

Modular Assembly of Arterial Organoids Enables Rapid Blood Perfusion to Ameliorate Ischemic Diseases

Corresponding Author: Professor Kai Wang

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

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

This study presents a scalable, chemically defined, dynamic bioreactor strategy to generate free-floating arterial organoid-like structures from human iPSCs and evaluates their therapeutic efficacy following local delivery in murine models of hindlimb ischemia and myocardial infarction. The integration of a manufacturing-oriented differentiation workflow with in vivo perfusion, functional recovery, and immune modulation analyses makes the work timely and potentially impactful for translational ischemia therapy. The in vivo perfusion and functional data are compelling and suggest advantages over single-cell transplantation. However, several central conceptual claims—particularly regarding pre-assembled arterial architecture and definitive arterial identity—appear stronger than the in vitro structural evidence currently supports. With additional architectural validation, quantitative lineage analysis, and improved clarity in several mechanistic aspects, the study would be substantially strengthened.

1. The evidence for pre-assembled vascular architecture prior to delivery requires further clarification. The data support the generation of vascular cell-enriched spheroids capable of forming perfused networks in vivo, yet convincing in vitro demonstration of pre-formed, lumenized, and interconnected vascular architecture within the organoids before transplantation remains limited. Marker co-expression and whole-mount staining confirm lineage specification but do not definitively establish continuous lumens, junctional continuity, or interconnected vascular geometry within individual organoids. High-resolution confocal z-stacks with orthogonal views and three-dimensional reconstruction would be necessary to substantiate claims of pre-assembled vascular units.
2. The assertion of arterial identity requires stronger validation. Although arterial-associated markers such as DLL4, CXCR4, and SOX17 are reported, these alone do not conclusively demonstrate stable arterial lineage commitment. Broader arterial-versus-venous gene set analysis, inclusion of additional arterial and venous markers, and quantitative comparison of lineage programs would strengthen the claim. It would also be important to determine whether arterial marker expression persists following in vivo engraftment. At present, the findings convincingly demonstrate vascular endothelial identity but do not yet fully establish arterial dominance.
3. The interpretation of apical-basal polarity appears overstated. The localization of podocalyxin at the outer surface and collagen IV internally is suggestive but insufficient to conclude true luminal polarization in the absence of orthogonal sections and junctional marker co-staining. Without clear visualization of a defined internal lumen, the pattern may reflect an endothelial shell with extracellular matrix deposition rather than bona fide apical-basal organization. Additional confocal z-stacks with orthogonal projections and co-staining for junctional markers would strengthen this conclusion, or the wording should be moderated.
4. The assessment of differentiation efficiency requires more rigorous quantification. The flow cytometry data do not clearly report the percentage of endothelial and mural cells within the total live singlet population, nor is the gating hierarchy fully described. The definition of “immature cells” is not provided, making it difficult to interpret population distributions. Explicit reporting of population frequencies and gating strategies is necessary to support claims of robust and homogeneous differentiation.
5. The reported differential expression of TAL1 between two-dimensional and three-dimensional conditions appears inconsistent with the presented quantification and should be revised unless supported by statistically significant differences.
6. The in vivo vessel characterization implies predominantly arterial structures based on α -SMA positivity, yet α -SMA expression alone does not establish arterial identity. Inclusion of additional lineage markers and quantitative analysis across multiple regions would improve interpretive rigor.
7. The macrophage analyses raise questions regarding temporal dynamics. Macrophage abundance appears to peak

several days after transplantation, yet mechanistic transcriptomic analyses focus primarily on the earliest time point. The rationale for this choice should be clarified, and additional time-point analyses would help determine whether the reported macrophage phenotype and VEGFA induction persist or evolve. Furthermore, the terminology describing depletion of “circulating macrophages” should be revised, as clodronate liposomes primarily target circulating monocytes and certain macrophage populations rather than all tissue-resident macrophages.

8. Longer-term durability of vascular integration would enhance translational relevance. Perfusion and integration are assessed for only a few weeks, and it remains unclear whether human-derived endothelial cells persist long term or are gradually replaced by host vasculature. Extended follow-up would strengthen the therapeutic implications.

9. The absence of a formal sample size calculation reduces statistical rigor, particularly given the functional endpoints assessed. Although common in exploratory preclinical studies, stronger justification of group sizes or retrospective power analysis would improve methodological transparency.

10. The single-cell RNA-sequencing analyses would benefit from greater transparency regarding integration quality, batch effect correction, and cluster stability metrics, given the mechanistic conclusions drawn from these data.

11. Given the emphasis on scalability and translational readiness, reporting batch-to-batch variability, differentiation reproducibility, and yield consistency would substantially strengthen the manufacturing claims.

Reviewer #3

(Remarks to the Author)

Authors developed a new model: homogeneous arterial organoids (AOs) from human iPSCs using 3D bioreactor system. They show that these spheroid AOs rapidly anastomosed with the host vasculature, enhancing perfusion recovery and preserving tissue function.

This work is very interesting in the context of ischemic disease, and was demonstrated with robust methodology.

However, one can wonder what is the difference with Blood Vessel Organoids (BVOs), that have been extensively studied and demonstrate enormous potential. BVOs are only mentioned once (ref 15), but no comparison has been discussed.

Through out this manuscript, it should have been compared and discussed, and even possibly added to the experimental set up.

Taken together, in the current state of the manuscript, the novelty of this work is not sufficient to be published in Nature Communications.

Reviewer #4

(Remarks to the Author)

Kong et al reported that human iPSC-derived arterial organoids exhibit therapeutic efficacy in murine models of hindlimb ischemia and myocardial infarction (MI). They showed that transplantation of AOs induces recruitment of VEGF-A-expressing macrophages, as a potential mechanism to promote tissue repair after hindlimb ischemia. Overall this is a well designed study with solid data supporting the robust therapeutic efficacy, particularly in preclinical MI settings. However, there are several fundamental issues regarding the animal models and the mechanism interpretation, in addition to concerns regarding the clarity and robustness of the work.

Major

1) The whole work depends on under-optimized models of athymic nude mice treated with human AOs. This immunocompromised model represents an issue for future human translation. Ideally the experiments should be performed in 1) treatment of mouse AOs (using similar strategy) in immunocompetent mice as a concept-of-proof and 2) treatment of human AOs in humanized mice.

2) Regarding the mechanism study, VEGF-A-producing macrophages do not essential to be reparative. This study was performed only in the hindlimb ischemic model. In contrast, recruited macrophages in MI are often CCR2+ with proinflammatory phenotypes and inhibit cardiac repair, while residential macrophages are more reparative. The authors should perform the related single-cell RNAseq analysis of residential and recruited macrophages/monocytes in MI model.

3) Fig. 1D, CD31 expression is not robust in the EC population, questioning how stable the endothelial phenotype/genotype in this iPSC-induced AO system. It also surprising to see CD31 is not expressed in the population of Arterial ECs (Fig. 2A vs Fig. 2D, PECAM1 expression). EC-tracing of the transplanted AOs in vivo is suggested in a long term condition.

4) Fig. 2. The transcriptional factor trajectory study is relatively descriptive. Most of data were based on co-relation study. Functional tests with knockdown of the identified transcriptional factors would be suggested.

5) Fig. 3E. Only small portion of vasculature was perfused, as shown to be Lectin+.

6) Fig. 3. What is the rationale of AO injection into kidney? The majority of the work has focused on ischemic diseases in hindlimb and heart. A consistent model would be suggested.

7) Fig. 4E, potential group bias. Ischemia induction in AO group seemed less severe than Vehicle group (at day 1), which may explain why AO group recovered quicker.

8) Fig. 5. The key information of MI study is missing. When echo was done after MI induction? What are the EF changes over time after treatment?

9) Fig. 6. The mechanism proposed seems to be descriptive. It is unclear how VEGF-A-producing macrophages will promote tissue repair (though it could stimulate angiogenesis), serving as the key mechanism. In addition, it is unclear the macrophage enrichment is due to infiltration or residential proliferation/migration. There was no phenotype analysis or immune profiling for macrophages. The key findings need to validate by flow cytometry.

Minor

1. Fig. 4H. It is suggested to include AO staining in tri-chrome Masson IHC, to show the tissue pathology around AOs.

2. Fig. 6B. HLI surgery itself seems to induce robust macrophage infiltration.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have addressed all my comments.

The authors have made a reasonable effort to address Reviewer #3's comments by expanding the discussion of the blood vessel organoid (BVO) literature, citing additional foundational studies, and clarifying the intended application of their arterial organoid platform. However, the response does not fully address the reviewer's central concern regarding novelty. Much of the rebuttal focuses on differences in application (therapeutic regeneration versus disease modeling), the use of a dual fluorescent reporter line, and downstream mechanistic studies following transplantation. While these are important strengths of the study, they do not clearly demonstrate how the arterial organoid platform itself represents a conceptual or technological advance over existing BVO systems. In particular, the response does not sufficiently explain why the homogeneous arterial composition, scalable bioreactor differentiation strategy, or other distinguishing features could not be achieved using established BVO methodologies, nor does it directly address the reviewer's suggestion of a more rigorous comparison with BVOs, either experimentally or through a deeper discussion of their biological and functional differences.

Reviewer #4

(Remarks to the Author)

Most of my previously raised concerns have been addressed. One more comment: Fig. R8D, the basal-line LDI images of control and AO treatment seem different, but the quantified results have no difference. This is inconsistent. Also suggest to include all data presented in the response letter in the figures or supplementary figures.

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A point-by-point response to the reviewer's comments
Nature Communications manuscript (NCOMMS-26-034510)

Reviewer #2

This study presents a scalable, chemically defined, dynamic bioreactor strategy to generate free-floating arterial organoid-like structures from human iPSCs and evaluates their therapeutic efficacy following local delivery in murine models of hindlimb ischemia and myocardial infarction. The integration of a manufacturing-oriented differentiation workflow with in vivo perfusion, functional recovery, and immune modulation analyses makes the work timely and potentially impactful for translational ischemia therapy. The in vivo perfusion and functional data are compelling and suggest advantages over single-cell transplantation. However, several central conceptual claims—particularly regarding pre-assembled arterial architecture and definitive arterial identity—appear stronger than the in vitro structural evidence currently supports. With additional architectural validation, quantitative lineage analysis, and improved clarity in several mechanistic aspects, the study would be substantially strengthened.

We sincerely thank the reviewer for the thoughtful assessment and for recognizing the significance and innovation of our study. We appreciate the constructive feedback and have addressed each point below.

***Q1** - The evidence for pre-assembled vascular architecture prior to delivery requires further clarification. The data support the generation of vascular cell-enriched spheroids capable of forming perfused networks in vivo, yet convincing in vitro demonstration of pre-formed, lumenized, and interconnected vascular architecture within the organoids before transplantation remains limited. Marker co-expression and whole-mount staining confirm lineage specification but do not definitively establish continuous lumens, junctional continuity, or interconnected vascular geometry within individual organoids. High-resolution confocal z-stacks with orthogonal views and three-dimensional reconstruction would be necessary to substantiate claims of pre-assembled vascular units.*

Response 1 – We thank the reviewer for this important comment. We agree that our current imaging data do not definitively demonstrate continuous lumen formation, junctional continuity, or fully interconnected vascular architecture throughout individual organoids prior to transplantation.

Our intention was to show that arterial organoids (AOs) contain organized vascular cell populations exhibiting endothelial cells (ECs) and mural cells (MCs) identities rather than to claim the existence of fully mature vascular networks before implantation. We have revised the manuscript (**Result section, Lines 161-165**) in the revised manuscript to moderate this interpretation and now describe AOs as vascular cell-enriched organoids containing organized ECs and MCs structures, rather than fully pre-assembled vascular units.

***Q2** - The assertion of arterial identity requires stronger validation. Although arterial-associated markers such as DLL4, CXCR4, and SOX17 are reported, these alone do not conclusively demonstrate stable arterial lineage commitment. Broader arterial-versus-venous gene set analysis, inclusion of additional arterial and venous markers, and quantitative comparison of lineage programs would strengthen the claim. It would also be important to determine whether arterial marker expression persists following in vivo engraftment. At present, the findings convincingly demonstrate vascular endothelial identity but do not yet fully establish arterial dominance.*

Response 2 – We thank the reviewer for this valuable comment. To spatially and quantitatively validate arteriovenous specification, we performed a blended feature mapping of the arterial and venous programs (**Figure R1**). As shown in the blended UMAP space, the endothelial population clearly bifurcates into a

committed arterial trajectory (red) and a committed venous trajectory (blue), with minimal co-expression, demonstrating mainly arterial lineage identity.

