Journal of the American Heart Association

ORIGINAL RESEARCH

Characterizing Stroke Clots Using Single-Cell Sequencing

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BACKGROUND: Ischemic stroke results in significant morbidity and mortality. By examining gene expression of cells comprising stroke clots, we aim to gain valuable insights into the underlying mechanisms of this disease and identify potential biomarkers of stroke cause.

METHODS: We employed single-cell RNA sequencing to analyze 10 clot samples from patients diagnosed with large vessel occlusion stroke. We aimed to identify and compare the immune cell compositions and gene expression profiles between stroke clots (atrial fibrillation vs carotid atherosclerosis). We also used Multi-marker Analysis of Genomic Annotation and genome-wide association studies summary statistics from the GIGASTROKE consortium to assess associations between genetic variants and cell type-specific gene expression within the stroke subtypes.

RESULTS: Our analysis revealed distinct immune cell populations, including monocytes, macrophages, dendritic cells, neutrophils, and T cells in both clot types. Notably, we observed significant differences in gene expression within the mononuclear phagocytic system cells between clots from patients with atrial fibrillation and carotid atherosclerosis. We identified specific genes associated with atherosclerosis and stroke-related processes, such as *CD74, HLA-DRB1*01, HTRA1, C1Q, CD81*, and *CR1* from patients with carotid atherosclerosis. In atrial fibrillation clots, CD8 T cells and natural killer cells show upregulated expression of genes such as *GZMH*, *GZMB*, *S100A4*, *FCGBP2*, *HLA-A*, *TIMP1*, *CLIC1*, and *IFITM2*, indicating their involvement in cytotoxic activities and potential tissue damage. The Multi-marker Analysis of Genomic Annotation approach highlighted significant genetic associations within leukocytes underscoring the potential roles of B cells, T cells, and macrophages in clot pathogenesis.

CONCLUSIONS: This study illuminates the immune and transcriptomic landscape within clots, offering potential biomarkers and lays the foundation for future research.

Key Words: immune cell profiling ■ single-cell RNA sequencing ■ stroke cause

Ischemic stroke, a leading cause of mortality and long-term disability, affects millions of individuals annually and requires early intervention for effective treatment.[1,2] Previous efforts studying clot composition and its interaction with stroke have provided important findings. For example, studies have investigated how

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Preprint posted on MedRxiv February 08, 2025. doi: https://doi.org/10.1101/2025.02.06.25321828.

This article was sent to Jacquelyn Y. Taylor, PhD, PNP-BC, RN, FAHA, FAAN, Associate Editor, for review by expert referees, editorial decision, and final disposition.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.125.041738

For Sources of Funding and Disclosures, see page 11.

JAHA is available at: www.ahajournals.org/journal/jaha

RESEARCH PERSPECTIVE

What Is New?

- Single-cell RNA sequencing of stroke clots reveals distinct immune cell profiles and gene expression signatures associated with different stroke causes (atrial fibrillation versus carotid atherosclerosis).

What Question Should Be Addressed Next?

- Future studies should investigate whether the identified immune signatures contribute causally to clot formation and stroke pathogenesis and explore their potential as diagnostic or therapeutic targets.

Nonstandard Abbreviations and Acronyms

DC	dendritic cells
MPS	mononuclear phagocyte system
NK	natural killer
scRNA-seq	single-cell RNA sequencing

the histological composition of clots is associated with periinterventional clot migration in patients with acute stroke.[3] These studies have demonstrated the impact of clot composition on technical and clinical success of thrombectomy, as well as its association with worse clinical outcomes.[4] Additionally, correlations between clot composition and stroke cause have been explored, revealing differences in clot composition between cardioembolic and large-artery atherosclerosis.[5] These analyses have shed light on the mechanical properties of clots, which can inform the choice of revascularization strategies and improve thrombectomy outcomes.[6–8] However, it is important to recognize the limitations inherent to traditional histological studies of clots. These methods, although useful for understanding the macroscopic and structural aspects of clots, often fall short in capturing the complex cellular dynamics and molecular interactions at play. Histological analyses typically provide a static snapshot of clot composition, lacking the depth to elucidate the intricate gene expression patterns and cellular behaviors that drive clot formation and evolution. In contrast, single-cell RNA (scRNA-seq) sequencing enables unbiased, high-resolution profiling of individual cells within a sample, allowing for the identification of rare cell populations, functional states, and intercellular communication networks.[9]

In this context, our study aims to investigate the interactions among immune cell types within clots associated with atrial fibrillation (AF) and carotid atherosclerosis (CA), employing scRNA-seq to provide a comprehensive understanding of the cellular composition and interactions within these clots.

METHODS

Code Availability

All data and scripts used for data analysis are available at GitHub (https://github.com/dadarenedo/human_clots).

Human Specimens and Patient Clinical Characteristics

We collected clot samples from 17 patients with large vessel occlusion stroke and deep vein thrombosis undergoing mechanical thrombectomy between October 2020 and July 2022 after written informed consent from patients or their surrogates. The study was overseen by the Yale School of Medicine and institutional review board approval was obtained. Mechanical thrombectomy was performed by 2 endovascular surgeons (C.M., R.H.) and the differential diagnosis between stroke causes was performed by 2 vascular neurologists (D.N., L.S.). Eligibility criteria included age ≥18 years, admission to Yale New Haven Hospital, and M1 or M2 occlusion for stroke cases. To protect the identity of the subjects, ages are reported as ranges and sex is not reported individually (Tables S1). For all analyses, 1 clot sample was collected per patient during mechanical thrombectomy. No intrapatient comparisons were performed. Clots were grouped according to clinical cause—AF, carotid disease, or venous thrombosis—and all comparisons represent interpatient analyses based on these aggregated groups.

Sample Collection

To minimize cellular degradation and maintain high cell viability during sample preparation, freshly isolated clots were promptly processed to reduce the time between collection and analysis. Clots were washed in PBS and digested with recombinant tissue plasminogen activator for 5 minutes, followed by 3 additional PBS washes to remove residual with recombinant tissue plasminogen activator. Red blood cells were eliminated by hypotonic lysis, and cells were then filtered to remove debris and undigested clot material using Flowmi Cell Strainers.

To ensure high-quality single-cell data, we assessed cell viability post isolation using a trypan blue exclusion test, confirming a viability rate >80%. Maintaining cell viability was a priority, and the use of PBS supplemented with 0.4% BSA helped to preserve

cell integrity. Only high-viability samples were used for scRNA-seq.

