

Perivascular Adipose Single-Cell Atlas Identifies CD55+ Adipose-derived Stem Cells as Vascular Remodeling Regulators in Atherosclerosis

Corresponding Author: Professor Peng Zhang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Using single cell RNA sequencing, Chen and colleagues have examined the atlas of perivascular adipose tissue from normal subjects and patients with symptomatic carotid stenosis. The authors identified two subsets of ADSCs. Through cell transplantation assays and bioinformation analysis, the authors believe that the CD55 positive ADSCs might play a vital role in atherosclerosis related vascular remodeling. Currently, the conclusion is not strongly supported by the data. There are several flaws which should be clarified and strengthened.

1. When identifying ADSC, why not use the canonical markers, such as PDGFRA and PDGFRB? In supplementary Table 1, these two genes were not enriched in the gene set of ADSC. Since CD142 might represent one marker of ADSC subset, it is necessary to check the expression of PDGFRA or PDGFRB in ADSC when conducting single-cell data analysis and flow cytometry assay.
2. In supplementary Figure 1E, the ratio of each cell type between two groups seems no significant difference. As indicated in Figure 1G, the percentages of ADSC and macrophage in CAS group vary a lot. Why?
3. The authors claimed that ADSCs can be divided into two groups by CD55 and CXCL14. However, as shown in Figure 2H, another ADSC subset, which exhibits no expression of CD55 and CXCL14, exist in both normal subjects and CAS patients.
4. In the transplantation assays described in Figure 3, why use ADSCs from normal C57 mice and patients with carotid artery stenosis. Additionally, the GFP expression is hard to see in Figure 3D.
5. Based on bioinformation analysis, the composition and functional alteration of individual immune cell type were depicted. Also, the interaction between ADSC and immune cells was investigated. However, these predictive data are not enough to demonstrate the central role of ADSC in vascular remodeling. More experimental data are needed to validate these findings.
6. The result section should be written without discussion contents.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

This is a valuable study that adds to our understanding of human PVAT in the context of atherosclerosis, and the scRNA-seq dataset itself is an important contribution to the field. The inclusion of validation using flow cytometry and/or IHC in an independent cohort is a definite strength, and the follow-up in vivo work on CD55⁺ ADSCs is intriguing. That said, the manuscript would benefit from a more streamlined narrative—at times it feels disjointed, and the main message gets a bit lost. In addition, some conclusions seem to infer causality from descriptive data, which would need more mechanistic support to be convincing. I hope the comments below are helpful in refining the manuscript further.

Major Points:

- The characterisation and validation of PVAT SVF heterogeneity in the context of atherosclerosis is a key strength. However, the interpretation is somewhat limited by the lack of discussion of relevant previous work—for instance, Angueira et al.'s study profiling CD55⁺ fibroblasts and preadipocytes in human and murine PVAT should be acknowledged and

compared.

- The in vivo data suggests a role for ASDCs in vascular remodelling following aortic ligation, but mechanistic insight is limited. Focusing on a single signalling pathway and providing functional validation would strengthen the study.
- The Introduction could be more tightly aligned with the specific aims and focus of the paper.

Minor Points:

Fig 1 / S1:

- It would be helpful to include per-sample QC metrics and cell numbers to allow the reader to assess consistency across samples.
- The integration strategy for combining datasets isn't clearly explained in the main text and should be clarified.
- Fig S1D/E: There's notable inter-individual variation in ADSC abundance—for example, one control and three CAS samples show markedly higher proportions. This heterogeneity should be addressed in the main text and ideally assessed statistically.
- Line 145: The observed increase in abundance doesn't equate to heterogeneous spatial distribution—this should be revised for clarity.
- Line 168: Please clarify whether "most significant changes" refers to fold change, adjusted p-values, or another metric.

Fig 2 / S2:

- The pseudotime analysis supports the idea that CD55⁺ ADSCs represent an earlier state, but the biological implications are not clearly discussed—especially in light of the relatively few DEGs identified.
- GPC3 should be shown on the UMAP, given that it's used as a defining marker.
- Do CD55 and GPC3 staining overlap? Some comparison in the text or figure would help clarify.
- Fig S2C/D: The pseudotime plot suggests three branches within the Cxcl14⁺ ADSCs—what might these represent functionally? Is there further heterogeneity?
- Fig 2D/E (line 194): The sentence is unclear and doesn't convincingly show involvement in angiogenesis.
- Fig 2F: This panel feels slightly circular—more discussion would help clarify what new insight is provided.
- The flow cytometry suggests an increase in CD55⁺ ADSCs in CAS—does this align with the scRNA-seq data?
- Including basic histological characterisation of the PVAT (e.g., adipocyte size, fibrosis) would provide helpful context and could be linked back to cellular composition or function.
- The link between ASDCs and angiogenesis needs more direct mechanistic evidence.
- The external validation datasets are a useful addition, but the way they're introduced in the text could be clearer.

Fig 3 / S3:

- The relationship between medial thickening and angiogenesis isn't well explained—are these part of the same process or distinct effects?
- To strengthen the claim that ASDCs influence neointima formation or angiogenesis, more mechanistic data is needed. Later in the manuscript, additional cell types and interactions are described—could some of these be validated in vivo or via co-localisation studies?
- Fig 3M/S3: It would be helpful to show a UMAP identifying plaque clusters. Do ASDCs appear within these clusters in the scRNA-seq dataset? Could transdifferentiation be tested in vitro?

Fig S4:

- The comparison of ASDCs across fat depots is a nice addition. One suggestion would be to integrate ASDCs across tissues and identify conserved versus depot-specific transcriptional programs using DEGs or marker analysis.

Fig4-7:

- Immune cell populations in inguinal and omental adipose tissue, as well as within atherosclerotic plaques, have been well characterised using scRNA-seq. Integrating this dataset with relevant publicly available datasets would help contextualise the findings and strengthen the proposed link between PVAT and plaque biology

(Remarks on code availability)

The source code for scRNAseq analysis was not provided. The methods display state of the art use of basic analysis packages. For some analysis additional figures would be useful to improve readability of the manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all my concerns. I have no more comments.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have addressed several of the previous comments, for example by adding new validation experiments (FGF2 ELISA, migration and tube formation assays, additional histology) and expanding methodological descriptions. However, major technical and interpretative concerns remain. The integration with public datasets is still insufficiently detailed, DEG

interpretation remains uncertain, and several mechanistic claims are incompletely supported.

Major points

1) Integration of scRNA-seq data with public datasets

The integration strategy with public datasets is insufficiently detailed, and the UMAPs suggest incomplete batch correction. Based on the provided code, non-standard parameter choices, such as using all genes as anchor features, mixed normalization methods, mismatched dimensionality reductions, and fixed k-values. This combined with missing adjustment for single-cell versus single-nucleus data, likely contributed to the poor dataset mixing.

2) DEG analysis and batch effects

DEGs were identified mainly with FindMarkers on SCT-normalized data without modeling batch, sample, or sequencing platform effects. Only minimal corrections (e.g., removeBatchEffect) were applied. DEGs may be dominated by technical variation. Please re-run differential expression using donor and batch as covariates and show per-sample heatmaps. Clarify which DEGs remain significant after correction.

3) FGF2 signaling and functional validation

The added ELISA and FGFR1 inhibitor assays strengthen the mechanistic link but remain incomplete:

Justify why FGF2 was prioritized—the Authors mention comparison of secretory profiles—was was tested? Is FGF2 in the DEG?

How does FGF2 secretions from CD55⁺ and CXCL14⁺ ADSCs relate to the minimal concentration needed for angiogenic effects. Authors should address whether CXCL14 CM media can induce the same effects.

For CXCL14 ADSCs, authors validate CXCL12 in the conditioned medium. CXCL14 levels in CM medium should be assessed. In addition, even though CXCL12-CXCR4 is claimed to be increased in CAS in Cellchat analysis, authors should explain high interaction probability in CTR samples and adjust the conclusion.

Clarify whether CXCL12-CXCR4 activity differs mechanistically from CXCL14 expression.

4) Lineage and plaque sequencing

The plaque scRNA-seq used in this analysis is not described in the Methods. Please provide details on plaque sample preparation, sequencing platform, and data processing, and sample QC. Clarify whether plaque and PVAT datasets were sequenced using the same chemistry. Matching PVAT and plaque samples could be of interest and strengthen the manuscript if interpreted appropriately.

How do CD55 to CXCL14 trajectory analysis correlate with the mitochondrial lineage findings—do these analyses agree or suggest distinct relationships?

5) Code and data reproducibility

The GitHub repository is helpful but incomplete, particularly regarding preprocessing of public datasets and plaque scRNA-seq data. Ensure that all figure-generating scripts and parameters are fully documented, and that every figure in the manuscript can be reproduced from the provided code and data.

In addition, the study uses two different sequencing platforms (10x Genomics and DNBelab), but the manuscript does not describe which samples were processed with which method or how potential platform-related batch effects were handled. Please clarify this in the Methods and ensure that integration and normalization steps account for cross-platform variability.

Minor points

- Provide per-sample QC plots (cell number, nGenes, nUMIs, %mito) after merging separate sequencing libraries in addition to the already provided QC matrix per sequencing batch.

- SCENIC (pySCENIC v0.10.2, mc9nr motif database) is described in the Methods, but no corresponding results or figures are presented. Either show the key transcription factor networks supporting ADSC heterogeneity or remove the section to avoid confusion.

- ScRNASeq composition: Ratio plots for immune subclusters are not informative and should be replaced with per-sample boxplots. Authors now provide explanation of Ro/e, however it is not mentioned in the methods or shown in the code how filtering on p-values was done.

- Authors insufficiently address the point of ADSC heterogeneity. In the provided code clusters are merged to achieve 2 main subtypes. This should be acknowledged in the text, the merging strategy should be shared and subcluster identification should be performed.

- Authors now provide additional IF staining for CD55 and GPC3. It would be useful to quantify coexpression (PDGFRA/B, CD142, GPC3, CD55). For example, regions in Fig S3I shows 2 distinct regions for the subsets. Are these images representative? Does this reflect subsets are niche specific? In addition, histological characterisation of the PVAT should be related to ADSC biology.

- Address the discrepancy between human and mouse transplantation data: in mice, CD55⁺ ADSCs increase CD31 staining, whereas in human experiments, CXCL14⁺ ADSCs appear to increase Isolectin B4 and human CD31 signals. In Figure 3, it is unclear which panels correspond to human or mouse ADSCs, and the figure legend does not match the text description. Please clarify which experiment each panel represents and how quantification was performed.

- Clarify all figure legends to ensure consistency and readability. Clearly specify, for example, which panels correspond to human vs. mouse ADSC experiments, and which mouse model was used for which panel. This will help readers interpret species, experimental model, and measurement context without referring back to the text.

- Avoid causal statements (“ADSCs promote angiogenesis”) when correlations are moderate ($R \approx 0.6$)

(Remarks on code availability)

The authors provide a GitHub repository with R code for their analyses, but the figures are only partially reproducible. All

analysis scripts are contained within a single large R file. A structured folder layout with separate scripts per figure or analysis step would substantially improve usability. Public data preprocessing (especially for external datasets and plaque scRNA-seq) is not included. I did not attempt to run the code.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have responded to the points made by the reviewer in detail and added relevant information, experimental data and explanations. I am of the opinion that the key finding of this paper is supported.

