

Characterization of the cellular components of mouse collecting lymphatic vessels reveals that lymphatic muscle cells are the innate pacemaker cells regulating lymphatic contractions

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This manuscript aims to identify the pacemaker cells in the lymphatic collecting vessels - the cells that initiate the autonomous action potentials and contractions needed to drive lymphatic pumping. Through the exemplary use of existing approaches (genetic deletions and cytosolic calcium detection in multiple cell types), the authors **convincingly** determine that lymphatic muscle cells are the origin of the action potential that triggers lymphatic contraction. The inclusion of scRNAseq and membrane potential data enhances a tremendous study. This **fundamental** discovery establishes a new standard for the field of lymphatic physiology.

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Abstract

Collecting lymphatic vessels (cLVs) exhibit spontaneous contractions with a pressure-dependent frequency, but the identity of the lymphatic pacemaker cell is still debated. By analogy to pacemakers in the GI and lower urinary tracts, proposed cLV pacemaker cells include interstitial cells of Cajal like cells (ICLC) or the lymphatic muscle (LMCs) cells themselves. Here we combined immunofluorescence and scRNAseq analyses with electrophysiological methods to examine the cellular constituents of the mouse cLV wall and assess whether any cell type exhibited morphological and functional processes characteristic of pacemaker cells: a continuous if not contiguous network integrated into the electrical syncytium; spontaneous Ca^{2+} transients; and depolarization-induced propagated

contractions. We employed inducible Cre (iCre) mouse models routinely used to target these specific cell populations including: c-kitCreER^{T2} to target ICLC; *Pdgfrβ*CreER^{T2} to target pericyte-like cells; *Pdgfra*CreERTM to target CD34⁺ adventitial cells and ICLC; and *Myh11*CreER^{T2} to target LMCs directly. These specific inducible Cre lines were crossed to the fluorescent reporter ROSA26mT/mG, the genetically encoded Ca²⁺ sensor GCaMP6f, and the light-activated cation channel rhodopsin2 (ChR2). c-KitCreER^{T2} labeled both a sparse population of LECs and round adventitial cells that responded to the mast cell activator compound 48-80. *Pdgfrβ*CreER^{T2} drove recombination in both adventitial cells and LMCs, limiting its power to discriminate a pericyte-specific population. *Pdgfra*CreERTM labeled a large population of interconnected, oak leaf-shaped cells primarily along the adventitial surface of the vessel. Of these cells, only LMCs consistently, but heterogeneously, displayed spontaneous Ca²⁺ events during the diastolic period of the contraction cycle, and whose frequency was modulated in a pressure-dependent manner. Optogenetic depolarization through the expression of ChR2 under control of *Myh11*CreER^{T2}, but not *Pdgfra*CreERTM or c-KitCreER^{T2}, resulted in propagated contractions upon photo-stimulation. Membrane potential recordings in LMCs demonstrated that the rate of diastolic depolarization significantly correlated with contraction frequency. These findings support the conclusion that LMCs, or a subset of LMCs, are responsible for mouse cLV pacemaking.

Impact

The presence and functionality of proposed pacemaker cells in collecting lymphatic vessels was tested with various mouse Cre models to drive expression of a recombination reporter ROSA26mT/mG, a genetically encoded Ca²⁺ sensor GCaMP6f, or the optogenetic tool channel-rhodopsin2. Lymphatic CD34⁺ adventitial cells co-express PDGFRα⁺ while vessel-associated cKit⁺ cells are mast cells; and neither cell type demonstrated pacemaking capability. *Myh11*CreER^{T2} identified lymphatic muscle cells that exhibited pacemaker behaviors such as pressure-dependent depolarization and calcium events during diastole and propagated contraction waves induced by focal, optical stimulation of channel-rhodopsin2.

Introduction

The spontaneous contractions of collecting lymphatic vessels (cLV) are an integral component to fluid and macromolecule homeostasis as they provide the force to transport fluid from the interstitial spaces back to the blood circulation (Scallan et al., 2016 [\[1\]](#)). In humans, spontaneous contractile activity is estimated to account for 2/3 of lymph transport (Engeset et al., 1977 [\[2\]](#)) and this function is significantly compromised in patients suffering from lymphedema, whose cLVs typically display weak and irregular or entirely absent contractile activity (Olszewski, 2002 [\[3\]](#)). *Ex vivo* studies, in which the intraluminal pressure can be precisely controlled, have refined our understanding of the pressure-dependent regulation of contraction frequency (Benoit et al., 1989 [\[4\]](#); Gashev et al., 2004 [\[5\]](#)), with some mouse cLVs displaying a 10-fold increase in contraction frequency over a 10 cmH₂O pressure gradient (Scallan and Davis, 2013 [\[6\]](#); Zawieja et al., 2018a [\[7\]](#)). The observation that cLVs, often cannulated at various lengths for *ex vivo* preparations, retain a consistently tunable contraction frequency points to the presence of a pacemaker cell(s) innate to the structure of the cLV wall and with a seemingly ubiquitous presence along the length of the vessel (Zawieja et al., 1993 [\[8\]](#); Castorena-Gonzalez et al., 2018b [\[9\]](#)). Furthermore, isolated cLVs typically display single pacemaker initiation sites unless damaged or electrically uncoupled by pharmacological inhibition of gap junctions or genetic deletion of *Gjc1* (Connexin 45, Cx45) (Behringer et al., 2017 [\[10\]](#); Castorena-Gonzalez et al., 2018b [\[9\]](#); Castorena-Gonzalez et al., 2020 [\[11\]](#)). In

sum, this suggests the pacemaker cell(s) is(are) likely both ubiquitous and continuous, to allow for electrical conduction via gap junctions, along the length of the cLV and prevent colliding contractile waves which would impair lymph transport.

Investigations into the cLV pacemaker identity have focused largely on cells termed interstitial cells of Cajal like cells (ICLC; or telocytes) (McCloskey et al., 2002 [↗](#); Briggs Boedtkjer et al., 2013 [↗](#)), as they display some morphological and cell marker expression profiles similar to the interstitial cells of Cajal (ICC), which are bona fide pacemakers in the gastrointestinal (GI) tract. ICC are classically identified by either methylene blue staining and expression of cKit, and coordinate GI smooth muscle contraction (Maeda et al., 1992 [↗](#); Ward et al., 1994 [↗](#); Ordog et al., 1999 [↗](#)). ICC also express the canonical Ca^{2+} activated chloride channel Anoctamin 1 (Ano1) (Gomez-Pinilla et al., 2009 [↗](#)), which is required for pacemaker activity (Hwang et al., 2009 [↗](#); Zhu et al., 2009 [↗](#); Singh et al., 2014 [↗](#)). Previous reports in sheep mesenteric lymphatic vessels identified a population of cKit⁺, vimentin⁺, ICLC in the vessel wall between the endothelial and LMC layer and running along the axis of the vessel (McCloskey et al., 2002 [↗](#)). Investigations in the human thoracic duct also identified a significant population of ICLC in close proximity to the lymphatic muscle cells (LMCs) evident by methylene blue staining, immunostaining for CD34, vimentin, and cKit, as well as the gold standard of electron microscopy (Briggs Boedtkjer et al., 2013 [↗](#)). However, neither study could determine if these cells had functional electrical communication with the LMCs or demonstrate either a membrane electrical clock or internal Ca^{2+} clock to drive the rhythmic lymphatic vessel contractions observed *ex vivo*. LMCs share a functional similarity to ICC in that they also display the *Ano1* mediated Ca^{2+} activated chloride current (Van Helden, 1993 [↗](#); Toland et al., 2000 [↗](#); Mohanakumar et al., 2018 [↗](#)) (Zawieja et al., 2019 [↗](#)), that regulates pacemaking. Spontaneous transient depolarizations, presumably *Ano1* dependent, were recorded in mesenteric cLVs from guinea pigs (Van Helden, 1993 [↗](#); von der Weid et al., 2008 [↗](#)) providing a mechanism for membrane potential instability to drive AP initiation. Furthermore, computational models have proposed LMC sarcoplasmic reticulum (SR) Ca^{2+} release as the oscillator mechanism driving pacemaking (Imtiaz et al., 2007 [↗](#)). SR Ca^{2+} release has also been implicated in pericyte regulation of arterioles (Hashitani et al., 2015 [↗](#); van Helden and Imtiaz, 2019 [↗](#)), in microvascular vasomotion (Boedtkjer et al., 2008 [↗](#); Aalkjaer et al., 2011 [↗](#); van Helden and Imtiaz, 2019 [↗](#)), and in the contraction waves of atypical muscle cells of the lower urinary tract (Grainger et al., 2022 [↗](#)).

