

Multi-parameter Tunable Synthetic Matrix for Engineering Lymphatic Vessels



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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I consider this manuscript to be high quality and focused on an underdeveloped research area. The introduction provides a nice overview of tissue engineering approaches and motivates the objective of the current study. I also found the figures to be comprehensively descriptive of the studies. I have included minor comments below for you to consider.

1. Line 154 – "... it was minimal in magnitude ...": I suggest deleting or editing this phrase. The phrase assumes that a small change is not important, yet we really do not know.

2. Discussion: I suggest adding text to identify or contextualize advantages of the presented approach. Consider the phrase, "Previously we reported that LYVE-1 and Prox-1 expression increased with decreasing substrate stiffness¹⁵, and now we report an additional method." I suggest elaborating on the advantages of using this additional "tunable" method for either probing physiology, evaluating treatments, or tissue engineering. This added text could serve to further substantiate the importance of the results for the reader.

3. Discussion: Also, consider – "Earlier benchmarking studies indicated that 8 million/mL LECs was a critical density required to form lymphatic tubes." I find it interesting that you set the cell density and evaluated parameter effects. I presume that altered parameters might influence tube formation. I suggest adding 2-3 sentences to the Discussion or methods to provide rationale for choosing cell density. It seems like you selected the cell density because it resulted in tube formation and, hence, allowed for parameter comparison studies.

4. Results/Discussion: Line 338 – "Additionally, a tumor suppressor, Folliculin, has been shown to be responsible for maintaining distinct blood and lymphatic vasculature and prevents vessel mismatch." I might have missed it, but I do not think Folliculin was added to your system. The relevance of this statement is not clear. Please edit to clarify your thinking. In addition, I am fascinated by Figure 7. I suggest adding more rationale or discussion related to the labeling method. If I understand correctly, you injected lectin into the foot pad and then harvested at a distal site. Labeling of human LECs would then suggest that these vessels are functional and connected to the draining mouse lymphatics in the footpad. Does this make sense? I think this is super intriguing and potentially novel. The potential finding raises questions about whether or not all human vessels are functional. Also, in Figure 7 there are a lot of green vessels. I assume some of these are blood vessels. Could this be the case? While the vessel identity and potential interaction between host blood vessels and human derived LECs is not totally surprising and could be expected based on recent examples of vessel interactions in the literature, the results are still convincing that something is going on. I think this is what is important and I suggest recognizing that the results are suggestive versus being conclusive. You probably do not know what is really going on. If you are convinced that the 2 GSI-lectin is lymphatic specific, then I suggest expanding on your rationale in the text.

5. Line 48 - "first ever". I suggest qualifying this claim. Is it the first 3D approach?

Reviewer #2 (Remarks to the Author):

Overview:

Engineered lymphatic vessels hold significant promise as a novel strategy for addressing lymphatic disorders. However, the optimal parameters for lymphatic engineering remain a challenge. This manuscript introduces a novel platform using hyaluronic acid (HA)-hydrogels to investigate lymphatic vessel formation within a synthetic matrix that incorporates both mechanical and biochemical cue. Notably, this study marks the first attempt at engineering lymphatic vessels without support cells, allowing independent modulation of parameters while keeping other factors fixed. The ability to investigate the effect of matrix composition, mechanics, and growth factors is a commendable aspect of the study, with potential implications for vascularizing organoids and transplantation therapies for lymphatic disorders. Nevertheless, several crucial points appear inadequate or missing, necessitating a major revision.

Major comments:

1. The expression of Flt4, Nrp2, and CD44 appears minimally or not different across % D.S. conditions alone (Figure 2). Given that 21% D.S. NorHA, along with optimal RGD, VEGFC, Ang2, and crosslinker concentration, promotes lymphatic tube formation compared to 21 %D.S. alone, it is essential to explore whether these multiple conditions impact Flt4, Nrp2, and CD44 expressions more significantly.
2. Consider addressing the dynamic modification of matrix mechanics to accelerate and sustain lymphatic formation. The study suggests that while less than 10% crosslinked gel is better for initiation, it is worse for maintenance, with the opposite observed for high crosslinked gel. Could a sequential approach be employed, initiating lymphatic formation with a lower crosslinked gel and subsequently transitioning to a higher crosslinked gel?
3. Evaluate the functionality (drainage) of the engineered lymphatic vessels. While the integration of mouse and human vasculature using Imaris imaging software is noted, consider injecting dye into the surrounding tissue and assessing lymphatic drainage function in the hydrogel construct, or vice versa.

Minor comments:

1. In the result of HA binding site availability influences LEC phenotype and vessel formation, it is mentioned that Nrp2 expression shows no differences in varied HA binding site availability (Figure 2F). However, the figure indicates significant differences (** or *) in Nrp2 expression, requiring clarification or correction.

Reviewer #3 (Remarks to the Author):

In their submission to Communications Biology, Alderfer et al., have developed a hyaluronic acid (HA)-hydrogel to closely investigate the influence of mechanical and biochemical cues on the formation of lymphatic vessels in a synthetic scaffold. The novelty of this work is engineering a 3D synthetic scaffold in which human lymphatic endothelial cells (LECs) can self-assemble in vitro into lymphatic vessels within 3 days and without the use of support cells. Overall, the text is very well-written, organized, and easy to follow. This work introduces an innovative approach to engineering lymphatic vessels, setting it apart from previous studies in the field.