(Remarks on code availability)

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Responses to Reviewer Critiques of Chen, et al. (NCOMMS-25-21637A).

The reviewers' comments are copied below, followed by our responses. Revised parts are marked in red in the manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Using single cell RNA sequencing, Chen and colleagues have examined the atlas of perivascular adipose tissue from normal subjects and patients with symptomatic carotid stenosis. The authors identified two subsets of ADSCs. Through cell transplantation assays and bioinformation analysis, the authors believe that the CD55 positive ADSCs might play a vital role in atherosclerosis related vascular remodeling. Currently, the conclusion is not strongly supported by the data. There are several flaws which should be clarified and strengthened.

Question (Q) 1. When identifying ADSC, why not use the canonical markers, such as PDGFRA and PDGFRB? In supplementary Table 1, these two genes were not enriched in the gene set of ADSC. Since CD142 might represent one marker of ADSC subset, it is necessary to check the expression of PDGFRA or PDGFRB in ADSC when conducting single-cell data analysis and flow cytometry assay.

Answer (A) 1: Thank you for your valuable comments. The marker genes for ADSCs listed in Supplementary Table 1 do include PDGFRA (avg_log2FC=4.157, pct.1= 0.764; pct.2= 0.012; p_val_adj=0, cluster=ADSC) and PDGFRB (avg_log2FC=3.085, pct.1= 0.512; pct.2= 0.012; p_val_adj=0, cluster=ADSC) (refer to **Supplementary Table 1**, sheet “Total DEG” rows 2010 and 2111). As suggested, we will supplement the UMAP visualization showing the expression distribution of PDGFRA and PDGFRB in the single-cell dataset. Due to space limitations in the main text, this figure will be provided in the supplementary materials for reference (**Supplementary Figure 3E and 3F**). We also performed immunofluorescence staining on paraffin-embedded sections of human PVAT from patients with carotid artery stenosis, which confirmed the co-localization of CD142 with PDGFRA and PDGFRB (**Supplementary Figure 3G**). Furthermore, we conducted Western blot analysis on sorted human ADSCs to examine the protein expression levels of PDGFRA and PDGFRB (**Supplementary Figure 4F**). Human smooth muscle cells were used as a control and showed negative expression for both markers, whereas the human ADSC samples tested positive for both.

We have also evaluated the widely reported mesenchymal stem cell markers CD34 and CD29 (ITGB1), whose expression patterns are consistent with ADSC characteristics (**Supplementary Figure 3B and 3C**). The selection of CD142 as an ADSC marker is based on studies by Schwalie et al. (*Nature*, 2018) and Merrick et al. (*Science*, 2019), both of which identified CD142 as a specific marker for ADSCs in human adipose tissue. Ultimately, our strategy for isolating ADSCs via flow cytometry using the CD45⁻CD142⁺ population was determined based on results from single-cell RNA sequencing analysis.

Q2. In supplementary Figure 1E, the ratio of each cell type between two groups seems no significant difference. As indicated in Figure 1G, the percentages of ADSC and

macrophage in CAS group vary a lot. Why?

A2: We appreciate this comment, which allows us to clarify the distinct analytical methods used in **Supplementary Figure 2B** and **Figure 1G**.

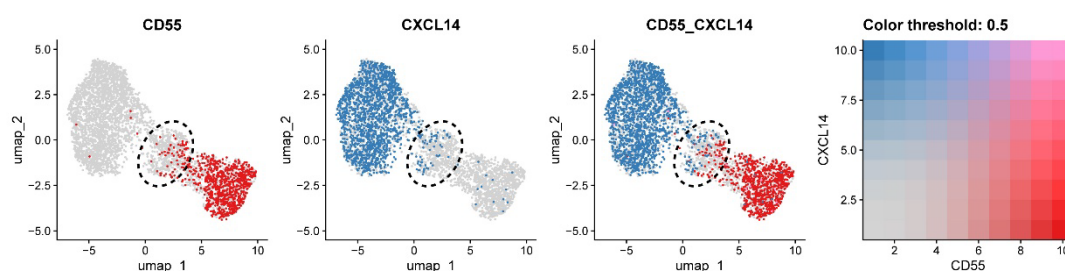
Supplementary Figure 2B displays the Ro/e (observed/expected cell ratio), a metric derived from chi-square testing that reflects the strength and direction of change in cell proportions between groups, rather than the absolute percentages (Lei Zhang et al. *Nature*, 2018). The expected values are calculated based on the control group's cell distribution. The Ro/e ratio is categorized into strength grades: '+++' (Ro/e >1, significant increase), '++' (0.8 < Ro/e ≤1, moderate increase), '+' (0.2 ≤ Ro/e ≤0.8, mild difference), '+/-' (0 < Ro/e <0.2, not significant), and '-' (Ro/e=0, no difference). The box plots show the median and interquartile range across samples within each group. We have also included the specific methods in the "Method" section (**Page 40 Line 880 to 885**).

In this figure, both ADSCs and macrophages in the CAS group received a '+++' rating, indicating a significant increase compared to the expected proportion. This is entirely consistent with the findings in Figure 1G (based on flow cytometry), which shows a substantial change in the absolute percentages of these cell types in the CAS group.

In summary, while the two figures employ different statistical representations (Ro/e ratio vs. absolute percentage), they both concordantly demonstrate a significant expansion of ADSCs and macrophages in the CAS group.

Q3. The authors claimed that ADSCs can be divided into two groups by CD55 and CXCL14. However, as shown in Figure 2H, another ADSC subset, which exhibits no expression of CD55 and CXCL14, exist in both normal subjects and CAS patients.

A3: We appreciate the opportunity to clarify this point. The reviewer is absolutely correct that our flow cytometry data (**Figure 2H**) clearly shows a population of cells that are double-negative for CD55 and CXCL14. The existence of this double-negative population is entirely consistent with our single-cell RNA sequencing (scRNA-seq) data. In our UMAP plot (**Response Figure 1**), we observe a continuum of expression for these markers rather than two completely discrete populations. The two ADSC subpopulations are not completely independent but may be developmentally related (**Supplementary Figure 3J&3K**). Consequently, we have defined them chiefly by their functional differences, using CD55 and CXCL14 as markers. The main text (**Page 9, Line 185 to 189**) has been revised accordingly to clarify this point.



Response Figure 1. Expressions of both CD55 and CXCL14 in ADSCs

The cells in the central region exhibit lower expression of both CD55 and CXCL14 (**Response**

Figure 1), representing a transitional state or the population identified by flow cytometry. The reason this double-negative group was not classified as a third distinct cluster in our scRNA-seq analysis is twofold:

1. Unsupervised clustering algorithm: Our clustering was based on the overall transcriptomic similarity of all genes across all cells. While the double-negative cells can be gated based on these two markers in flow cytometry, they did not form a separate cluster in the high-dimensional transcriptome space because they lack a unique and coherent gene expression signature that distinguishes them from the two main groups.

2. Biological function: Our subsequent functional experiments (e.g., adipogenic/osteogenic potential, cytokine secretion) were initiated based on the two transcriptomically-defined clusters. The double-negative population, from a functional perspective, did not demonstrate capabilities distinct enough to warrant classification as a unique third subpopulation.

In summary, the flow cytometry data provides a valuable orthogonal validation of the heterogeneity we identified by scRNA-seq. The double-negative population visible by flow is represented within our scRNA-seq dataset as cells with intermediate/low expression, which were not assigned a separate cluster due to the lack of a unique transcriptional identity and functional divergence.

Q4. In the transplantation assays described in Figure 3, why use ADSCs from normal C57 mice and patients with carotid artery stenosis. Additionally, the GFP expression is hard to see in Figure 3D.

A4: We appreciate your question. Firstly, to demonstrate that perivascular human-derived ADSCs can promote capillary formation, facilitate vascular remodeling, and subsequently migrate into the vasculature to exert functional effects during atherosclerosis, we conducted carotid artery partial ligation and ADSC transplantation experiments in nude mice using ADSCs obtained from patients with carotid artery stenosis.

Additionally, since C57 mice allow for convenient genetic modifications, thus providing a suitable model for subsequent mechanistic investigations and targeted intervention studies, we sought to confirm whether similar changes occur in murine-derived ADSCs.

In previous assessments of vascular thickness in C57 mice, we utilized Oil Red O staining for evaluation. However, as this method may not be fully accurate, we supplemented our analysis with Elastica Van Gieson (EVG) staining and re-evaluated the data. The updated results (**Figure 3B and 3C**) are consistent with the findings from the human ADSC intervention experiments in nude mice, thereby strengthening the evidence and enhancing the reliability of our conclusions.

Thank you for your reminder. We have now provided magnified views of the GFP images in **Figure 3D** and **Figure 3I**.

Q5. Based on bioinformation analysis, the composition and functional alteration of individual immune cell type were depicted. Also, the interaction between ADSC and immune cells was investigated. However, these predictive data are not enough to demonstrate the central role of ADSC in vascular remodeling. More experimental data are needed to validate these findings.

A5: We appreciate the reviewer's insightful comments. Regarding ADSC-immune cell interactions, we previously identified that CXCL14⁺ADSCs interact with macrophages via the CXCL12-CXCR4 axis (**Figure 7G&7I**). Using a migration assay with Raw264.7 cells (**Figure 7K-7M**), we found that CXCL14⁺ADSC-CM significantly enhanced macrophage migration compared to CD55⁺ADSC-CM ($P = 0.0002$). This effect was markedly attenuated by the CXCR4 inhibitor Plerixafor octahydrochloride ($P = 0.0004$), indicating that CXCL14⁺ADSCs facilitate macrophage recruitment primarily through the CXCL12-CXCR4 pathway. The role of ADSC-immune cell interactions in atherosclerosis remains a major focus of our future research.

In response, we conducted ADSC transplantation experiments, which provide support for the role of ADSC in influencing vascular remodeling and capillary formation (**Figure 3D and 3I**). Since our hypothesis proposes that CD55⁺ADSCs promote capillary formation leading to vascular remodeling, we compared the secretory profiles related to vasculature development between CD55⁺ADSCs and CXCL14⁺ADSCs, as well as between CD55⁺ADSCs from carotid artery stenosis (CAS) patients and controls. Through this analysis, we identified FGF2 as a key factor, highly expressed in CD55⁺ADSCs compared to CXCL14⁺ADSCs, and also upregulated in CD55⁺ADSCs from CAS patients compared to controls (**Supplementary Figure 6A**).

Existing literature strongly supports the pro-angiogenic role of FGF2: it has been shown to selectively stimulate angiogenesis in vivo without promoting other tissue growth (*Nature*. 1987 Jan 15-21;325(6101):257-9); contribute to vasa vasorum angiogenesis and plaque progression (*Circ Res*. 2009 Feb 13;104(3):337-45.); act as the first discovered pro-angiogenic molecule by activating FGFR1 signaling in endothelial cells (*Proc Natl Acad Sci U S A*. 2022 Jun 28;119(26):e2202631119.); and enhance the coupling between intimal hyperplasia and vasa vasorum proliferation in injured arteries (*J Clin Invest*. 1992 Feb;89(2):465-73.). These findings collectively suggest that FGF2 plays a significant role in angiogenesis and vascular remodeling.