Following cell isolation, scRNA-seq was performed using the 10× Genomics platform (3′ v3 chemistry). Illumina-ready libraries were sequenced on Illumina HiSeq 3000/4000 Systems, and sequence alignment was conducted using the Cell Ranger pipeline. In addition to unique molecular identifier filtering, standard quality control metrics were applied to further ensure data integrity.

Quality Control of Sequencing Data

Samples that had mean unique molecular identifier counts, and mean number of genes detected per cell <500 were excluded from further analysis. Cells that had <300 or >2500 features, as well as cells that contained >40% of reads from mitochondrial genes were considered low quality and removed from further analysis (Figure S1, Table S3).

scRNA-Seq Computational Pipelines and Analyses

The R package Seurat was used for normalization, data scaling, integration, clustering, dimensionality reduction, differential expression analysis, and visualization.[10] Principal component analysis was performed using variable genes, and the first 50 principal components were used to perform Uniform Manifold Approximation and Projection to embed the data set into 2 dimensions and to construct a shared nearest-neighbor graph that was used to further cluster the data set. The number of principal components was chosen based on inspection of the elbow plot and clustering stability across multiple resolutions. We employed the Louvain method to identify clusters.[11] The identification of cellular identity involved the use of Seurat's implementation of the Wilcoxon rank-sum test FindMarkers() to discover differentially expressed genes within each cluster. To control for multiple comparisons inherent to single-cell transcriptomic data, *P* values were adjusted using the Benjamini–Hochberg method to estimate the false discovery rate. Only genes with an adjusted *P* value (false discovery rate) <0.001 were considered statistically significant. Subsequently, these markers were compared with previously identified cell type-specific genes from prior data sets[12–17] (Figure S2, Table S4).

To obtain more granularity, the mononuclear phagocyte system (MPS) and T-cell cluster were further reclustered and annotated using the R package SingleR.[18] Reference transcriptomic data set specific for immune cells and for MPS were used to reannotate these clusters.[16,17] SingleR is a computational method that uses the Spearman test to correlate single-cell transcriptomes with reference transcriptomic data sets. SingleR identified CD4 T cells, CD8 T cells, dendritic cells (DCs), macrophages, monocytes, and natural killer (NK) cells among these clusters.

Gene Expression Analysis

The parent Seurat object was divided into individual objects based on their cell identity. Next, differential gene expression was calculated based on the nonparametric Wilcoxon rank-sum test between patients with CA stroke clots and AF stroke clots, and between venous clots (VTE clots) and arterial clots (a composite of both CA stroke clots and AF stroke clots) (Figures S3–S5, Tables S5–S20).

Adjusted *P* values were used in all downstream analyses and visualizations. Volcano plots were generated based on the log fold-change and adjusted *P* values to highlight the most statistically significant differentially expressed genes. The average log (fold-change) of each differentially expressed gene calculated by FindMarkers() was used to perform analysis with Ingenuity Pathway Analysis (Qiagen) and for Gene Ontology enrichment analysis (Tables S21, S22). To construct heatmaps of canonical pathways and predicted upstream regulators, genes were filtered to have a right-sided *P* value <0.05 by Ingenuity Pathway Analysis's implementation of Fisher's exact test, and the *Z* score of each pathway was plotted (Qiagen Inc., https://digitalinsights.qiagen.com/IPA). Gene Ontology enrichment analysis of genes with P_{adj} <0.001 was performed with python package gseapy.[19] (Figures S6–S13) Visualization of Ingenuity Pathway Analysis was performed using R package pheatmap.

Gene-Based and Gene-Set Analyses

We obtained genome-wide association studies (GWAS) summary statistics from the GIGASTROKE consortium, encompassing all ischemic strokes, as well as specific subtypes such as cardioembolic stroke and large artery strokes.[20] For control, we also used GWAS summary statistics for height and gastroesophageal reflux disease.[21,22] The Multi-marker Analysis of Genomic Annotation (MAGMA) approach was employed to assess the association between genetic variants and cell type-specific gene expression within the stroke subtypes.[23] To complement the GWAS analysis, we integrated single-cell transcriptomic data obtained from CA and AF clots, allowing us to explore the molecular landscape of relevant cell types.

Gene-level and gene-set analyses were performed using MAGMA v1.09b. Gene-based association testing was conducted using multiple linear regression models, which account for linkage disequilibrium between single-nucleotide variants to evaluate the joint effect of all single-nucleotide variants within a gene. For gene-set enrichment analysis, we used MAGMA's competitive testing framework to assess whether specific gene

sets were more strongly associated with each phenotype compared with other genes in the genome.

Cell Type Ligand–Receptor Interaction

Cell–cell interactions based on the expression of known ligand–receptor pairs in different cell types were calculated using CellPhoneDB version 2.1.2.[24] Only interactions with a *P* value <0.05 were considered statistically significant and included in downstream visualizations. To represent these interactions, we used multiple complementary approaches: (1) heatmaps displaying the number of significant interactions per cell type within each cause, (2) Circos plots illustrating significant interactions between cell types (Figures S14–S29), and (3) Venn diagrams identifying unique, significant interactions specific to each cause. This visualization strategy has been described elsewhere.[25,26] Results from this analysis were plotted using R packages pheatmap and circlize.[27]

RESULTS

We conducted scRNA-seq on 100 436 cells with an average of 5908 cells per sample from 17 clot samples from patients hospitalized with large intracranial vessel occlusion strokes (14) and deep vein thrombosis (3). Following quality control measures, we retained a total of 12 high-quality samples of clots for further analysis. (Figure 1A) The demographics and clinical features of these patients are listed in the . There were 6 (50%) women among the 12 patients profiled, and the mean age was 74.1 (SD 15.1).

Immune Cell Composition in Thrombotic Clots: A Single-Cell Analysis

Our findings revealed that the predominant immune cell population within the clots consisted mainly of cells belonging to the MPS, particularly monocytes, macrophages, and DCs. In addition, we observed abundant populations of neutrophils and T cells within the clots (Figure 1B).

