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From Heterogeneity to Hope: Uniting Efforts in Atherosclerosis Research and Therapeutic Development

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Abstract

The utilization of single cell technologies, lineage tracing mouse models, and advanced imaging techniques has significantly enhanced our understanding of the cellular landscape of atherosclerosis. These advancements have provided a clearer resolution of the diverse cellular states involved in the progression of atherosclerosis. However, this increased knowledge has also introduced greater complexity to current and future research, requiring a reevaluation of our approach to drug development. This review explores the impact of recent advancements in single cell technologies, which have allowed us to map the intricate cellular networks within atherosclerotic plaques. Additionally, we address the existing technological limitations that hinder our ability to identify the key cellular drivers of the disease and identify specific cell states, subsets, or surface antigens as potential drug targets for atherosclerosis.

Keywords: Single cell technologies; Cellular heterogeneity; Atherosclerosis; Endothelial cells; Vascular smooth muscle cells; Immune cells; Macrophages; T cells; B cells; Neutrophils; Plaque stability; Phenotypic modulation; Plasticity; Inflammatory mediators; Drug targets; Single cell sequencing

Introduction

The emergence of single cell technologies has propelled cardiovascular research, enabling us to comprehensively characterize the diverse cellular heterogeneity and complexity within atherosclerotic tissue from both human and mouse specimens. It is now widely acknowledged that major cell types found in atherosclerotic lesions, such as endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and immune cells (macrophages, T cells, B cells), exhibit a broad spectrum of heterogeneity and plasticity. Vascular ECs demonstrate remarkable heterogeneity based on their specific location within the vascular bed, as well as their dynamic phenotypes and ability to adapt to micro environmental changes [1]. Similarly, VSMCs display greater plasticity than previously recognized, undergoing phenotypic modulation in response to lipid exposure and cytokine stimulation within the atherosclerotic plaque [2]. Apart from transitioning from a contractile to a synthetic phenotype, VSMCs can also undergo trans differentiation into alternative phenotypes, such as macrophage-like foam cells, with differential effects on plaque stability.

The diverse range of immune cells present in plaques extends beyond macrophage polarization states and subpopulations, encompassing heterogeneous subsets of T cells and B cells. This intricate immune cell composition contributes to an imbalance between proinflammatory and pro-resolving mediators within the plaque microenvironment [3]. Recent clinical trials like CANTOS [4], COLCOT [5], and LoDoCo [6] have provided compelling evidence supporting the targeting of inflammation to reduce major cardiovascular events in humans. Consequently, studying the heterogeneity and functional complexity of immune cells in human atherosclerosis at the single cell level has gained significant interest, necessitating further insights to identify druggable targets within the plaque. In this review, we delve into the extensive heterogeneity and cellular plasticity exhibited by various cell populations in atherosclerotic lesions. We discuss the potential implications of these findings on future research and drug development, while also highlighting the need for novel technological advancements to identify actionable targets for atherosclerosis.

Breakthroughs in technology unveil previously unknown cellular states in atherosclerosis

Recent advancements in scientific techniques, such as single cell sequencing and other omics approaches (Figure 1), have significantly enhanced our understanding of the underlying mechanisms of atherosclerosis. Traditional methods like bulk RNA sequencing involve analyzing the average expression levels of genes across large cell populations or tissue sections. In contrast, single-cell RNA sequencing (scRNA-seq) enables the examination of individual cell transcriptomes. Initially, scRNA-seq involved manually isolating cells using micro-pipettes or fluorescence-activated cell sorting (FACS), but more recent developments include valve-based or droplet-based microfluidic devices.

Another powerful technique called Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) combines RNA sequencing with DNA-barcoded antibodies, allowing quantitative and qualitative assessment of surface proteins at the single-cell level. This approach utilizes droplet microfluidic devices to encapsulate individual cells in small droplets, enabling simultaneous measurement of cellular protein expression and transcriptomes. To study cell type-specific chromatin accessibility within a heterogeneous cellular population, the Single-cell assay for transposase-accessible chromatin with sequencing (ATAC-seq) has been developed. This technique employs Tn5 transposases and provides insights into the networks between promoters, enhancers, and transcription factors. It enables the analysis of gene activity and accessibility to genetic variants. Additionally, cytometry by time-of-flight (CyTOF) utilizes mass spectrometry to measure the abundance of metal isotope labels on antibodies and other