To validate this, we first quantified FGF2 levels in conditioned medium (CM) from CD55⁺ and CXCL14⁺ADSCs via ELISA. Results showed significantly higher FGF2 secretion in CD55⁺ADSC-CM (2222 ± 90.79 v.s. 1378 ± 36.00 pg/mL, $p = 0.0001$; **Supplementary Figure 5B**). We then performed a HUVEC tube formation assay treated with ADSC-CM. CD55⁺ADSC-CM markedly enhanced tube formation compared to CXCL14⁺ADSC-CM ($P = 0.0001$, **Supplementary Figure 6C-6E**). This pro-angiogenic effect was significantly inhibited by the FGFR1 inhibitor PD173074 (25 nM), indicating that CD55⁺ADSCs promote angiogenesis primarily through FGF2 secretion.

We further investigated the effect of ADSC-CM on human aortic smooth muscle cells (HASMCs), particularly regarding phenotypic switching—a central pathological process in neointima formation and vascular remodeling (*Circulation*. 2021 Oct 12;144(15):1244-1261. *Circulation*. 2017 Oct 10;136(15):1412-1433. **Supplementary Figure 6F-6H**). Treatment with FGF2 decreased the expression of contractile markers (ACTA2 and TAGLN). Administration of PD173074 reversed this effect by upregulating ACTA2 and TAGLN and suppressing MMP9 and OPN, suggesting that FGF2 promotes a synthetic phenotype in HASMCs. Similarly, CD55⁺ADSC-CM reduced ACTA2 and TAGLN. This effect was reversed by PD173074, confirming that CD55⁺ADSCs modulate smooth muscle cell phenotypic switching mainly via FGF2 secretion, thereby influencing neointima formation and vascular remodeling.

Q6. The result section should be written without discussion contents.

A6: We thank the reviewer for this insightful comment. We agree that the results section should present the findings without interpretation. We have revised the manuscript accordingly by moving all discussion elements from the Results section to the Discussion section (**Page 29 Line 638 to 644; 657 to 660; 702 to 714; 728 to 738; 747 to 750; 763 to 768**).

Reviewer #2 (Remarks to the Author):

This is a valuable study that adds to our understanding of human PVAT in the context of atherosclerosis, and the scRNA-seq dataset itself is an important contribution to the field. The inclusion of validation using flow cytometry and/or IHC in an independent cohort is a definite strength, and the follow-up in vivo work on CD55⁺ ASDCs is intriguing. That said, the manuscript would benefit from a more streamlined narrative—at times it feels disjointed, and the main message gets a bit lost. In addition, some conclusions seem to infer causality from descriptive data, which would need more mechanistic support to be convincing. I hope the comments below are helpful in refining the manuscript further.

Answer: We sincerely thank the reviewer for their positive evaluation of our work and for their constructive suggestions. We fully agreed that the manuscript would benefit from a more streamlined and logically cohesive narrative. In response, we added concise summary statements at the end of key paragraphs to improve transitions between sections and enhance readability. We also ensured that the Results section focused clearly on experimental procedures and outcomes, and moved all interpretive discussions to the Discussion section. Furthermore, we tempered our conclusions where necessary to avoid overinterpreting descriptive data and supplemented additional mechanistic experiments to further support our conclusions. We believe these revisions have significantly improved the clarity and rigor of the manuscript.

Major Points:

Question (Q) 1: The characterisation and validation of PVAT SVF heterogeneity in the context of atherosclerosis is a key strength. However, the interpretation is somewhat limited by the lack of discussion of relevant previous work—for instance, Angueira et al.'s study profiling CD55⁺ fibroblasts and preadipocytes in human and murine PVAT should be acknowledged and compared.

Answer (A) 1: We thank the reviewer for their comment regarding the characterization and validation of PVAT SVF heterogeneity as a key strength of our study. We also appreciate the suggestion to contextualize our findings within existing literature. We have also discussed the work by Angueira et al. profiling CD55⁺ fibroblasts and preadipocytes in human and murine PVAT in the Discussion section (**Page 30, Line 657 to 660**). We acknowledge that there is currently no universally accepted gold standard for annotating adipose-derived stromal cells at the single-cell transcriptomic level, which has led to the use of diverse terminology. Under our annotation criteria, the cell population identified by Angueira et al. as CD55⁺ fibroblasts could indeed be classified as CD55⁺ADSCs.

Furthermore, we have systematically compared our findings with those of other recent PVAT single-cell studies (*Science*. 2019 Apr 26;364(6438):eaav2501; *Nature*. 2020 Nov;587(7832):98-102; *Nature*. 2022 Mar;603(7903):926-933; *Nature*. 2025 Aug;644(8077):769-779; *Nat Metab*. 2020 Jan;2(1):97-109; *Nat Metab*. 2021 Apr;3(4):469-484; *Arterioscler Thromb Vasc Biol*. 2024 Jul;44(7):1628-1645). Specifically, we evaluated the consistency and divergence in differentially expressed genes (DEGs) and functional enrichment patterns between our dataset and others (**Supplementary Figure 7 and 10**). Our analysis reveals both shared and distinct biological processes, underscoring the context-dependent heterogeneity of PVAT.

The primary novelty and significance of our study lie in its specific focus on carotid atherosclerosis, which distinguishes it from previous PVAT atlas efforts that primarily described homeostatic or metabolic conditions. We believe this emphasis provides new mechanistic and translational insights into PVAT biology in the context of vascular disease.

Q2: The in vivo data suggests a role for ADSCs in vascular remodelling following aortic ligation, but mechanistic insight is limited. Focusing on a single signalling pathway and providing functional validation would strengthen the study.

A2: We appreciate the reviewer's insightful comments. Since our hypothesis proposes that CD55⁺ADSCs promote capillary formation leading to vascular remodeling, we compared the secretory profiles related to vasculature development between CD55⁺ADSCs and CXCL14⁺ADSCs, as well as between CD55⁺ADSCs from carotid artery stenosis (CAS) patients and controls. Through this analysis, we identified FGF2 as a key factor—highly expressed in CD55⁺ADSCs compared to CXCL14⁺ADSCs, and also upregulated in CD55⁺ADSCs from CAS patients compared to controls (**Supplementary Figure 6A**).

Existing literature strongly supports the pro-angiogenic role of FGF2: it has been shown to selectively stimulate angiogenesis in vivo without promoting other tissue growth (*Nature*. 1987 Jan 15-21;325(6101):257-9); contribute to vasa vasorum angiogenesis and plaque progression (*Circ Res*. 2009 Feb 13;104(3):337-45.); act as the first discovered pro-angiogenic molecule by activating FGFR1 signaling in endothelial cells (*Proc Natl Acad Sci U S A*. 2022 Jun 28;119(26):e2202631119.); and enhance the coupling between intimal hyperplasia and vasa vasorum proliferation in injured arteries (*J Clin Invest*. 1992 Feb;89(2):465-73.). These findings collectively suggest that FGF2 plays a significant role in angiogenesis and vascular remodeling.

To validate this, we first quantified FGF2 levels in conditioned medium (CM) from CD55⁺ and CXCL14⁺ADSCs via ELISA. Results showed significantly higher FGF2 secretion in CD55⁺ADSC-CM (2222 ± 90.79 v.s. 1378 ± 36.00 pg/mL, $p = 0.0001$; **Supplementary Figure 6B**). We then performed a HUVEC tube formation assay treated with ADSC-CM. CD55⁺ADSC-CM markedly enhanced tube formation compared to CXCL14⁺ADSC-CM ($P = 0.0001$, **Supplementary Figure 6C-6E**). This pro-angiogenic effect was significantly inhibited by the FGFR1 inhibitor PD173074 (25 nM, $P < 0.0001$), indicating that CD55⁺ADSCs promote angiogenesis primarily through FGF2 secretion.

We further investigated the effect of ADSC-CM on human aortic smooth muscle cells (HASMCs), particularly regarding phenotypic switching—a central pathological process in neointima formation and vascular remodeling (*Circulation*. 2021 Oct 12;144(15):1244-1261. *Circulation*. 2017 Oct 10;136(15):1412-1433. **Supplementary Figure 6F-6H**). Administration

with FGF2 decreased the expression of contractile markers (ACTA2 and TAGLN). Administration of PD173074 reversed this effect by upregulating ACTA2 and TAGLN, suggesting that FGF2 promotes a synthetic phenotype in HASMCs. Similarly, CD55⁺ADSC-CM reduced ACTA2 and TAGLN. This effect was reversed by PD173074, confirming that CD55⁺ADSCs modulate smooth muscle cell phenotypic switching mainly via FGF2 secretion, thereby influencing neointima formation and vascular remodeling.

In our analysis, we also identified interactions between ADSCs and immune cells as functionally relevant to disease progression. Specifically, we previously demonstrated that CXCL14⁺ADSCs interact with macrophages via the CXCL12–CXCR4 axis (**Figure 7J–7M**). Using a migration assay with Raw264.7 cells, we observed that conditioned medium (CM) from CXCL14⁺ADSCs significantly enhanced macrophage migration compared to CM from CD55⁺ADSCs ($P = 0.0002$). This promigratory effect was markedly attenuated by the CXCR4 inhibitor plerixafor octahydrochloride ($P = 0.0004$), indicating that CXCL14⁺ADSCs facilitate macrophage recruitment primarily through the CXCL12–CXCR4 pathway. The role of ADSC–immune cell crosstalk in atherosclerosis constitutes a major focus of our ongoing and future research.

Q3: The Introduction could be more tightly aligned with the specific aims and focus of the paper.

A3: We thank the reviewer for this constructive suggestion. We acknowledge that the Introduction now better highlights our focus on the role of ADSCs in atherosclerosis and includes relevant mechanistic evidence, thereby strengthening alignment with the study's objectives. Corresponding modifications have been made throughout the section (**Page 4 line 75 to 82; page 5 line 89 to 94; page 5 line 108 to 113**).

Minor Points:

Fig 1 / S1:

Q4: It would be helpful to include per-sample QC metrics and cell numbers to allow the reader to assess consistency across samples.

A4: We have consolidated all per-sample QC metrics and cell numbers into **Sheet “QC” and Sheet “Cell number”** of **Supplementary Table 1** to allow readers to better assess the quality and consistency of our single-cell sequencing data across all samples.

Q5: The integration strategy for combining datasets isn't clearly explained in the main text and should be clarified.

A5: We have now clarified in the methods section that our integration was performed using the Seurat pipeline with canonical correlation analysis (CCA) and 2000 highly variable genes to identify anchors and remove batch effects (**Page 39 Line 856 to 859**).

Q6: Fig S1D/E: There's notable inter-individual variation in ADSC abundance—for

example, one control and three CAS samples show markedly higher proportions. This heterogeneity should be addressed in the main text and ideally assessed statistically.

A6: We agree that inter-individual heterogeneity in cell abundance is a notable feature of single-cell RNA sequencing data, as well as in biology in general, and we appreciate the suggestion to address it more thoroughly.

Statistical Assessment: Our statistical analysis already accounted for this variability. The Mann-Whitney U test used in **Figure 1G** and the chi-square based Ro/e analysis in **Supplementary Figure 2B** were non-parametric tests that are robust to non-normally distributed data and the presence of outliers. They tested for differences in the overall distribution of values between groups rather than being overly influenced by individual sample variations. The consistent, statistically significant results ($P < 0.05$) from these tests confirmed that the group-level difference in ADSC abundance was robust despite the inter-individual variation within each group.