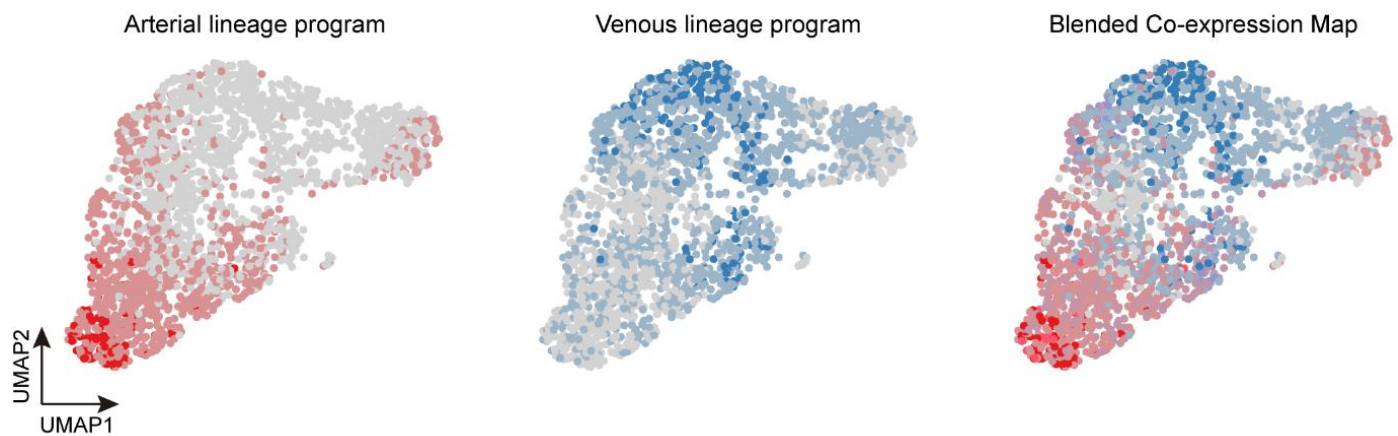


Figure R1. Blended feature mapping of arterial and venous lineage programs in ECs. Arterial lineage cells are shown in red, whereas venous lineage cells are shown in blue. Cells co-expressing both signatures are displayed as intermediate colors.

We further examined the vascular identity of engrafted AOs-derived vessels *in vivo*. Immunofluorescence staining demonstrated the presence of SOX17-positive human-derived endothelial structures, indicating persistence of arterial-associated characteristics after transplantation (**Figure R2A**). In addition, COUP-TFII-positive human-derived vessels were also observed (**Figure R2B**), suggesting that AOs-derived cells contribute to the formation of both arterial- and venous-like vascular structures within the regenerated tissue. These findings provide additional support for arterial specification of AOs while also highlighting the vascular plasticity and remodeling that occur following engraftment.

Accordingly, we have added these new data to **Figure S5** and revised the manuscript (**Result section, Lines 418-429; Supplemental Figures section, Lines 440-449**) to describe the emergence of both arterial- and venous-like vascular structures following engraftment.

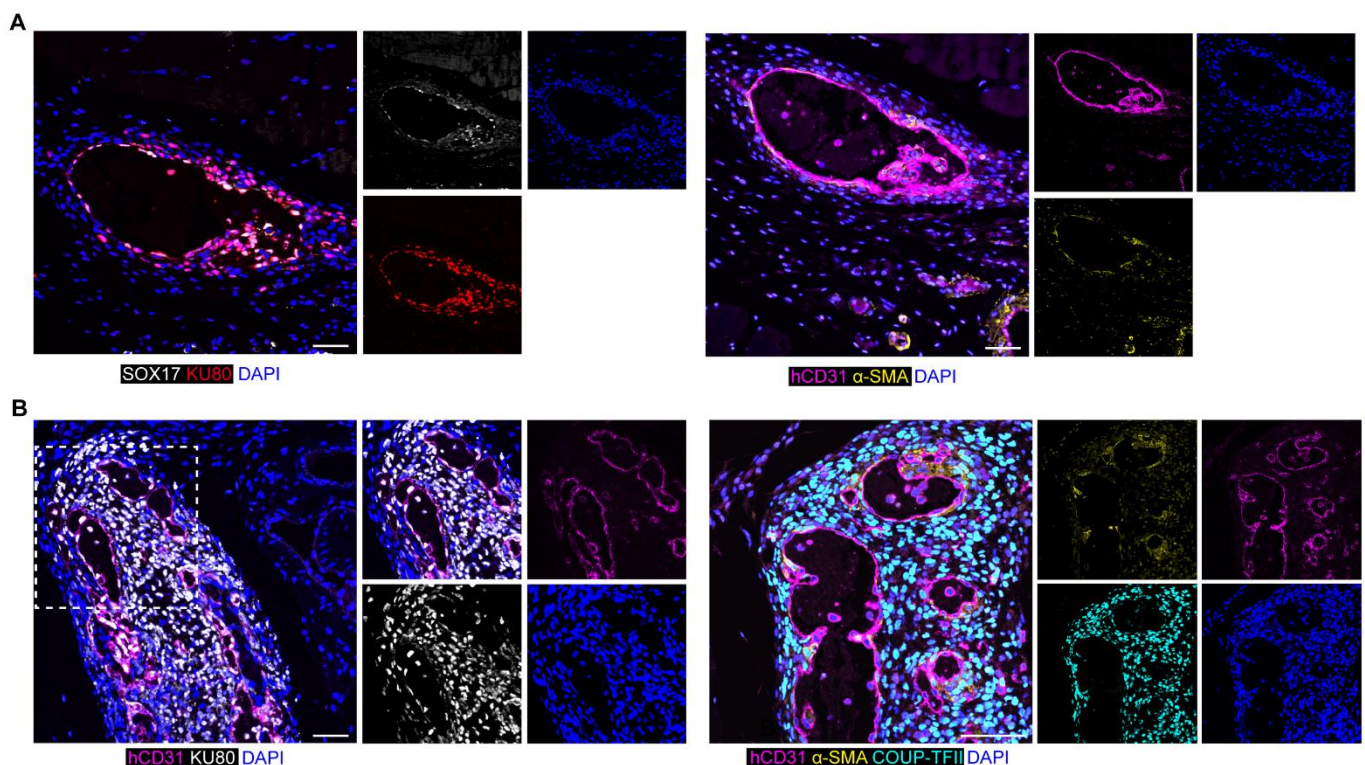


Figure R2. Arterial and venous identity of engrafted human-derived vessels. (A) Representative immunofluorescence images showing expression of the arterial-associated marker in human-derived vascular structures following AOs transplantation. Staining for arterial marker (SOX17⁺, white), human nuclear antigen (KU80⁺, red), human ECs (CD31⁺, magenta), MCs (α -SMA⁺, yellow), and nuclei counterstained with DAPI (blue). (B) Representative immunofluorescence images showing expression of the venous marker in human-derived vascular structures following AOs transplantation. Staining for venous marker (COUP-TFII⁺, cyan), human nuclear antigen (KU80⁺, white), human ECs (CD31⁺, magenta), MCs (α -SMA⁺, yellow), and nuclei counterstained with DAPI (blue). Scale bar: 100 μ m.

Q3 - The interpretation of apical–basal polarity appears overstated. The localization of podocalyxin at the outer surface and collagen IV internally is suggestive but insufficient to conclude true luminal polarization in the absence of orthogonal sections and junctional marker co-staining. Without clear visualization of a defined internal lumen, the pattern may reflect an endothelial shell with extracellular matrix deposition rather than bona fide apical–basal organization. Additional confocal z-stacks with orthogonal projections and co-staining for junctional markers would strengthen this conclusion, or the wording should be moderated.

Response 3 – We thank the reviewer for this thoughtful comment. We agree that the localization of podocalyxin and collagen IV, while suggestive of spatial organization within AOs, does not by itself definitively establish bona fide apical–basal polarity. To avoid overinterpretation of the data, we have revised the relevant text throughout the manuscript and moderated our conclusions. Specifically, rather than stating that ECs within AOs "developed polarized architecture", we now describe the observed distribution of podocalyxin and collagen IV as being "consistent with the emergence of apical–basal organization" under 3D culture conditions. We have also revised the comparison with native blood vessels to indicate that the observed pattern resembles certain aspects of vascular spatial organization, rather than definitively demonstrating mature vascular polarity (**Results section, Lines 165-173**).

Q4 - The assessment of differentiation efficiency requires more rigorous quantification. The flow cytometry data do not clearly report the percentage of endothelial and mural cells within the total live singlet population, nor is the gating hierarchy fully described. The definition of "immature cells" is not provided, making it difficult to interpret population distributions. Explicit reporting of population frequencies and gating strategies is necessary to support claims of robust and homogeneous differentiation.

Response 4 – We thank the reviewer for this helpful comment. We agree that the original flow cytometry visualization did not sufficiently describe the gating strategy and could lead to ambiguity regarding the definition of the "immature cell" population.

To address this concern, we have revised the figure presentation. Specifically, we replaced the t-SNE dimensionality-reduction plots with conventional flow cytometry dot plots to eliminate the confounding effects of extreme high-fluorescence values, which can distort population distributions in t-SNE visualizations. Furthermore, EC and MC differentiation efficiencies are now directly quantified using established lineage markers, including CD144, CD31, DLL4, CXCR4, and PDGFR β . The explicit gating hierarchy and population frequencies are now fully reported within the total live singlet population (**Figure R3**).

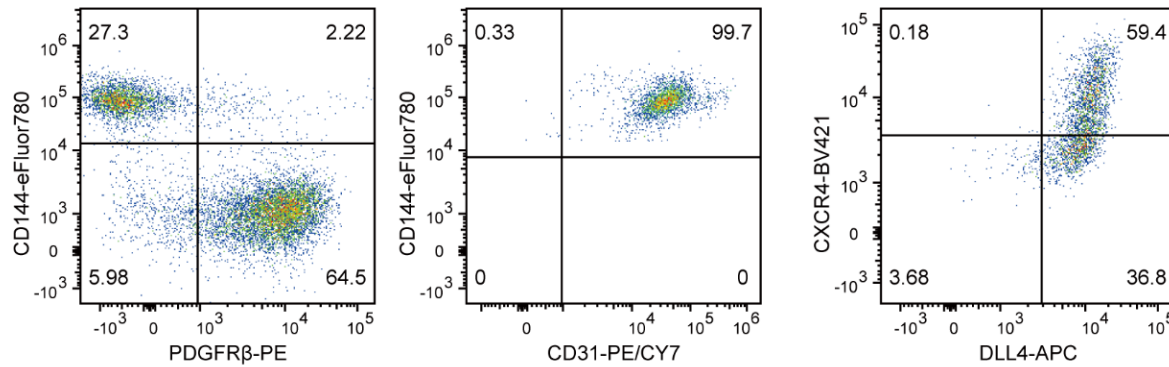


Figure R3. Flow cytometric assessment of AOs differentiation efficiency. Representative flow cytometry dot plots showing the frequencies of ECs and MCs populations within the live singlet population.

In addition, we have removed the term “immature cells” from the revised manuscript. In the original analysis, this designation referred to cells that did not express the ECs or MCs markers used for lineage assignment rather than a rigorously defined biological population. To avoid overinterpretation, these cells are no longer presented as a distinct cell type.

Q5 - The reported differential expression of TAL1 between two-dimensional and three-dimensional conditions appears inconsistent with the presented quantification and should be revised unless supported by statistically significant differences.

Response 5 – We thank the reviewer for carefully identifying this inconsistency. Upon re-examination of the original data and statistical analysis, we confirmed that TAL1 expression at differentiation day 4 was significantly higher in the 3D group than in the 2D group. The “n.s.” label shown in the original figure was introduced inadvertently during figure preparation and does not reflect the actual statistical result.

We have corrected the statistical annotation in the revised **Figure S2E** and revised the corresponding text (**Results section, Lines 285-292**) to accurately describe the significant upregulation of TAL1 under 3D differentiation conditions. These corrections do not affect the overall conclusions of the study.

Q6 - The in vivo vessel characterization implies predominantly arterial structures based on α -SMA positivity, yet α -SMA expression alone does not establish arterial identity. Inclusion of additional lineage markers and quantitative analysis across multiple regions would improve interpretive rigor.

Response 6 – We thank the reviewer for this insightful comment. We agree that α -SMA expression alone is insufficient to establish arterial identity.

Our intention in establishing the PECAM1-tdTomato and ACTA2-EGFP reporter iPSC line was not to classify the newly formed vessels as arterial structures based on α -SMA positivity. Rather, we used the dual reporter system to dynamically visualize the survival, engraftment, self-organization, and vascular network formation of AOs following transplantation. In this context, ACTA2/ α -SMA was used primarily as a mural cell marker to track the association of mural cells with AOs-derived endothelial structures and to assess vessel stabilization and maturation.

To avoid overinterpretation, we have revised the corresponding text (**Results section, Lines 332-336**) in the manuscript and now describe these vessels as AOs-derived vascular structures composed of ECs and MCs, rather than arterial vessels.

Regarding arterial identity, as noted above (Response to **Q2**), we further characterized the vascular identity of engrafted AOs-derived vessels using arterial and venous markers. These analyses demonstrated the presence of SOX17-positive and COUP-TFII-positive human-derived vascular structures *in vivo*, supporting the formation of a heterogeneous vascular network after transplantation. Therefore, α -SMA staining in Figure 3 should be interpreted as a marker of mural cell coverage and vessel maturation rather than definitive evidence of arterial identity.

Q7 - The macrophage analyses raise questions regarding temporal dynamics. Macrophage abundance appears to peak several days after transplantation, yet mechanistic transcriptomic analyses focus primarily on the earliest time point. The rationale for this choice should be clarified, and additional time-point analyses would help determine whether the reported macrophage phenotype and VEGFA induction persist or evolve. Furthermore, the terminology describing depletion of "circulating macrophages" should be revised, as clodronate liposomes primarily target circulating monocytes and certain macrophage populations rather than all tissue-resident macrophages.