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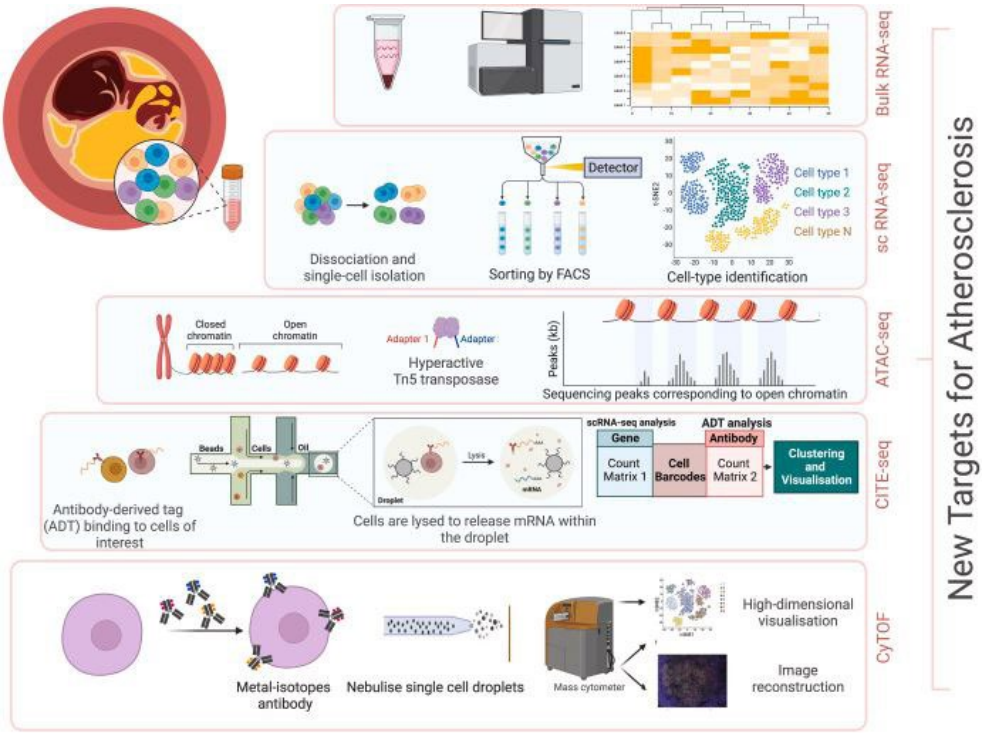

Figure 1: Exploration of single-cell technologies and omics approaches.

tags, allowing the detection of metal composition on individual cells. By determining the time-of-flight (TOF) of each metal atom, which is determined by its mass, CyTOF provides information about the metal atoms present on each cell. Overall, these innovative techniques have revolutionized our ability to probe and understand the cellular and molecular intricacies of atherosclerosis.

Emerging technologies reveal the diverse composition of cells within atherosclerotic plaques

Conventional histology has provided insights into the general composition of atherosclerotic plaques, depicting the classical view of a vulnerable plaque characterized by a thin cap, compromised smooth muscle cells, and infiltrating immune cells. However, recent advancements in single cell technologies have shed light on the remarkable biological diversity within plaque cells, highlighting the need for enhanced sub-phenotyping. These technological breakthroughs have revealed previously unrecognized subsets of cells among traditionally homogeneous cell types. Moreover, it has become evident that even within a single cell type, gene expression, transcriptomes, and protein profiles can vary significantly depending on factors such as cell activation, cell cycle, apoptosis, stress, or circadian phase [7]. Initially, microarray approaches were employed to examine the overall transcriptomes of atherosclerotic plaques or cell mixtures like peripheral blood mononuclear cells (PBMC) from patients [8]. Subsequently, deeper insights into transcriptomes were obtained through the sequencing of sorted cell populations using bulk RNA sequencing (RNA-seq). For instance, RNA sequencing analysis of macrophages isolated from murine atherosclerotic aortas revealed that foamy macrophages expressed fewer inflammatory genes compared to non-foamy macrophages, suggesting that foamy macrophages might possess reduced inflammatory potential than previously assumed [9]. Using a microfluidic platform for RNA sequencing on sorted CD4+ T cells from murine atherosclerotic aortas, Butcher et al. identified a subpopulation of interferon gamma (IFNγ)+ T helper 1 (Th1)/ regulatory T (Treg) cells, shedding light on Treg plasticity induced by atherosclerosis [10]. These approaches were precious in expounding the diversity in the transcriptome of atherosclerotic pillars and relating applicable pathways intertwined in atherosclerosis progression. Still, RNA- seq and its analysis have certain limitations when it comes to comprehending the miscellaneous nature of shrine towel. They give an aggregate transcriptome profile for all cells within the towel, without distinguishing the specific cells responsible for the signals or landing the goods of rare but pivotal cell populations. Although deconvolution styles have offered openings to overcome these limitations, their workflow still presents significant challenges [11,12].