Transcriptome Analysis of MPS Cells

We found that macrophages in carotid clots exhibited elevated expression levels of genes involved in antigen presentation (*HLA-DQB1*, *CD74*, and *HTRA1*) and immune regulation (*C1QA*, *CD81*, and *CR1*), suggesting their potential role in modulating immune responses and promoting inflammation within the atherosclerotic plaques (Figure 2A). Several studies support these findings, highlighting the involvement of these genes in atherosclerosis and related processes.[10–15] We observed upregulated expression of various genes in macrophage from AF clots, including genes associated with stress response (*HSPA1B* and *HMOX1*), extracellular matrix remodeling (*TGFBI* and *MMPs*), and immune regulation (*FCER1G* and *CCL2*) (Figure 2A). The observed gene expression patterns indicate that macrophages play a role in tissue remodeling and the inflammatory response within AF-associated clots. Notably, *TGFBI* has also been linked to the development of AF.[28] Gene expression analysis of monocytes in CA clots revealed upregulated expression of genes including *FN1, SPP1, FCER1G, MAP3K2, HSP90AA1, SLC25A37, ITM2B*, and *KMT2C* (Figure 2B). Of note, *SPP1* (osteopontin) plays a significant role in atherosclerosis progression, neointimal hyperplasia, and rapid coronary plaque progression.[29] Additionally, our findings revealed heightened expression of *FTL*, cathepsin D, and metalloproteinase-9 in monocytes from AF clots, all of which have been extensively discussed in the literature and associated with the pathogenesis of AF[30–33] (Figure 2B).

Among venous and arterial clots, macrophages from venous clots exhibited increased expression of genes involved in red blood cell breakdown, immune response regulation, calcium homeostasis, RNA degradation, and autophagy (*HBA1, HBA2, HBB, IFI30, LRMDA, MAML2, TPRG1, SLC8A1, RNASEK, CD83, CCL4*, and *GABARAP*). In contrast, macrophages from arterial clots showed increased expression of *FTL, ZNF804A, CTSL, TMCC1, RPS7, MT-ATP6, RPLP1, TYMP, SEPT7, SEPT2, MYL6, TXN, MINOS1*, and *RPL2* genes. These genes participate in various biological processes, including iron storage, transcriptional regulation, protein degradation, cell migration, nucleotide metabolism, cellular organization, muscle contraction, antioxidant activity, and protein synthesis (Figure 2D).

Additionally, monocytes from venous clots exhibited increased expression of *RERE, PID1, TNRC6B, ALCAM, HIP1, PUM2, UBAC2, CCL20, ZNF148, FN1, EML4, SGK3, MYCBP2, PAFAH1B1, ESYT2, CLU3, RALA, MTSS1*, and *SP3*. These genes are implicated in diverse biological functions including cell signaling, adhesion, cytoskeletal organization, immune response, and gene regulation, suggesting a role in venous thrombus formation and stability. In arterial clots, however, monocytes showed heightened expression of *CTSL, RPL11, HMOX1, RRPL9, SEC61G, CD74, PSAP, RPL26, FKBP1A, TPM4, RACK1, CTSB, RPL5, AIF1, TMBIM6*, and *CTSZ*. This gene set is associated with lysosomal activity, protein synthesis, oxidative stress response, antigen presentation, and cell survival, possibly reflecting the distinct mechanical and oxidative stress conditions in arterial thrombosis (Figure 2E).

Cytotoxicity and Tissue Remodeling: Gene Expression in Atrial Fibrillation Clots

We found that CD8 T cells and NK cells from AF clots exhibit upregulated expression of several genes,