Response 7 – We thank the reviewer for this insightful comment. We agree that temporal characterization of macrophage responses is important for understanding the mechanism of AOs-mediated tissue repair.

We would like to clarify that our scRNA-seq analysis included both Day 1 and Day 3 after AOs transplantation. As shown in the newly presented analyses (**Figure R4A, R4B**), we evaluated cellular composition and macrophage transcriptional profiles across all four groups (D1 HLI+Vehicle, D1 HLI+AO, D3 HLI+Vehicle, and D3 HLI+AO). Monocyte/macrophage abundance increased progressively from Day 1 to Day 3 (**Figure R4C**), indicating dynamic recruitment and expansion during the early phase of tissue repair.

Importantly, elevated *Vegfa* expression in macrophage subsets was observed not only at Day 1 but also at Day 3 following AOs treatment (**Figure R4D**). These findings suggest that AOs-induced VEGFA upregulation is sustained during the early regenerative phase rather than being restricted to the earliest time point.

To improve clarity, we have revised the corresponding text (**Results section, Lines 578-592**) to more explicitly describe the temporal dynamics of monocyte/macrophage accumulation and *Vegfa* expression following AOs transplantation.

We also appreciate the reviewer's comment regarding the terminology used in the clodronate liposome experiments. Our original wording was based on two independent assessments of depletion efficiency, including complete blood count analysis of peripheral blood and flow cytometric quantification of F4/80⁺ cells in muscle tissue. However, we agree that clodronate liposomes primarily reduce circulating monocytes and subsets of phagocytic macrophages rather than eliminating all tissue-resident macrophages. Therefore, we have revised the terminology throughout the manuscript to more accurately describe the affected monocyte/macrophage populations (**Expanded Methods section, Lines 199-207**).

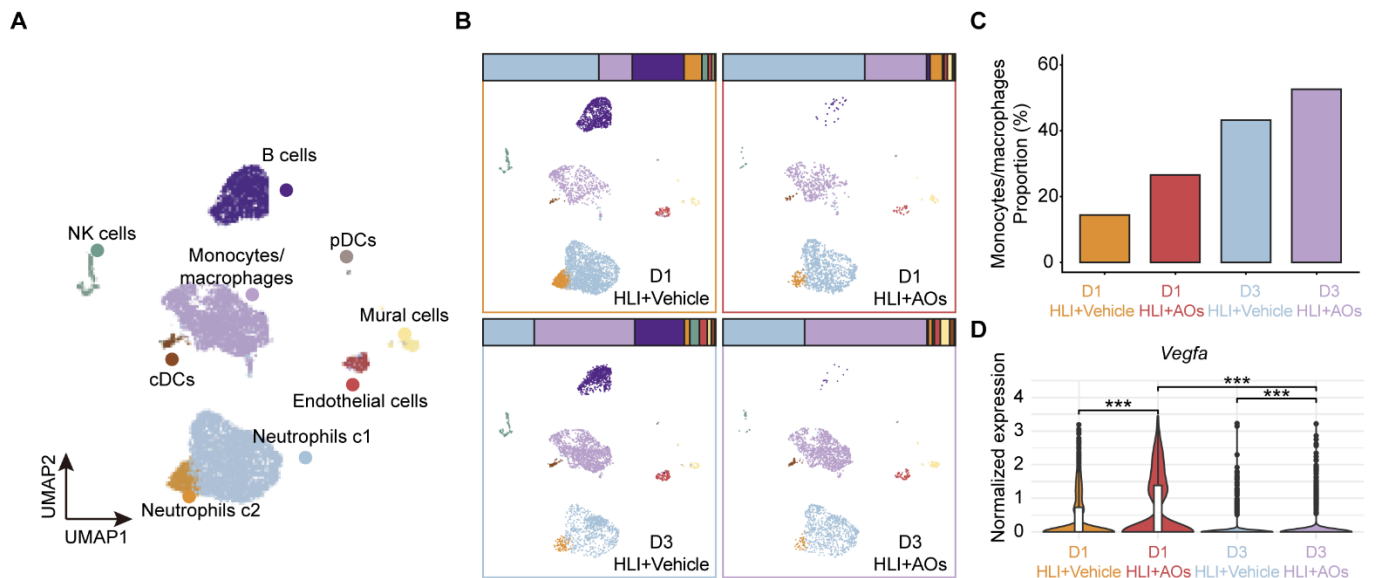


Figure R4. Temporal dynamics of monocyte/macrophage responses following AOs transplantation. (A) UMAP projection of single-cell transcriptomes from ischemic muscle tissues collected at Day 1 and Day 3 after treatment. (B) UMAP visualization of cellular populations in D1 HLI+Vehicle, D1 HLI+AOs, D3 HLI+Vehicle, and D3 HLI+AOs groups. (C) Proportions of monocyte/macrophage populations across the four experimental groups. (D) Violin plots showing *Vegfa* expression in macrophage subsets across the indicated groups.

Q8 - Longer-term durability of vascular integration would enhance translational relevance. Perfusion and integration are assessed for only a few weeks, and it remains unclear whether human-derived endothelial cells persist long term or are gradually replaced by host vasculature. Extended follow-up would strengthen the therapeutic implications.

Response 8 – We thank the reviewer for this important suggestion. To address the long-term durability of vascular integration, we performed additional longitudinal intravital imaging analyses of AOs transplanted under the kidney capsule and followed the grafts for up to 3 months after transplantation.

At 1 month post-transplantation, extensive AOs-derived vascular networks remained clearly detectable and were functionally perfused, as demonstrated by dextran labeling (**Figure R5A**). At 2 months, the vascular structures were still readily observed; however, dextran perfusion was largely absent, suggesting a decline in functional perfusion (**Figure R5B**). By 3 months, PECAM1-tdTomato-positive vascular networks were no longer detectable, while only weak residual ACTA2-EGFP signals remained. The transplantation site showed the presence of whitish tissue within the kidney subcapsular space (**Figure R5C**).

These findings suggest that AOs-derived vascular networks can persist and remain functionally perfused for extended periods after transplantation, but undergo substantial remodeling over time. The progressive loss of donor-derived endothelial signals indicates that human-derived vascular structures may eventually regress or be replaced by host-derived vasculature.

Accordingly, we have added these new data to **Figure S4** and revised the manuscript (**Result section, Lines 357-371; Supplemental Figures section, Lines 426-430**).

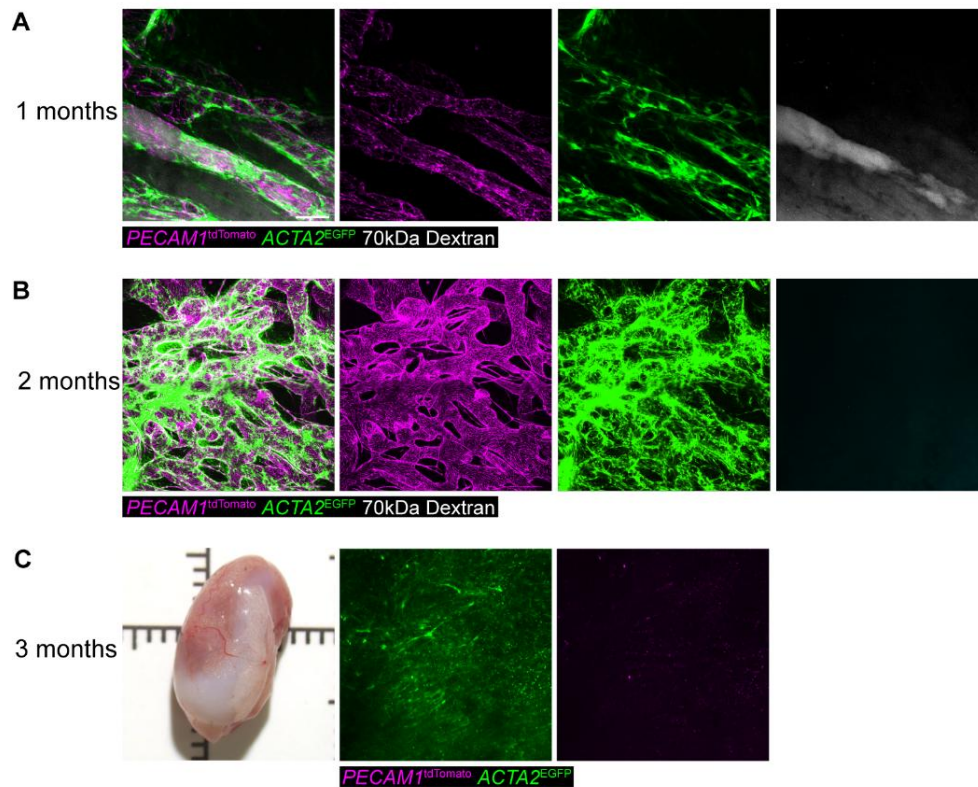


Figure R5. Long-term evaluation of AOs-derived vascular networks following kidney subcapsular transplantation. Representative intravital confocal microscopy images of AOs-derived vascular networks 1 month (**A**), 2 months (**B**) and 3 months (**C**) after transplantation under the kidney capsule. Scale bar, 100 μ m.

***Q9** - The absence of a formal sample size calculation reduces statistical rigor, particularly given the functional endpoints assessed. Although common in exploratory preclinical studies, stronger justification of group sizes or retrospective power analysis would improve methodological transparency.*

Response 9 – We thank the reviewer for this valuable comment. We agree that transparent reporting of sample size determination is important for ensuring methodological rigor.

We acknowledge that a formal a priori sample size calculation was not performed for this exploratory preclinical study. Sample sizes were determined based on our previous experience with similar animal models and organoid transplantation studies, as well as group sizes commonly reported in the related literature^{1–6}. These sample sizes consistently allowed detection of biologically meaningful differences in functional and histological endpoints.

To improve methodological transparency, we have now added a statement in the corresponding text (**Expanded Methods section, Lines 357-360**).

***Q10** - The single-cell RNA-sequencing analyses would benefit from greater transparency regarding integration quality, batch effect correction, and cluster stability metrics, given the mechanistic conclusions drawn from these data.*

Response 10 – We thank the reviewer for this valuable comment. To improve transparency regarding data integration and clustering robustness, we performed additional analyses.

Integration quality and batch effect correction: For the mouse scRNA-seq dataset, batch effects were corrected using Harmony. We now provide UMAP visualizations before and after integration (**Figure R6A**), showing improved mixing of cells across samples while preserving biological heterogeneity.

To quantitatively assess integration performance, we calculated the Local Inverse Simpson's Index (LISI). The median integration LISI (iLISI) increased from 1.51 to 2.41 after Harmony correction, indicating effective batch mixing, whereas the cell-type LISI (cLISI) remained close to 1.0, supporting preservation of distinct cell identities (**Figure R6B**).

Cluster stability assessment: To evaluate clustering robustness, we performed bootstrap subsampling by randomly selecting 80% of cells and repeating the clustering workflow. Cluster assignments were compared with the original clustering results using the Adjusted Rand Index (ARI), yielding a mean ARI of 0.908, indicating highly reproducible clusters (**Figure R6C**).

Clustree analysis demonstrated stable cluster structures across a range of clustering resolutions (0.1–1.0), supporting the robustness of the selected resolution (0.2) used for downstream analyses (**Figure R6D**).

These additional analyses have been incorporated into the revised manuscript (**Expanded Methods section, Lines 267-298**).

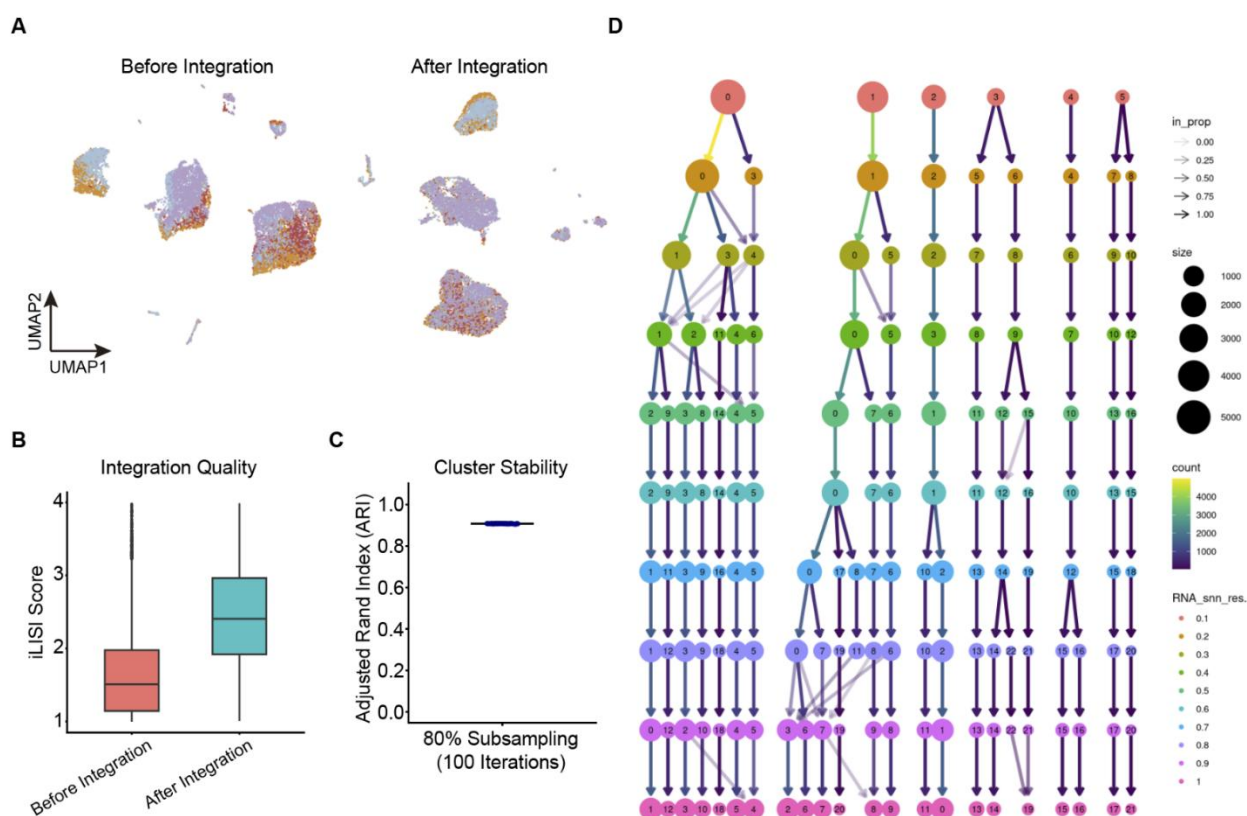


Figure R6. Assessment of integration quality and cluster stability for the mouse scRNA-seq dataset. (A) UMAP visualization of cells before and after Harmony integration, colored by sample identity. (B) Quantification of integration quality using the Local Inverse Simpson's Index (LISI), including integration LISI (iLISI) and cell-type LISI (cLISI). (C) Cluster stability analysis based on bootstrap subsampling (80% of cells per iteration). Cluster reproducibility was evaluated using the Adjusted Rand Index (ARI). (D) Clustering tree analysis generated using the clustree package across resolutions ranging from 0.1 to 1.0.