In order to achieve advanced resolution at the cellular position, there has been a rapid-fire shift from RNA- seq to single- cell RNA-sequencing (scRNA- seq) methodologies. While inflow cytometry and mass cytometry ways like cytometry by time of flight(CyTOF) can give single- cell resolution [13], they only incompletely prisoner cellular diversity, as they calculate on previous knowledge of cell phenotypes and labels to identify new or rare cell populations. For case, using CyTOF with a predefined panel of 35 antibodies, Cole et al. observed dynamic changes in the myeloid cell cube in atherosclerotic aortas from Apoe –/ – mice and demonstrated that high- fat feeding shifts myeloid cell subsets towards seditious monocyte- macrophage populations rather than resident macrophage phenotypes, indicating the remarkable malleability of shrine myeloid cells in response to a high- fat diet [14].

The emergence of scRNA- seq technologies has largely overcome these limitations by enabling transcriptome analysis of every cell in a sample [15]. Since the first RNA- sequencing study published in 2009, which employed a microfluidics- grounded platform for 96-well sorting, commercially available styles can now synopsize up to 10,000 individual

cells into oil painting driblets (drop- sequencing). Importantly, the small volumes needed by these ways significantly reduce the threat of external impurity. These styles have been successfully applied to dissect the cellular composition of atherosclerotic pillars in mice [16] and humans [17]. Through scRNA- seq, putatively analogous cell populations can be deconstructed into subpopulations with distinct transcriptional biographies that reflect different metabolic countries and unique natural functions [18]. For case, a study by Cochain et al. used single- cell analysis to reveal the phenotypic diversity of aortic macrophages in mouse atherosclerosis. Grounded on gene expression autographs, they linked a preliminarily uncelebrated macrophage population nominated TREM2hi, which represents macrophages amended for the touched off receptor expressed on myeloid cells 2 (Trem2) and is likely to have a technical function in lipid metabolism. To date, three separate scRNA- seq studies, including a dataset of mortal pillars, have singly linked a froth cell- suchlike population of TREM2 macrophages, demonstrating the robustness of the single- cell fashion despite variations in experimental design, towel type, or species. Lately, several studies have combined scRNA- seq technologies with mass cytometry, particularly to collude the vulnerable cell geography in atherosclerosis [19-21]. Through these combined approaches, Winkels et al. uncovered a largely different arterial leukocyte population in atherosclerosis, characterized by variations in face labels, gene expression programs, and cytokine stashing. T cells and myeloid cells were set up to dominate this population. Specially, they observed significant imbrication in vulnerable cell composition between mouse pillars and mortal carotid pillars. Inclusively, these styles have yielded new perceptivity into the pathogenesis of atherosclerosis. They've unveiled cellular diversity, stressed the presence of complex and/ or rare cell populations, linked new cell subsets, traced the circles of distinct cell lineages, uncovered nonsupervisory mechanisms, and established the functional applicability of lesional leukocytes in mortal atherosclerosis. Accordingly, these technologies have been necessary in constructing an atherosclerosis cell atlas for both mice and humans [21, 22].

The coming phase of single-cell platform analysis will involve the integration of broader multi-omics approaches, including DNA methylation by bisulfite conversion sequencing or TAB- seq, noncoding RNAs, chromatin availability by ATAC- seq (epigenomics), histone revision by chromatin immune precipitation sequencing, and protein expression situations by CITE- seq(also known as AB- seq). The integration of these different datasets with advanced bioinformatics approaches will enable more important analysis. These approaches have primarily been applied in studies fastening on the immunoprofiling of mortal atherosclerotic pillars. For case, Fernandez et al. combined scRNA- seq with CyTOF and CITE- seq to immunophenotype mortal carotid pillars. They set up that characteristic pillars displayed a distinct subset of CD4 T cells with transcriptional autographs associated with T cell activation, isolation, and prostration. Also, macrophages from these pillars displayed alternately actuated phenotypes, including subsets associated with shrine vulnerability. In another study, Depuydt et al. performed scRNA- seq combined with ATAC sequencing on mortal carotid atherosclerotic pillars to probe the connection between the transcriptional hand of vulnerable cells and epigenetic changes. They linked specific recap factors associated with the myeloid subpopulation and T cell cytokine biographies, pressing the significance of intercellular communication in complaint progression. Still, it's important to note that these scRNA- seq methodologies bear the medication of single- cell dormancies, which results in the loss of spatial information regarding the locales of different cell populations within the shrine. To overcome this limitation and gain perceptivity into the spatial association and intercellular communication within the shrine, spatial transcriptomics ways have surfaced. These ways have the eventuality to reveal the physical positions of colorful cell populations and study their relations. Several approaches can be employed to fantasize spatial gene expression in shrine towel. Spatial barcoding, in situ hybridization, and in situ sequencing are among the styles used to fantasize the spatial distribution of gene expression. New approaches have been introduced to recoup spatial information in a comprehensive manner, similar as seq FISH and Slide- seq ways [23,24]. These ways enable the identification of reiterations in their native spatial environment at near-single- cell resolution using a wide range of RNA examinations.