Presently, no investigations have clearly identified the cellular identities of possible pacemaker cells within the cLVs of the mouse. Mouse cLVs exhibit contractile parameters and conduction speed equivalent to those of human vessels (Castorena-Gonzalez et al., 2018b [↗](#)) and their simplified architecture, compared to larger mammals, in combination with the genetic tools developed for the mouse model, allowed us to test for a fundamental pacemaker cell in the cLV. In this study we utilized multiple genetic mouse models, confocal imaging of fluorescent reporters, cell specific expression of GCaMP6f for Ca^{2+} imaging, and optogenetic light-activated depolarization to both visualize and test the functional aspects of putative pacemaker cells, and membrane potential recordings in LMCs in pressure-challenged cLVs. We also performed immunostaining and single cell RNA sequencing (scRNAseq) of isolated cLVs to provide greater detail to the heterogeneous cellular populations found within the mouse cLVs. Despite identifying a significant population of CD34⁺Pdgfra⁺ adventitial cells along the length of mouse cLVs, the results of our functional studies support a myogenic origin of pacemaking in cLVs.

Results

Methylene Blue Staining Reveals a Minor Population of Cells in Mouse cLVs

Methylene blue staining was used to identify an ICLC population in the human lymphatic thoracic duct (Briggs Boedtkjer et al., 2013 [↗](#)). In our isolated and cleaned lymphatic inguinal axillary collecting vessels (IALVs), methylene blue stained a significant number of cells with variable density along the length of the IALV and heterogeneous cell morphologies (**Figure 1A-C** [↗](#)). A significant portion of the stained cells resembled lymphatic vessel-associated macrophages with an elongated shape, while other cells were smaller and circular (**Figure 1D-F** [↗](#)). Methylene blue also appeared to stain mast cells as there were large ovoid cells on the adventitia of the vessel with intracellular granules. In addition, methylene blue stained a minor population of cells that exhibited long and thin axon-like extensions which appeared to have a slight helical orientation, with a small central body and nucleus (**Figure 1C** [↗](#)). None of these cell populations were aligned with the longitudinal axis of the vessel that would permit efficient coupling or regulation across the circumferential LMCs required for coordinated propagation along the length of the vessel.

Immunofluorescence Imaging of IALVs Stained for ICLC, LEC, and LMC Markers

We next stained IALVs for the putative telocyte/ICLC markers cKIT, CD34, and the intermediate filament Vimentin, which have been previously utilized for ICLC identification in human and sheep lymphatic tissues (McCloskey et al., 2002 [↗](#); Briggs Boedtkjer et al., 2013 [↗](#)). Additionally, an antibody to the intermediate filament Desmin was used to label muscle cells (McCloskey et al., 2002 [↗](#)). IALVs stained with cKIT (**Figure 2B** [↗](#)) showed robust signal in large ovoid cells with a non-segmented circular nucleus (**Figure 2A** [↗](#)), characteristic of mast cells that were located in the outer part of the adventitia. Similarly, cKIT stained populations of elongated cells as well as circular cells with variable densities throughout the IALV wall, similar to methylene blue⁺ cell populations (**Figure 2B, J** [↗](#)). Staining for CD34 revealed a large population of cells that were seemingly contiguous along the length of the vessel. The CD34⁺ cells generally had multiple lobular processes and a “oak leaf” like appearance, typically a characteristic of fibroblasts, though some contained short, thin dendrite-like extensions (**Figure 2C, G, K** [↗](#)). The CD34⁺ cells were negative for Desmin (**Figure 2H** [↗](#)), which primarily stained the circumferential LMCs (**Figure 2F** [↗](#); note that the largely non-circumferential cell organization in this region is typical for a lymphatic endothelial valve site (Bridenbaugh et al., 2013a [↗](#))). Furthermore, CD34⁺ cells and cKIT⁺ cells were separate populations (**Figure 2D, L** [↗](#)). A Vimentin antibody labeled lymphatic endothelial cells (LECs) which exhibited a horizontal cobblestone morphology in parallel with the vessel axis (**Figure 2E, I** [↗](#)), while also co-labeling the majority of the CD34⁺ cells (**Figure 2H** [↗](#)) and cKIT⁺ cells (**Figure 2L** [↗](#)). Videos of the half vessel z-stacks are provided (Supplemental Movies 1-3 for **Figure 2D, H** [↗](#), and **L** [↗](#) respectively).

Of the cells stained in **Figure 2** [↗](#), the CD34⁺ population was intriguing due to its high density and distribution throughout the length of the IALV, which potentially would be conducive to effective regulation of LMC excitability. In addition to CD34 and Vimentin, PDGFRα staining is also commonly ascribed to both telocytes (Vannucchi et al., 2013 [↗](#); Xiao et al., 2013 [↗](#); Zhou et al., 2015 [↗](#)) as well as fibroblasts (Kimura et al., 2021 [↗](#); Clayton et al., 2022 [↗](#)). We performed immunofluorescence imaging for PDGFRα counterstained with CD34 and markers for LMCs, LECs, and pericytes. As noted in **Figure 2** [↗](#), CD34⁺ cells (**Figure 3A** [↗](#)) did not co-label LMCs (**Figure 3D** [↗](#)) which were smooth muscle actin⁺ (SMA, **Figure 3B** [↗](#)) and Calponin⁺ (**Figure 3C** [↗](#)). However, nearly all CD34⁺ (**Figure 3E** [↗](#)) cells were also PDGFRα⁺ (**Figure 3F** [↗](#), H). Occasionally some overlap of PDGFRα and SMA⁺ signal was noted (**Figure 3G** [↗](#), H). LECs staining with CD31 (PECAM, **Figure 3I** [↗](#)) revealed the expected rectangular elongated cobblestone morphology that was distinct from the PDGFRα⁺ cells (**Figure 3J, L** [↗](#)). Staining for Calponin also specifically labeled LMCs (**Figure 3K** [↗](#)) but not PDGFRα⁺ cells (**Figure 3L** [↗](#)). Lastly, we stained for PDGFRα, CD34,

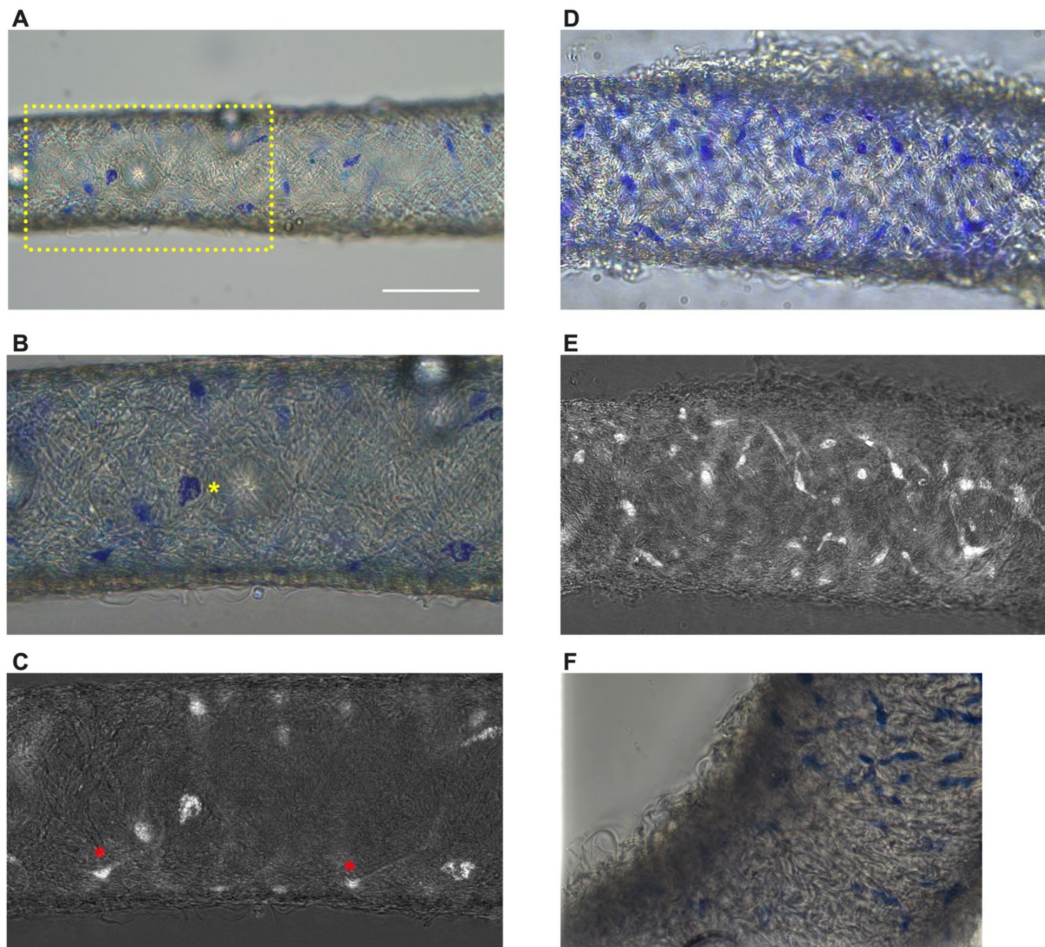


Figure 1

Methylene blue staining of isolated mouse IALVs

Representative image of an isolated and cleaned IALV after methylene blue staining which revealed cells of various morphology. (B) is the zoomed in image of the yellow dotted box in A which contained large ovoid cells with granular staining (B, yellow asterisks). Fine cellular extensions (red asterisks) stained by methylene blue in some cells were visualized with color channel separation and division (C). (D, E) Similar as B and C, but in a separate vessel which stained with a higher density of methylene blue stained cells some of which had limited cellular processes. F) Focal reconstruction from imaging a methylene blue stained IALV using an upright microscope and immersion objective.