Specific comments:

1. In figures 4 and 6, please add quantified data of the lymphatic vessel network for reading to assess the quality of these networks? Like vessel density? Branching? Or other morphological quantifications.
2. Additionally, in those networks, the images show that many LECs do not assemble into networks. Is it possible these cells are apoptosed and/or dysfunctional? Can they show the viability of these cells by running a live-dead assay?
3. The authors report that the lymphatic tubes can be cultured for up to 21 days in vitro – is there any supplementary data to support these claims? Please show the culture and some metric of stability of the vascular health for 21 days of culture.

Minor

Can the authors justify the choice of the sex of the mouse model? I think it's crucial that they state the rationale for choosing one sex in the study design over the other, i.e. a female mouse, and to acknowledge any limitations associated with that choice.

Response to Reviewers

Reviewer #1:

I consider this manuscript to be high quality and focused on an underdeveloped research area. The introduction provides a nice overview of tissue engineering approaches and motivates the objective of the current study. I also found the figures to be comprehensively descriptive of the studies. I have included minor comments below for you to consider.

We appreciate the reviewer's thoughtful critique of the manuscript. We are excited to hear that the reviewer found the study potentially impactful and beneficial to the field of lymphatic biology and tissue engineering. We have attempted to address all of the comments and concerns as described below. We thank the reviewer for the opportunity to greatly improve the clarity and scientific validity of the manuscript.

1. Line 154 – "... it was minimal in magnitude ...": I suggest deleting or editing this phrase. The phrase assumes that a small change is not important, yet we really do not know.

We deleted these phrases to avoid confusion. Please see below is the final version of the sentence in Line 154.

"There was a statistically significant difference in Flt4 expression between condition (Figure 2E), which could be explained by the differences in tube formation we observed across % D.S. conditions."

2. Discussion: I suggest adding text to identify or contextualize advantages of the presented approach. Consider the phrase, "Previously we reported that LYVE-1 and Prox-1 expression increased with decreasing substrate stiffness¹⁵, and now we report an additional method." I suggest elaborating on the advantages of using this additional "tunable" method for either probing physiology, evaluating treatments, or tissue engineering. This added text could serve to further substantiate the importance of the results for the reader.

We really appreciate the reviewer for this excellent suggestion. We have adjusted the wording in the first paragraph to further highlight the advantages of this multi-parameter tunable synthetic matrix.

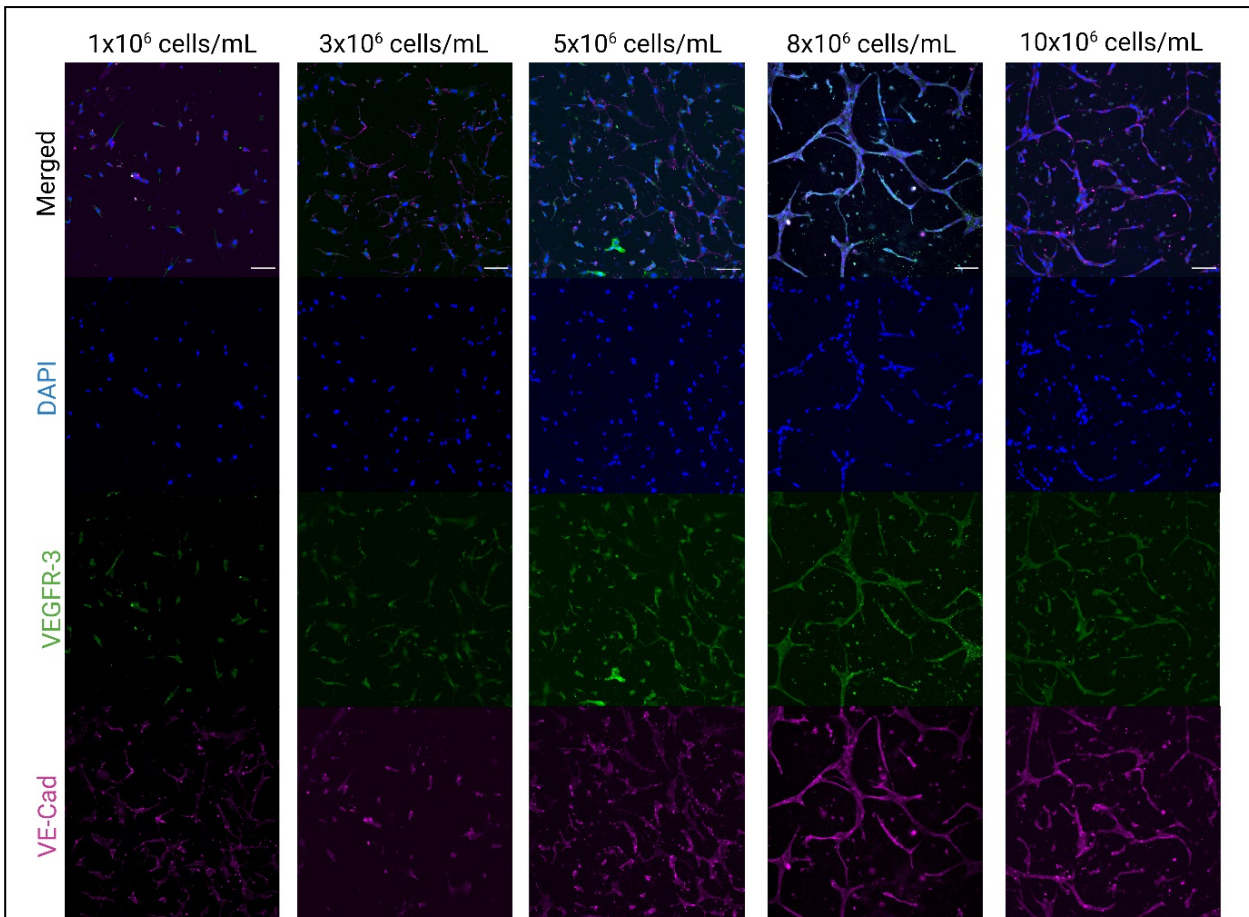
"Advancing to the current and more complex 3D NorHA system, we report an additional method for promoting the expression of key lymphatic markers through modulation of available HA binding sites within the synthetic matrix".