Advancements on the horizon: Emerging frontiers in single-cell technologies and bioinformatic tools

Despite remarkable progress, certain limitations persist in high-throughput techniques, scRNA-seq, in particular, faces challenges such as limited depth of reads per cell compared to bulk RNA-seq of sorted cells, where millions of reads per sample are typically sequenced. Shallow sequencing approaches in scRNA-seq may underrepresent transcriptional information, as higher sequencing depth enables the detection of a greater number of transcripts. Moreover, enzymatic and mechanical tissue dissociation required by all scRNA-seq methods can introduce artifacts [11,12]. To overcome these limitations, sequencing RNA of nuclei (snRNA-seq) has been proposed as a promising alternative. SnRNA-seq offers several advantages, including reduced dissociation bias, elimination of dissociation induced transcriptional stress responses and compatibility with frozen samples, allowing pooling of different samples collected at various time points [25]. However, snRNA-seq studies on atherosclerotic tissues have yet to be reported. It is important to acknowledge the limitations of CITE-seq. One such limitation is its incompatibility with intracellular staining, unlike flow cytometry. Additionally, the technical skill required for sample preparation in CITE-seq is higher compared to flow cytometry. The workflow necessitates access to multiple instruments, including a FACS sorter, single cell platforms, and next-generation sequencing sequencers. Presently, there is widespread accessibility to generate single-cell data due to the abundance of available technologies and their diverse commercial implementations. However, the analysis of such data often poses challenges in the field of bioinformatics. Numerous scRNA-seq datasets are already accessible to the scientific community for re-analysis or through online tools like PlaqView [26]. Additionally, a multitude of computational algorithms have been developed to handle the vast amount of gene expression data. Nevertheless, analyzing single-cell data appropriately is often complex due to the intricacy of the data, which can complicate the interpretation of the transcriptomic signal. Nevertheless, with the substantial volumes of data being generated, efficient computational and statistical workflows for data processing have been refined [27].

Current insights and future challenges in understanding cellular heterogeneity in atherosclerosis

The emergence of advanced technologies enabling the identification of multiple cellular parameters and spatial organization has significantly enhanced our understanding of atherosclerosis. These advancements have revealed a remarkable heterogeneity in cellular subsets, both locally within the plaques and systemically throughout the body. This heterogeneity encompasses not only different cell populations but also various stages of cell activation, further

complicating the intricate network of contributors involved in the initiation, progression, and exacerbation of atherosclerotic plaques and their clinical implications. Integrating these single-cell techniques with cell-specific lineage tracing, such as fate mapping studies, has provided deeper insights into the underlying mechanisms driving plaque formation. A diagrammatic representation is presented to illustrate the intricate and diverse cellular and molecular architecture of the atherosclerotic plaque, underscoring the complexity of the disease and the challenges in identifying effective therapeutic approaches. The major cell types within the plaque, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and immune cells, exhibit a high degree of heterogeneity and plasticity. Exploring the factors that drive the transition of plaque cells, such as VSMCs, towards a plaque-stabilizing phenotype could offer valuable insights for the development of novel therapeutic interventions. While there is considerable focus on targeting circulating inflammatory mediators like cytokines, there is also potential in identifying and targeting atherosclerosis-specific antigens, such as oxidation-specific epitopes, to treat atherosclerosis in a manner specific to the disease and its location. This approach holds promise for advancing the field of atherosclerosis treatment.

Cellular composition of the vascular wall

The utilization of mouse models incorporating vessel wall cell-specific lineage tracing, along with single cell technologies, has provided valuable insights into the migration of these cells into atherosclerotic plaques. Furthermore, these studies have emphasized the significant role of the remarkable plasticity exhibited by these cells in determining the stability of atherosclerotic plaques.