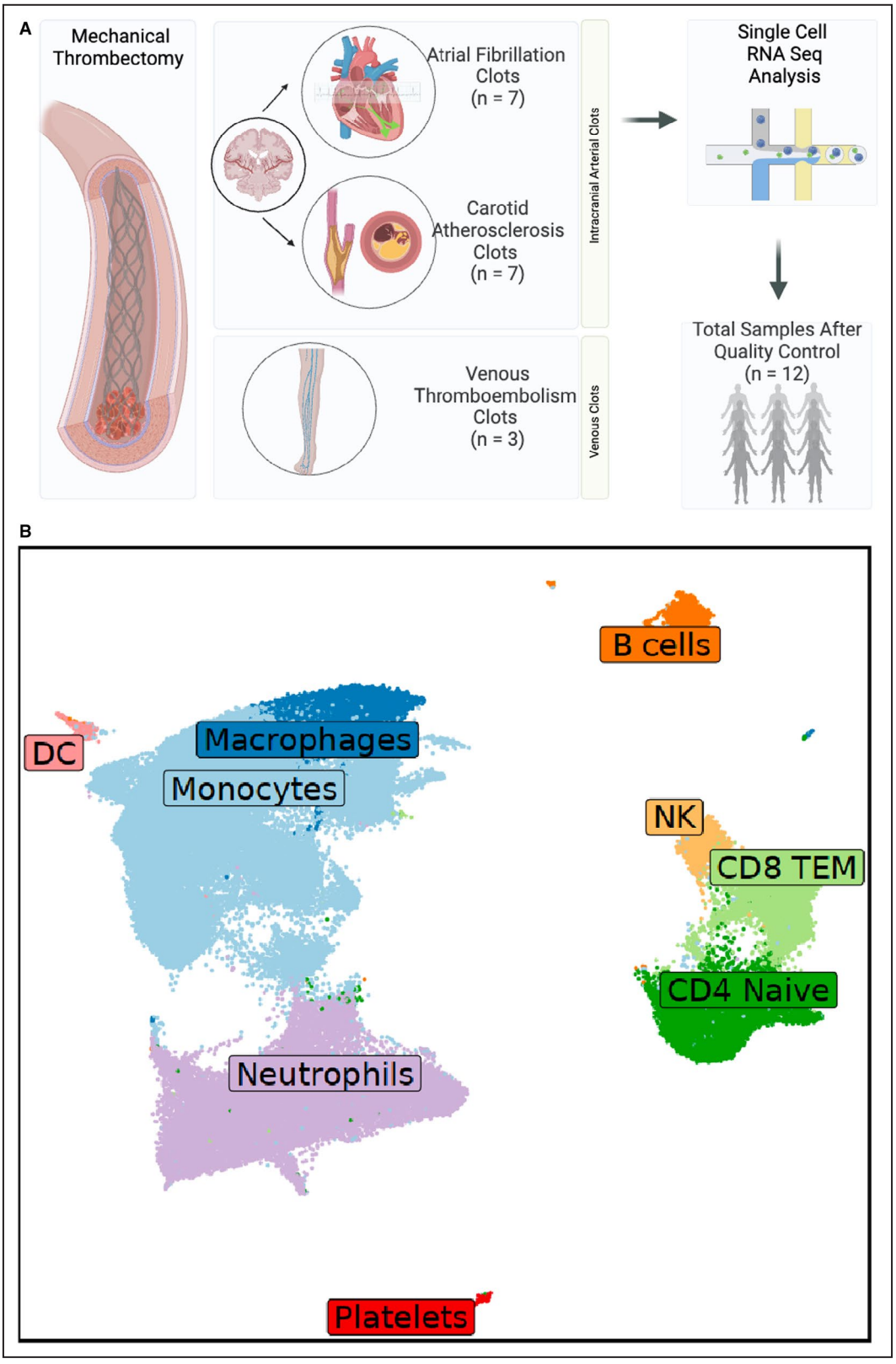

Figure 1. Study overview and immune cell type identification in stroke clots.
A, Schematic overview of the study, illustrating the workflow and key objectives. Figure created with BioRender.com. **B**, Uniform Manifold Approximation and Projection plot displaying the identified immune cell types within clots. DC indicates dendritic cells; NK, natural killer; and TEM, effector memory T cells.

Table. Demographics and Clinical Characteristics of Patients

Sample	Age, y	Location of occlusion	Side	Clinical diagnosis
AF1	60–69	M2	Left	Atrial fibrillation
AF2	70–79	M1	Left	Atrial fibrillation
AF3	80–89	M2	Left	Atrial fibrillation
AF4	70–79	M1	Right	Atrial fibrillation
AF5	80–89	M1	Right	Atrial fibrillation
AF6	90–99	M2	Left	Atrial fibrillation
CA1	70–79	M2	Left	Carotid atherosclerosis
CA2	80–89	M1	Left	Carotid atherosclerosis
CA3	80–89	M1–M2	Left	Carotid atherosclerosis
CA4	50–59	Internal carotid artery	Left	Carotid atherosclerosis
VTE1	30–39	Tibioperoneal trunk and left external iliac vein	Left	Deep vein thrombosis
VTE2	70–79	Pulmonary artery bifurcation	–	Pulmonary embolism

AF indicates atrial fibrillation clot; CA, carotid atherosclerosis clot; and VTE, venous thromboembolism clot.

including *GZMH*, *GZMB*, *S100A4*, *FCGBP2*, *HLA-A*, *TIMP1*, *CLIC1*, and *IFITM2*. The presence of effector markers like *GZMH* and *GZMB* suggests that these lymphocytes are involved in cytotoxic activities and may contribute to tissue damage within the AF clots (Figure 2C).

Ribosomal Biogenesis and Stress Response in CD8 T Cells From Arterial Clot

CD8 T cells from arterial clots exhibited differential expression of genes associated with ribosomal biogenesis and cellular stress response. Specifically, we observed the upregulation of ribosomal protein genes and heat shock protein genes, such as *HSPA1B*. These findings suggest the presence of ribosome biogenesis stress within these CD8 T cells (Figure 2F).

Pathway Expression in Clots

In AF clots, we observed upregulation of many pathways shared across multiple cell types. These included CDC42 signaling, associated with cellular morphology and dynamics, as well as remodeling of epithelial adherens junctions. Our findings also revealed the presence of pathways such as immunogenic cell death signaling, Th1 pathway, RhoA signaling, actin nucleation by ARP-WASP (actin-related protein-Wiskott–Aldrich Syndrome protein) complex, ILK (integrin-linked kinase) signaling, neutrophil extracellular trap signaling pathway, and hepatic fibrosis signaling pathways were upregulated in AF clots across all cell types. These observations underscore the intricate interplay between immune responses, cellular remodeling, and inflammatory processes in the pathogenesis of AF clots (Figure 3A).

In contrast, we found that the specific cause of CA clots led to distinct patterns of pathway expression. Mitochondrial dysfunction, granzyme A signaling, and sirtuin signaling were found downregulated in MPS cells from CA clots. EIF2 (eukaryotic initiation factor 2) signaling, suggesting increased protein translation, was strongly enriched in CD4 T cells from CA clots. In macrophages, Th1 pathway, multiple sclerosis signaling pathway, and phagosome formation were more enriched in the macrophages from CA clots, consistent with activation of macrophages in atherosclerotic lesions. CD8 T cells from CA clots showed upregulation of PD-1 (programmed cell death protein 1), PD-L1 (programmed cll death ligand-1) cancer immunotherapy and CTLA4 (cytotoxic T-lymphocyte associated protein 4) signaling suggesting negative regulation of these cells in atherosclerotic clots.

Comparing venous clots to arterial clots, we found upregulated pathways associated with mitochondrial dysfunction, RhoGDI signaling, sirtuin signaling pathway, coronavirus pathogenesis pathways, granzyme A signaling, human embryonic stem cell pluripotency, and role of NFAT (nuclear factor of activated T cells) in cardiac hypertrophy and cardiac hypertrophy signaling in venous clots (Figure 3B). Conversely, in arterial clots, we observed upregulated pathways related to oxidative phosphorylation, EIF2 signaling, crosstalk between DC and NKs, role of JAK (Janus kinase) family kinases in IL-6 (interleukin-6) type cytokine signaling, TNFR1 (tumor necrosis factor receptor 1) signaling, epithelial adhesion junction signaling, inflammatory cell death signaling pathway, PTEN (phosphatase and tensin homolog) signaling, VDR/RXR (vitamin D receptor/retinoid X receptor) activation, actin cytoskeleton signaling, CDC42 (cell division cycle 42) signaling, RhoA signaling, and neutrophil extracellular trap signaling pathway. These findings reveal potential alterations in cellular energetics, protein synthesis, immune cell communication, cytokine signaling, cell adhesion,