3. Discussion: Also, consider – "Earlier benchmarking studies indicated that 8 million/mL LECs was a critical density required to form lymphatic tubes." I find it interesting that you set the cell density and evaluated parameter effects. I presume that altered parameters might influence tube formation. I suggest adding 2-3 sentences to the Discussion or methods to provide rationale for choosing cell density. It seems like you selected the cell

density because it resulted in tube formation and, hence, allowed for parameter comparison studies.

We thank the reviewer for this excellent point. We initially benchmarked our studies using varying concentrations of LECs (1×10^6 , 3×10^6 , 5×10^6 , 8×10^6 , and 10×10^6 cells/mL). As the reviewer correctly pointed out, the degree of lymphatic tube formation depends on cell density. While increasing cell density resulted in more connected lymphatic vessels, 8×10^6 cells/mL was determined to be the threshold required for network formation. Therefore, subsequent studies were conducted using 8×10^6 cells/mL instead of 10×10^6 cells/mL in order to conserve the number of cells needed per hydrogel construct. We have included this data in **Supplementary Figure 3** and included additional sentences to explain this in the **Results**.

“While increasing the cell density beyond 8 million/mL resulted in qualitatively slightly more connected lymphatic networks, 8 million/mL LECs was determined to be a critical threshold and was selected for use in the subsequent parameter screenings, both to limit the cells required per hydrogel construct and to allow for systematic investigation of individual parameters.”



Supplementary Figure 3. To investigate how cell density affects lymphatic tube formation and to determine what cell density was a critical minimum for this system, hydrogels were fabricated with varying cell densities. At day 5, hydrogels were fixed and immunofluorescently stained to visualize tubes. A density of 8 million LECs/mL was determined to be the critical minimum.

4. Results/Discussion: Line 338 – “Additionally, a tumor suppressor, Folliculin, has been shown to be responsible for maintaining distinct blood and lymphatic vasculature and prevents vessel mismatch.” I might have missed it, but I do not think Folliculin was added to your system. The relevance of this statement is not clear. Please edit to clarify your thinking. In addition, I am fascinated by Figure 7. I suggest adding more rationale or discussion related to the labeling method. If I understand correctly, you injected lectin into the foot pad and then harvested at a distal site. Labeling of human LECs would then suggest that these vessels are functional and connected to the draining mouse lymphatics in the footpad. Does this make sense? I think this is super intriguing and potentially novel. The potential finding raises questions about whether or not all human vessels are functional. Also, in Figure 7 there are a lot of green vessels. I assume some of these are blood vessels. Could this be the case? While the vessel identity and potential interaction between host blood vessels and human derived LECs is not totally surprising and could be expected based on recent examples of vessel interactions in the literature, the results are still convincing that something is going on. I think this is what is important and I suggest recognizing that the results are suggestive versus being conclusive. You probably do not know what is really going on. If you are convinced that the 2 GSI-lectin is lymphatic specific, then I suggest expanding on your rationale in the text.

We really appreciate the reviewer for this excellent point. We cited the paper by Tai-Nagara et al to highlight that Folliculin, a tumor suppressor gene, is responsible for the separation between blood and lymphatic systems. Since we used NOD/SCID mice without altered folliculin gene, we would expect that there would be no mixture between the blood and lymphatic systems. The injected fluorescein-conjugated *Isolectin Griffonia simplicifolia* (GS-IB 4 isolectin) is specific to mouse ECs and the rhodamine-conjugated *Ulex Europaeus Agglutinin I* (UEA-1 lectin) stained the human LECs, since it is the only human ECs in the system. The GS-IB 4 isolectin stained both mouse blood and lymphatic ECs, contributing to the large amount of green vessels that the reviewer has noted. However, since we used mice without altered folliculin gene, we would expect that human LECs would only interact with mouse LECs. The reviewer raised an interesting point if all the implanted human LECs are functional. While there are many positive cells stained for human cells, we speculate that there may still many other human cells that are not functional. Nonetheless, as the reviewer currently pointed out that the results are suggestive versus being conclusive. Further genetic labelling and lineage tracing would probably be useful for future studies to elucidate the interactions and integration between human LECs and mouse LECs. We have included some sentences to highlight this important point in the **Discussion** section.

“The interaction between the engineered human lymphatic vessels and murine lymphatic vessels raises many interesting questions surrounding the fate of the engineered human cells, which could be further elucidated using genetic labeling and lineage tracing.”

“While the uptake of species-specific lectin dyes suggest that the engineered lymphatic networks can function and integrate with the host lymphatic vasculatures, future studies should explore the prospect of the lymphatic engineered approaches to drain fluid and how they interact with the host lymphatic vessels in clinically relevant lymphedema models^{54–56}.”

5. Line 48 - “first ever”. I suggest qualifying this claim. Is it the first 3D approach?

We thank the reviewer for this excellent point. While previous methods have reported the generation of lymphatic vessels in synthetic matrices, our study represents the first to report a 3D lymphatic tissue engineering approach to not require the use of support cells, such as fibroblasts or stem cells. We have included this clarification in the **Abstract** and **Discussion**.