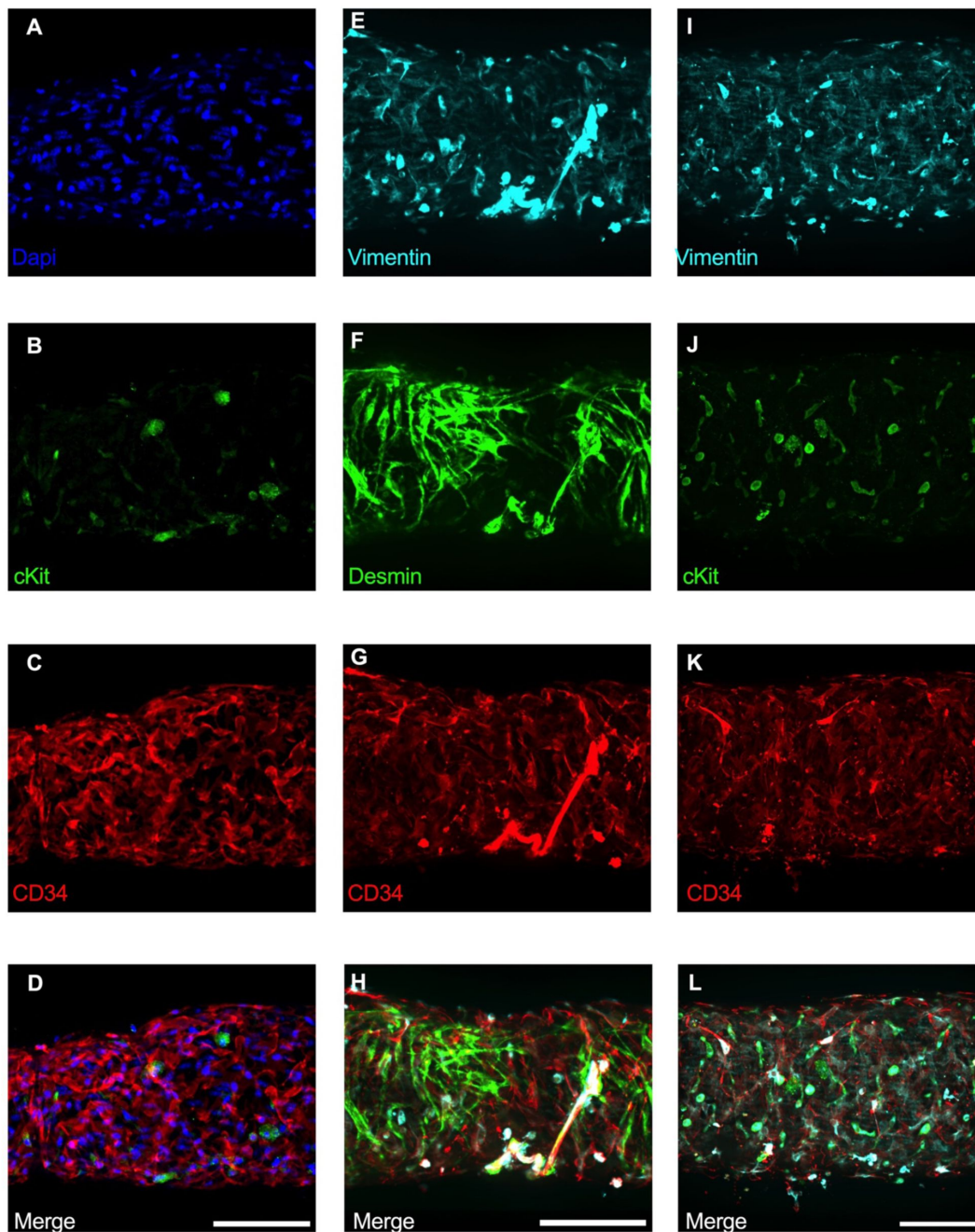


Figure 2

Staining Mouse IALVs for ICLC Markers

Representative immunofluorescent max projections of half vessel confocal image stacks imaged from mouse IALVs stained for ICLC markers. DAPI (A), cKit (B), and CD34 (C) and their merged image (D). Representative max projections of the intermediate filament vimentin (E), the intermediate filament desmin (F), CD34 (G) and their merged image (H). Representative max projection of vimentin (I), cKit (J), CD34 (K) and their merged image (L). Scale bar = 100 μ m for all images.

and PDGFR β , a commonly used pericyte marker (**Figure 3 M-P**). As above, CD34 and PDGFR α were highly colocalized (**Figure 3Q, R, T**), and many of the CD34 $^{+}$ and PDGFR α $^{+}$ cells were also PDGFR β $^{+}$ (**Figure 3P**). PDGFR β also stained some circumferential LMCs (**Figure 3Q**). During the imaging of mouse IALVs for these markers, we also observed that the lymphatic secondary endothelial valves were populated by elongated cells that stretched the length of the valve leaflet and were positive for CD34, PDGFR α , and PDGFR β , with varying intensities. These cells could be observed in most, if not all, the valves we assessed and found within both leaflets of the valve (**Figure 3R,S**). These cells had long, thin extensions that were branched, along with apparent dendrite extensions with a morphology that closely resembled those described of pericytes or telocytes (Popescu and Fausone-Pellegrini, 2010). PDGFR α $^{+}$ or CD34 $^{+}$ cells with this morphology were only observed in the valve leaflets, and thus seemed insufficient to regulate pacemaking as normal contractions are observed in CLVs without secondary valves (Van Helden, 1993; Gashev et al., 2002). Representative z-stacks demonstrating these valve-located “telocyte” shaped cells (**Figure 3R,S**) are provided as Supplemental Movies 4 and 5.

We next determined the degree of colocalization between the CD34 and PDGFR α signal given the significant overlap in their staining profile. Colocalization analysis of PDGFR α (**SuppFigure 1A**) and CD34 (**SuppFigure 1B**) and their colocalization (**SuppFigure 1C**) was determined with the FIJI BIOP-JaCoP tool. The Pearson’s coefficient was 0.83 (**SuppFigure 1D**) and Mander’s coefficient of overlap 0.80 was for the PDGFR α $^{+}$ signal and 0.87 for the CD34 signal (**SuppFigure 1E**). Colocalization between Myh11 and PDGFR α was significantly lower (**SuppFigure 1D**–F) with a Pearson’s coefficient of 0.30 (**SuppFigure 1G**), whereas the Mander’s coefficient for Myh11 overlap with PDGFR α was 0.077 and PDGFR α signal overlap with Myh11 was 0.043 (**SuppFigure 1H**). The high degree of colocalization CD34 and PDGFR α signal informed our use of the commercially available transgenic *PdgfraCreER*TM mouse model to target these cells. The vast majority of the PDGFR α $^{+}$ cells were located in the adventitial layer (**SuppFigure 2A–D**), which varied between 1–3 PDGFR α $^{+}$ cells thick (**SuppFigure 2E**). Under this layer, we observed only a single layer of largely circumferential LMCs stained by Myh11 (**SuppFigure 2B**) sitting atop a single layer of CD31 $^{+}$ LECs (**SuppFigure 2A**). We also observed occasional PDGFR α $^{+}$ cells or their extensions located in the sub-endothelial space (**SuppFigure 2E’, E’’**) positioned between the LECs and the LMCs.

