demonstrated the presence of a Th2 subset residing within the aorta [23■■]. In addition, natural killer T cells [24], innate lymphoid cells [25,26] and NK cells [24] were found in atherosclerotic aortae. Using *Cxcr6*^{GFP} mice, a CXCR6^{+} CD4 T-cell subset was identified [27], which was confirmed by scRNAseq [28■■]. The FACS approach identified only a few cell types per study. The main advantage of FACS is its low cost per cell and high throughput (Table 1). A 14-marker FACS panel resolved 12 different aortic leukocyte populations simultaneously (Table 1 and [23■■]) (Fig. 1).

THE IMMUNE CELL LANDSCAPE IN ATHEROSCLEROSIS ASSESSED BY MASS CYTOMETRY

Mass cytometry (CyTOF) allows for the simultaneous detection of up to 42 parameters based on metal-conjugated monoclonal antibodies with no spectral overlap [29]. These are clear advantages over FACS. We recently performed CyTOF to more comprehensively and precisely assess the immune landscape in the *Apoe*$^{-/-}$ model of atherosclerosis [23■■, 28■■]. We applied a 35-marker CyTOF panel followed by Pheno-Graph, a dimensionality reduction and unsupervised clustering algorithm [30]. Of the, at least, 32 billion possible marker combinations, the analysis grouped aortic leukocytes into 23 clusters (Table 2), including four B-cell clusters, five T-cell clusters, NK cells, eosinophils, neutrophils, three dendritic cell clusters, two monocyte clusters, three macrophage clusters and three yet unknown populations with unique marker combinations. Although the frequency of macrophages was unsurprisingly increasing in the aorta of *Apoe*$^{-/-}$ mice fed Western diet,

one B-cell population and a Ly-6C^{hi} CD8^{+} T-cell population both vanished [23■■]. Cole *et al.* [31■] identified 13 principal aortic immune populations in aortas of *Apoe*$^{-/-}$ mice including one population each of neutrophils, eosinophils, B cells, monocytes, macrophages, CD4^{+} T cells, CD8^{+} T cells, $\gamma\delta$ T cells, NK cells, ILCs, plasmacytoid dendritic cells and two populations of dendritic cells. Subclustering of only myeloid cells uncovered 20 different populations of which subsets of CD169^{+}CD206^{+} macrophages (one expressing CD209b and the other not) and common dendritic cells were significantly reduced in the aortae of cholate-free high-fat-fed *Apoe*$^{-/-}$ mice [31■].

BEFORE SINGLE-CELL RNA SEQUENCING

Trogan *et al.* [32] dissected macrophage-rich areas of atherosclerotic plaques from *Apoe*$^{-/-}$ mice by laser-capture microdissection (LCM) and analyzed the expression of several macrophage-related genes by quantitative PCR. Microarray analysis of LCM-dissected CD68^{+} cells from shoulder regions of human plaques showed 72 genes, among them *IRF5* and *CSF1*, to be upregulated compared with in-vitro stimulated THP1 cells [33]. LCM was used to generate the first aortic transcript atlas of wild-type and atherosclerotic *Apoe*$^{-/-}$ mice [34■,35■,36]. Although not single-cell, this LCM effort yielded transcriptional profiles of the plaque, media, adventitia and adventitia harboring arterial tertiary lymphoid organs (ATLOs). LCM and microarrays also showed differences in the transcriptional landscape of atherosclerotic plaques obtained by carotid endarterectomy from symptomatic and nonsymptomatic patients [37■].

Next-generation sequencing allows for a deeper insight into the entire transcriptome without having to decide *a priori* which transcripts should be measured. This methodology was first used in atherosclerosis to determine the transcriptional differences of effector T cells (Teff), Tregs and a newly defined population of CCR5^{+} Teff cells appearing at later stages of atherosclerosis [38]. FACS-sorting the cells requires a-priori knowledge of the phenotype (surface markers) of the cell type to be analyzed. Thus, bulk transcriptomics is less suitable for discovery purposes of yet undefined cell types.

SINGLE-CELL RNA SEQUENCING

The first application of single-cell transcriptomics in atherosclerosis focused on T cells and described a population called Th1/Tregs [39■■], which is very similar to the aforementioned CCR5^{+} Teff cells. This group used the Fluidigm C1 single microfluidics chip device (Fluidigm, California, USA) for capturing and preparing libraries of 270 cells of interest sorted by FACS.

Microfluidic devices encapsulating single cells in solvent droplets embedded in silicone oil recently became commercially available (10× Genomics, Pleasanton, California, USA), allowing for massively parallel transcriptome assessment of single cells (Drop-Seq) [40]. This technology, used in two recent publications, is now robust, reproducible and economical [23■■,28■■]. At this time, new scRNAseq techniques are being described every year, and the field is not settled. There are significant differences in sensitivity, coverage, precision, reproducibility, cost, time and scalability [41].

Most of the scRNAseq techniques enrich only for the 3′ ends of the transcripts; however, a switching mechanism at the 5′ end of the RNA template, (SMART)-Seq and its derivatives [42,43], produces reads spanning entire transcripts. Although the former can be suitable for studies exploring the heterogeneity of various tissues, only the latter can be used to study splicing isoforms, structural variation and single-nucleotide polymorphisms.