Reviewer #2:

Overview:

Engineered lymphatic vessels hold significant promise as a novel strategy for addressing lymphatic disorders. However, the optimal parameters for lymphatic engineering remain a challenge. This manuscript introduces a novel platform using hyaluronic acid (HA)-hydrogels to investigate lymphatic vessel formation within a synthetic matrix that incorporates both mechanical and biochemical cue. Notably, this study marks the first attempt at engineering lymphatic vessels without support cells, allowing independent modulation of parameters while keeping other factors fixed. The ability to investigate the effect of matrix composition, mechanics, and growth factors is a commendable aspect of the study, with potential implications for vascularizing organoids and transplantation therapies for lymphatic disorders. Nevertheless, several crucial points appear inadequate or missing, necessitating a major revision.

We appreciate the reviewer’s thoughtful critique of the manuscript. We are excited to hear that the reviewer found the study potentially impactful and beneficial to the field of lymphatic biology and tissue engineering. We have attempted to address all the comments and concerns as described below. We thank the reviewer for the opportunity to greatly improve the clarity and scientific validity of the manuscript.

Major comments:

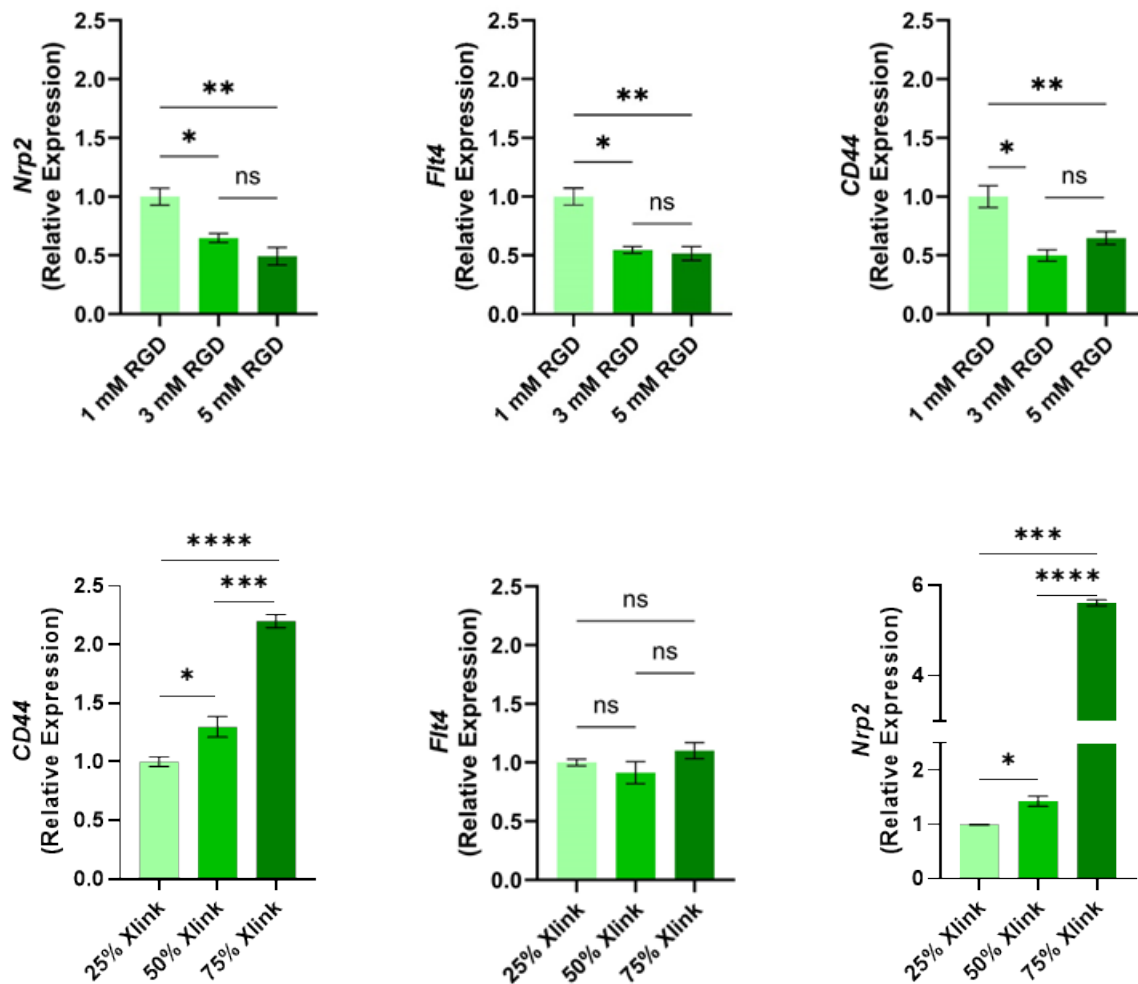
1. The expression of *Flt4*, *Nrp2*, and *CD44* appears minimally or not different across % D.S. conditions alone (Figure 2). Given that 21% D.S. NorHA, along with optimal RGD, VEGFC, Ang2, and crosslinker concentration, promotes lymphatic tube formation compared to 21 %D.S. alone, it is essential to explore whether these multiple conditions impact *Flt4*, *Nrp2*, and *CD44* expressions more significantly.