Use of iCre-Mediated Recombination of Rosa26mT/mG to Delineate and Characterize Specific IALV Cell Types

After confirming the presence of Vimentin $^{+}$, cKit $^{+}$, and CD34 $^{+}$ PDGFR α $^{+}$ positive cells within the mouse IALV, we sought to further investigate these cell populations by using constitutive and inducible Cre recombinase expressing mouse lines. IALVs from the constitutively active *PdgfraCre*-ROSA26mTmG and *Ng2Cre*-ROSA26mTmG mice had GFP fluorescence in the majority of LMCs as well as in the fibroblast-shaped cells found within the IALV wall (**Figure 4 A,B**). While informative of expression of the LMC progenitor cells, neither constitutive Cre would be useful in delineating cell types. In contrast to the constitutively active *PdgfraCre*, the tamoxifen inducible *PdgfraCreER*TM line drove significant recombination in only the fibroblast-shaped cells previously stained with CD34 and PDGFR α but not in LMCs or LECs (**Figure 4C**). *Pdgfr β CreER*^{T2}, commonly used to label pericytes, drove recombination in both a minor population of the LMCs and the fibroblast-shaped cells. *cKitCreER*^{T2}, which capably drives recombination in the ICC of the GI (Baker et al., 2016), drove recombination only in a small population of irregularly spaced large ovoid cells on the surface of the IALV (**Figure 4E**), although recombination in 1 or 2 LECs could occasionally be detected (not shown). Finally, *Myh11CreER*^{T2} drove recombination in nearly all LMCs which were largely circumferentially oriented with dendrite-like, cell-cell contacts visible between them and without significant GFP fluorescence in either LECs or the fibroblast-shaped CD34 $^{+}$ PDGFR α $^{+}$ cell population (**Figure 4F**). Additionally, some LMCs maintained the bipolar shape but had secondary extensions forming a “Y” shape in which an adjacent LMC typically filled

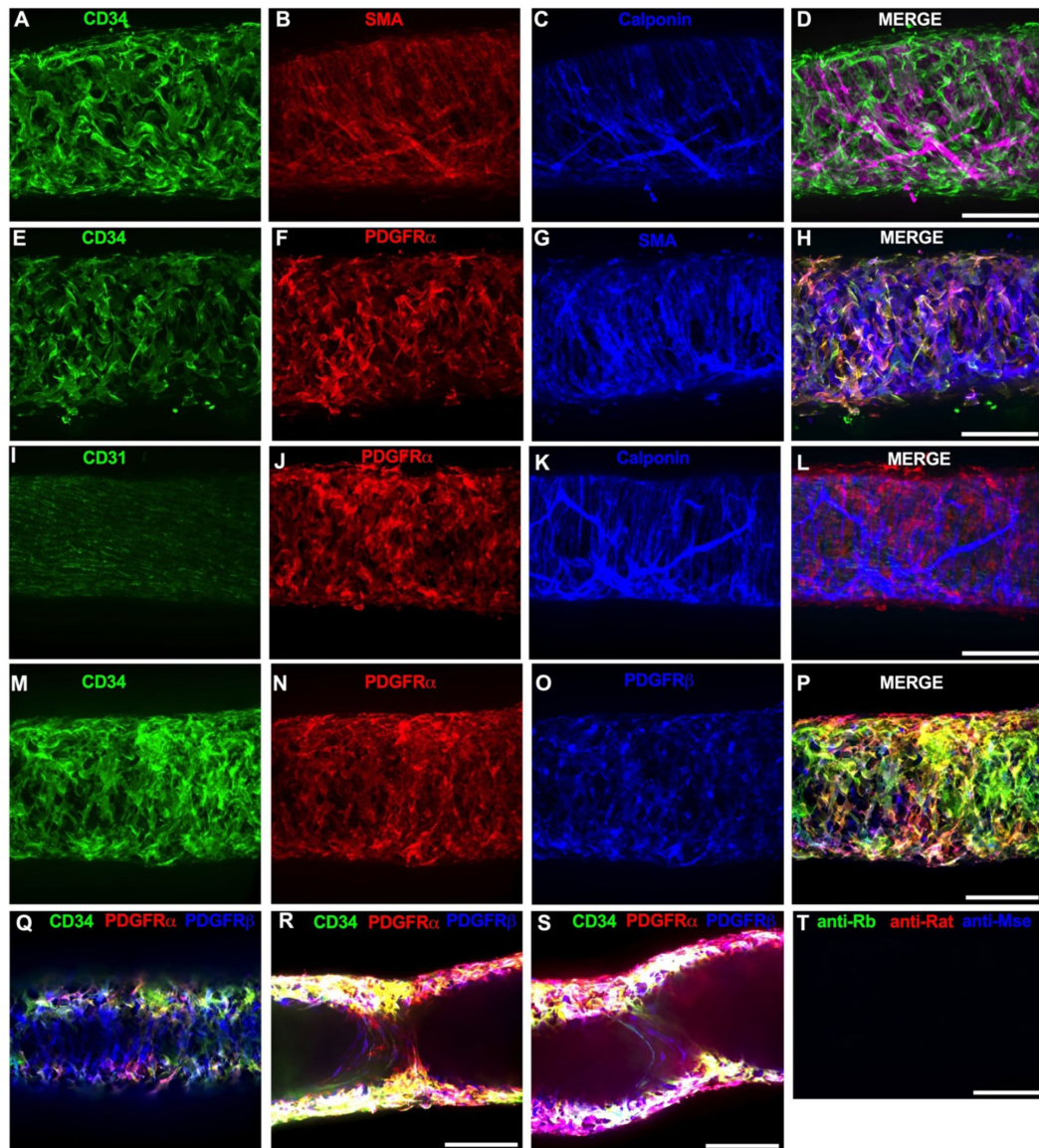


Figure 3

Immunofluorescence Labeling of Mouse IALVs with Markers for ICLC, LMC, LEC, and Immune Cell Populations

We stained isolated mouse IALVs with cellular markers used to differentiate various cell types observed in cLVs. Half vessel image stacks were taken with confocal microscopy and the resulting representative max projections are shown. (A) CD34 stained cells and LMC staining with SMA (B) and calponin (C) and the corresponding merged (D) image. There was significant overlap in (E) CD34 staining along with the fibroblast marker PDGFR α compared to LMC staining with SMA (G) and the merged (H) image. The endothelial marker CD31 (I) to delineate LECs with PDGFR α staining (J), and the LMC marker calponin (K) with the merged image (L) revealed 3 separate populations of cells. PDGFR β (O) stained many cells that were CD34 (M) and PDGFR α (N) positive, as seen in the merge imaged (P), in addition to PDGFR β signal detected in the LMC layer (Q). Max projections of only the luminal frames of a z-stack at lymphatic valve locations revealed PDGFR β , CD34, and PDGFR α labeling in bipolar shaped cells with long extensions that traveled throughout the valve leaflets (V, W). d Control IALV (Y) stained only with secondary antibody. Scale bar = 100 μ m for all images.

the inner void. A very minor population of recombined cells in the *Myh11CreER^{T2}*-ROSA26mTmG IALVs were smaller and irregularly patterned with multiple fine axon-like projections or ruffled edges (**Figure 4F** [↗](#)).

To complement the morphological and cell density findings obtained with confocal microscopy, we digested IALVs from the iCre-ROSA26mTmG lines, and the *Prox1-eGFP* line as a control, into single cell suspensions and sorted the respective GFP⁺ populations (**Figure 4G-J** [↗](#)) for RT-PCR profiling (**Figure 4K** [↗](#)). We first focused on determining the molecular fidelity of the sorted cells based on the gene promoters used to drive each “iCre” model to discern cellular overlap. In agreement with the confocal images, sorted GFP⁺ cells from *PdgfrβCreER^{T2}*-ROSA26mTmG IALVs expressed *Pdgfrβ* but also *Myh11* and *Pdgfra*. In contrast, GFP-sorted cells from *PdgfraCreERTM* IALVs expressed *Pdgfra* and *Pdgfrβ*, but with no detectable expression of *Myh11*. GFP⁺ cells from sorted *Myh11CreER^{T2}*-ROSA26mTmG IALVs had high expression for *Myh11* as well as *Pdgfrβ*, but did not express *Pdgfra*. IALVs from *cKitCreER^{T2}*-ROSA26mTmG mice were not pursued for FACS due to the exceptionally sparse recombination observed along the IALV.