A known shortcoming of Drop-Seq is the random encapsulation of cells into the drops, so abundant cell types are sequenced more often than rare ones. Another inherent shortcoming is the occurrence of doublets, which can be mitigated by reducing the number of cells per microfluidic channel [44], but this increases the cost per cell.

IMMUNE CELL HETEROGENEITY IN ATHEROSCLEROSIS ASSESSED BY SINGLE-CELL RNA SEQUENCING

scRNAseq identified 11– 13 distinct immune cell populations (Table 2) based on SEURAT, an algorithm that provides unsupervised identification of cells based on similarity in their transcriptomes [45]. The Drop-Seq approach detected an average of 1004–1873 genes per cell, which is state of the art and accounts for about 10% of the transcriptome [23■■,28■■]. Increasing sequencing depth would not detect more genes, as saturation was already reached at 1 million reads per cell. Genomic gating by Seq-Geq (FlowJo LLC, Ashland, California, USA) of scRNAseq data increased the B-cell diversity from two populations to three, which is the same number found by mass cytometry and FACS (Table 2). A B1-like cell subset was enriched for tumor necrosis factor signaling and produced large amounts of CCL5, suggesting a functional role to recruit more immune cells to the aorta [23■■]. Furthermore, two B2-like subsets, both producing IFNγ and granuolcyte monocyte colony stimulating factor, were identified.

Macrophages are resident in almost all healthy tissues [46] including healthy mouse aortae [2]. The transcriptomes of FACS-sorted macrophages have been reported [47,48], but the two recent publications [23■■,28■■] are the first to show evidence of macrophage heterogeneity in atherosclerotic mouse aortae. All macrophages express the adhesion G-protein-coupled receptor, F4/80, and most also express the Fc*g* receptor-1 (CD64) and the receptor tyrosine kinase, MerTK [48,49]. In healthy mouse aortae, only a single population of macrophages was found by scRNAseq [28■■], which corresponded to previously defined resident vascular macrophages [2]. In atherosclerotic *Ldlr*$^{-/-}$ aortae, two additional subsets of macrophages were uncovered, one of which uniquely expressed TREM2 and another resembled an inflammatory macrophage phenotype that may be specific for atherosclerosis [28■■]. In atherosclerotic *Apoe*$^{-/-}$ mice, CyTOF identified three macrophage subsets that expressed CX3CR1, CD11c and CD103, respectively (Table 2 and [23■■]). Genomic gating of scRNAseq data for *Adgre1* (F4/80) and *Cd68* revealed four macrophage subsets. It is not known if and how these correspond to the subsets seen in aortae of *Ldlr*$^{-/-}$ mice.

Direct comparison of the principal immune cell populations across the single-cell transcriptomes reveals similar proportions of NK cells and a higher abundance of T cells in aortae of *Apoe*$^{-/-}$ mice, whereas macrophages, although increasing upon Western diet feeding in both mice, are more predominant in *Ldlr*$^{-/-}$ mice (Fig. 2). Significantly, the

frequency of B cells is higher in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice fed chow diet compared with *Ldlr*$^{-/-}$ mice fed a cholesterol-rich diet. This suggests a substantial dynamic range of aortic immune cells across the different models. However, at this point, it is unclear whether a difference in digestion protocols (digest for 60 min [23■■] vs. 40 min [28■■]) might favor the isolation of lymphocytes or myeloid cells.

GENE SIGNATURES AS PROGNOSTIC MARKERS

Single-cell technologies, like CyTOF and scRNAseq, have provided promising advances in many diseases and disease models. Spitzer *et al.* [50] observed a multiorgan immune system response during cancer treatment and found that specific cell-type abundance predicted effective cancer therapy. Immune cell signatures before surgery predict recovery [51]. Activation and responsiveness states of B-cell sub-sets in the bone marrow were found to predict relapse in patients with B-cell precursor acute lymphoblastic leukemia [52]. Gene signatures from bulk transcriptomic data have shown promise as prognostic or diagnostic indicators of disease [53–55]. Gene expression deconvolution algorithms, such as CIBERSORT [56], allow for the in-silico dissection of biopsies from patients by probing the transcriptome of individual leukocyte signatures and estimating their respective abundance [57]. We extracted 11 signatures from the single-cell transcriptomes of the murine aortic leukocytes and tested whether these signatures had predictive value in the BiKE cohort [37■] (Fig. 1). The BiKE study contains bulk transcriptomes from 100 human atherosclerotic plaques from carotid endarterectomies. As in mouse lesions, the predominant signature is derived from macrophages (55%), whereas T cells, B cells and monocytes make up 40% of the lesional immune cells, and NK cells are the smallest population (Fig. 2). From all 11 single-cell transcriptomes identified in atherosclerotic mouse aortae, the memory T-cell population correlated negatively and significantly with ischemic event-free survival of the patient [23■■].

Although single-cell methods require dissection and digestion, which means cellular spatial information is lost, we were able to gain insight into the spatial distribution of some immune cell populations by genetically deconvolving the individual mouse immune cell signatures with bulk transcriptomes derived by LCM of murine aortae (lesion, media, adventitia and adventitia with ATLOs) (Fig. 2 and [23■■]). This confirmed that the majority of aortic B cells reside in the adventitia, whereas macrophages, T cells and monocytes populate the plaque (Fig. 2 and [23■■]). This approach also corrects for the under-sampling of macrophages caused by the digestion and mechanical disruption of the tissue.