Q11 - Given the emphasis on scalability and translational readiness, reporting batch-to-batch variability, differentiation reproducibility, and yield consistency would substantially strengthen the manufacturing claims.

Response 11 – We thank the reviewer for this valuable suggestion. We agree that demonstrating differentiation reproducibility across independent stem cell lines is important for supporting the scalability and translational potential of the AOs platform.

To address this point, we evaluated AOs differentiation using four independent stem cell lines, including two human embryonic stem cell (ESC) lines and two induced pluripotent stem cell (iPSC) lines. For each cell line, three independent differentiation experiments were performed. Following AOs generation, organoids were dissociated into single-cell suspensions and analyzed by flow cytometry.

As shown in Figure R4, all four cell lines consistently generated AOs containing comparable proportions of CD144⁺ ECs and PDGFR β ⁺ MCs (**Figure R7A**). No significant differences were observed among the cell lines. CD144⁺ ECs accounted for approximately 20–40% of the total population, whereas PDGFR β ⁺ mural cells represented approximately 60% across all differentiation batches (**Figure R7B**).

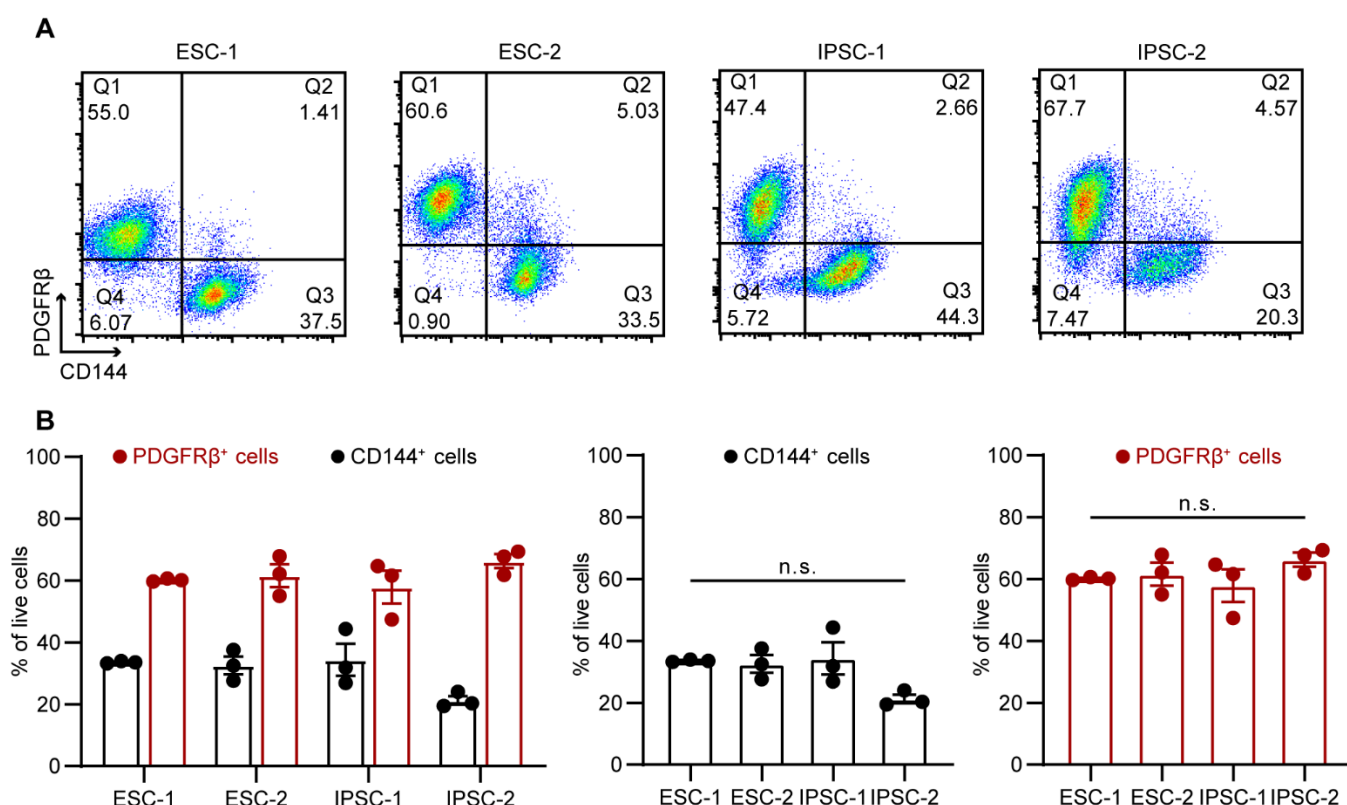


Figure R7. Reproducibility of AOs differentiation across independent pluripotent stem cell lines. (A) Representative flow cytometry plots showing CD144⁺ ECs and PDGFR β ⁺ MCs in AOs generated from two human ESC lines and two human iPSC lines. (B) Quantification of CD144⁺ ECs and PDGFR β ⁺ MCs frequencies across independent differentiation batches. For each cell line, three independent differentiation experiments were performed. Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA. Significance is indicated as n.s., not significant.

These results demonstrate robust differentiation reproducibility across multiple stem cell sources and support the scalability and manufacturing consistency of the AOs generation protocol. Accordingly, we have added these new data to **Figure S1** and revised the manuscript (**Result section, Lines 152-159; Supplemental Figures section, Lines 380-385**).

Reviewer #3

Authors developed a new model: homogeneous arterial organoids (AOs) from human iPSCs using 3D bioreactor system. They show that these spheroid AOs rapidly anastomosed with the host vasculature, enhancing perfusion recovery and preserving tissue function. This work is very interesting in the context of ischemic disease, and was demonstrated with robust methodology. However, one can wonder what is the difference with Blood Vessel Organoids (BVOs), that have been extensively studied and demonstrate enormous potential. BVOs are only mentioned once (ref 15), but no comparison has been discussed. Throughout this manuscript, it should have been compared and discussed, and even possibly added to the experimental set up. Taken together, in the current state of the manuscript, the novelty of this work is not sufficient to be published in Nature Communications.

Response 12– We sincerely thank the reviewer for this thoughtful and important comment. We fully agree that the pioneering studies from Wimmer and colleagues established blood vessel organoids (BVOs) as a landmark platform for studying human vascular biology, disease modeling, and vascular development. In the revised manuscript, we have expanded the manuscript (**Discussion section, Lines 665-674**) and now cite and discuss multiple foundational studies in this field, including the original BVO report (Wimmer et al., *Nature*, 2019)⁷, the subsequent protocol for BVO generation (Wimmer et al., *Nature Protocols*, 2019)⁸, and recent single-cell analyses of BVO developmental trajectories (Nikolova et al., *Cell*, 2025)⁹.

These studies collectively demonstrated the remarkable capacity of iPSCs to self-organize into vascular organoids and provided important insights into vascular development. Our work was directly inspired by these advances. While both systems contain vascular cell populations, they differ in several important aspects.

First, the primary objective of BVOs was to establish a human vascular organoid model for studying diabetic vasculopathy and other vascular diseases, whereas our study focuses on developing a homogeneous arterial organoid platform specifically optimized for therapeutic vascular regeneration in ischemic tissues.

Second, our AOs system was generated using a scalable 3D bioreactor-based differentiation strategy, resulting in highly homogeneous arterial organoids enriched for arterial endothelial and smooth muscle cell populations. In contrast, previously reported BVOs were primarily developed as complex vascular networks for disease modeling applications.

Third, we established a dual fluorescent reporter iPSC line (PECAM1-tdTomato/ACTA2-EGFP), which enabled direct visualization and longitudinal tracking of endothelial and smooth muscle cell behaviors following transplantation. This allowed us to monitor graft survival, vascular network formation, and host-graft integration *in vivo*, providing mechanistic insights that were not addressed in previous BVO studies.

Fourth, beyond demonstrating therapeutic efficacy in two independent hindlimb ischemia models, we further investigated the cellular mechanisms underlying AOs-mediated repair and identified a VEGFA-high macrophage population that contributes to tissue regeneration. These findings extend the study from organoid generation and transplantation toward understanding the regenerative microenvironment elicited by AOs therapy.

We acknowledge the reviewer's concern regarding novelty and have revised the manuscript to more clearly position our work relative to the landmark BVO studies. We hope that the additional discussion and clarification will help highlight the conceptual and translational advances provided by the present study.

Reviewer #4

Kong et al reported that human iPSC-derived arterial organoids exhibit therapeutic efficacy in murine models of hindlimb ischemia and myocardial infarction (MI). They showed that transplantation of AOs induces recruitment of VEGF-A-expressing macrophages, as a potential mechanism to promote tissue repair after hindlimb ischemia. Overall this is a well designed study with solid data supporting the robust therapeutic efficacy, particularly in preclinical MI settings, However, there are several fundamental issues regarding the animal models and the mechanism interpretation, in addition to concerns regarding the clarity and robustness of the work.

Major Points:

Q13 - The whole work depends on under-optimized models of athymic nude mice treated with human AOs. This immunocompromised model represents an issue for future human translation. Ideally the experiments should be performed in 1) treatment of mouse AOs (using similar strategy) in immunocompetent mice as a concept-of-proof and 2) treatment of human AOs in humanized mice.

Response 13 – We thank the reviewer for this insightful comment. We agree that evaluating AOs efficacy in models with a more physiologically relevant immune system is important for future clinical translation.

To address this concern, we performed additional validation studies using a humanized immune mouse model (Hu-PBMC-NPG), in which human peripheral blood mononuclear cells (PBMCs) were engrafted into NPG mice prior to experimentation. Human immune cell reconstitution was confirmed by flow cytometric analysis provided by the vendor, showing an average human CD45⁺ cell proportion of approximately 35% and a residual mouse CD45⁺ cell proportion of approximately 65% within circulating mononuclear cells (**Figure R8A**)

Humanized mice were subjected to hindlimb ischemia and subsequently treated with either human AOs or vehicle control. Consistent with our findings in athymic nude mice, AOs-treated animals exhibited significantly improved blood perfusion recovery compared with controls (**Figure R8B-E**), supporting the therapeutic efficacy of human AOs in the presence of a partially reconstituted human immune system.

Interestingly, we also observed notable differences between the humanized mouse model and athymic nude mice. In contrast to the severe limb necrosis frequently observed in nude mice, humanized mice displayed substantially milder tissue necrosis following hindlimb ischemia (**Figure R8F**), suggesting that immune reconstitution may influence the progression of ischemic injury.

Furthermore, longitudinal bioluminescence imaging demonstrated a progressive decline in AOs-derived signals after transplantation (**Figure R8G-H**), indicating that immune-mediated rejection of allogeneic human AOs may still occur despite the immunodeficient background of the host mice. These findings further highlight the importance of immune compatibility in future translational applications.

We agree that evaluation of mouse-derived AOs in fully immunocompetent mice would provide an additional complementary approach. However, generation and characterization of murine AOs analogous to the human AO platform established in this study would require substantial methodological development and is beyond the scope of the current work.

Importantly, the therapeutic efficacy of human AOs was further validated in a PBMC-humanized mouse model. Although AOs transplantation significantly improved perfusion recovery, longitudinal bioluminescence imaging revealed progressive loss of graft-derived signals, suggesting that immune-mediated rejection

remains a potential barrier for future clinical translation. These findings highlight the need for strategies to enhance immune compatibility in future studies.