We revised the main text to explicitly acknowledge this observed heterogeneity (**Page 29 Line 636 to 637**). We discussed that the variation in ADSC proportions among individuals could arise from multiple factors, such as individual genetic background, age, BMI, the precise anatomical location of tissue sampling and the tissue dissociation efficiency, all of which are known influencers of stromal cell populations.

We believe this addition will enrich the discussion and provide a more nuanced interpretation of our data. Thank you for prompting this important clarification.

Q7: Line 145: The observed increase in abundance doesn't equate to heterogeneous spatial distribution—this should be revised for clarity.

A7: We agree that an increase in abundance does not necessarily imply a heterogeneous spatial distribution. Accordingly, we have revised the sentence on **line 145** by removing the word "spatial" to more accurately describe our findings.

Q8: Line 168: Please clarify whether "most significant changes" refers to fold change, adjusted p-values, or another metric.

A8: We have revised the text at **line 165 and line 637** to explicitly state that the selection was based on cell proportion percentage change from 3.32% in Control group to 6.67% in CAS group.

Fig 2 / S2:

Q9: The pseudotime analysis supports the idea that CD55⁺ ADSCs represent an earlier state, but the biological implications are not clearly discussed—especially in light of the relatively few DEGs identified.

A9: We thank the reviewer for this insightful comment. **Supplementary Figure 3J**, which performed pseudotime analysis, revealed a potential lineage differentiation relationship between CD55⁺ ADSCs and CXCL14⁺ ADSCs. To further corroborate this finding, we

employed CytoTRACE analysis (**Supplementary Figure 3K**) to independently evaluate the differentiation status and stemness of these two subpopulations.

The CytoTRACE result clearly demonstrates that CD55⁺ ADSCs (green) exhibit a significantly higher median score compared to CXCL14⁺ ADSCs (orange), indicating that CD55⁺ ADSCs are less differentiated and retain a more primitive, stem cell-like state.

We agree with the reviewer that the biological implications of this differentiation hierarchy warrant deeper investigation. However, the precise mechanistic underpinnings of this lineage relationship were beyond the scope of the current study. In the revised discussion (**Page 35 Line 763 to 768**), we have more explicitly highlighted this as a focus for future research, particularly to elucidate how the primitive state of CD55⁺ADSCs contributes to their enhanced functional potency in vascular remodeling.

Q10: GPC3 should be shown on the UMAP, given that it's used as a defining marker.

A10: Given the importance of GPC3 as a defining marker for the CXCL14⁺ADSC population, we have now included its expression pattern on the UMAP plot (**Supplementary Figure 3H**).

Q11: Do CD55 and GPC3 staining overlap? Some comparison in the text or figure would help clarify.

A11: To directly address the co-localization of these markers, we have performed additional immunofluorescence staining for CD142 (a general ADSC marker), CD55, and GPC3 (**Supplementary Figure 3I**). Our results show that, within the CD142-positive ADSC population, CD55 and GPC3 exhibit only minimal co-localization. This finding is consistent with our flow cytometry data presented in **Figure 2H**. We have added corresponding descriptions of these results in the manuscript.

Furthermore, as included in **Supplementary Table 1 (Sheet “ADSC DEG” Line 6)**, GPC3 is among the most significantly upregulated genes in CXCL14⁺ ADSCs compared to CD55⁺ ADSCs (avg_log2FC = 2.574, pct.1 = 0.933, pct.2 = 0.344, p_val_adj = 0), reinforcing its distinct expression pattern and supporting the limited overlap between CD55 and GPC3 at the expression level.

Q12: Fig S2C/D: The pseudotime plot suggests three branches within the Cxcl14⁺ ASDCs—what might these represent functionally? Is there further heterogeneity?

A12: The primary focus of our study was to identify and functionally validate the two major ADSC subpopulations defined by CD55 and CXCL14, which exhibit distinct roles in vascular remodeling.

Regarding the three branches within the CXCL14⁺ADSCs on the pseudotime plot, we agree that they may suggest further heterogeneity or intermediate states within this population. Although these three branches exhibited heterogeneity in their differentiation relationships, they showed similar gene expression pattern (**Figure 2A**) and all highly expressed genes related to chemotaxis (**Figure 2B and 2F**), which was the most crucial function of the CXCL14⁺ADSC

subpopulation, and we are cautious in over-interpreting these branches without additional functional validation.

We fully agree that this is an important and interesting question. While elucidating the functional significance of these substates falls beyond the scope of the current manuscript, we have now highlighted this observation in the **Discussion section (Page 35 Line 763 to 768)** as a key direction for our future research. We will aim to isolate and characterize these potential subpopulations functionally to determine if they represent distinct transitional or functional states.

Q13: Fig 2D/E (line 194): The sentence is unclear and doesn't convincingly show involvement in angiogenesis.

A13: To clarify, within the Gene Ontology (GO) Biological Process hierarchy, the term "vasculature development" (GO:0001944) serves as a parent category that encompasses more specific processes, including "angiogenesis" (GO:0001525). As illustrated in the GO structure, angiogenesis is defined as a child term under vasculature development, indicating that angiogenesis is an integral and specific subtype of vasculature development.

To make this relationship unequivocally clear to the reader, we have revised the text on **line 195** to state: '...was significantly enriched for terms related to vasculature development (including angiogenesis) ...' This revision, coupled with our functional tube formation assay data, we believe, convincingly demonstrates the involvement in angiogenesis."

Q14: Fig 2F: This panel feels slightly circular—more discussion would help clarify what new insight is provided.

A14: We thank you for your thoughtful comment. **Figure 2D/E** (enrichment bar plots) display the most significantly enriched biological pathways within each ADSC subpopulation, which can be regarded as a form of "qualitative screening" for cellular functions. However, these panels do not directly compare the activity of the same pathway between the two cell groups.

In a complementary manner, **Figure 2F** (functional scoring plot) provides a quantitative activity comparison of pre-selected functional pathways—including "positive chemotaxis" and "positive regulation of vascular development"—between the two ADSC subpopulations. This analytical approach enables a "direct comparison" of the same functional pathway across different cell groups, unambiguously addressing questions such as "which subpopulation exhibits stronger chemotactic functionality" and "which shows weaker pro-angiogenic potential." Specifically, it demonstrates that CXCL14⁺ADSCs are more inclined toward chemotaxis-related functions, whereas CD55⁺ADSCs exhibit a stronger tendency toward vascular development-related processes. More importantly, it offers statistically supported quantitative evidence for these functional biases, thereby providing a data-driven hypothesis and clear direction for subsequent functional experiments, such as cell migration and angiogenesis assays.

We have accordingly revised the corresponding descriptions and discussions in the main text (**Page 10, Line 201 to 209**) and figure legend (**Figure 2F**).

Q15: The flow cytometry suggests an increase in CD55⁺ ADSCs in CAS—does this align with the scRNA-seq data?

A15: We thank the reviewer for raising this important point regarding the concordance between our flow cytometry and scRNA-seq data. We have added a new panel (**Supplementary Figure 3N**) showing the proportions of CD55⁺ and CXCL14⁺ADSCs derived from the scRNA-seq analysis. In our dataset, the apparent increase in CD55⁺ADSCs in CAS did not achieve statistical significance. We acknowledge that this appears to differ from the flow cytometry findings. This discrepancy can be attributed to the fundamental technical differences between the two methods; scRNA-seq is subject to cell capture biases and typically requires filtering out subpopulations with low cell counts (e.g., <30 cells) to ensure analytical rigor, which can diminish statistical power for quantifying population abundances. Consequently, flow cytometry is widely regarded as a more reliable and quantitative tool for this specific purpose. The compelling and statistically significant result from flow cytometry on a larger sample set provides robust orthogonal validation, confirming the true expansion of the CD55⁺ADSC subpopulation in CAS.

Q16: Including basic histological characterisation of the PVAT (e.g., adipocyte size, fibrosis) would provide helpful context and could be linked back to cellular composition or function.

A16: We have now performed histological characterization (H&E and Sirius Red staining) on PVAT from 85 cases to assess adipocyte size and fibrosis. Our results demonstrate that adipocyte size was significantly larger in the CAS group compared to controls ($1976 \pm 480.4 \mu\text{m}^2$ vs. $1476 \pm 613.1 \mu\text{m}^2$, $P = 0.0159$, **Supplementary Figure 3O&3P**). Adipocyte hypertrophy is known to be associated with promoted inflammation and fibrosis (*Nature reviews Endocrinology*, 21(5), 272–288.). Regarding fibrosis, the PVAT from CAS patients exhibited a significantly higher fibrosis area percentage ($2.952 \pm 2.213\%$ vs. $1.587 \pm 0.727\%$, $P=0.0311$, **Supplementary Figure 3Q&3R**). Further analysis revealed a marked increase in the deposition of type I collagen (the major fibrillar collagen) in the CAS group ($P = 0.0178$), while the change in type III collagen was not statistically significant ($P = 0.7092$). These histological alterations indicate substantial PVAT remodeling during atherosclerosis progression. PVAT fibrosis, characterized predominantly by type I collagen accumulation, has been linked to disease advancement. A prior study has correlated CT attenuation values (Hounsfield units) with PVAT fibrosis (*Eur Heart J.* 2019;40(43):3529-3543), suggesting that fibrosis is associated with elevated local inflammation, collectively contributing to disease pathogenesis. The histological evidence provided in our study offers crucial contextual information for understanding the role of PVAT in the mechanisms underlying AS.

Q17: The link between ASDCs and angiogenesis needs more direct mechanistic evidence.

A17: As we mentioned in Question 2, our hypothesis proposes that CD55⁺ADSCs promote capillary formation leading to vascular remodeling, we compared the secretory profiles related to vasculature development between CD55⁺ADSCs and CXCL14⁺ADSCs, as well as between CD55⁺ADSCs from carotid artery stenosis (CAS) patients and controls. Through this analysis, we identified FGF2 as a key factor—highly expressed in CD55⁺ADSCs compared to CXCL14⁺ADSCs, and also upregulated in CD55⁺ADSCs from CAS patients compared to

controls (**Supplementary Figure 6A**).

To validate this, we first quantified FGF2 levels in conditioned medium (CM) from CD55⁺ and CXCL14⁺ADSCs via ELISA. Results showed significantly higher FGF2 secretion in CD55⁺ADSC-CM (2222 ± 90.79 v.s. 1378 ± 36.00 pg/mL, $p = 0.0001$; **Supplementary Figure 6B**). We then performed a HUVEC tube formation assay treated with ADSC-CM. CD55⁺ADSC-CM markedly enhanced tube formation compared to CXCL14⁺ADSC-CM ($P=0.0001$, **Supplementary Figure 6C-6E**). This pro-angiogenic effect was significantly inhibited by the FGFR1 inhibitor PD173074 (25 nM), indicating that CD55⁺ADSCs promote angiogenesis primarily through FGF2 secretion.

Q18: The external validation datasets are a useful addition, but the way they're introduced in the text could be clearer.

A18: As suggested, we have created a separate supplementary table (**Supplementary Table 3**) that provides a clear summary of the external validation datasets used in this study, including their tissue origin, sequencing technology, and sample size (**Line 369 and 539**).

Fig 3 / S3:

Q19: The relationship between medial thickening and angiogenesis isn't well explained—are these parts of the same process or distinct effects?