We really appreciate the reviewer for this excellent suggestion. We have performed additional experiments to investigate the influence of various RGD peptide concentrations (1 mM, 3 mM, and 5 mM) and MMP-sensitive peptide concentrations (25%, 50%, and 75%) on the expression of key lymphatic markers *Flt4*, *Nrp2*, and *CD44*. With increasing RGD peptide concentrations, we observed a decrease in *Flt4*, *Nrp2*, and *CD44* expression. Conversely with increasing MMP-sensitive peptide concentrations, we observed an increase in *Flt4*, *Nrp2*, and *CD44* expression. While these trends deviate from what was hypothesized based on the morphology observed in hydrogels with

corresponding conditions, it's important to note that RNA was isolated at day 5 in order to remain consistent with the methodology used for **Figure 2**. However, day 5 aligns with the time point when network formation reaches a maximum, so it is possible that by day 5, LECs in less optimal gel conditions are upregulating expression of these key markers in an attempt to signal lymphangiogenesis, while in the optimal conditions network formation has already occurred and early signaling for lymphangiogenesis has subsided. In gels with less optimal conditions, perhaps RNA is not being translated, resulting in higher RNA-level expression of these markers but a lower level of network formation relative to the optimal gel conditions and corresponding to the morphology observed. We have included this additional data in the **Results** and **Discussion** to highlight the tunability of our system.

*“Analysis of key lymphatic markers by qRT-PCR at day 5 demonstrated an inverse trend compared to the observed morphology, where increasing RGD peptide concentrations decreased the expression of CD44, Flt4, and Nrp2 (**Supplementary Figure 5A**). Due to RNA being isolated at peak network formation, we hypothesize that by day 5, LECs in sub-optimal gel conditions are upregulating expression in an attempt to signal lymphangiogenesis, but it might not be translated. Future studies should more extensively investigate the kinetics of key lymphatic marker expression in correspondence with morphology.”*

*“Interestingly and contrary to results in our prior 2D hydrogel system where Flt4 expression increased with decreasing substrate stiffness¹⁵, in this 3D system Flt4 expression increased with increasing matrix stiffness (**Supplementary Figure 5B**), despite LECs failing to self-assemble in these stiffer environments. This data may suggest that LECs try to upregulate key genes to compensate for the higher crosslinking density and stiffer environment. In order to confirm that LECs could not self-assemble in stiffer environments, even given a longer time frame to remodel their environment, 75% crosslinked gels were cultured for up to 21 days but tubes failed to develop (data not shown), suggesting that Flt4 might not be translated into VEGF-C in these stiffer environments and is a point of investigation in future studies.”*



Supplementary Figure 5. To investigate the influence of RGD (**A**) and MMP-sensitive peptide (**B**) concentrations on the expression of key lymphatic markers, hydrogels were lysed at day 5 and RNA was purified for real-time qRT-PCR. Differential gene expression was analyzed using unpaired t-tests. Significance levels were set at ns>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

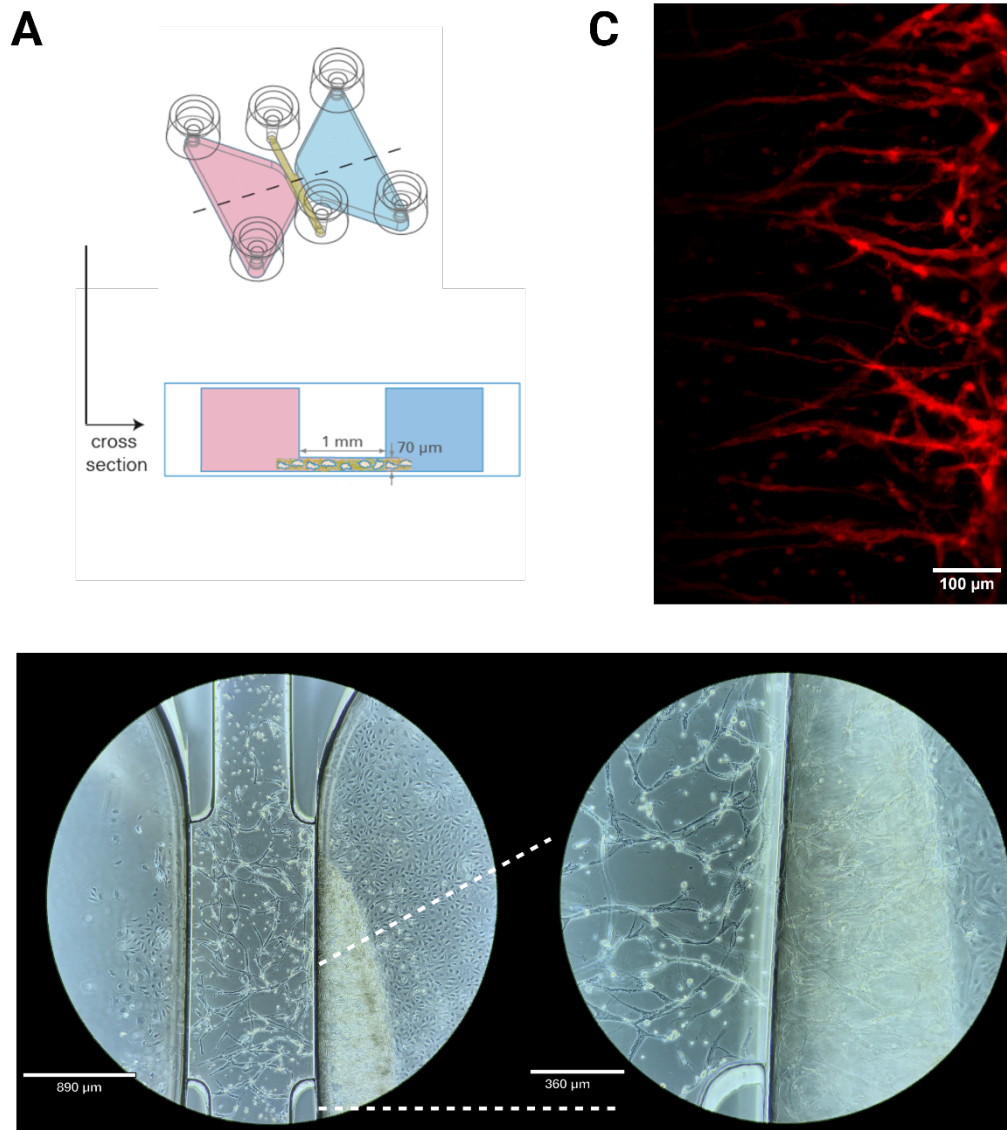
2. Consider addressing the dynamic modification of matrix mechanics to accelerate and sustain lymphatic formation. The study suggests that while less than 10% crosslinked gel is better for initiation, it is worse for maintenance, with the opposite observed for high crosslinked gel. Could a sequential approach be employed, initiating lymphatic formation with a lower crosslinked gel and subsequently transitioning to a higher crosslinked gel?