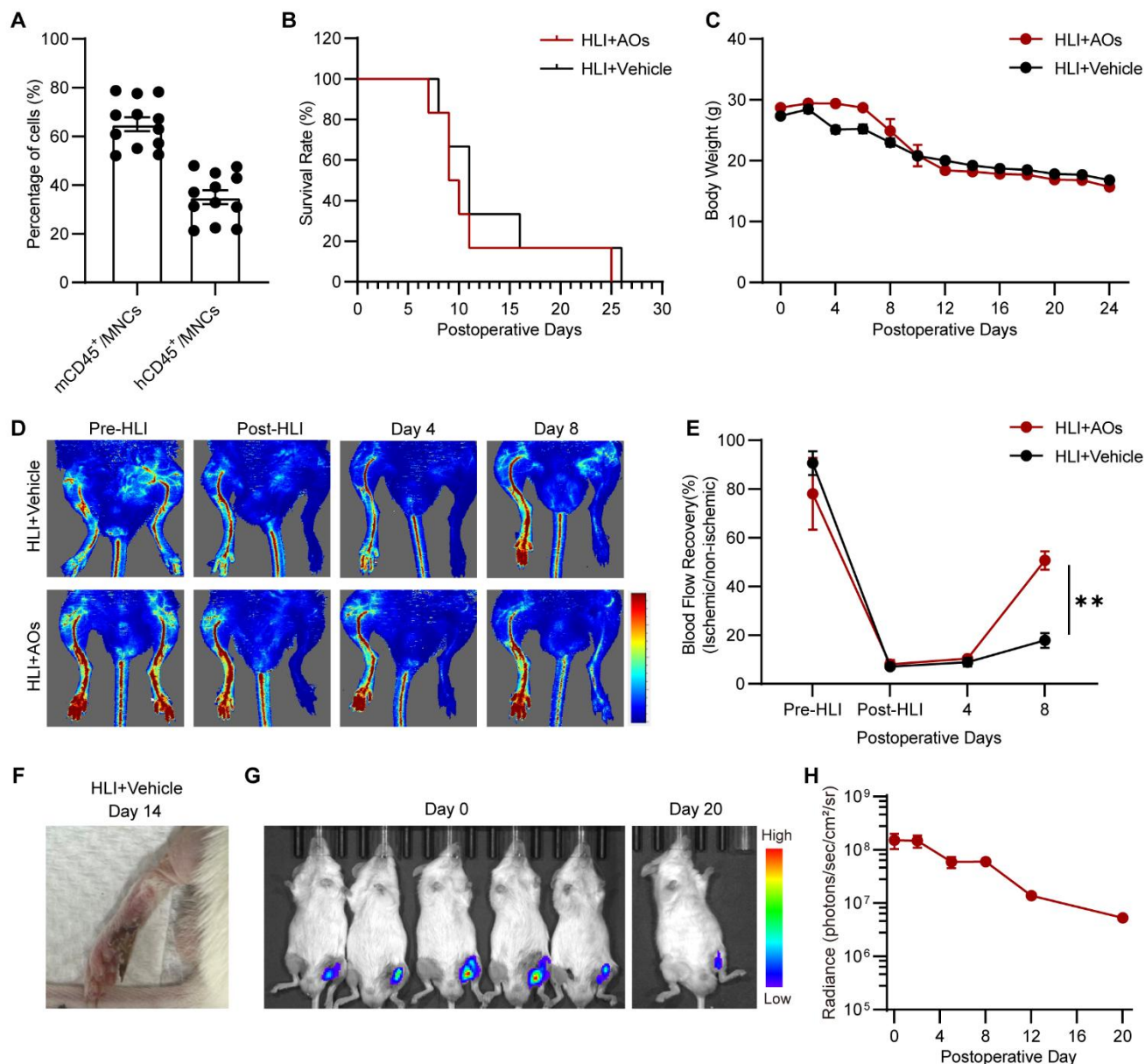


Figure R8. Therapeutic evaluation of human AOs in a humanized hindlimb ischemia model. (A) Flow cytometric assessment of human immune cell reconstitution in Hu-PBMC-NPG mice before experimentation, showing the proportions of human and mouse CD45+ cells within circulating mononuclear cells, n = 12 mice. (B) Kaplan–Meier survival analysis of humanized mice following hindlimb ischemia and treatment with vehicle or AOs. (C) Longitudinal body weight changes after treatment, n = 6 mice. (D) Representative laser Doppler perfusion images of ischemic hindlimbs in vehicle- and AOs-treated humanized mice. (E) Quantification of blood perfusion recovery following treatment, n = 6 mice. (F) Representative photographs showing progression of ischemic limb injury in humanized mice. (G) Representative bioluminescence images of transplanted AOs after implantation into humanized mice. (H) Quantification of bioluminescence signal intensity over time, demonstrating progressive loss of AO-derived signals following transplantation. Data are presented as mean $\pm$ SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, versus control.

Accordingly, we have added these new data to **Figure S8** and revised the manuscript (**Result section, Lines 594-624; Supplemental Figures section, Lines 487-501**).

Q14 - Regarding the mechanism study, VEGF-A-producing macrophages do not essential to be reparative. This study was performed only in the hindlimb ischemic model. In contrast, recruited macrophages in MI are often CCR2+ with proinflammatory phenotypes and inhibit cardiac repair, while residential macrophages are

more reparative. The authors should perform the related single-cell RNAseq analysis of residential and recruited macrophages/monocytes in MI model.

Response 14 – We thank the reviewer for these insightful comments. To further characterize macrophage responses following AOs transplantation in the MI model, we performed flow cytometric analyses of immune cell populations isolated from infarcted hearts at Days 1, 3, and 5 after treatment (**Figure R9A**). Consistent with our observations in the hindlimb ischemia model, AOs transplantation significantly altered macrophage dynamics within the injured tissue. Notably, the proportion of total macrophages was significantly increased in the AOs-treated group compared with the MI+Vehicle group at Day 3 post-transplantation (**Figure R9B**).

Interestingly, AOs transplantation induced dynamic changes in CCR2⁺ macrophages. Day 3, CCR2⁺ macrophages were significantly reduced compared with vehicle-treated controls. This difference was no longer evident by Day 5.

To further validate these findings, we analyzed single-cell transcriptomic datasets generated from infarcted hearts following AOs transplantation. Consistent with the flow cytometric results, macrophages from the AOs-treated group exhibited significantly lower expression of *Ccr2* at Day 3 compared with the MI+Vehicle group (**Figure R9C**). Together, these observations suggest that although AOs transplantation initially alters macrophage recruitment dynamics, it is subsequently associated with reduced CCR2-related inflammatory signaling during cardiac repair.

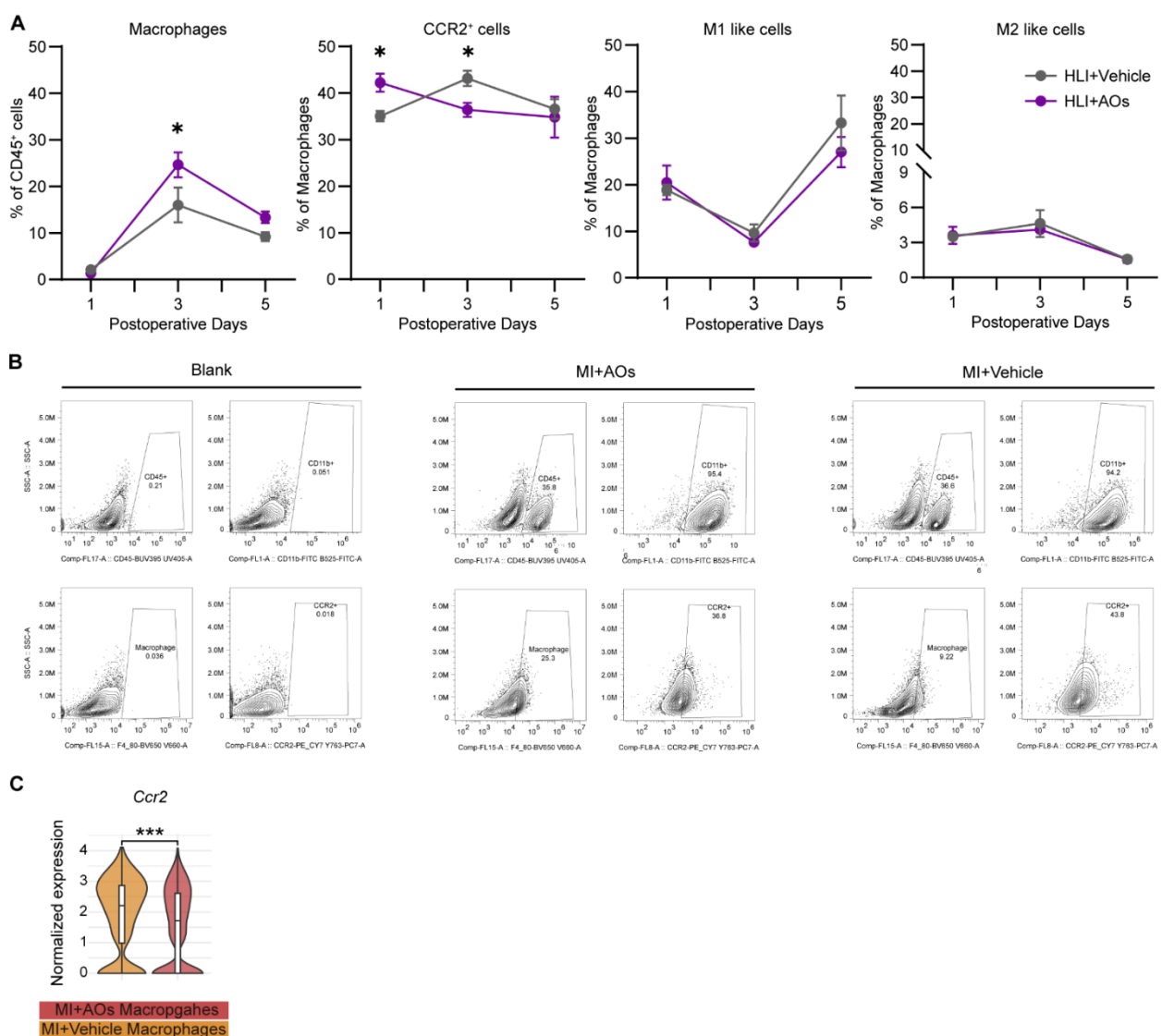


Figure R9. AOs transplantation modulates CCR2-associated macrophage responses in the infarcted heart. (A) Flow cytometric quantification of total macrophages, CCR2⁺ macrophages, M1-like macrophages, and M2-like macrophages in infarcted hearts collected at Days 1, 3, and 5 after AOs transplantation or vehicle treatment. Data are expressed as mean ± SEM. n = 3 mice. (B) Representative flow cytometry plots from Blank, MI+Vehicle, and MI+AOs groups at Day 3 after myocardial infarction. Cardiac immune cells were sequentially gated as CD45⁺ leukocytes, CD11b⁺ myeloid cells, F4/80⁺ macrophages, and CCR2⁺ macrophages. (C) Violin plot showing *Ccr2* expression levels in macrophage populations identified by scRNA-seq from infarcted hearts at Day 3 after treatment. Significance is indicated as n.s., not significant; **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

Accordingly, we have added these new data to **Figure S7** and revised the manuscript (**Result section, Lines 535-548; Supplemental Figures section, Lines 476-485**).

To further address this concern, we performed additional scRNA-seq analyses of infarcted hearts at Day 3 following AOs transplantation (**Figure R10**). Unbiased clustering identified nine major cell populations, including immune cells, ECs, and MCs (**Figure R10A, R10B**). Comparative analysis between MI+Vehicle and MI+AOs groups revealed a marked increase in macrophage abundance following AO treatment (**Figure R10C**).

To investigate how AOs treatment influences cellular communication within the infarct microenvironment, we performed CellChat analysis. Compared with vehicle-treated hearts, AOs-treated hearts exhibited substantial remodeling of intercellular communication networks, particularly among macrophages, ECs, and MCs (**Figure R10D**). Pathway analysis further demonstrated enrichment of extracellular matrix-associated signaling pathways, including Laminin, Collagen, and Galectin signaling, whereas several inflammation-associated pathways were relatively reduced (**Figure R10E**).

Consistent with these observations, macrophages from AOs-treated hearts exhibited increased expression of extracellular matrix remodeling and cell–matrix interaction genes, including *Lamc1*, *Itgb1*, *Col1a2*, *Sdc4*, and *Lgals9* (**Figure R10F**), suggesting enhanced participation in tissue remodeling processes.

Given the reviewer's specific concern regarding recruited versus resident macrophages, we next performed subclustering analysis of cardiac macrophages (**Figure R10G**). This identified four macrophage subsets with distinct transcriptional characteristics. AO treatment increased the abundance of the C0 macrophage population while reducing C1 and C2 populations (**Figure R10H**). Module score analysis revealed that C0 was enriched for resident, reparative, and M2-associated gene signatures, whereas C1 and C2 exhibited stronger recruited macrophage and inflammatory transcriptional programs (**Figure R10I**). Consistent with these findings, flow cytometric and scRNA-seq analyses demonstrated reduced CCR2-associated macrophage signatures following AOs treatment (**Figure R9**).