The endothelium, serving as a critical barrier between the bloodstream and tissues, exhibits a heterogeneous nature that plays a pivotal role in maintaining vascular homeostasis. Endothelial cell dysfunction, characterized by increased permeability, leucocyte adhesion, and reduced nitric oxide production, represents a crucial event in the initiation of atherosclerosis. While endothelial cell heterogeneity can be observed at the organ level, it also exists within specific vascular beds, varies between sexes and with aging, and is influenced by local micro environmental cues. One well-studied factor contributing to local changes in endothelial function within atherosclerotic lesions is the transcription factor Krüppel-like factor 2 (KLF2) [28]. Under conditions of high laminar shear stress, KLF2 promotes endothelial quiescence by upregulating anti-inflammatory and anti-thrombotic proteins while down regulating pro-inflammatory and pro-thrombotic proteins. However, atherosclerotic lesion sites, exposed to disturbed shear stress, exhibit low expression of KLF2, leading to endothelial cell activation and propagation of the inflammatory response [29]. These localized alterations in shear stress regions significantly contribute to the heterogeneity observed within the endothelium at atherosclerotic lesion sites. Notably, Kluza et al., employing (immuno) scanning electron microscopy imaging, have elegantly demonstrated the diverse landscape of atherosclerotic endothelium [30]. Interestingly, early atherosclerotic lesions in Apoe^{+} mice display junctional abnormalities and large transcellular pores. However, as atherosclerotic plaques progress, the endothelium seems to undergo regeneration, characterized by increased junctional integrity and reduced immune cell adhesion. It is intriguing to speculate that this regenerative response and junctional stabilization might represent a protective mechanism aimed at stabilizing the atherosclerotic lesion through intimal remodeling.

Insights from scRNA-seq studies

Several scRNA-seq studies have provided compelling evidence of the highly heterogeneous endothelial landscape within plaque tissues. Furthermore, these studies have revealed that endothelial cells (ECs) can undergo transient and reversible activation, a state that may influence plaque stability. Of particular interest is a specific EC cluster that exhibits signs of endothelial-to-mesenchymal transition. While it was previously believed that myofibroblast-like cells (characterized by the expression of alpha smooth muscle cell actin (ACTA2)) exclusively originated from vascular smooth muscle cells (VSMCs), recent data indicate that 20-40% of the ACTA2+ cells arise from non-VSMC sources, including ECs. By integrating bulk RNA-seq analysis with in vitro models, it has been revealed that this transition is regulated in an IL-1β and TGFβ-mediated manner. This notion is further supported by histological intraplaque analysis, which demonstrates co-expression of both ACTA2 and CD34, suggesting that transdifferentiation of ECs occurs within atherosclerotic lesions.

Discussion

Furthermore, the intricate and diverse nature of the plaque necessitates innovative strategies in basic research for the identification of potential therapeutic targets in atherosclerosis. While single cell transcriptomics have been instrumental in deciphering the cellular networks within the plaque, additional insights into cell activation and intercellular interactions are crucial for identifying the key drivers of the disease. The next step forward would involve the adoption of novel single cell technologies and advanced bioinformatic tools, enabling a comprehensive exploration of the functional complexity of the cells within the plaque microenvironment. Integrating cell-specific fate mapping, single cell omics, and human genetics will further contribute to establishing a causal relationship between cellular heterogeneity and the development of atherosclerosis.

Conclusion

The application of single cell technologies has emerged as a powerful tool for unraveling the intricate and heterogeneous cellular architecture of atherosclerotic plaques. These advancements have not only enhanced our understanding of atherogenesis, but have also challenged conventional notions regarding the plasticity of certain cell types, thereby shaping the direction of future research. Single cell transcriptomics has played a pivotal role in mapping the intricate cellular and molecular networks within plaques, leading to the development of single cell atlases for both human and experimental atherosclerosis. However, the true impact of these technologies lies in their potential to drive the development of novel therapeutic approaches to combat atherosclerosis. To achieve this, we propose a shift away from conventional strategies targeting circulating molecules that lack disease or site specificity. Instead, we advocate for the adoption of innovative technologies that enable the study of cell activation and cell-cell interactions, allowing for a comprehensive understanding of the functional complexity of plaque cells. By embracing this approach, we can pave the way for more personalized and tailored therapeutic solutions for atherosclerosis.

The discovery of highly plastic and heterogeneous cells within the atherosclerotic plaque introduces a new layer of complexity to future investigations in atherosclerosis research. Traditional approaches relying on cell markers for histological analysis of plaques are no longer sufficient to accurately assess the cellular composition or plaque stability, as vascular wall cells and macrophages can express

overlapping markers. To overcome this challenge, the use of lineage tracing techniques combined with the analysis of transcriptional or proteomic signatures offers a more reliable method to identify specific cell subsets and their progeny.

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Conflict of Interest

Author declares no conflict of interest.

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