LIMITATIONS

Although CyTOF and scRNAseq provide a wealth of information, they both share the need for making single-cell suspensions by enzymatic and mechanical tissue disruption. Although dead cells are excluded by staining in CyTOF, cell death does not affect all cells equally, and this distorts the proportions. Large, branched cells like macrophages are systematically undersampled, as they are more likely to be destroyed by the enzymatic and mechanical dissociation [58]. As mentioned above, this can be corrected by running the scRNAseq-based gene signatures on bulk transcriptomes of LCM material using CIBERSORT.

Contamination with circulating leukocytes can be controlled by injecting an anti-CD45 antibody to stain intravascular leukocytes and exclude them from subsequent analysis.

CyTOF is a destructive method, which means the cells analyzed cannot be recovered. CyTOF is currently limited by 42 lanthanide isotopes that are commercially available, but more metals are likely to become available soon. The most severe drawback of CyTOF is that it depends on user-driven selection of markers.

scRNAseq is truly hypothesis-free, but the spatial location of the interrogated cells is usually lost. An attempt to circumvent this limitation is the use of a photoconvertible fluorochrome to label a subset of immune cells in a known location before harvesting [59]. Downstream analysis of scRNAseq is more complex than bulk RNA sequencing due to the increased technical variability among individual cells, which can be attributed to the cell-cycle state [60], the burstiness of gene expression [61], the drop-out probability of low-expressed genes and the different PCR efficiency across the cells and across transcripts within the same cell. The lack of depth in scRNAseq transcriptomes appears to be fundamental and, in part, represents the fact that rare transcripts may not be in the cell at the time of harvest (stochastic dropouts). This can be overcome, partly, by constructing synthetic transcriptomes from the cells found in each t-distributed stochastic neighbor embedding cluster.

CONCLUSION

The immune system plays a crucial role in the progression and modulation of atherosclerosis. High-modality technologies including CyTOF and scRNAseq uncovered an unexpected diversity among aortic leukocytes. Furthermore, signatures constructed from single-cell transcriptomes can be useful to predict secondary ischemic events [23■■]. Although both CyTOF and scRNASeq were capable of detecting the principle immune subsets, an integration of single-cell protein and transcriptome information may uncover a potentially even larger heterogeneity among aortic immune subsets. Also, a single immune cell atlas of human plaques is yet missing. The information gained by these data will help to specifically tailor new therapeutic strategies for altering the immune response in atherosclerosis.

Acknowledgements

Financial support and sponsorship

H.W. was supported by DFG award (GZ WI 4811/1-1), K.L. was supported by grants HL115232, HL88093 and HL121697 from the National Heart, Lung, and Blood Institute.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

■ of special interest

■■ of outstanding interest

1. Hansson GK, Libby P, Tabas I. Inflammation and plaque vulnerability. J Intern Med 2015; 278:483–493. [PubMed: 26260307]
2. Ensan S, Li A, Besla R, et al. Self-renewing resident arterial macrophages arise from embryonic CX3CR1(+) precursors and circulating monocytes immediately after birth. Nat Immunol 2016; 17:159–168. [PubMed: 26642357]
3. Galkina E, Kadl A, Sanders J, et al. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. J Exp Med 2006; 203:1273–1282. [PubMed: 16682495] ■■ The first study assessing leukocyte diversity in atherosclerotic aortas by flow cytometry.
4. Butcher MJ, Herre M, Ley K, Galkina E. Flow cytometry analysis of immune cells within murine aortas. J Vis Exp 2011; 53:2848.
5. Shankman LS, Gomez D, Cherepanova OA, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat Med 2015; 21:628–637. [PubMed: 25985364]
6. Feil S, Fehrenbacher B, Lukowski R, et al. Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. Circ Res 2014; 115:662–667. [PubMed: 25070003]
7. Robbins CS, Hilgendorf I, Weber GF, et al. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. Nat Med 2013; 19:1166–1172. [PubMed: 23933982] ■ The study highlights the contribution of macrophage proliferation to atherosclerosis.
8. Majesky MW, Horita H, Ostriker A, et al. Differentiated smooth muscle cells generate a subpopulation of resident vascular progenitor cells in the adventitia regulated by Klf4. Circ Res 2017; 120:296–311. [PubMed: 27834190]
9. Koltsova EK, Garcia Z, Chodaczek G, et al. Dynamic T cell-APC interactions sustain chronic inflammation in atherosclerosis. J Clin Invest 2012; 122:3114–3126. [PubMed: 22886300]
10. Drechsler M, Megens RT, van Zandvoort M, et al. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. Circulation 2010; 122:1837–1845. [PubMed: 20956207]
11. Swirski FK, Libby P, Aikawa E, et al. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. J Clin Invest 2007; 117:195–205. [PubMed: 17200719]
12. Paulson KE, Zhu SN, Chen M, et al. Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. Circ Res 2010; 106:383–390. [PubMed: 19893012]
13. Daissormont IT, Christ A, Temmerman L, et al. Plasmacytoid dendritic cells protect against atherosclerosis by tuning T-cell proliferation and activity. Circ Res 2011; 109:1387–1395. [PubMed: 22021930]
14. Doring Y, Manthey HD, Drechsler M, et al. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. Circulation 2012; 125:1673–1683. [PubMed: 22388324]
15. Cochain C, Koch M, Chaudhari SM, et al. CD8+ T cells regulate monopoiesis and circulating Ly6C-high monocyte levels in atherosclerosis in mice. Circ Res 2015; 117:244–253. [PubMed: 25991812]
16. Vu DM, Tai A, Tatro JB, et al. GammadeltaT cells are prevalent in the proximal aorta and drive nascent atherosclerotic lesion progression and neutrophilia in hypercholesterolemic mice. PLoS One 2014; 9:e109416. [PubMed: 25313857]
17. Gjurich BN, Taghavie-Moghadam PL, Galkina EV. Flow cytometric analysis of immune cells within murine aorta. Methods Mol Biol 2015; 1339:161–175. [PubMed: 26445788]
18. Smith E, Prasad KM, Butcher M, et al. Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. Circulation 2010; 121:1746–1755. [PubMed: 20368519]
19. Clement M, Guedj K, Andreata F, et al. Control of the T follicular helper-germinal center B-cell axis by CD8(+) regulatory T cells limits atherosclerosis and tertiary lymphoid organ development. Circulation 2015; 131:560–570. [PubMed: 25552357]
20. Maganto-Garcia E, Tarrio ML, Grabie N, et al. Dynamic changes in regulatory T cells are linked to levels of diet-induced hypercholesterolemia. Circulation 2011; 124:185–195. [PubMed: 21690490]