The methodology used for isolation of cardiac immune cells has now been added to the revised manuscript (**Expanded Methods, Lines 187-197**). Collectively, these results suggest that AOs transplantation reshapes the cardiac macrophage compartment by promoting resident reparative macrophage programs while reducing recruited inflammatory macrophage states, thereby supporting tissue remodeling and vascular regeneration after myocardial infarction.

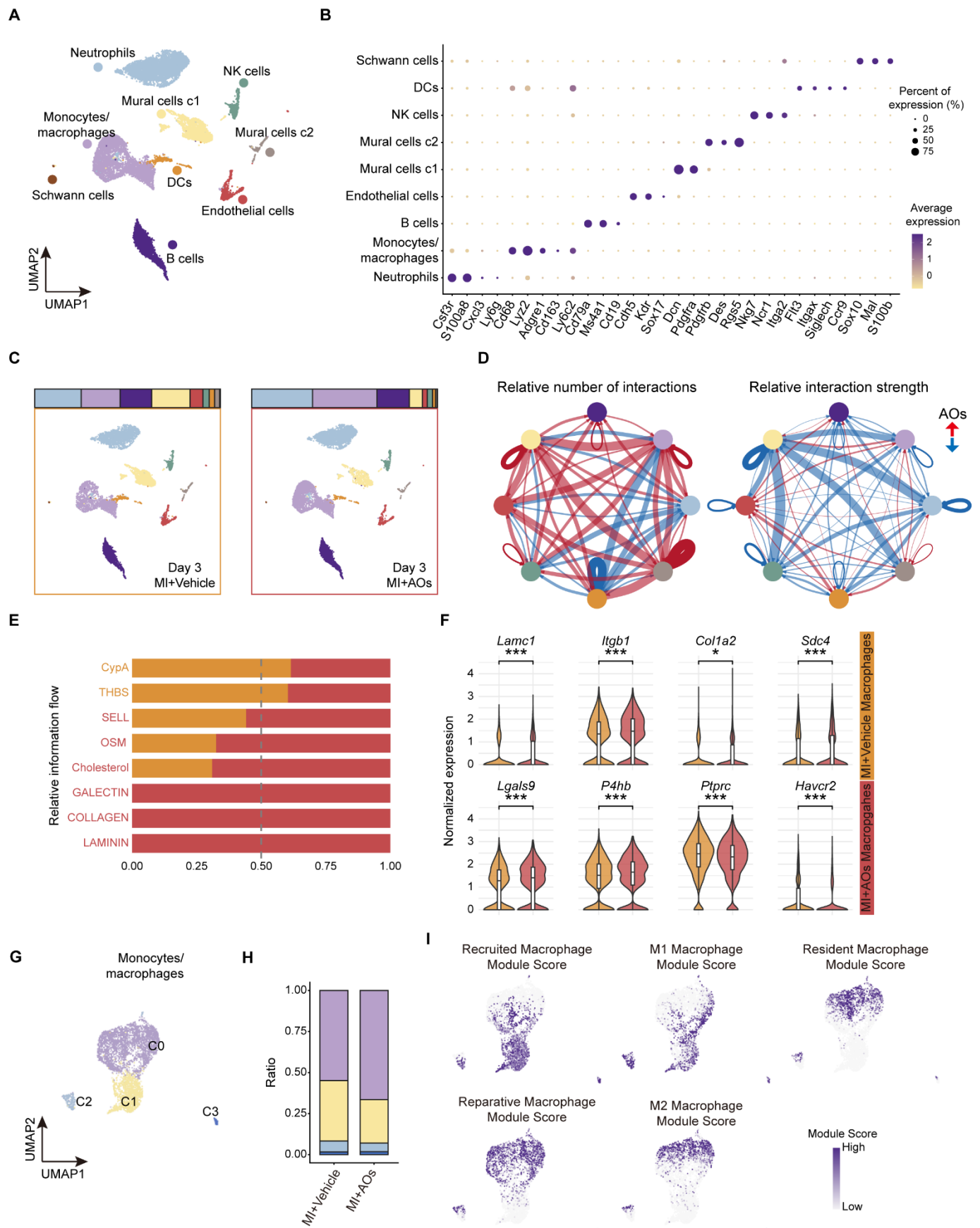


Figure R10. Single-cell transcriptomic analysis of infarcted hearts following AO transplantation. (A) UMAP visualization of single-cell transcriptomes from infarcted hearts collected 3 days after AOs transplantation, colored according to annotated cell populations. (B) Dot plot showing the expression of representative marker genes across major cell populations. Dot size indicates the percentage of expressing cells, and color intensity indicates average expression level. (C) UMAP plots of MI+Vehicle and MI+AOs groups shown separately, together with quantitative analysis of the relative proportions of major cell populations. (D) Cell-cell

communication networks inferred by CellChat analysis comparing MI+Vehicle and MI+AOs groups. Line thickness indicates interaction strength or interaction number between cell populations. (E) Relative contributions of signaling pathways identified by CellChat analysis in MI+Vehicle and MI+AOs groups. (F) Violin plots showing the expression of selected extracellular matrix- and immune-related genes in macrophage populations. (G) UMAP visualization of macrophage subclusters identified by reclustering analysis. (H) Relative proportions of macrophage subclusters in MI+Vehicle and MI+AOs groups. (I) Module score analysis showing resident macrophage, recruited macrophage, reparative macrophage, M1-associated, and M2-associated transcriptional signatures across macrophage subclusters.

Q15 - Fig. 1D, CD31 expression is not robust in the EC population, questioning how stable the endothelial phenotype/genotype in this iPSC-induced AO system. It also surprising to see CD31 is not expressed in the population of Arterial ECs (Fig. 2A vs Fig. 2D, PECAM1 expression). EC-tracing of the transplanted AOs *in vivo* is suggested in a long term condition.

Response 15 – We thank the reviewer for these insightful comments.

Regarding the concern about the robustness and stability of endothelial differentiation, we have revised the flow cytometry analyses and now provide direct quantification of ECs and MCs populations using established lineage markers, including CD144, CD31, DLL4, CXCR4, and PDGFR β (**Figure R11A**).

In addition, differentiation reproducibility was evaluated across four independent pluripotent stem cell lines (two ESCs and two iPSCs), demonstrating consistent generation of CD144⁺ ECs and PDGFR β ⁺ MCs across independent differentiation batches (**Figure R11B, R11C**). These results support the robustness and reproducibility of the endothelial phenotype within our AOs system.

Regarding the apparent discrepancy between **Figure 2A** and **Figure 2D**, PECAM1 expression is indeed present within the arterial endothelial cell cluster. In **Figure 2D**, the arterial endothelial population identified in **Figure 2A** shows clear PECAM1 expression, consistent with its endothelial identity. To improve clarity, we have highlighted the arterial endothelial cell cluster to facilitate direct comparison between the annotated cluster and its corresponding PECAM1 expression pattern (**Figure R11D**). We believe this concern may arise from the difficulty of visually matching specific clusters between the cell-type annotation UMAP and the gene expression UMAP.

Finally, to address the reviewer's suggestion regarding long-term ECs tracing, we performed additional longitudinal intravital imaging studies following kidney capsule transplantation and monitored AOs-derived vascular networks for up to 3 months (**Figure R11E, R11F and R11G**). AOs-derived PECAM1-tdTomato-positive vascular networks remained detectable and perfused at 1 month, persisted structurally at 2 months, and gradually diminished by 3 months. These findings provide additional insight into the long-term fate and remodeling of AOs-derived vasculature *in vivo*.

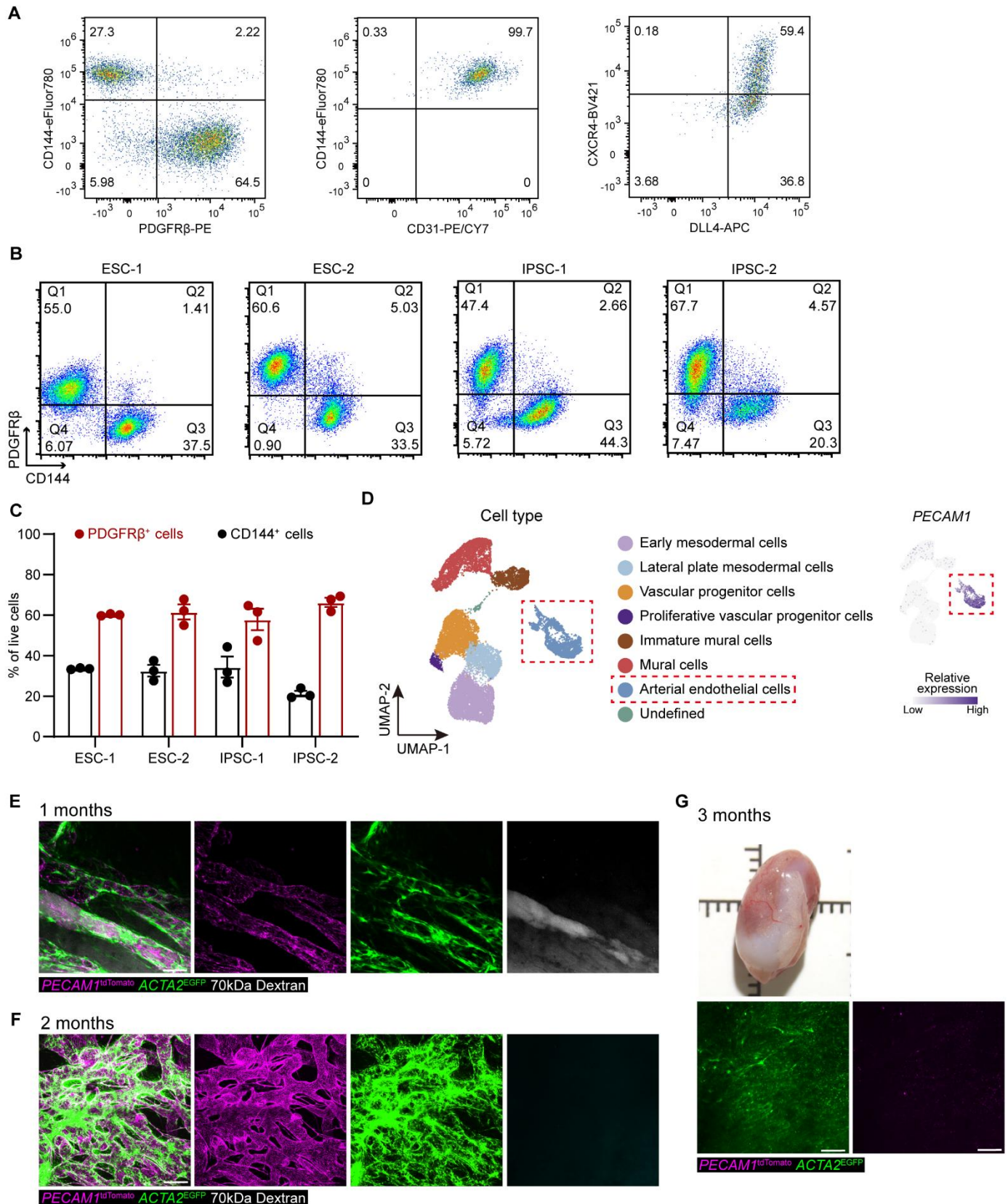


Figure R11. (A) Representative flow cytometry dot plots showing the frequencies of ECs and MCs populations within the live singlet population. (B) Representative flow cytometry plots showing CD144⁺ ECs and PDGFRβ⁺ MCs in AOs generated from two human ESC lines and two human iPSC lines. (C) Quantification of CD144⁺ ECs and PDGFRβ⁺ MCs frequencies across independent differentiation batches. (D) The arterial endothelial cell cluster is highlighted with a red dashed outline to facilitate direct comparison between the annotated cluster in Figure 2A and its corresponding PECAM1 expression pattern. (E) Long-term evaluation of AOs-derived vascular networks following kidney subcapsular transplantation. Representative intravital confocal microscopy images of AOs-derived vascular networks 1 month (E), 2 months (F) and 3 months (G) after transplantation under the kidney capsule. Scale bar, 100 μm.

Q16 - Fig. 2. The transcriptional factor trajectory study is relatively descriptive. Most of data were based on co-relation study. Functional tests with knockdown of the identified transcriptional factors would be suggested.

Response 16 – We thank the reviewer for this insightful suggestion. We agree that the original transcription factor trajectory and regulon analyses were primarily correlative and would benefit from direct functional validation.

To address this concern, we performed loss-of-function studies targeting the five top-ranked transcription factors identified by SCENIC analysis, namely *SOX17*, *TAL1*, *KLF2*, *ERG*, and *ETV2*. First, qPCR analysis confirmed efficient knockdown of each transcription factor in iPSCs prior to differentiation (**Figure R12A**). Subsequently, both control and knockdown iPSCs were differentiated into AOs using the same protocol. The differentiation efficiencies of ECs (*CD144*⁺) and MCs (*PDGFRβ*⁺) were evaluated by flow cytometry (**Figure R12B**).

As shown in the revised figure, knockdown of *SOX17*, *TAL1*, *KLF2*, *ERG*, or *ETV2* significantly decreased the proportion of *CD144*⁺ ECs while concomitantly increasing the proportion of *PDGFRβ*⁺ MCs compared with the control group (**Figure R12C**). These findings provide direct functional evidence that the identified transcription factors are required for endothelial lineage commitment during AOs development and support the conclusions drawn from the single-cell regulatory network analysis.