We thank the reviewer for this excellent point. The dynamic modification of matrix mechanics seems to be very important for lymphatic tube formation. As the reviewer correctly pointed out, sequential crosslinking methods can be used to further crosslink the hydrogels, so we can further evaluate the influence of higher crosslinking gel in lymphatic tube stabilization¹. In fact, our research group just recently published a similar strategy, where we used guest-host interactions to study lymphatic tube formation². We found that LECs can form lymphatic tube networks more rapidly in viscoelastic hydrogels. Further exposure to UV light (365 nm) caused dimerization of the guest-host molecules to form covalent bonds. Consequently, these dynamics hydrogels can be used to study hydrogels and mechanical properties can influence lymphatic network formation. We have included this as part of the **Discussion**.

“By using a UV-driven crosslinking system, this hydrogel platform allows for sequential cross-linking steps and could serve as a novel platform to investigate the effects of a stiffening matrix. For example, LECs could be encapsulated in optimal conditions, allowed to self-assemble in a partially crosslinked (<100%) system, and then additional crosslinker could be swelled in to stiffen the matrix and to investigate genetic and morphological changes on developed vessels after matrix stiffening occurs. Recent advances in dynamic hydrogels can also be used to investigate the influence of viscoelastic properties on lymphatic tube formation⁵³.”

3. Evaluate the functionality (drainage) of the engineered lymphatic vessels. While the integration of mouse and human vasculature using Imaris imaging software is noted, consider injecting dye into the surrounding tissue and assessing lymphatic drainage function in the hydrogel construct, or vice versa.

We thank the reviewer for this excellent point. We have performed additional experiments to demonstrate that the engineered lymphatic networks are perfusable and able to drain fluids. We encapsulated LECs in NorHA hydrogel within a perfusion chip. After 5 days of culture, robust lymphatic networks were formed within the center channel and we added FluoSpheres Polystyrene Microspheres (1µm in diameter, red fluorescent) in one side channel to test perfusion. Imaging lymphatic draining *in vivo* can be technically very challenging, but these perfusion chips allow for observation and assessment of lymphatic transport in real time via confocal microscopy. While this perfusion chip does not recapitulate all the *in vivo* physiology, it suggests that the engineered lymphatic networks are functional and perfusable. Of note, we are still in the early process of benchmarking a tail lymphedema model, which we believe can be used to assess lymphatic drainage function of the engineered lymphatic networks in the future. While we agree with the reviewer that assessing lymphatic drainage is important, we envision this to be part of future studies with a longer timepoint using clinically relevant lymphedema models^{3,4}.



Supplementary Figure 8. (A) The perfusion chip, μ -Slide Chemotaxis (ibidi) was used to investigate the perfusability of the engineered lymphatic networks. A schematic of the perfusion chip was adapted from the user manual. The gels and cell mixture were placed in the middle channel and pushed toward the reservoir for better contact to the media and beads solution. (B) At day 5 after culture, bright field images show robust lymphatic networks formed in the middle of the channel. FluoSpheres Polystyrene Microspheres (1 μ m in diameter, red fluorescent) were added to one side of the reservoirs. (C) After 12 hrs of incubation, the perfusion chip was analyzed using confocal microscopy. Fluorescent images (580/605 nm) indicate lymphatic networks were perfused with FluoSpheres Polystyrene Microspheres.

Minor comments:

1. In the result of HA binding site availability influences LEC phenotype and vessel formation, it is mentioned that Nrp2 expression shows no differences in varied HA binding site availability (Figure 2F). However, the figure indicates significant differences (** or *) in Nrp2 expression, requiring clarification or correction.

We appreciate the reviewer for this critical suggestion. While the figure indicates significant differences (** or *) in *Nrp2* expression, there is very small differences in magnitude compared to the differences in other genes investigated (*LYVE-1*, *Prox1*, and *Pdpr*). We have edited the sentence to clarify this point in **Results** section.

“Collectively, the data suggests that while the availability of HA influences the expression of Flt4 and Nrp2, it has a more significant impact on the expression of LYVE-1, Prox-1, and Pdpr.”

Reviewer #3:

Remarks to the Author:

In their submission to Communications Biology, Alderfer et al., have developed a hyaluronic acid (HA)-hydrogel to closely investigate the influence of mechanical and biochemical cues on the formation of lymphatic vessels in a synthetic scaffold. The novelty of this work is engineering a 3D synthetic scaffold in which human lymphatic endothelial cells (LECs) can self-assemble in vitro into lymphatic vessels within 3 days and without the use of support cells. Overall, the text is very well-written, organized, and easy to follow. This work introduces an innovative approach to engineering lymphatic vessels, setting it apart from previous studies in the field.