21. Frostegard J, Ulfgren AK, Nyberg P, et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis 1999; 145:33–43. [PubMed: 10428293]
22. Schulte S, Sukhova GK, Libby P. Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis. Am J Pathol 2008; 172:1500–1508. [PubMed: 18467709]
23. Winkels H, Ehinger E, Vassallo M, et al. Atlas of the immune cell repertoire in mouse atherosclerosis defined by single-cell RNA-sequencing and mass cytometry. Circ Res 2018; 122:1675–1688. [PubMed: 29545366] ■■ The first study assessing leukocyte diversity in atherosclerotic aortae by single-cell RNA sequencing (scRNAseq) and mass cytometry.
24. Rombouts M, Ammi R, Van Brussel I, et al. Linking CD11b ( ) dendritic cells and natural killer T cells to plaque inflammation in atherosclerosis. Mediators Inflamm 2016; 2016:6467375. [PubMed: 27051078]
25. Engelbertsen D, Foks AC, Alberts-Grill N, et al. Expansion of CD25 innate lymphoid cells reduces atherosclerosis. Arterioscler Thromb Vasc Biol 2015; 35:2526–2535. [PubMed: 26494229]
26. Newland SA, Mohanta S, Clement M, et al. Type-2 innate lymphoid cells control the development of atherosclerosis in mice. Nat Commun 2017; 8:15781. [PubMed: 28589929]
27. Galkina E, Harry BL, Ludwig A, et al. CXCR6 promotes atherosclerosis by supporting T-cell homing, interferon-gamma production, and macrophage accumulation in the aortic wall. Circulation 2007; 116:1801–1811. [PubMed: 17909108]
28. Cochain C, Vafadarnejad E, Arampatzi P, et al. Single-cell RNA-seq reveals the transcriptional landscape and heterogeneity of aortic macrophages in murine atherosclerosis. Circ Res 2018; 122:1661–1674. [PubMed: 29545365] ■■ A pioneering study uncovering macrophage heterogeneity in atherosclerotic aortae by scRNAseq.
29. Spitzer MH, Nolan GP. Mass cytometry: single cells, many features. Cell 2016; 165:780–791. [PubMed: 27153492]
30. Levine JH, Simonds EF, Bendall SC, et al. Data-driven phenotypic dissection of AML reveals progenitor-like cells that correlate with prognosis. Cell 2015; 162:184–197. [PubMed: 26095251]
31. Cole JE, Park I, Ahern D, et al. Immune cell census in murine atherosclerosis: cytometry by time of flight illuminates vascular myeloid cell diversity. Cardiovasc Res 2018; doi: 10.1093/cvr/cvy109. [Epub ahead of print]■ An important study examining aortic myeloid cell diversity by mass cytometry.
32. Trogan E, Choudhury RP, Dansky HM, et al. Laser capture microdissection analysis of gene expression in macrophages from atherosclerotic lesions of apolipoprotein E-deficient mice. Proc Natl Acad Sci U S A 2002; 99:2234–2239. [PubMed: 11842210]
33. Tuomisto TT, Korkeela A, Rutanen J, et al. Gene expression in macrophage-rich inflammatory cell infiltrates in human atherosclerotic lesions as studied by laser microdissection and DNA array: overexpression of HMG-CoA reductase, colony stimulating factor receptors, CD11A/CD18 integrins, and interleukin receptors. Arterioscler Thromb Vasc Biol 2003; 23:2235–2240. [PubMed: 14576072]
34. Grabner R, Lotzer K, Dopping S, et al. Lymphotoxin beta receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia of aged ApoE–/– mice. J Exp Med 2009; 206:233–248. [PubMed: 19139167] ■ This is a valuable resource providing bulk transcriptomes of different layers of the murine aorta at different stages of atherosclerosis.
35. Beer M, Doepping S, Hildner M, et al. Laser-capture microdissection of hyperlipidemic/ApoE(−)/(−) mouse aorta atherosclerosis. Methods Mol Biol 2011; 755:417–428. [PubMed: 21761324] ■ This is a valuable resource providing bulk transcriptomes of different layers of the murine aorta at different stages of atherosclerosis.
36. Yin C, Mohanta S, Ma Z, et al. Generation of aorta transcript atlases of wild-type and apolipoprotein E-null mice by laser capture microdissection-based mRNA expression microarrays. Methods Mol Biol 2015; 1339: 297–308. [PubMed: 26445797]
37. Folkersen L, Persson J, Ekstrand J, et al. Prediction of ischemic events on the basis of transcriptomic and genomic profiling in patients undergoing carotid endarterectomy. Mol Med 2012; 18:669–675. [PubMed: 22371308] ■ The study encompasses bulk transcriptomes of 126 human plaques and patient follow-up information.