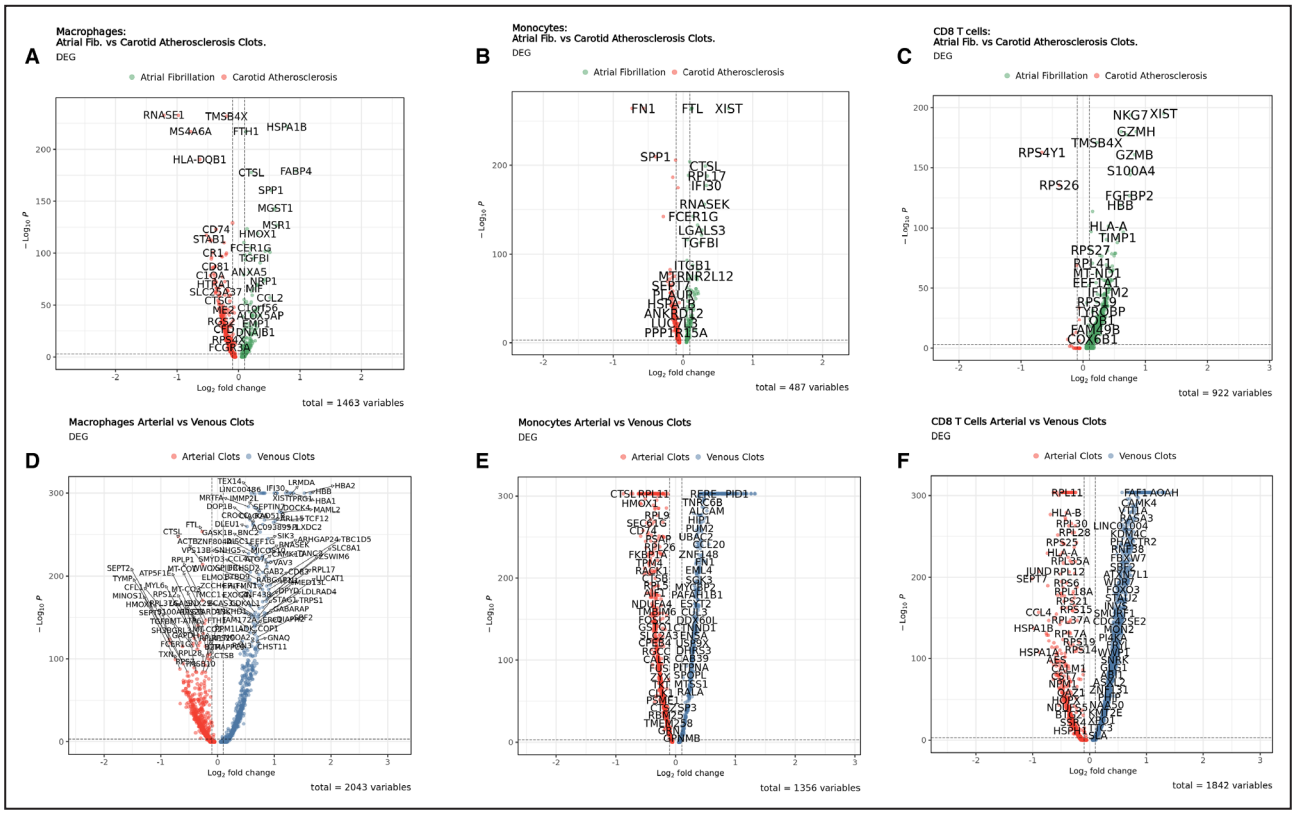

Figure 2. Volcano plots.
A, Macrophages DEG between atrial fibrillation clots and carotid atherosclerosis clots. **B**, Monocytes DEG between atrial fibrillation clots and carotid atherosclerosis clots. **C**, CD8 T cells DEG between atrial fibrillation clots and carotid atherosclerosis clots. **D**, Macrophages DEG between venous and arterial clots. **E**, Monocytes DEG between venous and arterial clots. **F**, CD8 T cells DEG between venous and arterial clots. DEG indicates differential gene expression.

inflammatory responses, and cytoskeletal dynamics within arterial clots.

Receptor-Ligand Insights

We investigated the receptor-ligand interactions within various cell populations. In AF clots, we observed a higher number of significant interactions ($P<0.05$) involving DC compared with other cell types (Figure 4A). Notably, significant interactions were found between DC and DC, DC and monocytes, and DC and platelets. In CA clots, we found a higher prevalence of significant interactions involving NK cells and neutrophils. Specifically, significant interactions were observed between NK cells and NK cells, NK cells and neutrophils, and neutrophils and neutrophils (Figure 4B). Venous clots exhibited a distinct pattern of significant interactions, with a notable emphasis on interactions involving DC. The most prominent interactions observed were DC–DC interactions, as well as interactions between DC and neutrophils, NK cells, and platelets (Figure 4C). Additionally, we investigated the presence of unique interactions among these cells in each stroke type. In AF, we identified specific interactions among DC–DC that included VCAM1-ITGA4/ITGB7 (vascular cell adhesion molecule 1/integrin alpha-4/integrin beta-7), JAG1 (jagged-1)-NOTCH2/NOTCH4, and CD274-PDCD1 (programmed cell death protein 1). These interactions hold biological significance, as they implicate molecules involved in cell adhesion, immune signaling, and immune checkpoint regulation. Furthermore, in carotid clots, the unique interactions among DC–DC encompassed intriguing pairs such as TGFB1-ACVR1 (transforming growth factor beta-activin A receptor, type I), VEGFA-FTL1 (vascular endothelial growth factor A-ferritin light chain 1), IL10-IL10RA, CXCL1-CX3CR1 (C-X-C motif chemokine ligand 1-CX3C motif chemokine receptor 1), and adhesion G protein-coupled receptor E5 (ADGRE5)-CD55/LPAR2/LPAR3 (lysophosphatidic acid receptor 2/3). These interactions suggest potential involvement of these molecules in modulating inflammatory responses, angiogenesis, and cellular signaling pathways specifically within the context of CA-related thrombosis (Figure 4D,E).