A19: We thank the reviewer for raising this important point, which allowed us to identify and correct a limitation in our initial analysis.

In our original submission, the assessment of vascular thickening in the C57 mouse model relied on Oil Red O staining, which is primarily optimized for lipid quantification rather than precise structural morphometry. This methodological limitation likely led to the ambiguous interpretation that medial thickening was the predominant feature in that model, which appeared inconsistent with the clear neointimal (intimal) thickening observed in our nude mouse model using human ADSCs.

To address this definitively, we have now re-evaluated all C57 mouse sections using **Elastica Van Gieson (EVG) staining**, the gold standard for distinguishing elastic laminae and measuring vascular layers. **The new analysis consistently shows neointimal formation** as the primary response in both mouse models (**Figure 3B**). This unified finding strengthens our central conclusion that ADSC transplantation promotes a vascular remodeling response characterized by intimal thickening.

Regarding the reviewer's question on the relationship between intimal thickening and angiogenesis: they represent distinct yet integrally linked processes in vascular pathological remodeling. Intimal hyperplasia primarily arises from smooth muscle cell proliferation, migration, and phenotypic switching, while angiogenesis refers to the expansion of the vasa vasorum or microvasculature. Importantly, these processes are synergistically promoted by common molecular mediators. **In our study, we identified FGF2—secreted by CD55⁺ADSCs—as one such key mediator that concomitantly stimulates both angiogenesis and the synthetic phenotypic switch in smooth muscle cells.** Existing evidence indicates that

FGF2 not only induces angiogenesis but also directly enhances smooth muscle cell migration and proliferation, thereby contributing to neointima formation (*Arterioscler Thromb Vasc Biol.* 1997;17(3):490-497; *Cardiovasc Res.* 2009;81(2):319-326). **Thus, FGF2 serves as a mechanistic link between these two processes:** by promoting the growth of microvessels; and by directly acting on smooth muscle cells, it stimulates their transition to a synthetic, pro-inflammatory phenotype, resulting in extracellular matrix deposition and intimal thickening. Our *in vitro* findings that CD55⁺ADSC-derived FGF2 simultaneously promotes endothelial tube formation and smooth muscle synthetic activation provide functional support for this dual role and offer a plausible mechanism through which this subpopulation contributes to concerted vascular remodeling.

We have revised the manuscript to clarify this point and to present the updated, consistent morphological data.

Q20: To strengthen the claim that ADSCs influence neointima formation or angiogenesis, more mechanistic data is needed. Later in the manuscript, additional cell types and interactions are described—could some of these be validated in vivo or via co-localisation studies?

A20: Since our hypothesis proposes that CD55⁺ADSCs promote capillary formation leading to vascular remodeling, we compared the secretory profiles related to vasculature development between CD55⁺ADSCs and CXCL14⁺ADSCs, as well as between CD55⁺ ADSCs from carotid artery stenosis (CAS) patients and controls. Through this analysis, we identified FGF2 as a key factor—highly expressed in CD55⁺ADSCs compared to CXCL14⁺ADSCs, and also upregulated in CD55⁺ADSCs from CAS patients compared to controls (**Supplementary Figure 6A**).

To validate this, we first quantified FGF2 levels in conditioned medium (CM) from CD55⁺ and CXCL14⁺ADSCs via ELISA. Results showed significantly higher FGF2 secretion in CD55⁺ ADSC-CM (2222 ± 90.79 v.s. 1378 ± 36.00 pg/mL, $p = 0.0001$; **Supplementary Figure 6B**). We then performed a HUVEC tube formation assay treated with ADSC-CM. CD55⁺ADSC-CM markedly enhanced tube formation compared to CXCL14⁺ADSC-CM ($P = 0.0001$; **Supplementary Figure 6C-6E**). This pro-angiogenic effect was significantly inhibited by the FGFR1 inhibitor PD173074 (25 nM), indicating that CD55⁺ADSCs promote angiogenesis primarily through FGF2 secretion.

In our analysis, we also identified interactions between ADSCs and immune cells as functionally relevant to disease progression. Specifically, we previously demonstrated that CXCL14⁺ ADSCs interact with macrophages via the CXCL12–CXCR4 axis (**Supplementary Figure 6F-6J**). Using a migration assay with Raw264.7 cells, we observed that conditioned medium (CM) from CXCL14⁺ADSCs significantly enhanced macrophage migration compared to CM from CD55⁺ADSCs ($P = 0.0002$). This promigratory effect was markedly attenuated by the CXCR4 inhibitor plerixafor octahydrochloride ($P = 0.0004$), indicating that CXCL14⁺ADSCs facilitate macrophage recruitment primarily through the CXCL12–CXCR4 pathway. The role of ADSC–immune cell crosstalk in atherosclerosis constitutes a major focus of our ongoing and future research.

Q21: Fig 3M/S3: It would be helpful to show a UMAP identifying plaque clusters. Do ASDCs appear within these clusters in the scRNA-seq dataset? Could transdifferentiation be tested in vitro?

A21: In response, we have now included a UMAP plot displaying the plaque cell clusters, along with a dot plot showing the marker genes used for annotation (**Supplementary Figure 4I&4J**).

Since we hypothesized that ADSC in PVAT affects the structural stability of plaques by promoting capillary neogenesis, we performed mitochondrial lineage analysis to observe whether ADSC in PVAT shares a lineage differentiation relationship with endothelial cells within the plaques. The results were positive (**Figure 3M and Supplementary Figure 5**). Furthermore, to directly test the potential for transdifferentiation in a functional setting, we performed an *in vivo* cell fate tracing experiment. We isolated ADSCs from the PVAT of patients with carotid artery stenosis, labeled them with GFP lenti-virus, and transplanted them around the ligated left carotid artery of nude mice. Immunofluorescence analysis revealed the presence of GFP⁺ cells within the neointimal area, with a subset of these cells co-localizing with human-specific CD31 (endothelial marker), suggesting that perivascular ADSCs can migrate into vascular remodeling sites and differentiate into endothelial cells (**Figure 3I**). While *in vitro* transdifferentiation assays would indeed be interesting, our *in vivo* findings provide direct evidence supporting the functional integration and endothelial differentiation potential of human ADSCs in a relevant disease model.

Fig S4:

Q22: The comparison of ASDCs across fat depots is a nice addition. One suggestion would be to integrate ASDCs across tissues and identify conserved versus depot-specific transcriptional programs using DEGs or marker analysis.

A22: To identify both conserved and depot-specific transcriptional features of ADSCs, we integrated our scRNA-seq data with publicly available datasets from multiple adipose depots, including PVAT (from *Nature Metabolism*, 2021 and *ATVB*, 2024), subcutaneous adipose tissue (from *Science*, 2019; *Nature Metabolism*, 2020; and *Nature*, 2020; *Nature*, 2022; *Nature*, 2025), visceral adipose tissue (from *Nature Metabolism*, 2020; *Nature*, 2022), and deep neck BAT (from *Nature*, 2020). Our integrated analysis successfully identified the two conserved ADSC subpopulations (CXCL14⁺ and CD55⁺) across all datasets (**Supplementary Figure 7B**), with only modest variation in their relative abundances (**Supplementary Figure 7C**). Notably, differential gene expression analysis revealed that our dataset (from carotid artery stenosis patients) showed even greater DEGs with other PVAT datasets and shared fewer divergence from subcutaneous and visceral adipose tissues (**Supplementary Figure 7D**), suggesting that the transcriptional landscape of ADSCs in our study may be influenced more strongly by the disease-specific microenvironment of carotid artery stenosis than by depot origin. Functional enrichment analysis further supported this notion: CXCL14⁺ADSCs from our dataset exhibited upregulation of inflammatory pathways—such as response to interferon-alpha, macrophage activation, and acute inflammatory response—compared to those from other depots. In contrast, CD55⁺ADSCs showed enrichment in processes related to osteoblast proliferation, cellular response to growth factor stimulus, and mesenchymal cell proliferation (**Supplementary Figure 7E**). These findings suggest that the transcriptional profiles of CXCL14⁺ ADSCs and CD55⁺ADSCs in carotid stenosis patients are likely shaped more significantly by pathological stimuli than by innate anatomical differences.

Fig4-7:

Q23: Immune cell populations in inguinal and omental adipose tissue, as well as within atherosclerotic plaques, have been well characterised using scRNA-seq. Integrating this dataset with relevant publicly available datasets would help contextualise the findings and strengthen the proposed link between PVAT and plaque biology.

A23: To contextualize our immune cell data within existing knowledge, we integrated our scRNA-seq dataset with publicly available datasets from multiple adipose depots, including PVAT (from *Nature Metabolism*, 2021 and *ATVB*, 2024), subcutaneous adipose tissue (SAT; from *Science*, 2019; *Nature Metabolism*, 2020; and *Nature*, 2020), visceral adipose tissue (VAT; from *Nature Metabolism*, 2020), and deep-neck adipose tissue (from *Nature*, 2020).

Our comparative analysis revealed that the proportions of CD4⁺ and CD8⁺ memory T cells in our dataset were similar to those in other PVAT datasets but higher than those typically found in SAT (**Supplementary Figure 10A–10B**). In contrast, SAT showed a increased proportion of CD8⁺ effector T cells. While macrophages constituted the majority of myeloid immune cells in other PVAT datasets (**Supplementary Figure 10C–10D**), the proportions of macrophages, cDC1, and cDC2 in our data were comparable to those in SAT and VAT. Notably, we observed a significant increase in plasmacytoid dendritic cells (pDCs) in our dataset, which may be attributed to both the disease state (carotid atherosclerosis) and anatomical location. Regarding B cell subsets, the proportions of naïve B, unswitched memory B, switched memory B, and plasma cells in our data were similar to those in SAT (**Supplementary Figure 10E–10F**). In the NK cell compartment, compared to coronary PVAT (*ATVB*, 2024), our data showed an increase in NK_{tr} cells, while the proportions of NK_{CD56dim} and NK_{CD56bright} cells were similar (**Supplementary Figure 10E–10F**).

Overall, the proportions of T and NK cell subsets in our data were consistent with those in other PVAT datasets, whereas clear differences were observed in B cell and myeloid cell subsets. These findings suggest that the anatomical location of adipose tissue may differentially influence immune cell composition during atherosclerosis progression. This represents an important direction for future research, particularly given known differences in the pathological mechanisms of atherosclerotic plaques among carotid, coronary, and lower extremity arteries.

Reviewer #2 (Remarks on code availability):

Q24: The source code for scRNAseq analysis was not provided. The methods display state of the art use of basic analysis packages. For some analysis additional figures would be useful to improve readability of the manuscript.

A24: We have now created a GitHub repository containing the complete source code and analytical pipelines for our scRNA-seq analysis. The repository includes detailed documentation of all state-of-the-art computational methods and packages used in this study. Access is available at: <https://github.com/SongMei233/PVAT>.

Additionally, to enhance manuscript readability, we have expanded supplementary figures to better visualize key analytical steps (**Supplementary Figure 1C-1E**). These revisions improve transparency and reproducibility of our work.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all my concerns. I have no more comments.

Reviewer #2 (Remarks to the Author):

The authors have addressed several of the previous comments, for example by adding new validation experiments (FGF2 ELISA, migration and tube formation assays, additional histology) and expanding methodological descriptions. However, major technical and interpretative concerns remain. The integration with public datasets is still insufficiently detailed, DEG interpretation remains uncertain, and several mechanistic claims are incompletely supported.