Characterization of the cellular constituents of the mouse IALVs by scRNAseq and FACS-RT-PCR

The results from the immunofluorescence staining, ROSA26mTmG reporter imaging, and FACS-RT-PCR experiments suggested both LMCs and AdvCs express *Pdgfrβ*. To provide further clarity and detail to the cellular populations within the mouse cLV wall, and potential subsets within those broad cell types we performed scRNAseq on our isolated and cleaned inguinal axillary cLVs from male and female mice. The resulting UMAP projection (**Figure 5A** [↗](#)) revealed a host of cell types which had 3 main clusters which corresponded to LECs, LMCs and AdvCs (**Figure 5A** [↗](#)). We assessed the expression of genes that correspond to the markers from our earlier immunofluorescence staining as well as cell identification markers commonly used within the literature to identify each cell cluster (**Figure 5B** [↗](#)). Cell identity was confirmed by commonly used markers (**Figure 5B** [↗](#)) and the top differentially expressed genes (**SuppFigure 3A** [↗](#)). Dot plots for the LEC markers *Prox1* (**Figure 5C** [↗](#)) and *Flt4* (**Figure 5D** [↗](#)), LMC markers *Myh11* (**Figure 5E** [↗](#)) and *Cnn1* (**Figure 5F** [↗](#)), and the AdvCs markers *Pdgfra* (**Figure 5G** [↗](#)) and *Lumican* (**Figure 5H** [↗](#)) were quite specific for labelling their respective cell clusters. Very few *Kit* (**Figure 5I** [↗](#)) expressing cells were observed in accordance with our imaging results. *Pdgfrβ* was observed in both LMC and AdvC clusters (**Figure 5J** [↗](#)) while the remaining cell clusters were of immune origin as they expressed the gene encoding the hematopoietic marker *CD45* (**Figure 5K** [↗](#)). Notably, the previous genes suggested to identify LMCs in a previous scRNASeq study (Kenney et al., 2022 [↗](#)), *Dpt*, *Pi16*, and *Ackr3*, were largely absent in LMCs and instead were expressed in a minor population of AdvCs (SuppFigure3B). We provide a further sub-clustering breakdown of the LECs (**SuppFigure 4** [↗](#)), LMCs (SuppFigure5), AdvCs (**SuppFigure 6** [↗](#)), and a detailed expression profile of the immune cell clusters (**SuppFigure 7** [↗](#)). Further assessment of the LEC subcluster included a putative lymphatic endothelial “up valve” cell population in sub cluster 8 which expressed high levels of *Prox1*, *Cldn11*, *Itga9*, *Gja4*, and *Neo1* and “down valve” population in cluster 6 which expressed *Clu*, *Adm*, *Gja4* and *Lypd6* (**SuppFigure 4C** [↗](#)) which map well to a previous RNAseq dataset (Gonzalez-Loyola et al., 2021 [↗](#); Petkova et al., 2023 [↗](#); Yoon et al., 2024 [↗](#)). The top differentially expressed genes in the putative down valve population in cluster 8 included *Irx3*, *Neo1*, *Tub*, *Ano4*, and *Fxyd2* and we noted *Cacna1c*, *Fgf14*, and *Irf1* in the up-valve cluster 6. Analysis of the LMC subclusters did not reveal any significant differences in known pacemaking associated genes *Ano1* or *Itpr1* expression. However, we provide an overview of the typical ion channel families expressed in LMCs in **SuppFigure 5B-I** [↗](#). The AdvC cells could be further subclustered into multiple populations (**SuppFigure 6A,C** [↗](#)) with little evidence of LMC gene contamination as these cells lacked *Myh11*, *Kcnma1*, and *Tagln* though despite expression of *Cacna1c*, *Ano1*, and *Cx45*. Over 75% of AdvCs expressed *Pdgfra* (**SuppFigure 6** [↗](#)) and 65% of the total AdvCs expressed both *Pdgfra* and *CD34*. Our immunofluorescent colocalization of *Pdgfra* and *CD34* was also supported as 72% of *Pdgfra* expressing AdvCs also co-expressed *CD34* (**SuppFigure**

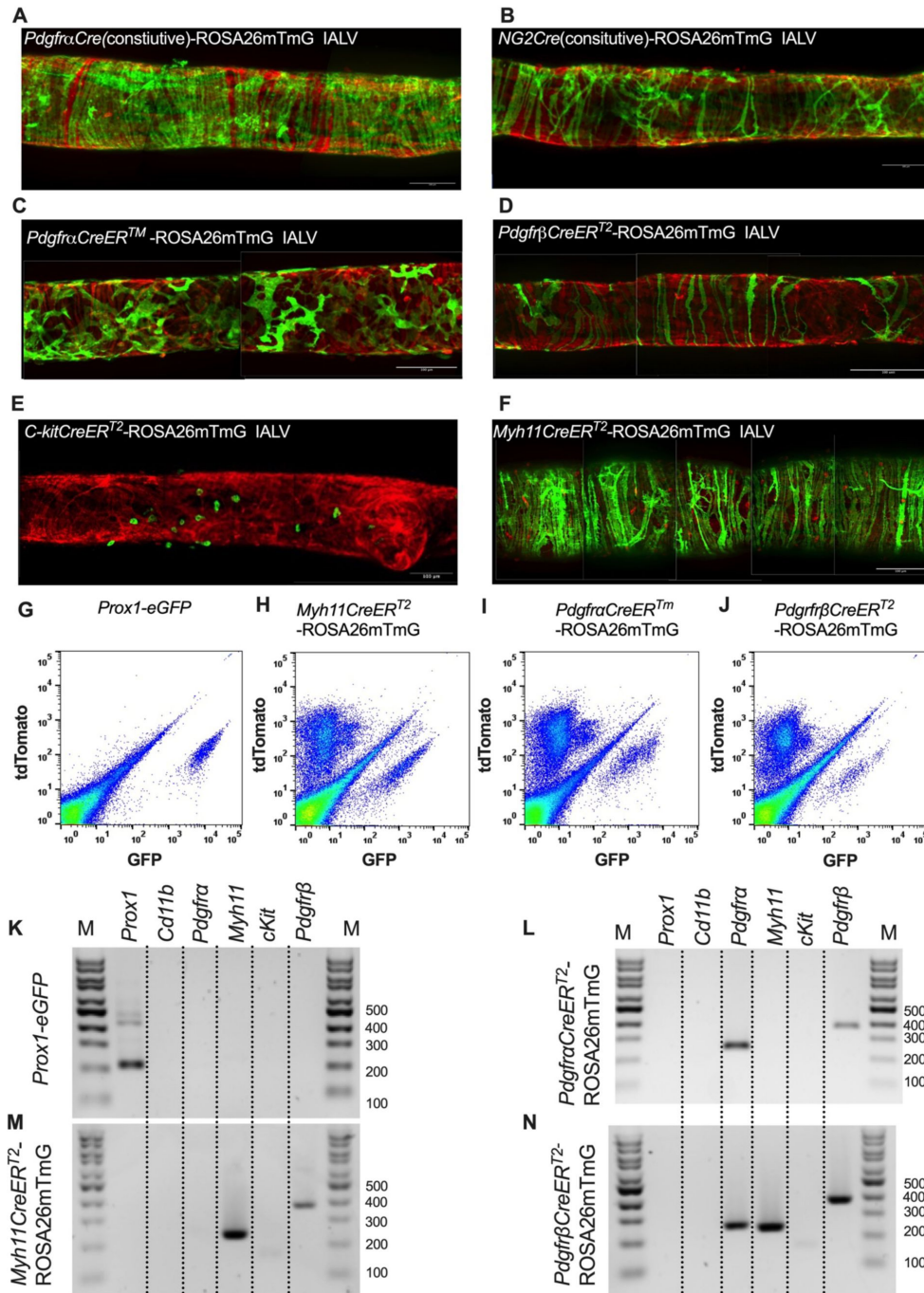


Figure 4

iCre-ROSA26mTmG Labelling and Fidelity to Target Putative Pacemaker Cell Populations

Stitched montages of serial max projections of GFP and tdTomato signal from live IALVs isolated from *Pdgfra*Cre-ROSA26mTmG (A), *Ng2*Cre-ROSA26mTmG (B), *Pdgfra*CreERTM-ROSA26mTmG (C), *Pdgfrβ*CreER^{T2}-ROSA26mTmG (D), *cKit*CreER^{T2}-ROSA26mTmG (E), and *Myh11*CreER^{T2}-ROSA26mTmG (F). IALVs were digested into single cells and GFP⁺ cells were purified via FACS from *Prox1*-eGFP (G), *Myh11*CreER^{T2}-ROSA26mTmG (H), *Pdgfra*CreERTM-ROSA26mTmG (I), and *Pdgfrβ*CreER^{T2}-ROSA26mTmG (J) mice. Representative gels demonstrating RT-PCR products corresponding to the respective genes used in the promoter of each specific transgene employed to drive either eGFP or Cre mediated recombination of ROSA26mTmG from each GFP⁺ sorted population (K-N) to assess fidelity. Images are representative of IALVs from at least 3 separate mice. FACS and RT-PCR was repeated at least 3 times for each mouse.

6D [↗](#)). The vast majority of AdvCs expressing *Pdgfrβ* (**SuppFigure 6E** [↗](#)) or *Cspg4* (**SuppFigure 6F** [↗](#)) also expressed *Pdgfra*. Expression of *Ano1*, *Cx45*, and *Cacna1c*, was also observed in some of the AdvCs and most of these cells also co-expressed *Pdgfra* supporting further use of the *PdgfraCreERTM* line (**SuppFigure 6G-I** [↗](#)).