38. Li J, McArdle S, Gholami A, et al. CCR5+ T-bet+ FoxP3+ effector CD4 T cells drive atherosclerosis. Circ Res 2016; 118:1540–1552. [PubMed: 27021296]
39. Butcher MJ, Filipowicz AR, Waseem TC, et al. Atherosclerosis-driven Treg plasticity results in formation of a dysfunctional subset of plastic IFNgamma+ Th1/Tregs. Circ Res 2016; 119:1190–1203. [PubMed: 27635087] ■■ The first study using single-cell transcriptomics in atherosclerosis research.
40. Zheng GX, Terry JM, Belgrader P, et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun 2017; 8:14049. [PubMed: 28091601]
41. Ziegenhain C, Vieth B, Parekh S, et al. Comparative analysis of single-cell RNA sequencing methods. Mol Cell 2017; 65:631–643.e34. [PubMed: 28212749]
42. Ramskold D, Luo S, Wang YC, et al. Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. Nat Biotechnol 2012; 30:777–782. [PubMed: 22820318]
43. Picelli S, Bjorklund AK, Faridani OR, et al. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods 2013; 10:1096–1098. [PubMed: 24056875]
44. Macosko EZ, Basu A, Satija R, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell 2015; 161:1202–1214. [PubMed: 26000488]
45. Satija R, Farrell JA, Gennert D, et al. Spatial reconstruction of single-cell gene expression data. Nat Biotechnol 2015; 33:495–502. [PubMed: 25867923]
46. Amit I, Winter DR, Jung S. The role of the local environment and epigenetics in shaping macrophage identity and their effect on tissue homeostasis. Nat Immunol 2016; 17:18–25. [PubMed: 26681458]
47. Cortese R, Gileles-Hillel A, Khalyfa A, et al. Aorta macrophage inflammatory and epigenetic changes in a murine model of obstructive sleep apnea: potential role of CD36. Sci Rep 2017; 7:43648. [PubMed: 28240319]
48. Rahman K, Vengrenyuk Y, Ramsey SA, et al. Inflammatory Ly6Chi monocytes and their conversion to M2 macrophages drive atherosclerosis regression. J Clin Invest 2017; 127:2904–2915. [PubMed: 28650342]
49. Gautier EL, Shay T, Miller J, et al. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nat Immunol 2012; 13:1118–1128. [PubMed: 23023392]
50. Spitzer MH, Carmi Y, Reticker-Flynn NE, et al. Systemic immunity is required for effective cancer immunotherapy. Cell 2017; 168:487–502.e15. [PubMed: 28111070]
51. Gaudilliere B, Fragiadakis GK, Bruggner RV, et al. Clinical recovery from surgery correlates with single-cell immune signatures. Sci Transl Med 2014; 6:255ra131.
52. Good Z, Sarno J, Jager A, et al. Single-cell developmental classification of B cell precursor acute lymphoblastic leukemia at diagnosis reveals predictors of relapse. Nat Med 2018; 24:474–483. [PubMed: 29505032]
53. Buscher K, Ehinger E, Gupta P, et al. Natural variation of macrophage activation as disease-relevant phenotype predictive of inflammation and cancer survival. Nat Commun 2017; 8:16041. [PubMed: 28737175]
54. Burel JG, Peters B. Discovering transcriptional signatures of disease for diagnosis versus mechanism. Nat Rev Immunol 2018; 18:289–290. [PubMed: 29629714]
55. Ng SW, Mitchell A, Kennedy JA, et al. A 17-gene stemness score for rapid determination of risk in acute leukaemia. Nature 2016; 540:433–437. [PubMed: 27926740]
56. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. Nat Methods 2015; 12:453–457. [PubMed: 25822800]
57. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat Med 2015; 21:938–945. [PubMed: 26193342]
58. McArdle S, Mikulski Z, Ley K. Live cell imaging to understand monocyte, macrophage, and dendritic cell function in atherosclerosis. J Exp Med 2016; 213:1117–1131. [PubMed: 27270892]
59. Medaglia C, Giladi A, Stoler-Barak L, et al. Spatial reconstruction of immune niches by combining photoactivatable reporters and scRNA-seq. Science 2017; 358:1622–1626. [PubMed: 29217582]