Major points

1) Integration of scRNA-seq data with public datasets

The integration strategy with public datasets is insufficiently detailed, and the UMAPs suggest incomplete batch correction. Based on the provided code, non-standard parameter choices, such as using all genes as anchor features, mixed normalization methods, mismatched dimensionality reductions, and fixed k-values. This combined with missing adjustment for single-cell versus single-nucleus data, likely contributed to the poor dataset mixing.

Response: Thank you for this valuable comment. In our initial submission, we integrated our immune-cell dataset with multiple public datasets and performed batch-effect correction using the FastMNN workflow. In response to the reviewer's suggestion, we systematically evaluated alternative integration strategies for combining our data with public references and found that Seurat's standard IntegrateData pipeline provided the most robust and consistent performance. We therefore adopted this approach in the revised analysis. Specifically, we selected 2,000 highly variable genes as anchor features, applied a unified SCT normalization across all datasets, and specified CCA as the dimensionality reduction method when identifying anchors. We also standardized the k-value to 40 for anchor finding.

After applying the revised integration workflow, the UMAPs demonstrate markedly improved dataset mixing compared with the FastMNN results. Furthermore, we systematically evaluated batch-effect removal using kBET (**Supplementary Figure 11 A, D, G and J**), which showed that the majority of cells mixed well across batches.

We have now added a detailed description of these updated procedures in the Methods section and improved the clarity of the corresponding code in our GitHub repository.

2) DEG analysis and batch effects

DEGs were identified mainly with FindMarkers on SCT-normalized data without modeling batch, sample, or sequencing platform effects. Only minimal corrections (e.g., removeBatchEffect) were applied. DEGs may be dominated by technical variation. Please re-run differential expression using donor and batch as covariates and show per-sample heatmaps. Clarify which DEGs remain significant after correction.

Response: Thank you for pointing out this important issue. As the reviewer notes, our initial

differential expression analysis relied on FindMarkers on SCT-normalized data and did not explicitly model batch, donor, or platform effects. To address this, we have re-performed the differential expression analysis using RUV-III-NB for normalization of the single-cell RNA-seq data. Using pseudo-replicates, RUV-III-NB explicitly accounts for potential associations with biological signals while removing unwanted variation, thereby mitigating effects attributable to batch, sample, or sequencing platform ([Nucleic Acids Res. 2022 Sep 9;50\(16\):e96.](#)). Differentially expressed genes were then identified using FindMarkers on the RUV-III-NB-normalized data. We subsequently re-ran all functional enrichment analyses using the DEGs obtained after batch correction to ensure that the inferred biological changes reflect true biological variation rather than technical noise (**Supplementary Figure 8C and D**). In addition, as requested, we have updated the DEG heatmaps to per-sample heatmaps (**Supplementary Figure 8A and B**), providing a more accurate representation of inter-sample variability.

The revised manuscript now includes a detailed description of these updated methods, and we have also improved the clarity and organization of the corresponding code in our GitHub repository.

3) FGF2 signaling and functional validation

The added ELISA and FGFR1 inhibitor assays strengthen the mechanistic link but remain incomplete:

3.1) Justify why FGF2 was prioritized— the Authors mention comparison of secretory profiles – was tested? Is FGF2 in the DEG?

Response: Thank you for raising this point. The selection was indeed based on a systematic comparison of secretory profiles, as mentioned in the text (**Line 367**). To prioritize candidate secreted factors, we focused specifically on genes that (i) belong to the GOBP_VASCULATURE_DEVELOPMENT gene set and (ii) encode secreted proteins. Within this predefined space, we compared genes that were upregulated in CD55⁺ADSC relative to CXCL14⁺ADSC, as well as genes that were upregulated in CD55⁺ADSC (CAS condition) compared with the control group. By intersecting these two comparisons and restricting the analysis to secreted, vasculature-related genes, FGF2 emerged as the most selectively and consistently overexpressed candidate. As a classic pro-angiogenic factor, it offered a clear and compelling rationale for prioritizing it for functional validation. We agree that presenting these supporting data is important and have therefore included the full results of the comparative analyses as **Supplementary Figure 6A and B**. We appreciate the opportunity to clarify this rationale.

3.2) How does FGF2 secretions from CD55⁺ and CXCL14⁺ ADSCs relate to the minimal concentration needed for angiogenic effects. Authors should address whether CXCL14 CM media can induce the same effects.

Response: Thank you for this important question. We appreciate the opportunity to clarify the relationship between FGF2 concentrations in our conditioned media (CM) and the minimal concentration required for angiogenic effects, as well as the efficacy of CXCL14⁺ADSC CM.

The reviewer rightly points out the need to consider the biologically effective concentration of FGF2. As cited by Chang et al. ([Proc Natl Acad Sci U S A. 2004 Aug 10;101\(32\):11658-63.](#)), the proliferation of Human Microvascular Blood Endothelial Cells (HMBECs) increases significantly at FGF2 concentrations between 0.5 and 1.5 ng/mL, with no significant effect

observed at 0.25-0.5 ng/mL. In our study, the FGF2 concentration in CXCL14⁺ADSC CM was approximately 1.5 ng/mL, and in CD55⁺ADSC CM, it was approximately 2.2 ng/mL. Both concentrations are within or above the range (0.5-1.5 ng/mL) shown to be effective for stimulating endothelial cell proliferation, supporting their biological relevance for angiogenic effects.

Regarding whether CXCL14⁺ADSC CM can induce angiogenic effects, our experimental data (**Supplementary Figure 6C-6E**) provide direct evidence. The results demonstrate that CM from CXCL14⁺ADSCs does indeed promote tube formation *in vitro*, although its effect is weaker than that of CD55⁺ADSC CM (**Line 378**). Importantly, the tube-forming capability of CXCL14⁺ADSC CM was significantly stronger than that of the blank control medium.

As suggested, we added the following details to the Methods section to ensure reproducibility: "For CM collection, cells were seeded at a 2.2×10^6 cell number in 10-cm dishes with 10 mL of medium. CM harvesting began 48 hours post-seeding. The medium was replaced with fresh medium, and the CM was collected after 24 hours. This process was repeated to collect two batches of CM for analysis. All collected CM was aliquoted and stored at -80°C until use." (**Line 1234**).

3.3) For CXCL14 ADSCs, authors validate CXCL12 in the conditioned medium. CXCL14 levels in CM medium should be assessed. In addition, even though CXCL12-CXCR4 is claimed to be increased in CAS in Cellchat analysis, authors should explain high interaction probability in CTR samples and adjust the conclusion.

Response: We appreciate the reviewer's suggestion. In our revised manuscript, we have quantified CXCL14 levels in the conditioned medium (CM) derived from both CXCL14⁺ADSCs and CD55⁺ADSCs. The results confirm that CXCL14⁺ADSCs secrete significantly higher levels of CXCL14 compared to CD55⁺ADSCs. This data has been incorporated into the manuscript (**Figure 7K**).

We agree that the initial conclusion required refinement. As rightly pointed out, the CellChat analysis indicates interaction probabilities in control (CTR) samples, although the inferred pathway activity is markedly elevated in the CAS group. This suggests a basal level of CXCL12-CXCR4 signaling under physiological conditions, which is amplified during disease. Furthermore, we acknowledge that our *in vitro* macrophage migration assay was not conducted within an atherosclerotic model context. Therefore, we have toned down and refined the related conclusion throughout the manuscript. The revised conclusion now reads: "suggesting that elevated pathway activity during disease states may amplify chemotactic cell recruitment." (**Line 634**). We believe this formulation more accurately reflects our data, acknowledging the baseline activity while highlighting the significant upregulation and its potential functional consequence in the pathological context.

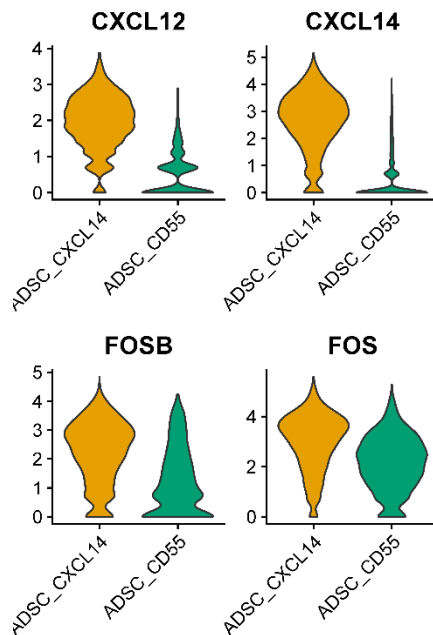
3.4) Clarify whether CXCL12-CXCR4 activity differs mechanistically from CXCL14 expression.

Response: We sincerely thank the reviewer for raising this insightful and important question regarding the mechanistic relationship between CXCL12-CXCR4 activity and CXCL14 expression. This is indeed a complex aspect of chemokine biology, and we appreciate the opportunity to clarify our perspective based on the existing literature and our own findings.

The reported functions of CXCL12 and CXCL14 exhibit both overlap and apparent

contradiction. CXCL12, signaling predominantly through CXCR4, is a well-established potent chemoattractant for lymphocytes and plays significant roles in atherosclerosis ([Circulation. 2017 Jul 25;136\(4\):388-403.](#)). In contrast, CXCL14, which can also interact with CXCR4 ([Cardiovasc Res. 2021 Feb 22;117\(3\):903-917.](#)), is known to chemoattract monocytes, immature dendritic cells, neutrophils, and NK cells ([J Inflamm \(Lond\). 2016 Jan 5:13:1.](#)). Notably, CXCL14 is highly expressed in obese adipose tissue, where it promotes the accumulation of pro-inflammatory CD11c⁺ macrophages ([Gut. 2018 Jul;67\(7\):1317-1327.](#)). Studies have confirmed that ox-LDL upregulates CXCL14 expression via the Activator Protein 1 (AP-1) transcription factor pathway. CXCL14, in turn, promotes THP-1 monocyte migration, macrophage lipid uptake, and smooth muscle cell migration and proliferation, primarily through the ERK1/2 pathway, thereby contributing to foam cell formation and atherosclerosis progression ([J Cardiovasc Transl Res. 2020 Apr;13\(2\):215-224.](#)). Literature on their interaction presents a nuanced picture. Some reports suggest that CXCL12 and CXCL14 can have additive or even synergistic chemotactic effects, for instance, in guiding the migration of immature myeloid dendritic cells ([Blood. 2009 Jun 4;113\(23\):5848-56.](#)). However, other studies indicate that high levels of CXCL14 can antagonize CXCL12 binding to CXCR4, enhance sequestration of CXCL12 by CXCR7, and thereby reduce CXCR4 signaling ([Eur J Cancer. 2015 Nov;51\(17\):2624-33.](#)). Synthetic CXCL14 has been shown to be as effective as recombinant protein in inhibiting CXCL12-mediated cell migration ([EBioMedicine. 2017 Oct;24:247-256.](#)). These seemingly conflicting reports likely underscore the high context-dependence of chemokine functions, which can vary with tissue microenvironment, disease state, and cellular composition.