While scRNASeq highlighted the depth of heterogeneity of the cellular composition of the mouse cLV, we wanted to validate the actual recombined cell populations from our iCre-ROSA26mTmG models. We profiled each iCre driven recombination of ROSAmTmG via FACS-purified cells and RT-PCR for common markers for endothelial cells, muscle cells, and pericytes. *Nos3* (eNOS) expression was observed only in the *Prox1-eGFP* sorted cells, and LECs also expressed *Vim*, *Mcam*, and had weak but detectable signal for *CD34* (**Figure 6A** [↗](#)). *Myh11CreER^{T2}* sorted cells showed expression of smooth muscle actin (*Acta2*), the alpha subunit of the L-type voltage gated Ca^{2+} channel *Cacna1c* (*Cav1.2*), *Desmin* (*Des*), *Mcam*, and *Vimentin* (*Vim*, **Figure 6B** [↗](#)). In addition to the genes expressed under *Myh11CreER^{T2}* recombination, *Cdh5*, *CD34*, and *Cspg4* (*Ng2*) were detected in cells sorted from *PdgfrβCreER^{T2}* IALVs (**Figure 6C** [↗](#)). As expected, the GFP⁺ cells sorted from *PdgfraCreERTM* IALVs expressed mRNA for *CD34*, weak signal for *Cspg4*, and *Vimentin*, but not *Desmin*, *Acta2*, nor the pericyte marker *Mcam* (**Figure 6D** [↗](#)). The *Cacna1c* was expressed in cells FACS purified from both *PdgfrβCreER^{T2}* and *Myh11CreER^{T2}* IALVs and sorted cells from *PdgfraCreERTM* IALVs without any evidence that *Myh11* expressing muscle cells contaminated the latter. These findings confirmed the separate cell populations achieved with *PdgfraCreERTM* and *Myh11CreER^{T2}* mediated recombination, at least as it pertains to the ROSA26mTmG reporter. These findings were largely validated by our scRNASeq dataset. *Cdh5* (**Figure 6E** [↗](#)) and *Nos3* (**Figure 6F** [↗](#)) were almost exclusively expressed in our LEC clusters while *Acta2* (**Figure 6G** [↗](#)) was highly expressed in the LMC cluster. We also observed that *Cacna1c* was highly expressed in the LMCs (**Figure 6H** [↗](#)) and some AdvCs. *Cd34* was highly expressed in the AdvCs but was also observed in LECs (**Figure 6I** [↗](#)) although we did not observe a signal in our earlier immunofluorescence staining (**Figure 3** [↗](#)). *Cspg4* was observed in a minor population of AdvCs (**Figure 6J** [↗](#)). The intermediate filament *Vim* (**Figure 6K** [↗](#)) was ubiquitously across all clusters expressed but *Des* was primarily expressed in LMCs and some subsets of AdvCs (**Figure 6K,L** [↗](#)). The endothelial and pericyte marker *Mcam* (also referred to as CD146) was expressed in LECs and LMCs but was largely absent in AdvCs (**Figure 6M** [↗](#)). We followed up the identification of *Cav1.2* expression in the *PdgfraCreERTM* sorted cell population by assessing the expression of other genes involved in either electrical conduction (*Cx45*) (**Figure 6N** [↗](#)) or pacemaking (*Ano1*) (**Figure 6O** [↗](#)) of IALVs. Expression of *Ano1* and *Cx45* was observed in *PdgfraCreERTM* ROSA26mtmG FACS-purified cells (**Figure 6P** [↗](#)).

Inducible Deletion of Either Cav1.2, Ano1, and Cx45 with *PdgfraCreERTM* Did Not Affect cLV Pacemaking

The expression of the genes critically involved in cLV function—*Cav1.2*, *Ano1*, and *Cx45*—in the *PdgfraCreERTM*-ROSA26mTmG purified cells and scRNAseq data prompted us to generate *PdgfraCreERTM*-*Ano1^{fl/fl}*, *PdgfraCreERTM*-*Cx45^{fl/fl}*, and *PdgfraCreERTM*-*Cav1.2^{fl/fl}* mice for contractile tests. We isolated popliteal cLVs and tested their pacemaker and contractile function in response to a physiological pressures range of 0.5-10 cmH₂O, under normal conditions. However, we did not detect any significant differences in pacemaking nor contractile function as assessed by contraction frequency, ejection fraction, and vessel tone in popliteal cLVs studied from *PdgfraCreERTM*-*Ano1^{fl/fl}* mice (**Figure 7A-C** [↗](#)) or *PdgfraCreERTM*-*Cx45^{fl/fl}* mice (**Figure 7D-F** [↗](#)). There was no difference in contraction frequency in cLVs from *PdgfraCreERTM*-*Cav1.2^{fl/fl}* mice, however, we noted a mild but statistically significant increase in ejection fraction at the lowest pressure, 0.5 cmH₂O (**Figure 7H** [↗](#)). Additionally, vessels isolated from *PdgfraCreERTM*-*Cav1.2^{fl/fl}* mice also had a statistically significant increase in vessel tone (**Figure 7I** [↗](#)) noted at the 2-way level although we did not resolve significance at any specific pressure with this sample. No difference in normalized contraction amplitude, fractional pump flow, or diastolic diameters were observed (**SuppFigure**

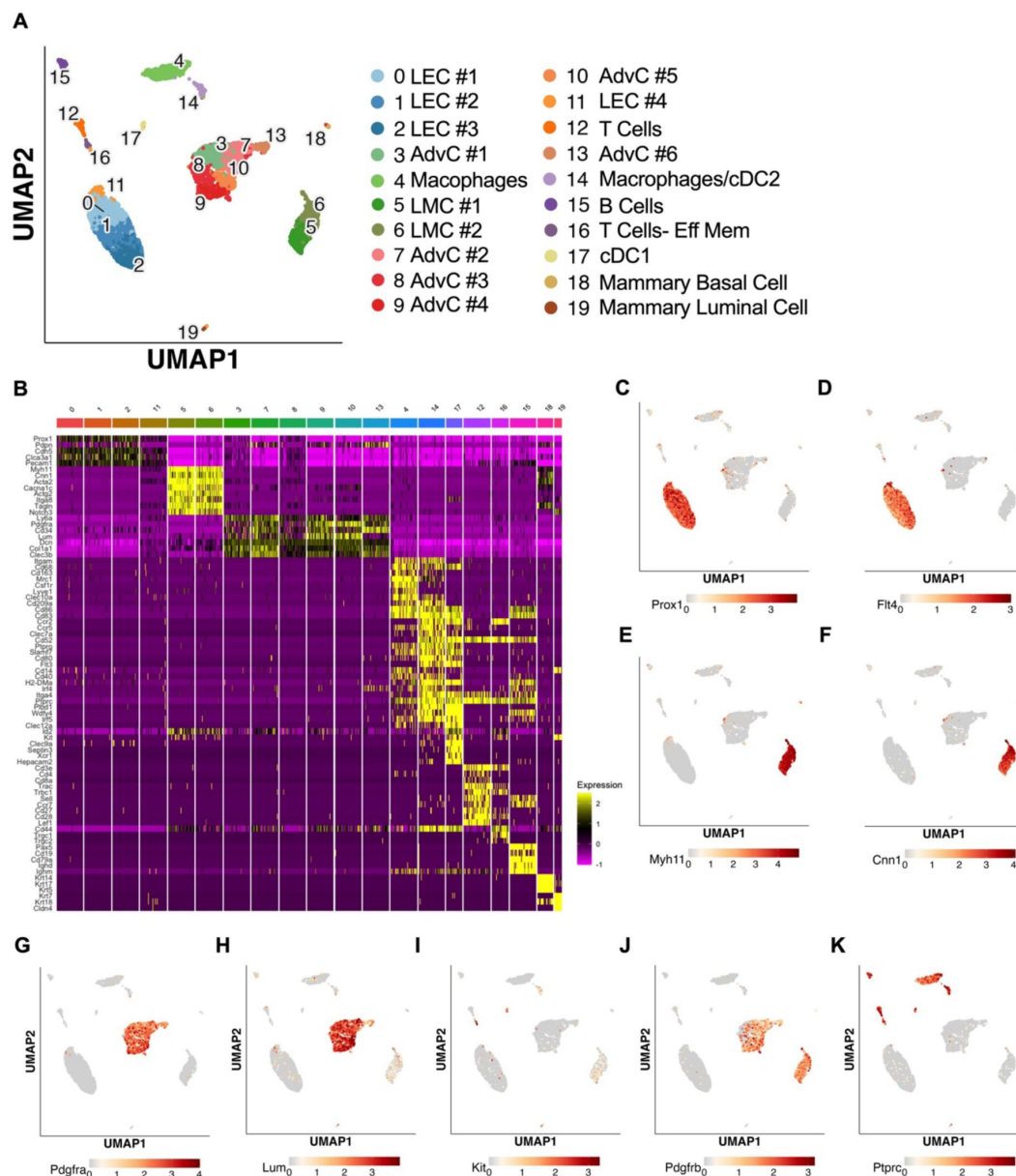


Figure 5

scRNAseq analysis of mouse IALVs from ROSA26mTmG mice.