60. Buettner F, Natarajan KN, Casale FP, et al. Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden sub-populations of cells. Nat Biotechnol 2015; 33:155–160. [PubMed: 25599176]

61. Suter DM, Molina N, Gatfield D, et al. Mammalian genes are transcribed with widely different bursting kinetics. Science 2011; 332:472–474. [PubMed: 21415320]

KEY POINTS

- Mass cytometry and scRNAseq are powerful, high-parametric technologies for uncovering leukocyte diversity and yet unknown leukocyte populations.
- Both methodologies revealed an unexpected diversity among aortic leukocytes.
- scRNAseq reveals leukocyte subset signatures, which can be used to deconvolve bulk transcriptomes to assess the relative contribution of immune cells within the assessed tissue.
- Additional technological advances will provide a deeper look into the transcriptome, simultaneous integration of protein information and high-throughput capacity.

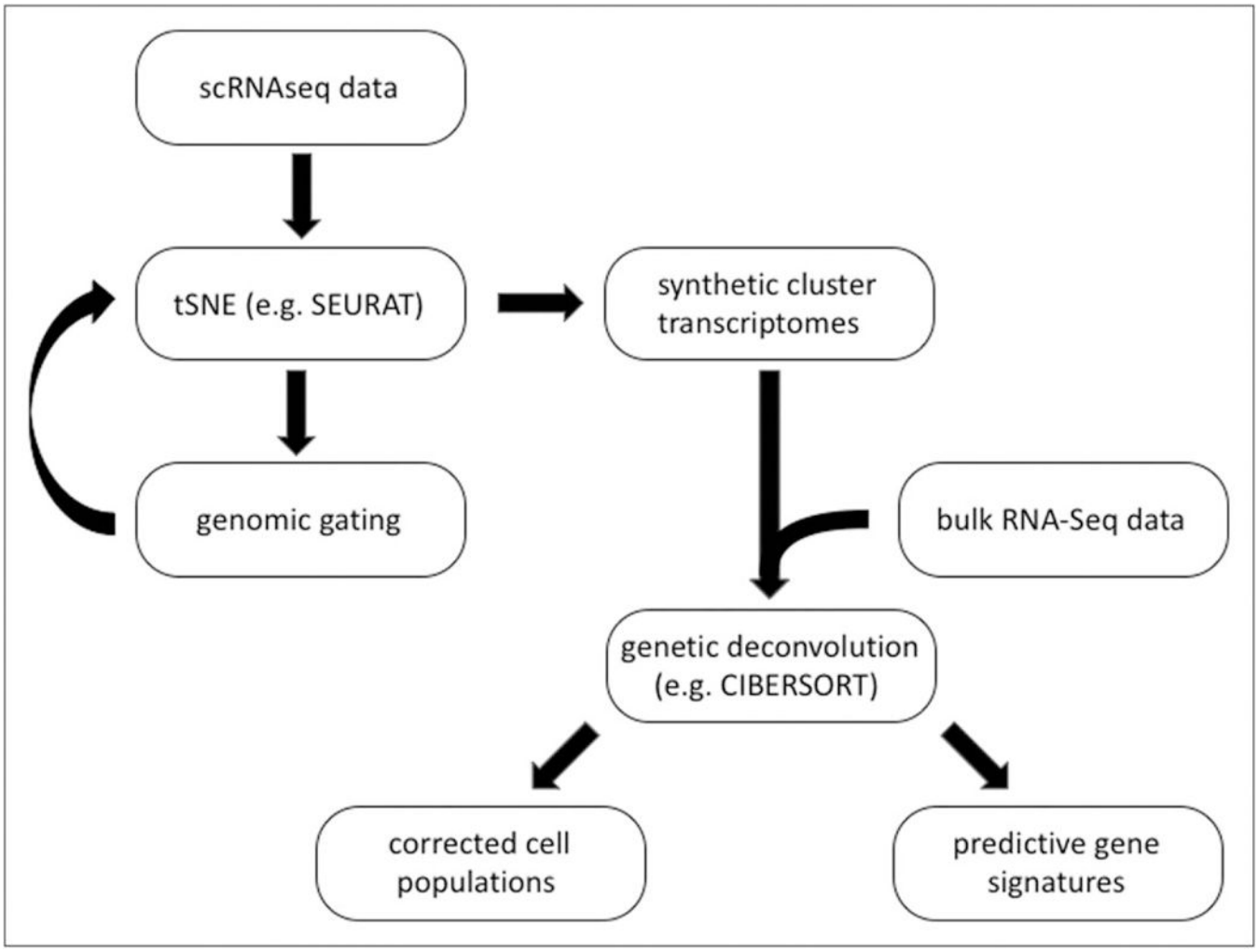

FIGURE 1.
Proposed analysis workflow of single-cell RNA sequencing data. The prognostic value of gene signature requires data sets including patient follow-up information.