We appreciate the reviewer's thoughtful critique of the manuscript. We are excited to hear that the reviewer found the study potentially impactful and beneficial to the field of lymphatic biology and tissue engineering. We have attempted to address all the comments and concerns as described below. We thank the reviewer for the opportunity to greatly improve the clarity and scientific validity of the manuscript.

Specific comments:

1. In figures 4 and 6, please add quantified data of the lymphatic vessel network for reading to assess the quality of these networks? Like vessel density? Branching? Or other morphological quantifications.

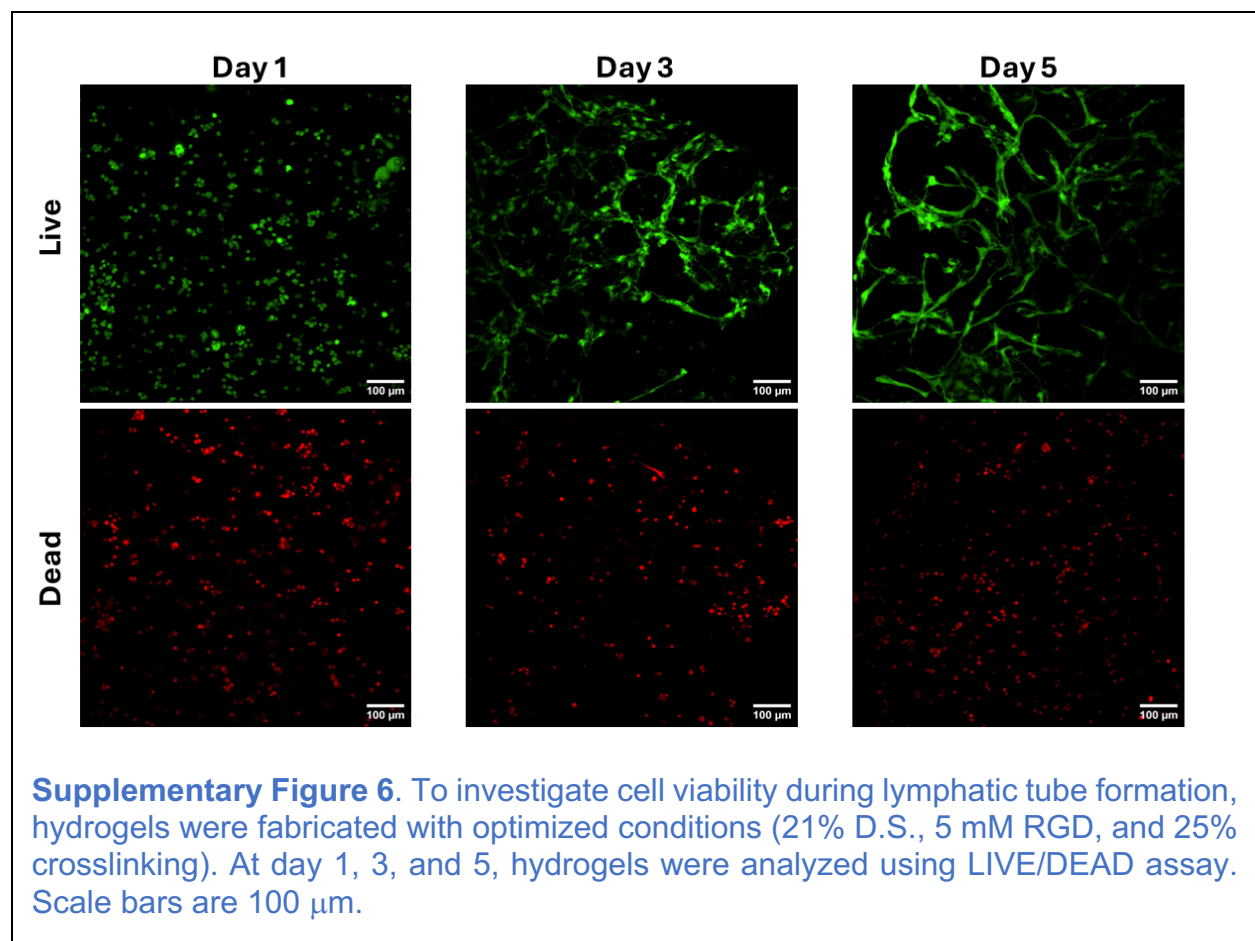
We thank the reviewer for this excellent point. While we agree that morphological quantifications can be useful, we found that quantifying tube networks in 3D hydrogels can be very technically challenging, due to the networks being variable thicknesses throughout the hydrogels which makes normalizing analyzed areas difficult, as well as

imaging through a thick (~3mm) hydrogel and variable signal intensities due to variable imaging depths to locate the maximum network area. Therefore, we opted for a qualitative approach where we scanned the entire hydrogel at a lower magnification to locate the maximum network area and then imaged the hydrogel constructs to assess qualitatively for network connectedness.

2. Additionally, in those networks, the images show that many LECs do not assemble into networks. Is it possible these cells are apoptosed and/or dysfunctional? Can they show the viability of these cells by running a live-dead assay?