IALVs were cleaned and isolated from 8 ROSA26mTmG mice and digested into a single cell suspension for scRNAseq analysis with the 10X platform. A) UMAP of the various cell populations that compromise the mouse IALV though some mammary epithelia contamination was present (populations 18,19). B) Heat map of commonly used genes for cell identification for each of the cell clusters. Dot plots to assess cell cluster expression of the genes shown in [Figure 4](#) using a dot plot for the LEC markers *Prox1* (C) and *Flt4* (D, VEGFR3), LMC markers *Myh11* (E) and caponin1 (F, *Cnn1*), fibroblast markers *Pdgfra* (G) and *Lum* (H, *Lumican*), ICC marker *Kit* (I), the pericyte and smooth muscle precursor marker (*Pdgfrb*), and the hematopoietic marker *Ptprc* (K, CD45).

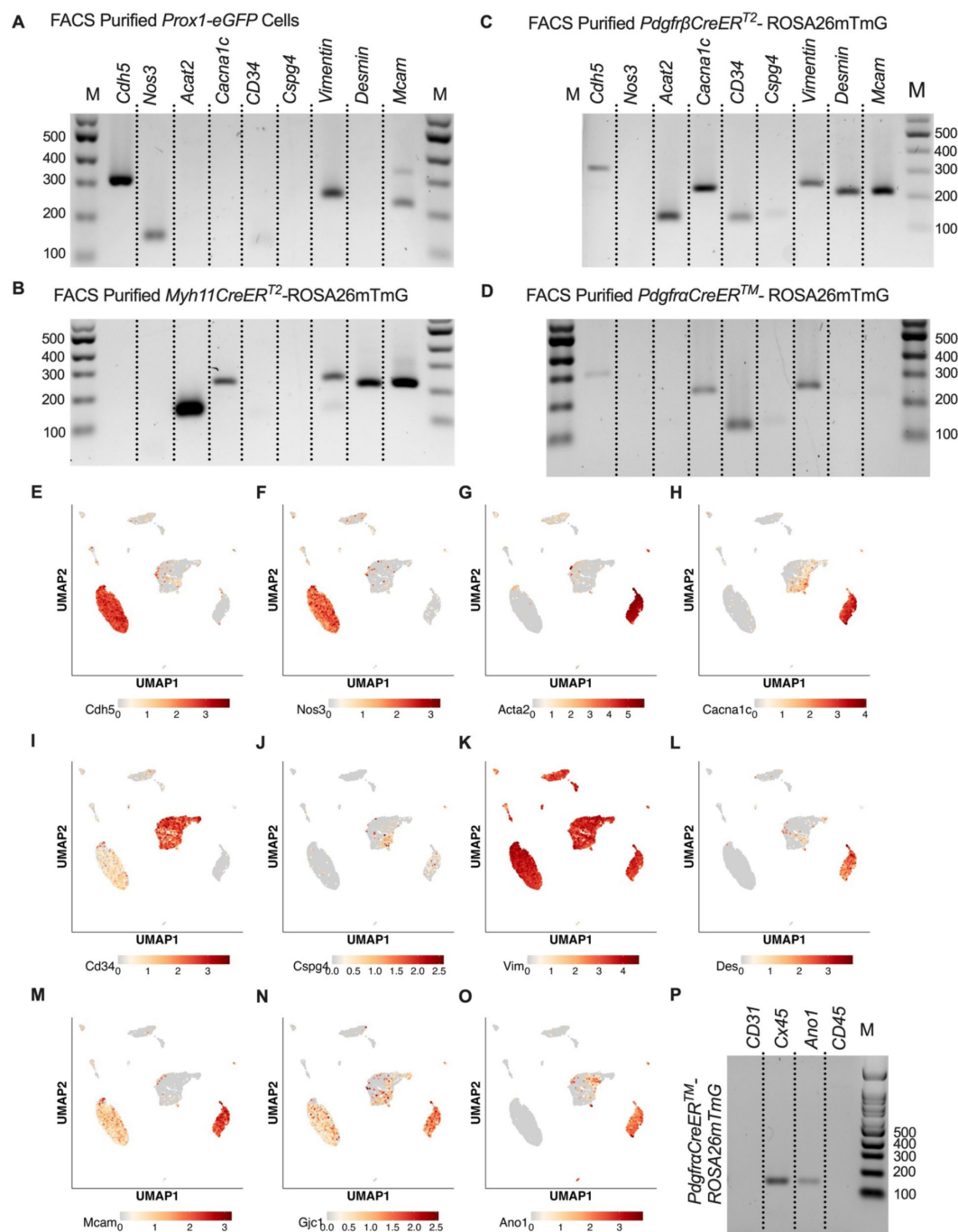


Figure 6

RT-PCR Profiling of FACS Purified Cells from iCre-ROSA26mTmG

Expanded RT-PCR profiling of genes to discriminate LECs, LMCs, and other cell types in our GFP⁺ sorted cells from *Prox1-eGFP* (A), *Myh11CreER^{T2}-ROSA26mTmG* (B), *PdgfrβCreER^{T2}-ROSA26mTmG* (C), and *PdgfraCreERTM-ROSA26mTmG* (D). Dot plots for the genes assessed in A-D in our IALV scRNAseq analysis confirmed those results. In addition to a population of AdvCs expressing *Cacna1c*, we also noted expression of *Cx45* (N) which was also observed in LECs) and *Ano1* (O) in the AdvC clusters. We confirmed this expression using GFP⁺ cells sorted from *PdgfraCreERTM-ROSA26mTmG* IALVs for RT-PCR (P) and ruled out hematopoietic or LEC contamination. All RT-PCRs were performed 2-4 times for each gene over each sorted cell population collected from different mice.

8). In total, despite the presence of transcript for these critical genes in *Pdgfra*⁺ cells, *PdgfraCreER*TM mediated deletion of *Cx45*, *Cav1.2* or *Ano1* failed to recapitulate previous reports of the significant contractile defects using the *Myh11CreER*^{T2} line (Castorena-Gonzalez et al., 2018b; Zawieja et al., 2019; To et al., 2020; Davis et al., 2022).

PDGFRα⁺ Adventitial Fibroblasts Express Markers Associated with Multipotency

Despite the lack of cLV pacemaking deficits in the *PdgfraCreER*TM genetic knockout lines, we were curious to discern further insight into the role or function of the PDGFRα⁺ CD34⁺ cells since they comprise a significant portion of the lymphatic cLV wall. We performed RT-PCR on FACS purified cells from *Prox1-eGFP*, *Myh11CreER*^{T2}-ROSA26mTmG, and *PdgfraCreER*TM-ROSA26mTmG IALVs for markers multipotency including Krüppel-like factor 4 (*Klf4*), stem cell antigen 1 (*Sca1*, also referred to as *Ly6a*), and *Gli1* with *CD34* and *Pdgfra* used to assess purity. Recombined (GFP⁺) cells from *Myh11CreER*^{T2}-ROSA26mTmG had weak expression of *Klf4* and *Gli1* but were negative for *Ly6a* (SuppFigure 9A). *PdgfraCreER*TM recombined cells strongly expressed *Klf4*, *Ly6a*, and *Gli1* (SuppFigure 9A). LECs sorted from *Prox1-eGFP* IALVs were positive for *Klf4*, weak for *Ly6a*, and positive for *CD34* but negative for *Gli1* and PDGFRα (SuppFigure 9B). The unrecombined population (tdTomato⁺) cells in the *Myh11CreER*^{T2}-ROSA26mTmG IALVs (SuppFigure 9B) showed expression for all the markers as expected. *PdgfraCreER*TM recombined cells also expressed the mesenchymal stromal cell markers *CD29*, *CD105*, and *CD44* (SuppFigure 9C, positive control in 9D). However, expression of these genes was not homogenous across all the AdvCs population based on our scRNAseq analysis (SuppFigure 9E-J). We performed immunofluorescence staining for one of these multipotent markers, *Ly6a* (SuppFigure 9K) in the adventitial cells with PDGFRα (SuppFigure 9L) and counter staining for LMCs with MYH11 (SuppFigure 9M). The morphology and staining pattern of *Sca1* overlapped significantly with PDGFRα staining and not *Myh11* staining (SuppFigure 9N, Supplemental Movie 6).