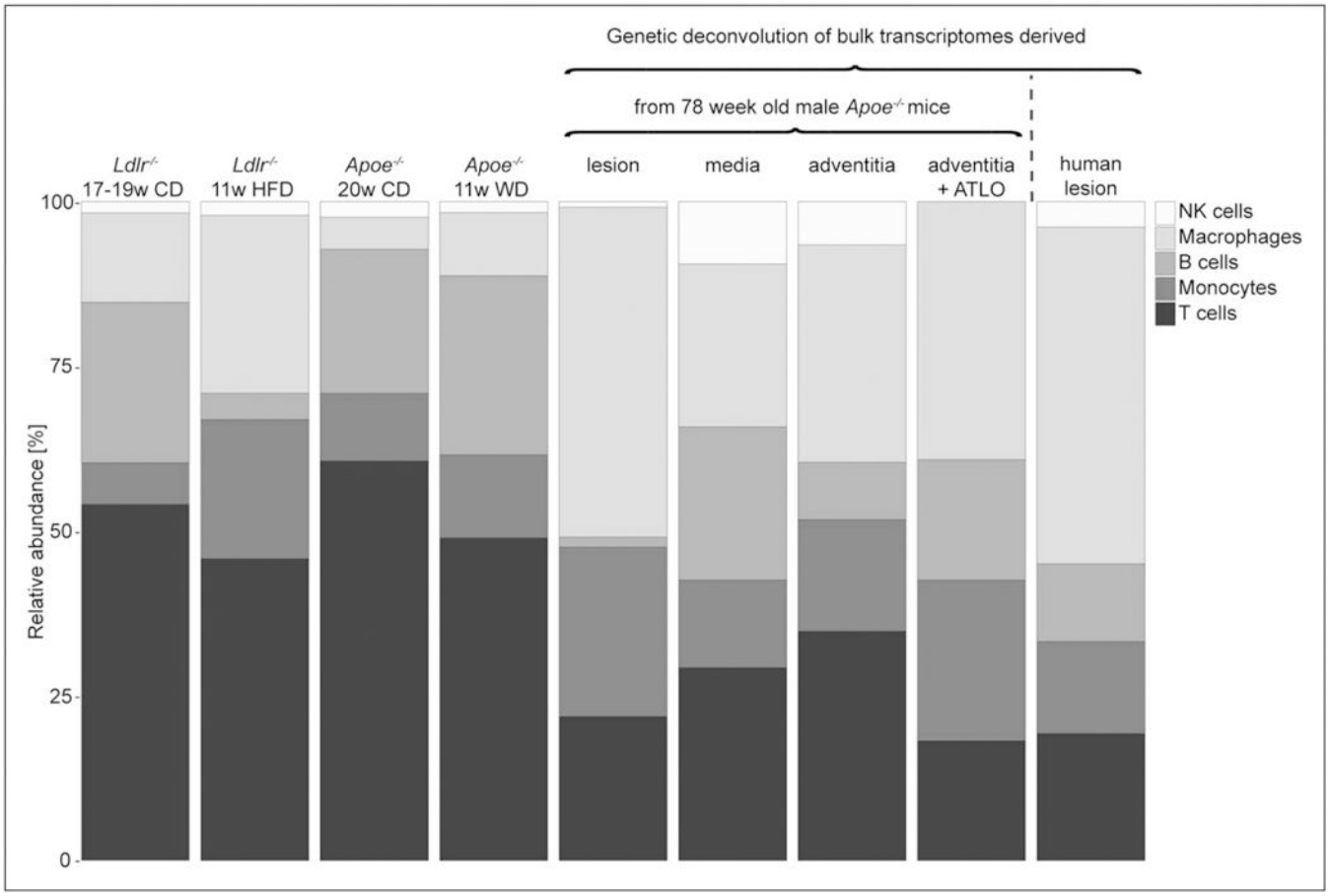

FIGURE 2.

Relative presence of principal immune populations (NK cells, macrophages, B cells, monocytes, T cells) in the aortae of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ identified by single-cell RNA sequencing [23■■,28■■]. Bulk transcriptomes derived by laser capture microdissection from lesion, media, adventitia, and adventitia with artery tertiary lymphoid organ from 78-week old *Apoe*$^{-/-}$ mice (GSE21419) were genetically deconvolved with the single-cell transcriptome signatures of the principal immune cell populations in the aorta of *Apoe*$^{-/-}$ mice [23■■]. Similarly, bulk transcriptomes of 126 human lesions obtained by carotid endarterectomy were genetically deconvolved with the same single-cell transcriptome signatures [23■■].

Table 1.

Comparison of single-cell methods

Methodology	Flow cytometry	Mass cytometry	Single-cell RNA sequencing (10× Genomics drop-seq)
Parameters	4–18	<42	~1000–1800
Cells, *n*	Millions	1 million	Up to 20000
Actual cells analyzed	~5000	~5000	555/909/2077 Winkels *et al.* [23■■] 327/854/1219 Cochain *et al.* [28■■]
Cell types detectable	12 (with 14 markers)	23 (with 35 markers)	11–13 (with 1004–1873 genes/cell)
Cost/cell	~0.005 cents/cell (with 1 mio events and ~250$/mAB equaling 100 tests)	~0.034 cents/cell (with 1 mio events and 400$/mAB equaling 50 tests)	~16–20 cents

Prices exclude salary and equipment costs.