Genetic Insights Into Clot Formation in Atrial Fibrillation and Carotid Clots

We examine the expression of known genetic risk variants for stroke in leukocytes from the different clot types. Our comprehensive analysis using the

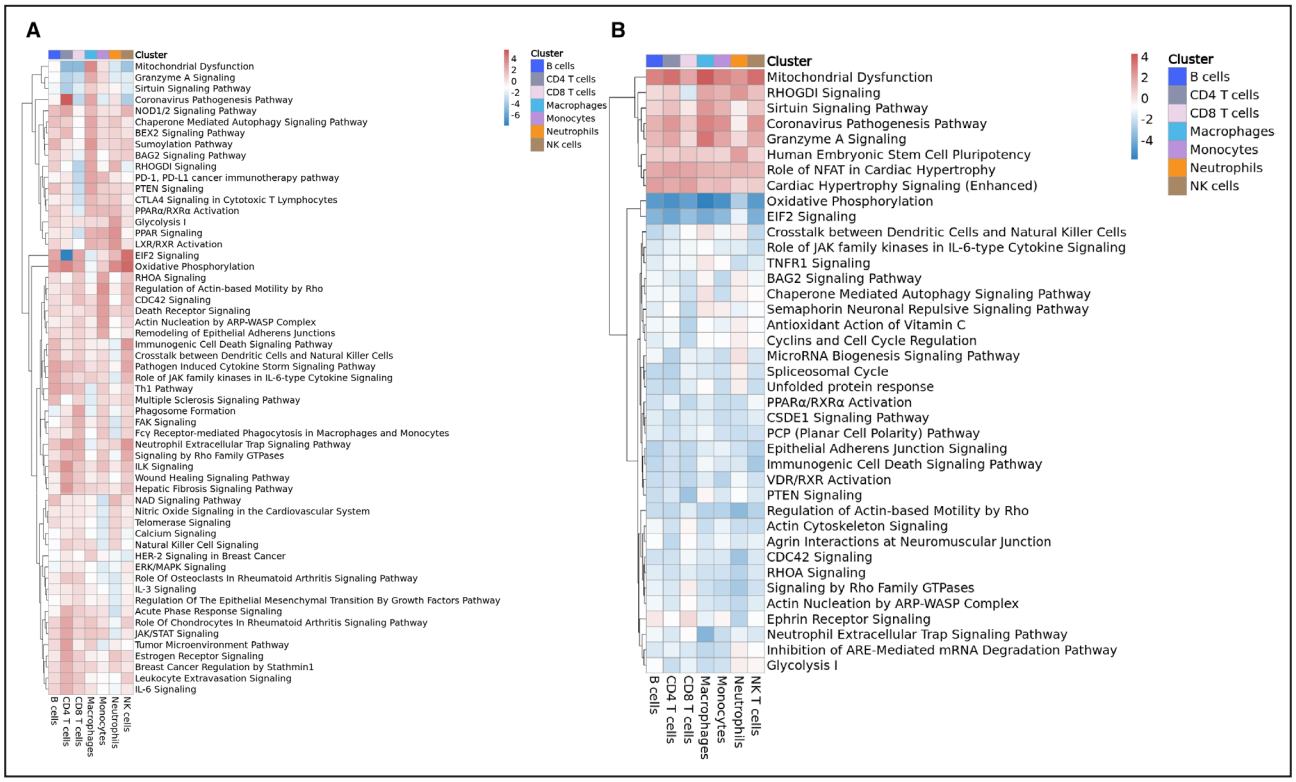

Figure 3. Ingenuity pathway analysis of differential gene expression results.
A, Heatmap with IPA of DEG between carotid atherosclerosis clots and atrial fibrillation clots for all clusters (cell types). Red upregulated in AF stroke clots. **B**, Heatmap with IPA of DEG between venous and arterial clots. Red upregulated in venous clots. AF indicates atrial fibrillation; DEG, differential gene expression; IPA, Ingenuity Pathway Analysis; and NK, natural killer.

GIGASTROKE trans-ancestry GWAS data set uncovered significant associations within the genetic landscape of clot formation in AF and carotid clots (Figure 5). By employing MAGMA, we ranked gene expression specificity for each cell type and observed intriguing patterns in the context of clot pathogenesis (Figure 5A). In atrial clots, B cells, CD4 T cells, CD8 T cells, macrophages, monocytes, and DCs surpassed the predetermined *P* value threshold, indicating their potential roles in the intricate mechanisms of stroke development (Figure 5B). Similarly, in carotid clots, B cells, CD8 T cells, CD4 T cells, NK cells, and macrophages exhibited significant associations, underscoring their relevance in the genetic factors governing carotid clot formation (Figure 5C). As controls, we used gene sets for gastrointestinal reflux and height and failed to find significant enrichment in the leukocytes from the clots. These findings shed light on the active participation of these specific cell types in particular T cells and their interactions in the complex molecular processes underlying stroke.

DISCUSSION

In our study, we employed scRNA-seq analysis to investigate the cellular composition, transcriptome and interactions within stroke clots. Our analysis revealed the predominant presence of immune cells belonging to the MPS within the clots, including monocytes, macrophages, and DCs. Neutrophils and T cells were also observed in abundance. Interestingly, we found variations in the composition of immune cell populations between different causes of stroke (cardioembolic versus carotid disease). Carotid atherosclerosis clots showed enrichment of B cells, CD4 T cells, DCs, and macrophages, whereas AF clots exhibited enrichment of CD8 T cells, monocytes, NK cells, and neutrophils. This suggests distinct immune responses and mechanisms associated with different causes.

Furthermore, the analysis of gene expression patterns provided insights into potential stroke biomarkers. We identified specific genes associated with atherosclerosis and stroke-related processes, such as *CD74, HLA-DRB1*01, HTRA1, C1Q, CD81*, and *CR1*. In addition, we observed differential gene expression patterns in CD8 T cells and NK cells from AF clots, indicating their involvement in cytotoxicity, tissue remodeling, and antigen presentation.

Comparative analysis of arterial and venous clots revealed distinct gene expression profiles in macrophages, suggesting unique molecular characteristics and potential roles in clot formation and associated