Our study provides novel observations within the specific context of human carotid artery stenosis perivascular adipose tissue (PVAT). Our single-cell RNA sequencing analysis revealed the co-expression of CXCL12 and CXCL14 in CXCL14⁺ADSC in this pathological niche (**Response Figure 1**). Furthermore, SCENIC analysis indicated that their expression is potentially co-regulated by transcription factors from the same AP-1 family (FOSB for CXCL12 and FOS for CXCL14, **Response Figure 1**). Prior studies report co-expression and tightly related activities of FOS and FOSB, supporting a model of coordinated transcriptional regulation ([J Biol Chem. 2014 Feb 21;289\(8\):5025-39.](#)). This shared regulatory architecture, coupled with their co-expression, leads us to hypothesize that in the inflammatory environment of carotid stenotic PVAT, CXCL12 and CXCL14 may be co-upregulated by common upstream signals and act cooperatively rather than antagonistically in shaping the immune cell landscape. We acknowledge that this specific hypothesis requires future research.



Response Figure 1. Gene expression of CXCL12, CXCL14 and their candidate transcription factors in ADSC subsets. Violin plots show the expression levels of (top) the chemokine genes CXCL12 and CXCL14, and (bottom) their respective candidate transcription factors FOSB and FOS, as identified by SCENIC analysis, in the CXCL14⁺ADSC and CD55⁺ADSC subpopulations.

Supporting the functional relevance of this pathway in our system, our functional experiments showed that conditioned medium from CXCL14⁺ADSCs promoted macrophage migration/recruitment more potently than that from CD55⁺ADSCs. Critically, this pro-migratory effect was effectively inhibited by a CXCR4 antagonist (**Figure 7L-7N**). This key result demonstrates that, within our experimental model, the migratory signal, whether primarily driven by CXCL12, CXCL14, or both—converges on CXCR4, validating it as the central actionable node in this specific context.

We wish to clarify that while the CXCL14⁺ADSC subset and the interplay between CXCL12/CXCL14 are interesting findings, the primary focus of our study is the identification and functional characterization of the CD55⁺ADSC subpopulation as a major disease-relevant stromal subset since the flow cytometry confirmed a significant increase in the number of CD55⁺ADSCs in the CAS group. The detailed mechanistic dissection of the CXCL12/CXCL14 axis, though highly promising, extends beyond the scope of the current work. This has been addressed in the Discussion section (**Line 787**). Our data presented here offer valuable preliminary clues and a compelling rationale for future investigations into this complex chemokine interaction in vascular disease.

4) Lineage and plaque sequencing

The plaque scRNA-seq used in this analysis is not described in the Methods. Please provide details on plaque sample preparation, sequencing platform, and data processing, and sample QC. Clarify whether plaque and PVAT datasets were sequenced using the same chemistry. Matching PVAT and plaque samples could be of interest and strengthen the manuscript if interpreted appropriately.

Response: We thank the reviewer for highlighting the need for methodological detail regarding

the plaque scRNA-seq data, which was previously omitted. The following information has now been added to the Methods section.

Plaque sample preparation: Plaque specimens were processed within 1 h of surgical resection. Each specimen was thoroughly rinsed in DMEM, weighed, and digested in DMEM supplemented with 10% FBS, collagenase type IV (Sigma, C5138; 1 mg/mL), DNase (Sigma, DN25; 0.3 mg/mL), hyaluronidase (Sigma, H3506; 0.3 mg/mL), collagenase type XI (Sigma, C7657; 0.3 mg/mL), and collagenase type II (Sigma, C6885; 0.3 mg/mL) for 1 h at 37 °C. The digested tissue was sequentially filtered through 70 µm and 40 µm cell strainers. Dead cells were removed using a dead cell removal kit (Miltenyi Biotec 130-090-101) according to the manufacturer's protocol (**Line 904**).

Data processing, and sample QC: The plaque data was processed the same as PVAT samples as described in “Single-cell RNA-seq data preprocessing and quality control” of Methods. Briefly, cells with the gene number less than 400 or the proportion of mitochondrial genes greater than 15% were removed. To remove doublets, cells with greater than 8000 genes were first removed. Then we used DoubletFinder to further detect the doublets. Seurat objects from all samples were integrated using canonical correlation analysis (CCA) with 2000 features to identify integration anchors and remove batch effects (**Line 947**).

Sequencing platform and chemistry: Finally cells in each sample were loaded, and libraries were constructed using a Chromium single-cell kit (Control1, Control2, CAS1, CAS2, CAS3 and CAS4 of PVAT) or DNBelab C Series Single-Cell Library Prep Set (Control3_1, Control3_2, CAS5_1, CAS5_2, CAS6_1, CAS6_2, CAS7_1 and CAS7_2 of PVAT; CAS5_1, CAS5_2, CAS6_1, CAS6_2, CAS7_1 and CAS7_2 of Plaque). Single-cell suspensions were prepared according to the protocol of Chromium™ Single Cell 3' Solution (V3 chemistry) and DNBelab C Series Single-Cell Library Prep Set (MGI, 940-000507-00), respectively. Among them, PVAT and plaques of CAS5, CAS6, CAS7 and Control3 are matched samples from same patient (**Line 924**).

4.1) How do CD55 to CXCL14 trajectory analysis correlate with the mitochondrial lineage findings—do these analyses agree or suggest distinct relationships?

Response: Thank you for this important question. The two analyses are complementary and partially agree:

The trajectory analysis suggests a potential differentiation sequence within the ADSC compartment (from CD55⁺ADSC to CXCL14⁺ADSCs). The mitochondrial lineage analysis tests whether ADSCs are precursors to plaque endothelial cells (ECs).

Our supplemental mitochondrial lineage analysis (**Figure 3M and Supplementary Figure 5**) confirms a clonal relationship between CD55⁺ADSCs and CXCL14⁺ADSCs, while lineage analysis cannot determine the exact differentiation order or quantify the degree of contribution. We have added this clarification to the revised Results section (**Line 356**).

Therefore, these two analyses are not simply consistent but rather work in a stepwise manner to construct a more complete chain of evidence, pseudotime analysis and lineage analysis confirm the lineage between these two ADSC subpopulations, and trajectory analysis further provides a plausible hypothesis for the direction of differentiation. We have incorporated this explanation into the revised Discussion sections (**Line 778**).

5) Code and data reproducibility

The GitHub repository is helpful but incomplete, particularly regarding preprocessing of public datasets and plaque scRNA-seq data. Ensure that all figure-generating scripts and parameters are fully documented, and that every figure in the manuscript can be reproduced from the provided code and data.

Response: Thank you for pointing this out. We acknowledge that the initial version of our GitHub repository did not fully document the preprocessing steps for the public datasets and the plaque scRNA-seq data. To address this, we have substantially expanded the repository to ensure complete reproducibility. Specifically, we have added fully annotated preprocessing scripts, provided the complete preprocessing workflow for the plaque scRNA-seq dataset, uploaded all figure-generating scripts and ensured that every figure in the manuscript can be reproduced end-to-end.

5.1) In addition, the study uses two different sequencing platforms (10x Genomics and DNBelab), but the manuscript does not describe which samples were processed with which method or how potential platform-related batch effects were handled. Please clarify this in the Methods and ensure that integration and normalization steps account for cross-platform variability.

Response: Thank you for raising this important point. We apologize for not clearly describing the sequencing platforms and the corresponding batch-handling strategy in the initial submission. In our analysis, when integrating the datasets generated from 10x Genomics and DNBelab, we applied a unified workflow using Seurat's IntegrateData pipeline. Specifically, we selected 2,000 highly variable genes as anchor features, used SCT normalization consistently across all samples, and specified CCA as the dimensional reduction method when identifying anchors.

To evaluate cross-platform effects, we generated UMAP embeddings stratified by sequencing platform (**Supplementary Figure 1G**). These visualizations show that 10x Genomics-derived and DNBelab-derived cells mix well, with no evidence of major platform-driven segregation. We further quantified this using kBET, which demonstrated that the majority of cells are well mixed across platforms, indicating that platform-related batch effects were effectively mitigated. We have added a detailed description of platform assignment, preprocessing, and integration in the revised Methods section, and we have updated our GitHub repository to include clear, reproducible code reflecting all steps of the cross-platform integration workflow.

Minor points

- Provide per-sample QC plots (cell number, nGenes, nUMIs, %mito) after merging separate sequencing libraries in addition to the already provided QC metrix per sequencing batch.

Response: Thank you for the suggestion. We have now added per-sample QC plots (cell numbers, nGenes, nUMIs, and %mito) for all individual libraries after merging. These figures are included in the revised **Supplementary Figure 1A**.

- SCENIC (pySCENIC v0.10.2, mc9nr motif database) is described in the Methods, but no corresponding results or figures are presented. Either show the key transcription factor networks supporting ADSC heterogeneity or remove the section to avoid confusion.

Response: We thank the reviewer for this careful observation. Following the reviewer's suggestion, and as the analysis was not central to the main conclusions of this study, we have removed the mention of the SCENIC analysis from the Methods section to prevent any potential confusion. The manuscript has been updated accordingly.

- ScRNASeq composition: Ratio plots for immune subclusters are not informative and should be replaced with per-sample boxplots. Authors now provide explanation of Ro/e, however it is not mentioned in the methods or shown in the code how filtering on p-values was done.

Response: We thank the reviewer for these constructive suggestions. We agree that per-sample boxplots more clearly illustrate the composition changes across conditions. As suggested, we have replaced the ratio plots in the main figure with boxplots showing the proportions of key immune subclusters, including T cell, B cell, NK cell and myeloid cell subsets (**Figure 4D&4L, Figure 5C, Figure 6C&6I**).

We sincerely apologize for the lack of detail in our initial submission. Please find below a detailed description of the statistical testing and p-value filtering process for our spatial association analysis, which we have now added to the revised manuscript under the Methods section (**Line 979**).

1. Statistical Testing & P-value Adjustment:

In our analysis, the enrichment or depletion of a cell type within a specific group was statistically assessed using a two-sided Fisher's exact test. This test was performed for every cell type and group combination, comparing the observed distribution against the null hypothesis of random distribution. The resulting raw p-values from all tests were then corrected for multiple comparisons using the Benjamini-Hochberg (BH) method to control the false discovery rate (FDR). The outputs include the raw p.value, the odds ratio, and the adj.p.value.

2. Significance Filtering in Analysis:

In our study, we interpreted an FDR-adjusted p-value (adj.p.value) < 0.05 as statistically significant. Cell-type and group pairs meeting this criterion were considered to have significant group enrichment or depletion. While our core analysis function returns all results (as shown in the code), this significance threshold was rigorously applied in the downstream steps for result interpretation and drawing biological conclusions, ensuring that only robust associations were highlighted.

3. Code Implementation:

The complete workflow is implemented in R. The key steps are contained within two functions in our publicly available scripts: (1) The main wrapper function `do.tissueDist()` (in script [Roe calculation.R]) orchestrates the analysis and visualization. (2) The core statistical routine `test.dist.table()` performs the Fisher's exact test, calculates the odds ratio (OR), and applies the Benjamini-Hochberg p-value adjustment (see the line `adj.p.value:=p.adjust(p.value,"BH")`). The returned `data.table` contains all p-values and corrected values for explicit filtering.

- Authors insufficiently address the point of ADSC heterogeneity. In the provided code clusters are merged to achieve 2 main subtypes. This should be acknowledged in the text, the merging strategy should be shared and subcluster identification should be

performed.