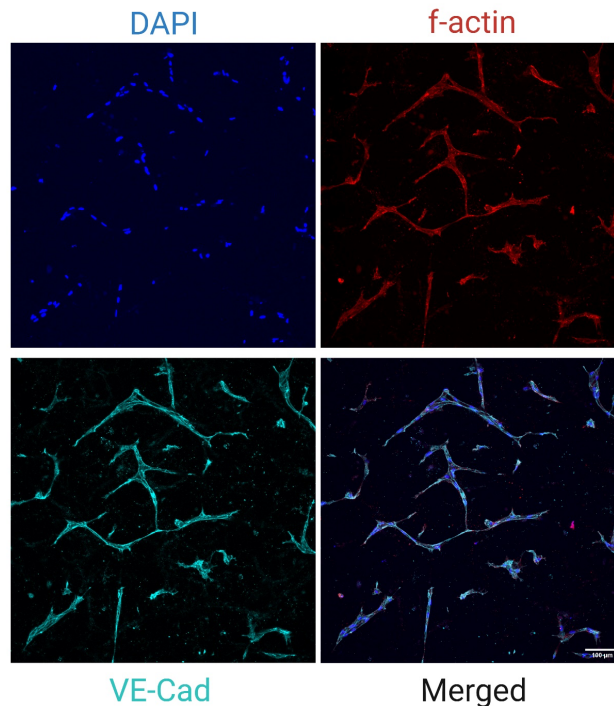
We thank the reviewer for this excellent point. We have performed an additional live/dead assay to evaluate the viability of the cells in the gels. We observed that most of the cells that formed tubes were alive, while the majority of the cells that do not form tubes were dead cells (**Supplementary Figure 6**). We have included a sentence to clarify this point in the **Results** section.

“Using a LIVE/DEAD stain across different time points of network self-assembly, we observe that healthy LECs assemble into tubes while many of the cells that do not contribute to tubes were dead, possibly as a result of the UV crosslinking process (Supplementary Figure 6).”



3. The authors report that the lymphatic tubes can be cultured for up to 21 days *in vitro* – is there any supplementary data to support these claims? Please show the culture and some metric of stability of the vascular health for 21 days of culture.

We thank the reviewer for this important point. We have included lymphatic tubes at day 22 as part of **Supplementary Figure 7**. We used morphological inspection and immunostaining to show the stability of the lymphatic vessel.



Supplementary Figure 7. Hydrogels fabricated with the optimized parameters were cultured long-term and visually inspected daily to monitor how long they were stable for. Media was replaced daily. After 22 days of culture, vessels could still be observed and hydrogels were fixed for immunofluorescent staining. Staining for DAPI, f-actin, and VE-Cadherin outlined the vessels and demonstrated that vessels were still throughout the hydrogel after 3 weeks of *in vitro* culture. Scale bar: 100μm.

Minor comment:

Can the authors justify the choice of the sex of the mouse model? I think it's crucial that they state the rationale for choosing one sex in the study design over the other, i.e. a female mouse, and to acknowledge any limitations associated with that choice.

We thank the reviewer for this important point. Since most secondary lymphedema cases occur due to surgical resection to remove breast cancer in female patients, we wanted to study if we can use the engineered lymphatic networks in this context. Accordingly, we used a murine mammary gland model, which has been previously used in the context of patient-derived tumor xenograft models of breast cancers. Nonetheless, the reviewer correctly pointed out that choosing this model using female mouse may introduce some limitations. In the future, we hope to follow up this study using a different lymphedema model (i.e., tail vein lymphedema), where we can study the engineered lymphatic networks in the context of sex-differences. We have included limitations of the current study in the **Discussion** section.

"It is also important to note that the current study was done in a murine mammary gland model using female mice. While this model mimics the clinical case of secondary lymphedema in breast cancer patients, it precludes sex as a biological variable."⁵

References

1. Vega SL, Kwon MY, Song KH, et al. Combinatorial hydrogels with biochemical gradients for screening 3D cellular microenvironments. *Nature Communications*. 2018;9(1):614.
2. Fan F, Su B, Kolodychak A, et al. Hyaluronic Acid Hydrogels with Phototunable Supramolecular Cross-Linking for Spatially Controlled Lymphatic Tube Formation. *ACS Appl. Mater. Interfaces*. 2023;15(50):58181–58195.
3. Hadamitzky C, Zaitseva TS, Bazalova-Carter M, et al. Aligned nanofibrillar collagen scaffolds – Guiding lymphangiogenesis for treatment of acquired lymphedema. *Biomaterials*. 2016;102:259–267.
4. Michalaki E, Rudd JM, Liebman L, et al. Lentiviral overexpression of VEGFC in transplanted MSCs leads to resolution of swelling in a mouse tail lymphedema model. *Microcirculation*. 2023;30(2–3):e12792.
5. McClain AK, Monteleone PP, Zoldan J. Sex in cardiovascular disease: Why this biological variable should be considered in in vitro models. *Science Advances*. 10(19):eadn3510.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

Thank you for addressing my comments.

Reviewer #2 (Remarks to the Author):

Thank you for your prompt and thorough revision of your manuscript titled “Multi-parameter Tunable Synthetic Matrix for Engineering Lymphatic Vessels.” I appreciate the effort and time you have dedicated to addressing the comments and suggestions provided in the initial review.

The inclusion of more comprehensive data on gene expressions under complex conditions, dynamic modifications, and functional evaluations of the engineered lymphatic vessels significantly enhances the manuscript. These additions provide potential implications for vascularizing organoids and transplantation therapies for lymphatic disorders, making the study more promising.

The overall quality of the manuscript has substantially improved, and the revisions have strengthened its clarity and scientific rigor.

I am pleased to recommend the manuscript for acceptance in its current form.

Once again, I commend you on the improvements made and look forward to seeing this important work published.

Reviewer #3 (Remarks to the Author):

The authors have addressed the comments.