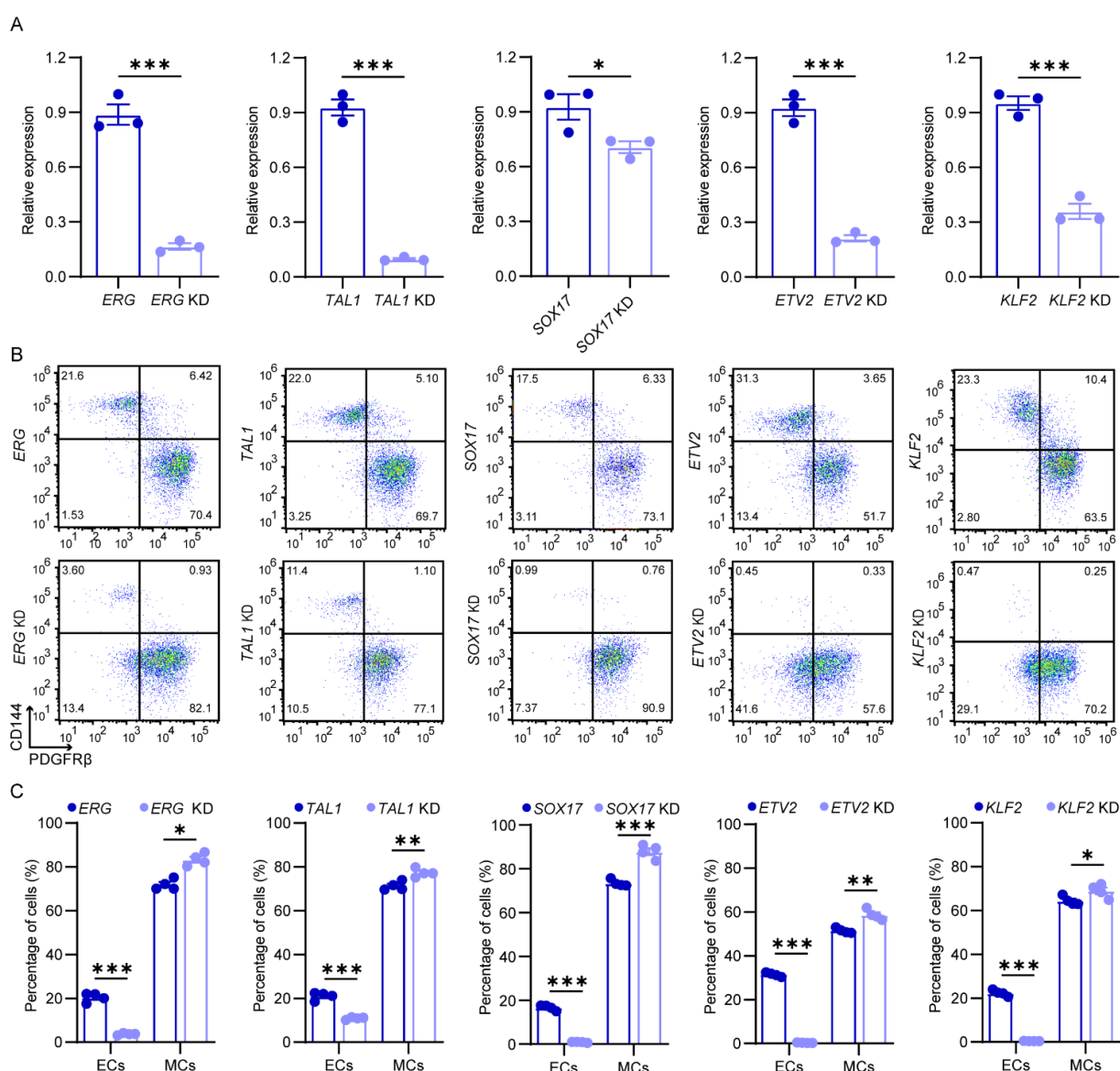


Figure R12: Functional validation of transcription factors identified by single-cell regulatory network analysis. (A) PCR analysis confirming the knockdown efficiency of *SOX17*, *TAL1*, *KLF2*, *ERG*, and *ETV2* in iPSCs. Gene expression levels were normalized to GAPDH and presented relative to the control group. (B) Representative flow cytometry plots showing the proportions of CD144⁺ ECs and PDGFRβ⁺ MCs in AOs derived from control and transcription factor-knockdown iPSCs. (C) Quantification of flow cytometry results shown in (B). Data are presented as mean ± SEM from four independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, versus control.

The corresponding data have been added to **Figure S2** and described in the revised manuscript (**Result section, Lines 257-269; Supplemental Figures section, Lines 380-385**).

Q17 - Fig. 3E. Only small portion of vasculature was perfused, as shown to be Lectin+.

Response 17 – We thank the reviewer for this insightful comment. We agree that only a subset of AOs-derived vascular structures appeared fully perfused in the representative image shown in **Figure 3E**.

Our intention was not to suggest that all AOs-derived vessels had achieved complete perfusion by this time point. Rather, **Figure 3E** was intended to demonstrate the ability of AOs-derived vascular networks to establish functional connections with the host circulation. Indeed, the coexistence of lectin-positive/dextran-positive vessels and lectin-positive/dextran-low vessels likely reflects different stages of vascular integration and maturation following transplantation.

Importantly, dextran-positive perfused AOs-derived vessels were detectable as early as Day 7 after transplantation and remained evident at 3 weeks post-transplantation (**Figure S3E**), indicating that functional anastomosis with the host circulation occurs rapidly and is maintained over time. We agree that the proportion of fully perfused vessels may remain limited during the early stages of graft integration.

Q18 - Fig. 3. What is the rationale of AO injection into kidney? The majority of the work has focused on ischemic diseases in hindlimb and heart. A consistent model would be suggested.

Response 18 – We thank the reviewer for this important comment. We agree that the rationale for using the kidney capsule transplantation model should be more clearly explained.

The kidney capsule experiment was not intended as a therapeutic model but rather as a well-established platform to evaluate the *in vivo* survival, vascularization, maturation, and host integration of transplanted organoids under a highly vascularized and stable physiological environment. Kidney capsule transplantation has been widely used in the organoid field because it provides robust host vascular support and enables reliable assessment of graft survival and structural development following transplantation^{2,7,10}.

In our study, the kidney capsule model served as a proof-of-concept system to determine whether AOs could survive, establish vascular networks, and integrate with host tissues *in vivo*. In contrast, the hindlimb ischemia and myocardial infarction models were specifically employed to evaluate the therapeutic efficacy of AOs in ischemic disease settings. Therefore, these models address complementary biological questions rather than representing alternative therapeutic models.

To clarify this rationale, we have added additional explanation in the revised manuscript (**Results section, Lines 651-655**) and included relevant references describing kidney capsule transplantation as a standard approach for *in vivo* organoid maturation and vascularization studies.

Q19 - Fig. 4E, potential group bias. Ischemia induction in AO group seemed less severe than Vehicle group (at day 1), which may explain why AO group recovered quicker.

Response 19 – We thank the reviewer for this careful observation. We agree that the presentation of the longitudinal perfusion data in the original Figure 4E may visually suggest minor differences among groups at Day 1 time points.

To improve clarity, we have now separately displayed perfusion ratios as bar graphs in the revised manuscript (**Figure R13**). These data were derived from the same measurements shown in Figure 4E. As shown in the revised figures, no significant differences were observed among the experimental groups either before hindlimb ischemia induction or at Day 1 after surgery, indicating comparable initial ischemic severity across groups.

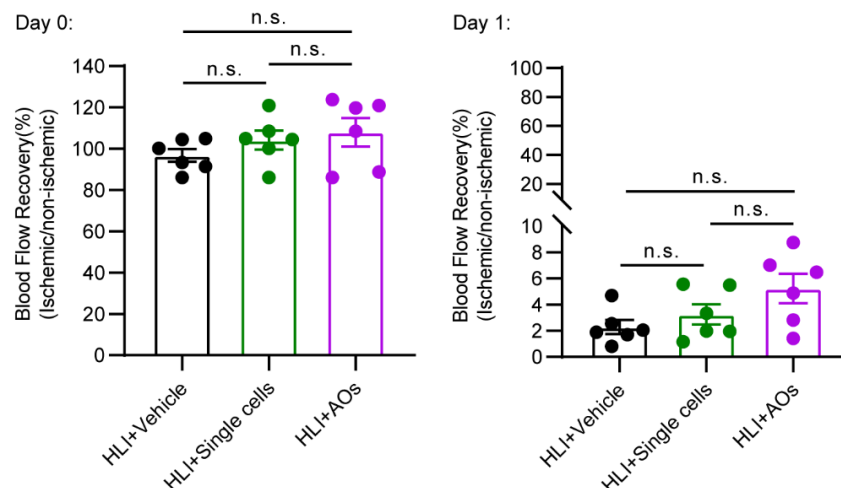


Figure R13: Comparable initial ischemic severity among experimental groups. Quantification of hindlimb perfusion ratios before surgery (baseline) and Day 1 after hindlimb ischemia induction, derived from the data presented in Figure 4E. No significant differences were observed among groups. Data are presented as mean ± SEM, n = 6 mice.

Therefore, the enhanced perfusion recovery observed in the AOs-treated group at later time points is unlikely to be attributable to differences in the initial extent of ischemic injury.

Q20 - Fig. 5. The key information of MI study is missing. When echo was done after MI induction? What are the EF changes over time after treatment?

Response 20 – We thank the reviewer for this important comment. We apologize that the timing of echocardiographic assessment was not clearly described in the original manuscript.

In the present study, cardiac function was evaluated by echocardiography at 2 weeks after myocardial infarction and treatment, which was selected as the predefined endpoint for assessing therapeutic efficacy. We have now clarified the timing of echocardiographic measurements in the manuscript (**Result section, Lines 475-479, Lines 1012-1014; Expanded Methods section, Lines 118-119**) sections of the revised manuscript.

Q21 - Fig. 6. The mechanism proposed seems to be descriptive. It is unclear how VEGF-A-producing macrophages will promote tissue repair (though it could stimulate angiogenesis), serving as the key mechanism. In addition, it is unclear the macrophage enrichment is due to infiltration or residential proliferation/migration. There was no phenotype analysis or immune profiling for macrophages. The key findings need to validate by flow cytometry.

Response 21 – We thank the reviewer for this important comment. We agree that our original description may have overstated the mechanistic significance of VEGFA-expressing macrophages. To substantiate this, we performed a cell-cell communication analysis using our scRNA-seq dataset. The results demonstrate a robust paracrine signaling axis primarily driven by macrophage-derived *Vegfa* acting on ECs receptors (*Kdr/Flt1*), which functionally couples the immune microenvironment to the vascular phenotypes observed in our model (**Figure R14A**).

To definitively address whether the macrophage enrichment is driven by peripheral infiltration or local proliferation, we performed a comprehensive transcriptomic profiling of the macrophage subclusters using our scRNA-seq dataset. Specifically, we conducted a Module Score analysis to evaluate established multi-gene transcriptional signatures for resident, recruited, reparative, M1-associated, and M2-associated macrophages across all subclusters (**Figure R14B, R14C**).

Our analysis revealed that macrophage subcluster enriched in the AOs group exhibited profoundly higher module scores for the "recruited macrophage" and "reparative/M2-associated" signatures, while scoring remarkably low for "resident" and "M1-associated" signatures (**Figure R14D**).