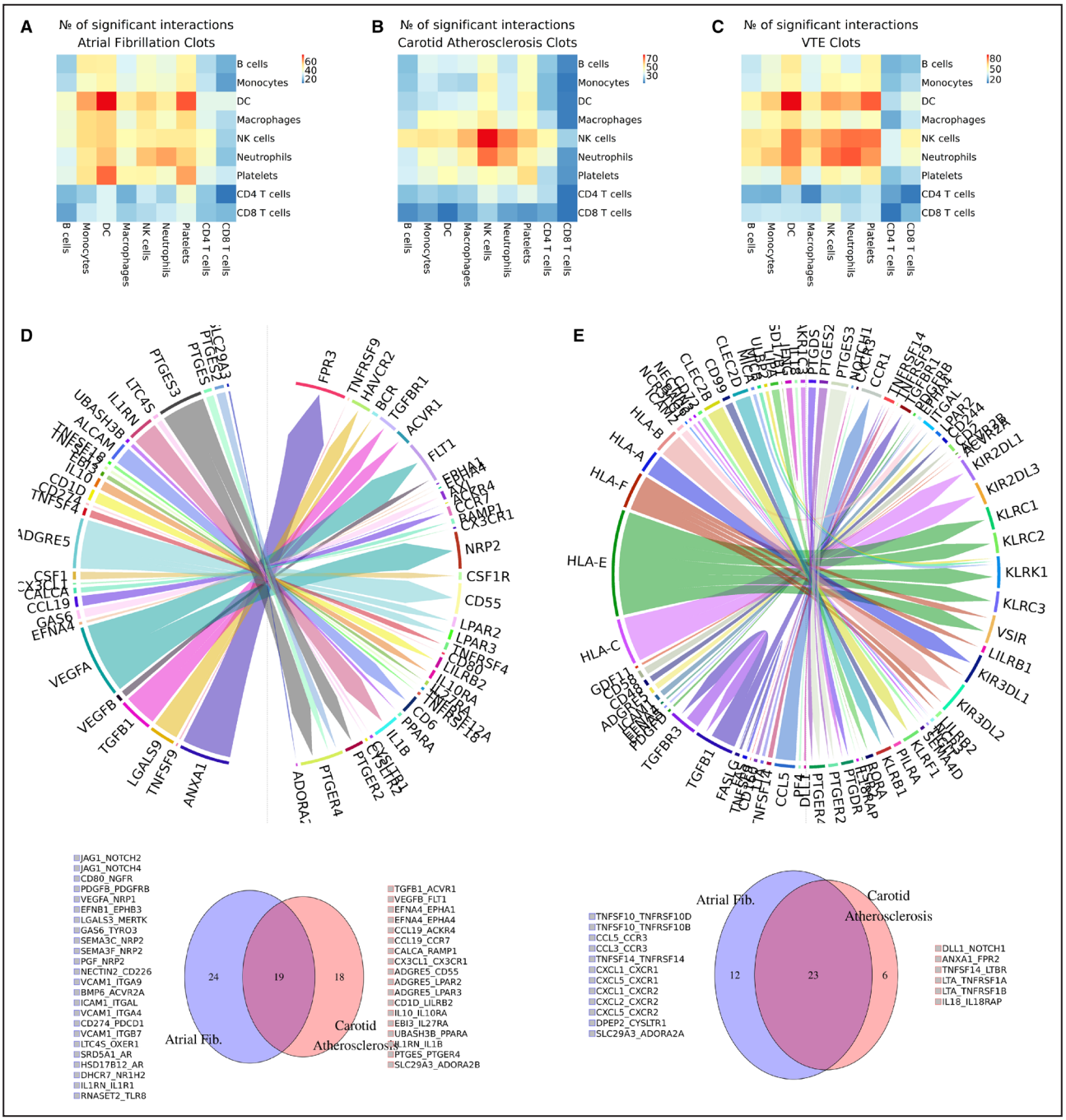

Figure 4. Interactions among immune cell types in atrial fibrillation and carotid atherosclerosis clots.
A, Heatmaps displaying the number of significant interactions among cell types in AF clots. **B**, Heatmaps displaying the number of significant interactions among cell types in carotid atherosclerosis clots. **C**, Heatmaps displaying the number of significant interactions among cell types in venous clots. **D**, Circos plot illustrating significant interactions between DC within AF clots and Venn diagram showing the number of significant interactions shared and the number of unique interactions between causes in DC cells. **E**, Circos plot illustrating significant interactions between NK cells within AF clots and Venn diagram showing the number of significant interactions shared and the number of unique interactions between causes in NK cells. *The legend accompanying the Venn diagrams provides the names of specific unique interactions. AF indicates atrial fibrillation; DC, dendritic cell; NK, natural killer; and VTE, venous thromboembolism.

pathophysiological processes. Additionally, pathway analysis unveiled dysregulated pathways associated with mitochondrial dysfunction, immune response, cellular stress response, and inflammatory processes in both AF and CA clots. Moreover, our study explored cell–cell interactions within the clot, which enabled us