Table 2.

Comparison of aortic leukocyte population of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice identified by single-cell RNA sequencing and mass cytometry

Publication	Cochain *et al.* [28]	Cochain *et al.* [28]	Cochain *et al.* [28]	Cochain *et al.* [28]	Winkels *et al.* [23]	Winkels *et al.* [23]	Winkels *et al.* [23]	Winkels *et al.* [23]
Method	ScRNAseq	scRNAseq	scRNAseq	scRNAseq	scRNAseq	scRNAseq	CyTOF	CyTOF
Model	*Ldlr*$^{-/-}$	*Ldlr*$^{-/-}$	*Ldlr*$^{-/-}$	*Apoe*$^{-/-}$	*Apoe*$^{-/-}$	*Apoe*$^{-/-}$	*Apoe*$^{-/-}$	*Apoe*$^{-/-}$
Age	17–19 weeks	17–19 weeks	26–28 weeks	20 weeks	8 weeks	20 weeks	20 weeks	20 weeks
Diet	CD/11-week HFD	11-week HFD	20-week HFD	12-week WD	CD	CD/12-week WD	CD	12-week WD
Subsets, *n*	13	12	9	13	5	11 (+1)	23	21
B	B	B	B	B (1)	B	B (1)	B (1)	
B				B (2)		B (2)	B (2)	B (2)
B						B (3) (by genomic gating)	B (3)	B (3)
B							B (4)	B (4)
Memory T cells					Memory T cells	Memory T cells		
Th17						Th17 cells		
Th2						Th2 cells		
CD4$^+$ T cells							Ly6-C^{hi}	Ly6-C^{hi}
CD4$^+$ T cells							Ly6-C^{neg}	Ly6-C^{neg}
γδ-T Cells							γδ-T Cells	γδ-T Cells
T cells				T cells (1)				
T cells				T cells (2)				
T cells				T cells (3)				
T cells	CXCR6$^+$	CXCR6$^+$	CXCR6$^+$	CXCR6$^+$				
CD8$^+$ T cells	CD8 T cells	CD8 T cells	CD8 T cells	CD8 T cells		CD8 T cells		
CD8$^+$ T cells							Ly6-C^{hi}	
CD8$^+$ T cells							Ly6-C^{neg}	Ly6-C^{neg}
NK cells	NK	NK	NK	NK	NK	NK	NK	NK
Monocytes	Monocytes	Monocytes			Monocytes	Monocytes	Ly6-C^{lo}	Ly6-C^{lo}
Monocytes						Ly6-C$^+$	Ly6-C$^+$	Ly6-C$^+$
Dendritic cells	MoDC/DC	MoDC/DC	MoDC/DC	MoDC/DC			MHC-II^{hi} CD117^{hi}	MHC-II^{hi} CD117^{hi}

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Publication	Cochain *et al.* [28■]	Cochain *et al.* [28■]	Cochain *et al.* [28■]	Cochain *et al.* [28■]	Winkels *et al.* [23■]	Winkels *et al.* [23■]	Winkels *et al.* [23■]	Winkels *et al.* [23■]
Dendritic cells							$CD103^{hi}$ $MHC\text{-}II^{lo}$	$CD103^{hi}$ $MHC\text{-}II^{lo}$
Dendritic cells							$CD117^{med}$ $MHC\text{-}II^{med}$	$CD117^{med}$ $MHC\text{-}II^{med}$
Granulocytes	Granulocytes	Granulocytes					Neutrophils	Neutrophils
Granulocytes							Eosinophils	Eosinophils
Macrophages	Resident-like	Resident-like	Resident-like	Macrophages	Macrophages 1	Macrophages	$F4/80^{hi}$ $CD64^{med}$	$F4/80^{hi}$ $CD64^{med}$
Macrophages	Inflammatory	Inflammatory	Inflammatory		Macrophages 2		$F4/80^{med}$ $CD64^{hi}$	$F4/80^{med}$ $CD64^{hi}$
Macrophages	$TREM2^{hi}$	$TREM2^{hi}$	$TREM2^{hi}$				$CD11c^{med}$ $F4/80^{lo}$	$CD11c^{med}$ $F4/80^{lo}$
Myeloid	Mixed/mast cells			Mixed/mast cells				
Unknown				Myeloid cells/monocytes				
Unknown				Mixed/proliferating cells				
Unknown		Mixed cells						
Unknown	Mixed/T cells 1	Mixed T cells						
Unknown	Mixed/T cells 2							
Unknown						CD4/CD8	CD4/CD8	CD4/CD8
Unknown			Mixed T/B cells					
Unknown							$CD4^{lo}CD25^{hi}$ $CD43^{hi}$	$CD4^{lo}CD25^{hi}$ $CD43^{hi}$
Unknown							$TCR\beta^{lo}IgM^{lo}$ $CD11c^{lo}$	$TCR\beta^{lo}IgM^{lo}$ $CD11c^{lo}$

CD, chow diet; CyTOF, mass cytometry; DC, dendritic cell; HFD, high-fat diet; MoDC, monocyte-derived dendritic cell; scRNAseq, single-cell RNA sequencing; WD, Western-type diet.