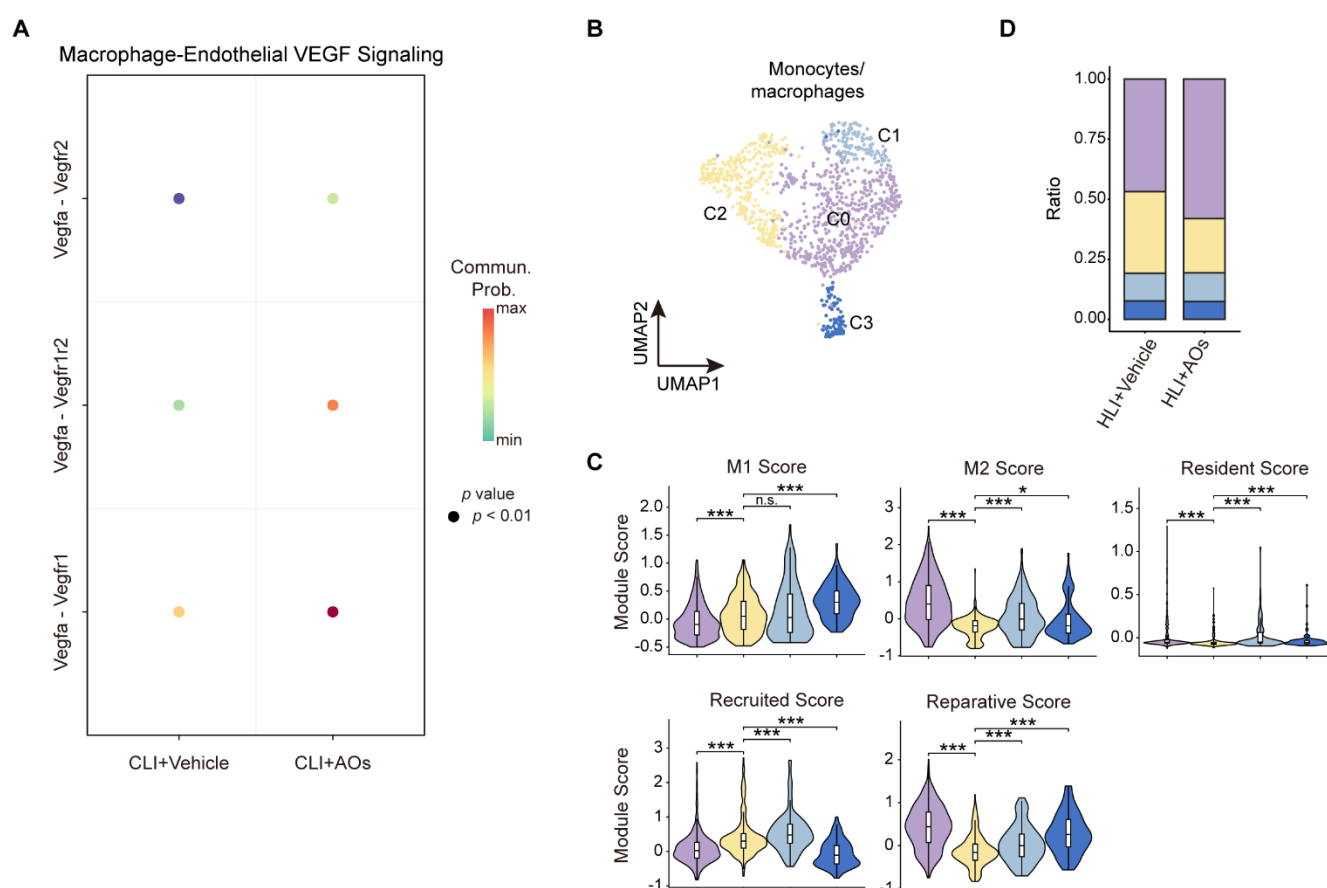


Figure R14. Characterization of macrophage heterogeneity and cell-cell communication following AOs treatment. (A) Cell-cell communication analysis showing predicted VEGF signaling interactions between macrophages and ECs in ischemic muscle tissues. Ligand-receptor pairs were inferred from scRNA-seq data. (B) UMAP visualization of macrophage subclusters identified by reclustering analysis. Four macrophage populations (C0–C3) were identified based on their transcriptional profiles. (C) Violin plots showing module scores for resident macrophage, recruited macrophage, reparative macrophage, M1-associated, and M2-associated transcriptional signatures across macrophage subclusters. (D) Quantification of the relative abundance of macrophage subclusters (C0–C3) in HLI+Vehicle and HLI+AOs groups.

To strengthen the characterization of macrophage populations, we performed additional flow cytometric analyses of ischemic muscle tissues at Days 1 and 3 following AOs transplantation (**Figure R15**). These

analyses revealed a significant increase in M2-like macrophages and a reduction in MHC-II⁺ M1-like macrophages at Day 1 in AOs-treated tissues, whereas CCR2⁺ populations remained unchanged. By Day 3, the early increase in M2-like macrophages was no longer observed. These findings indicate that macrophage polarization is dynamically regulated during the early regenerative phase rather than being persistently shifted toward a single phenotype.

Importantly, the lack of significant differences in CCR2⁺ macrophages suggests that AOs treatment does not substantially enhance CCR2-associated inflammatory monocyte recruitment. However, we acknowledge that the current data do not allow us to distinguish between local proliferation and other mechanisms contributing to macrophage accumulation.

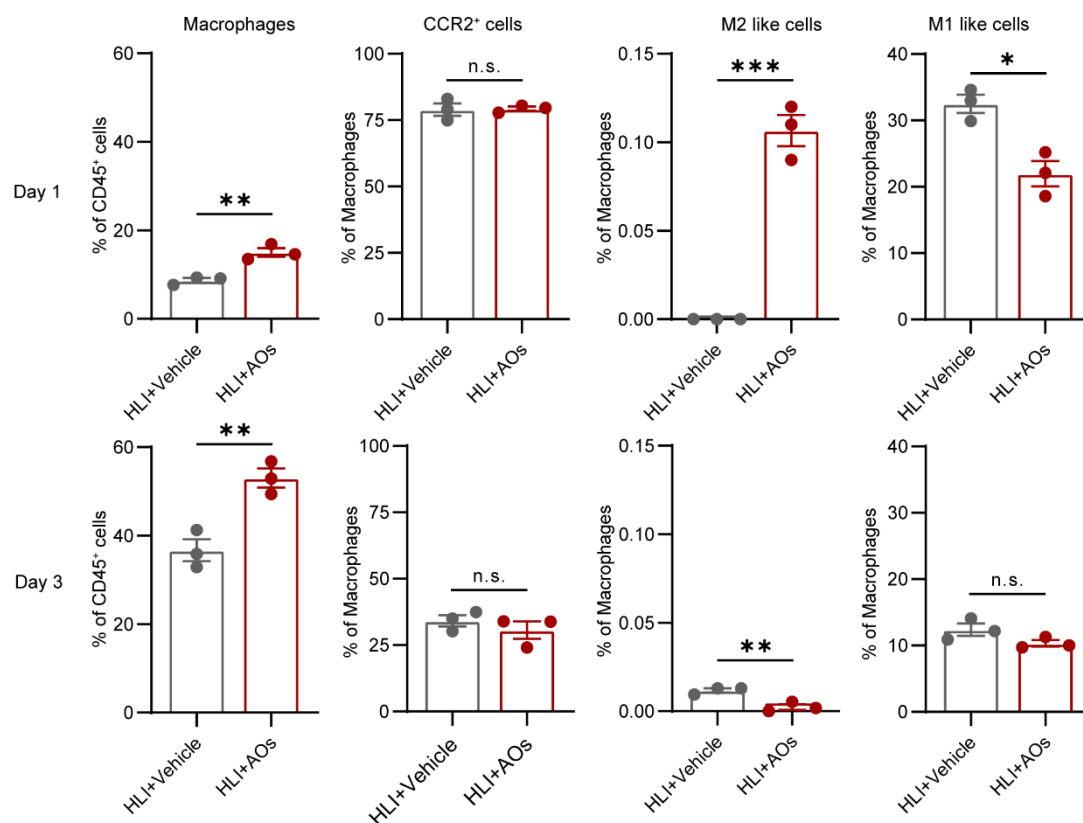


Figure R15. Flow cytometric characterization of macrophage phenotypes following AOs transplantation in HLI mouse model. Quantification of CCR2⁺, M1-like and M2-like macrophage populations at Day 1 and Day 3. Data are presented as mean $\pm$ SEM, n = 4 mice. Statistical significance was determined using a two-tailed unpaired Student's t-test. Significance is indicated as n.s., not significant; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

We further agree that our data establish an association between VEGFA-expressing macrophages and AOs-mediated repair rather than a definitive causal mechanism. Accordingly, we have revised the manuscript (**Results section, Lines 562-571**) to moderate the interpretation and describe VEGFA-high macrophages as a potential pro-regenerative population associated with AOs-mediated vascular repair.

Minor Points:

Q22 - Fig. 4H. It is suggested to include AO staining in tri-chrome Masson IHC, to show the tissue pathology around AOs.

Response 22 – We thank the reviewer for this helpful suggestion. We would like to clarify that **Figure 4H** was intended to assess the overall extent of fibrosis within the ischemic tissue rather than the local tissue pathology surrounding individual AOs grafts. Because Masson's trichrome staining alone does not distinguish host-derived and AOs-derived vascular structures, the precise location of AOs-derived vascular networks cannot be identified directly in **Figure 4H**.

To further address the reviewer's concern, we have included representative H&E images in Supplementary **Figure S4** comparing tissues receiving single-cell suspensions or AOs transplantation. Regions of interest have been highlighted to facilitate evaluation of the local tissue architecture surrounding the transplanted grafts (**Supplemental Figures Section, Lines 436-437**). These data further support the improved preservation of tissue structure following AOs transplantation.

Q23 - Fig. 6B. HLI surgery itself seems to induce robust macrophage infiltration.

Response 23 – We thank the reviewer for this important observation. We agree that hindlimb ischemia itself induces a substantial inflammatory response characterized by macrophage infiltration into ischemic tissue.

Our intention was not to suggest that macrophage recruitment was exclusively triggered by AOs transplantation. Rather, the comparison between the HLI+Vehicle and HLI+AOs groups demonstrates that AOs treatment further enhanced macrophage accumulation beyond the level induced by ischemia alone. As shown in **Figure 6B**, macrophage abundance was significantly increased in the AOs-treated group during the early post-transplantation period (Days 1–3), whereas macrophage frequencies became comparable between groups at later time points (Days 5–6).

To avoid overinterpretation, we have revised the text to clarify that hindlimb ischemia itself induces macrophage infiltration and that AOs transplantation further augments this early macrophage response (**Results section, Lines 510-514**).

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A point-by-point response to the reviewer's comments
Nature Communications manuscript (NCOMMS-26-007624B)

Reviewer #2

The authors have addressed all my comments.

The authors have made a reasonable effort to address Reviewer #3's comments by expanding the discussion of the blood vessel organoid (BVO) literature, citing additional foundational studies, and clarifying the intended application of their arterial organoid platform. However, the response does not fully address the reviewer's central concern regarding novelty. Much of the rebuttal focuses on differences in application (therapeutic regeneration versus disease modeling), the use of a dual fluorescent reporter line, and downstream mechanistic studies following transplantation. While these are important strengths of the study, they do not clearly demonstrate how the arterial organoid platform itself represents a conceptual or technological advance over existing BVO systems. In particular, the response does not sufficiently explain why the homogeneous arterial composition, scalable bioreactor differentiation strategy, or other distinguishing features could not be achieved using established BVO methodologies, nor does it directly address the reviewer's suggestion of a more rigorous comparison with BVOs, either experimentally or through a deeper discussion of their biological and functional differences.

We thank the reviewer for this insightful comment and appreciate the opportunity to further clarify the conceptual and technological advances of our arterial organoid (AO) platform. We agree that the novelty of this study should not be solely interpreted from its downstream applications, such as therapeutic vascular regeneration or disease modeling, but rather from the intrinsic properties and engineering principles of the organoid system itself.

In the revised manuscript, we have expanded the Discussion section to provide a more comprehensive comparison between our arterial organoids and previously reported blood vessel organoid (BVO) systems, including their developmental trajectories, cellular compositions, scalability, and functional objectives (Lines 614-627). We have also clarified that our AO platform represents a complementary advancement beyond existing BVO approaches rather than simply a different application of established vascular organoid technologies.

Specifically, while previous BVO systems have provided important advances in generating vascular networks containing endothelial and mural cell populations, most reported BVOs are primarily optimized for the formation of generic vascular structures, angiogenic modeling, or developmental studies. In contrast, our AO platform was specifically designed to reconstruct an arterial vascular module with defined arterial identity and functional maturation. Several features distinguish our approach:

(1) Arterial specification as the core design principle.

Unlike conventional BVO systems that mainly rely on spontaneous vascular organization, our differentiation strategy was engineered to promote arterial lineage commitment through controlled modulation of mesoderm induction and vascular specification. The resulting AOs exhibited sustained arterial transcriptional programs, including enhanced expression of arterial-associated regulators and markers, indicating a stable arterial identity rather than a mixed vascular phenotype.

(2) Defined and enriched arterial cellular composition.

Our AOs display a relatively homogeneous arterial architecture, consisting predominantly of arterial endothelial cells and mural cells arranged into a coordinated vascular unit. This compositional control provides a key advantage for applications requiring arterial reconstruction, where the identity and functional

properties of vascular components are critical determinants of therapeutic outcome.

(3) Scalable and reproducible bioreactor-based manufacturing.

A major limitation of many existing organoid platforms is variability arising from spontaneous self-organization and small-scale production. By implementing a bioreactor-based differentiation strategy, our approach enables scalable generation of AOs with improved batch consistency, providing a potential foundation for future translational applications.

(4) Functional validation as an integrated vascular module.

Beyond demonstrating vascular marker expression, we showed that AOs rapidly connected with host circulation after transplantation and improved blood perfusion in ischemic tissues. Furthermore, our study revealed a macrophage-mediated mechanism regulating AO vascular integration, providing mechanistic insight into how engineered arterial tissues interact with the host microenvironment.

We thank the reviewer again for highlighting this important point, which has helped us improve the clarity of the manuscript regarding the conceptual advance and significance of our arterial organoid platform.

Reviewer #4

Most of my previously raised concerns have been addressed. One more comment: Fig. R8D, the basal-line LDI images of control and AO treatment seem different, but the quantified results have no difference. This is inconsistent. Also suggest to include all data presented in the response letter in the figures or supplementary figures.

Response 12– We thank the reviewer for this helpful suggestion. Following the reviewer's recommendation, we have incorporated the relevant supporting data presented during the revision process into the revised manuscript. Key validation experiments and quantitative analyses have now been included in the Supplementary Information (Supplementary Figures 1, 2, 5, 7, 8), ensuring that these data are available to readers in the published version.

In addition, for Fig. R8D, we have replaced the representative baseline LDI images with more representative images from each group to better reflect the quantitative analysis. The baseline perfusion analysis confirmed no statistically significant difference between groups before AO transplantation.

Revised Fig. R8D: Representative laser doppler perfusion images of ischemic hindlimbs in vehicle- and AOs-treated humanized mice.