Response: We thank the reviewer for raising this important point. We acknowledge that in our analysis, the subclusters identified within the ADSC population were merged to define the two main subtypes (CXCL14⁺ADSC and CD55⁺ADSC) presented in the study. This merging strategy was biologically guided, aiming to group transcriptionally similar clusters that shared key functional markers and to focus the subsequent functional analysis on the most distinct and prevalent subpopulations with clear relevance to the disease context.

Our approach is supported by established literature on ADSC heterogeneity. Studies in both mice and humans have consistently identified major stromal subsets. Merrick *et al.* ([Science. 2019 Apr 26;364\(6438\):eaav2501.](#)) defined DPP4⁺CD55⁺ interstitial progenitors and ICAM1⁺ committed preadipocytes in human adipose tissue, with CD142 expressed across both groups—a finding consistent with our data. In our study, the CD55⁺ADSC subset aligns closely with the described DPP4⁺ progenitor phenotype, while the CXCL14⁺ADSC subset is distinct from the ICAM1⁺ preadipocytes. The emergence of this CXCL14⁺ population is likely linked to the unique anatomic and pathological microenvironment of the carotid artery. Other studies involving human adipose tissue have also reported CD55 ([Nat Metab. 2021 Apr;3\(4\):469-484.](#)) and CXCL14 ([Nat Metab. 2020 Jan;2\(1\):97-109.](#)) as marker genes for ADSCs or closely related stromal subpopulations. This pattern is further supported by work in mouse aortic PVAT, which also describes two major ADSC subsets: Dpp4⁺ADSC (marked by *Cd55*) and Col4a2/Icam1⁺ADSC (marked by *Cxcl14*) ([Diabetes. 2022 Feb 1;71\(2\):321-328.](#)).

To transparently illustrate the underlying heterogeneity, we have now provided the unsupervised UMAP visualization showing five initial ADSC subclusters (**Supplementary Figure 3A**). The distinct expression patterns of *CD55* and *CXCL14* across these subclusters (**Supplementary Figure 3B-3C**) validate the biological basis for our final merged classification into two major groups. This approach allows us to balance the resolution of heterogeneity with the clarity needed for downstream functional interpretation.

- Authors now provide additional IF staining for CD55 and GPC3. It would be useful to quantify coexpression (PDGFRA/B, CD142, GPC3, CD55). For example, regions in Fig S3I shows 2 distinct regions for the subsets. Are these images representative? Does this reflect subsets are niche specific?

Response: We thank the reviewer for the suggestion to quantify the co-expression of CD55 and GPC3 via immunofluorescence (IF). We would like to clarify the respective roles of the IF and flow cytometry data in our study.

The IF staining presented in **Supplementary Figure 3L** was performed primarily to provide *in situ* spatial evidence that the two ADSC subpopulations, defined by distinct marker sets, indeed exist within the tissue architecture. The images shown are representative of the staining pattern observed across multiple sections.

In direct response to the reviewer's request, we performed a semi-quantitative co-localization analysis of CD55 and GPC3 signals on the IF sections. The area of co-localized signal constituted $8.93\% \pm 5.90\%$ (mean $\pm$ SD, n=11 sections) of the total CD55-positive area. This minor degree of overlap could stem from technical factors (e.g., non-specific binding, signal spillover) or rare biological instances (e.g., certain cell types like erythrocytes express both markers). Critically, this low co-localization percentage is consistent with the clear separation observed in the flow cytometry data (**Figure 2H**). This result confirms that the CD55 and GPC3 signals are largely distinct at the tissue level.

In addition, histological characterisation of the PVAT should be related to ADSC biology.

Response: We thank the reviewer for this suggestion. In the revised manuscript, we have repositioned the histological data (H&E and Masson staining) from the section focusing on ADSCs to the initial subsection describing the general pathological changes in PVAT.

While ADSCs are a component of PVAT, the observed large-scale pathological changes, such as adipocyte hypertrophy and tissue-level fibrosis, are complex outcomes of the broader PVAT inflammatory microenvironment involving multiple cell types (e.g., macrophages, as highlighted in our data). Our findings do not provide direct evidence that ADSCs are the primary drivers of these specific global tissue alterations. Presenting these histopathological findings at the outset provides essential context, establishing the overall diseased state of the tissue before delving into the detailed, single-cell resolution analysis of its cellular constituents, including ADSC subpopulations.

We believe this repositioned description offers a more logical flow: it first establishes the organ/tissue-level phenotype (pathological remodeling of PVAT, **Line 166**) and then investigates the cellular and molecular-level mechanisms underlying this phenotype, which includes the detailed biology of ADSCs and other cell types. This allows the role of specific ADSC subpopulations to be discussed later within the clearer context of the overall diseased tissue.

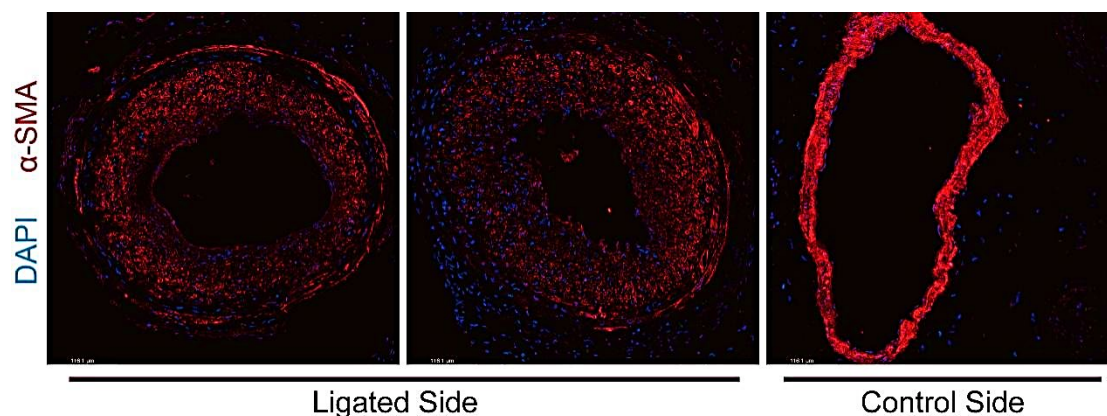
We hope this repositioning and explanation address the reviewer's concern. We are happy to reconsider if the reviewer has further suggestions regarding the logical connection.

- Address the discrepancy between human and mouse transplantation data: in mice, CD55⁺ ADSCs increase CD31 staining, whereas in human experiments, CXCL14⁺ ADSCs appear to increase Isolectin B4 and human CD31 signals. In Figure 3, it is unclear which panels correspond to human or mouse ADSCs, and the figure legend does not match the text description. Please clarify which experiment each panel represents and how quantification was performed.

Response: We thank the reviewer for this critical observation regarding the apparent discrepancy between the human and mouse transplantation data. The reviewer is correct to note a difference: in the *in vivo* C57 mouse model, CD55⁺ADSC transplantation led to a greater increase in CD31⁺ area (**Figure 3E**), whereas in the nude mouse stem cell transplantation experiment, following the intervention with human-derived ADSCs, the CXCL14⁺ADSC group showed a higher CD31-positive area compared to the CD55⁺ADSC group (**Figure 3L**). It is important to note that the CD31 signal in the CXCL14⁺ADSC group here is not capillary-shaped but rather appears as a diffusely expressed pattern (**Figure 3I**). Previous studies have also reported this kind of diffuse distribution of CD31 signal in the neointima, considered to be

associated with EndoMT ([Cardiovasc Res. 2023 Jul 4;119\(7\):1606-1618.](#)), as described in our Results (**Line 345**) and Discussion (**Line 763**). This is also why we supplemented the IF staining for isolectin B4 (**Figure 3K**) in the nude mouse samples. To further confirm whether the neointima in the nude mice samples truly exhibits potential EndoMT-like changes, we performed α -SMA immunofluorescence staining on samples from the CXCL14⁺ADSC group.

As shown in the **Response figure 2**, ligated carotid artery sections from the CXCL14⁺ADSC group displays robust α -SMA expression within the neointima with a positive staining in the media of the control side serving as an internal positive control. This finding is consistent with an EndoMT-like phenotype.



Response Figure 2. Immunofluorescence for α -SMA in the CXCL14⁺ADSC transplantation group indicates EndoMT in the neointima.

While this observation provides valuable mechanistic insight, we have chosen to present it primarily in this response. The central focus of the present study remains the functional characterization of the CD55⁺ADSC subpopulation as the major disease-relevant subset. A comprehensive investigation into the EndoMT pathway specifically driven by CXCL14⁺ADSCs, while highly interesting, constitutes a complex endeavor that merits dedicated future research.

- Clarify all figure legends to ensure consistency and readability. Clearly specify, for example, which panels correspond to human vs. mouse ADSC experiments, and which mouse model was used for which panel. This will help readers interpret species, experimental model, and measurement context without referring back to the text.

Response: Thank you for this valuable suggestion. We agree that clarifying the experimental context in the figure legends is crucial for reader comprehension. Accordingly, we have thoroughly revised all figure legends to ensure consistency and readability.

Specifically, we have now explicitly stated the species origin (human vs. mouse) and the specific color used in the relevant figure panels. For instance, the legends for panels in **Figure 2G, 2J, 3D, 3I and 3J** now specify the corresponding experiment, target and color. Furthermore, details regarding species and models have been added to the legends for **Figure 3B–3M**.

- Avoid causal statements (“ADSCs promote angiogenesis”) when correlations are moderate ($R \approx 0.6$)

Response: We appreciate the reviewer’s attention to this important nuance. We agree that the language should precisely reflect the nature of the evidence. In response, we have revised the

text on Line 243 to replace the causal phrasing with a more accurate description “Higher numbers of ADSCs are associated to increased capillary formation.” in **Line 254**.

Reviewer #2 (Remarks on code availability):

The authors provide a GitHub repository with R code for their analyses, but the figures are only partially reproducible. All analysis scripts are contained within a single large R file. A structured folder layout with separate scripts per figure or analysis step would substantially improve usability. Public data preprocessing (especially for external datasets and plaque scRNA-seq) is not included. I did not attempt to run the code.

Response: Thank you for this helpful feedback. We agree that the initial structure of the GitHub repository limited usability and reproducibility. In the revised version, we have substantially reorganized and expanded the repository:

- 1. Restructured folder layout:** Each major analysis step (preprocessing, integration, clustering, DEG analysis, pseudobulk, trajectory, etc.) now resides in a dedicated directory.
- 2. Separate scripts per figure:** All figures in the manuscript now have individual, self-contained scripts, each linked to the specific intermediate objects required for reproducibility.
- 3. Expanded preprocessing documentation:** We have added complete preprocessing pipelines for all external public datasets and for the plaque scRNA-seq dataset, including raw-data loading, QC, filtering, and normalization.
- 4. Improved reproducibility:** Every figure can now be regenerated directly from the provided scripts and data objects following the step-by-step instructions.

We believe these revisions make the codebase significantly more transparent, modular, and reproducible.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Q1: The authors have responded to the points made by the reviewer in detail and added relevant information, experimental data and explanations. I am of the opinion that the key finding of this paper is supported.

A1: Thank you very much for your positive feedback and for your valuable suggestions throughout the review process. We appreciate your time and expertise in helping us improve our manuscript.