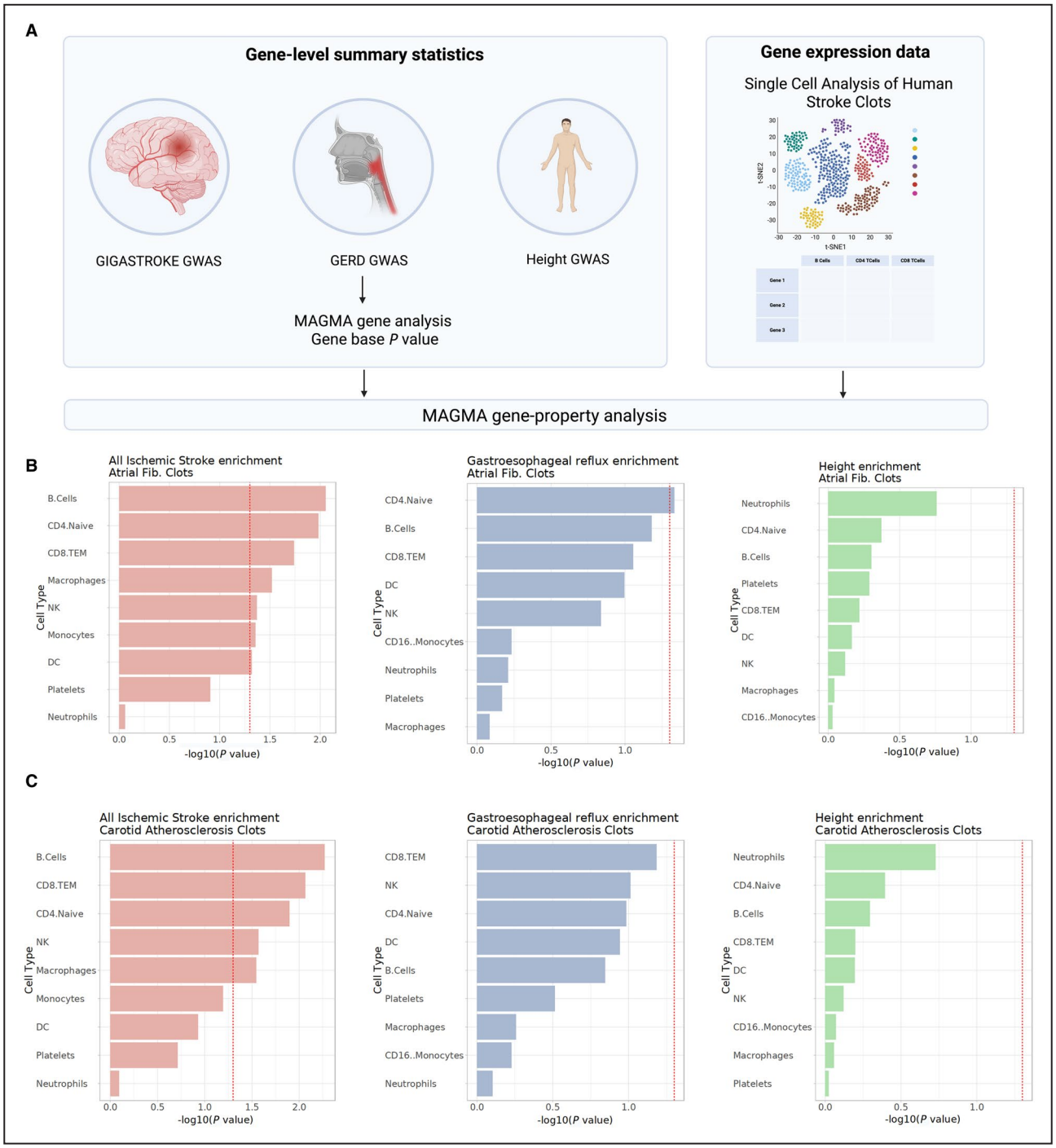

Figure 5. MAGMA workflow and cell-type analysis in human stroke clots.
A, Schematic representation of the MAGMA workflow using the GIGASTROKE, GERD and height GWAS. **B**, Bar plots depict the relationship between various cell types in atrial fibrillation. Clots and *P* values obtained from the MAGMA gene analysis for each different GWAS. The red lines represent the *P* value threshold of <0.05, indicating statistically significant associations. **C**), Bar plots depict the relationship between various cell types in carotid atherosclerosis clots and the *P* values obtained from the MAGMA gene analysis for each different GWAS. The red lines represent the *P* value threshold of <0.05, indicating statistically significant associations. GERD indicates gastroesophageal reflux disease; GWAS, genome-wide association studies; and MAGMA, Multimarker Analysis of Genomic Annotation.

to identify potential communication networks and interactions between immune cells and other cell types present in the clots. The heterogeneity in immune cell composition and gene expression across stroke clots in our study likely reflects differences in stroke pathogenesis, timing of clot retrieval, and individual

patient factors. Cardioembolic and atherothrombotic strokes, for example, are known to elicit distinct immune responses.[34]

Our findings highlight several pathways and cell populations that are not only implicated in the pathophysiology of vascular disease but are also amenable to pharmacologic targeting. For instance, some of the inflammatory and fibrotic pathways we identified—such as IL-6 signaling, immune checkpoint pathways, MMP (matrix metalloproteinase) activity, and TGF-β signaling—could represent future therapeutic targets using drugs that are already clinically available or under investigation. Tocilizumab, an IL-6 receptor inhibitor, has shown promise in improving endothelial function despite its effects on lipid profiles.[35] Statins, widely used for lipid lowering, also upregulate anti-inflammatory mediators like IL-10, potentially contributing to plaque stabilization and vascular repair.[36] Doxycycline, a broad-spectrum MMP inhibitor, has been explored in the context of abdominal aortic aneurysms and may offer therapeutic benefit by modulating extracellular matrix remodeling.[37] Additionally, immune checkpoint regulators such as ipilimumab (anti-CTLA4), nivolumab (anti-PD-1), and atezolizumab (anti-PD-L1), though primarily used in oncology, have been associated with cardiovascular immune modulation and may inform future strategies for targeting immune pathways in vascular disease.[38] Lastly, although TGF-β inhibitors remain largely experimental, their role in fibrosis and atherosclerosis is actively being studied and may hold potential for future intervention.[39] Together, these insights underscore the translational relevance of our findings and suggest that several of the dysregulated pathways identified in thrombus-associated immune cells could be modulated with existing or emerging therapies.

Our results can also be aligned with proteomic analyses of stroke clots. For example, Mai et al. identified TGF-β1 as a key fibrosis-related protein enriched in the stiff regions of arterial thrombi, suggesting its role in thrombus resistance to lysis.[40] Consistently, our single-cell data show activation of TGF-β–associated transcriptional programs in immune cells, particularly macrophages, supporting a role for this pathway in thrombus remodeling.

It is important to note that although our study provides valuable insights, there are limitations to consider. The sample size was relatively small, and further validation with larger cohorts is necessary to confirm the observed findings. Moreover, the analysis focused on scRNA-seq data, and functional validation of the identified biomarkers and pathways is required.

Although our MAGMA-based GWAS analysis provides valuable insights into the association between genetic variants and cell-specific gene expression, we acknowledge that direct experimental validation of these links is necessary. Future studies should focus on experimentally testing these associations to better understand the biological mechanisms underlying the genetic signals identified. Another limitation of our study is the lack of confirmatory validation of identified differential expression analysis and pathway enrichments through techniques such as quantitative polymerase chain reaction or immunohistochemistry. Future studies should incorporate these approaches to further validate and strengthen the findings from scRNA-seq analysis.

In summary, our study highlights the utility of scRNA-seq in characterizing the immune landscape of clots associated with stroke. The findings shed light on the cellular composition, gene expression profiles, and pathways involved in different causes, providing insights into potential biomarkers and therapeutic targets. Further research and validation are needed to translate these findings into clinical applications that can improve the diagnosis, treatment, and management of stroke and venous thrombosis.

ARTICLE INFORMATION

Received May 22, 2025; accepted July 28, 2025.

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Sources of Funding

This work was supported by the NIH under award number R21NS119992: “Single cell transcriptomics to determine stroke etiology and outcomes after thrombectomy.” Dr Matouk served as principal investigator, with Drs Navaratnam and Sansing as coinvestigators.

Disclosures

Dr Matouk is a consultant for Penumbra, Terumo Neurovascular, and Boston Scientific.

Supplemental Material

Figures S1–S29
Tables S1–S22

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