Optogenetic Stimulation of iCre-driven Channel Rhodopsin 2 to Induce Test Light-Stimulated Depolarization Induced Lymphatic Contraction

We next used optogenetic methods to test whether the cell populations recombined by either *cKitCreER*^{T2}, *PdgfraCreER*TM, or *Myh11CreER*^{T2} could elicit a coordinated contraction. The Chr2-tdTomato construct appeared more sensitive to recombination than ROSA26mTmG, in some cases resulting in LMC expression of Chr2-tdTomato in *PdgfraCreER*TM and *CkitCreER*^{T2} popliteal cLVs based on morphology. Care was taken to image each vessel for tdTomato (Figure 8A,C,E) prior to stimulation at its respective sites under brightfield conditions for diameter tracking (Figure 8B,D,F) to ensure fidelity of the cell types and morphologies observed in Figure 3 and Figure 4. As with ROSA26mTmG, *CkitCreER*^{T2} drove the Chr2-tdTomato expression primarily in large ovoid cells found on the adventitia of the vessel. Cells were stimulated by positioning an optical laser fiber (tip diameter 2-3 mm) near a Chr2⁺ cell, with an illumination field of 10-50 mm. Localized photo-stimulation of these cells did not initiate coordinated contractions (Figure 8G-J,S). Similarly, photo-stimulation of Chr2-tdTomato expressing cells driven by *PdgfraCreER*TM failed to initiate a coordinated contraction (Figure 8K-N, T). In contrast, localized photo-stimulation of LMCs, using *Myh11CreERT2* to express Chr2-tdTomato, resulted in a propagated contraction in the popliteal vessel (Figure 8O-R, U). In total, only 3.25% of photo-stimulation events for *cKitCreER*^{T2}-Chr2-TdTomato and 3.03% of photo-stimulation events for *PdgfraCreER*TM-Chr2-tdTomato were associated with a contraction, while 88.5% of photo-stimulation events for *Myh11CreER*^{T2}-Chr2-tdTomato induced contractions (Figure 8V). The optogenetic triggering of contractions observed in *PdgfraCreER*TM-Chr2-tdTomato and *cKitCreER*^{T2}-Chr2-TdTomato vessels is likely due to the happenstance of spontaneous contractions occurring during the time and proximity of optogenetic stimulation (see Methods). As a control, we also used non-induced (no

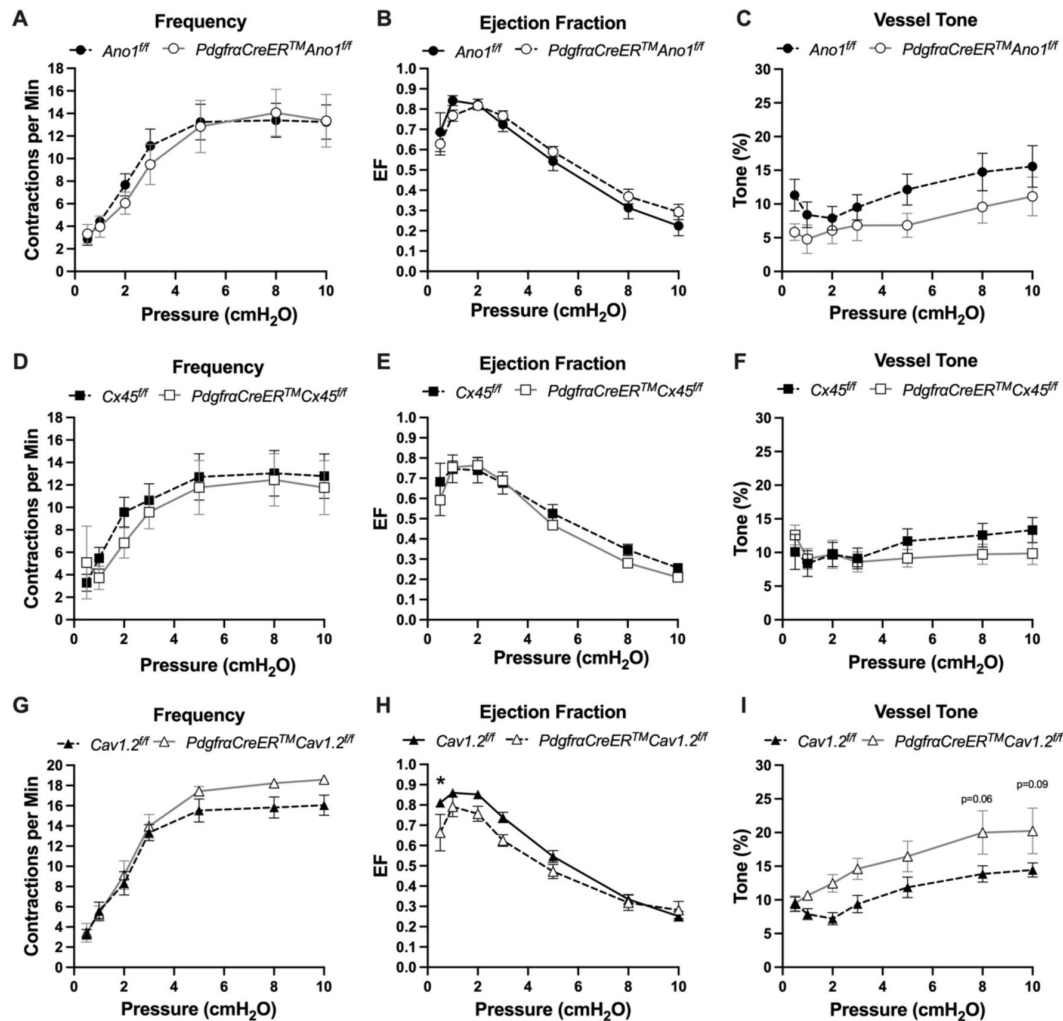


Figure 7

Isobaric contractile Assessment of popliteal cLV from *PdgfraCreERTM* driven deletion of *Ano1*, *CX45*, and *Cav1.2*

Summary of the contractile parameters recorded from popliteal cLVs in *PdgfraCreERTM-Ano1^{fl/fl}*, *PdgfraCreERTM-CX45^{fl/fl}* mice, *PdgfraCreERTM-Cav1.2^{fl/fl}* mice. Contraction frequency (A, D, G), ejection fraction (B, E, H), and vessel tone (C, F, I) were assessed. No statically significant differences observed in cLVs isolated from *PdgfraCreERTM-Ano1^{fl/fl}* and *PdgfraCreERTM-CX45^{fl/fl}* mice across these three parameters. Mean and SEM shown, n=6 popliteal vessels from 3 mice *PdgfraCreERTM-Ano1^{fl/fl}* mice and n=10 popliteal vessels from 6 mice *Ano1^{fl/fl}* mice. Mean and SEM shown, n=5 popliteal vessels from 3 mice *PdgfraCreERTM-CX45^{fl/fl}* mice and n=8 popliteal vessels from 11 mice *CX45^{fl/fl}* mice. Mean and SEM shown, n=6 popliteal vessels from 3 mice *PdgfraCreERTM-Cav1.2^{fl/fl}* mice and n=9 popliteal vessels from 20 mice *Cav1.2^{fl/fl}* mice. The contractile data from control *Cav1.2^{fl/fl}* vessels was previously published but was separated by sex (Davis et al., 2022) while they are combined here. * Denotes significance at $p < 0.05$ which $0.10 > p > 0.05$ are reported as text. Normalized contraction amplitude, fractional pump flow, end diastolic diameter can be found in **Supp Figure 8**.

tamoxifen) *Myh11CreER^{T2}*-Chr2-tdTomato cLVs and contractions were associated with only 7% of photo-stimulation events, in line with the *PdgfraCreERTM* and *cKitCreER^{T2}* results (**Figure 8V** [↗](#)). As mast cells are not ascribed any tissue specific pacemaking behavior, these similar low percentages observed between these three groups are suggestive of random coincidence. Brightfield videos of the photo-stimulation and representative traces for *cKitCreER^{T2}*-Chr2-TdTomato, *PdgfraCreERTM*-Chr2-tdTomato, *Myh11CreER^{T2}*-Chr2-tdTomato are provided in Supplemental Movies 7-9.

Confocal Ca²⁺ Imaging of GCaMP6f Expression

Driven by *cKitCreER^{T2}*, *PdgfraCreERTM*, and

Myh11CreER^{T2} Over the Lymphatic Contraction Cycle

Subcellular calcium transients are observed in many pacemaker cells. We imaged IALVs from *cKitCreER^{T2}*-GCaMP6f mice, which primarily resulted in expression of GCaMP6f in the large ovoid cells in the adventitia (**Figure 9A** [↗](#)), although we occasionally observed GCaMP6f expression in both LEC and LMCs (**Figure 9A** [↗](#)) as depicted in the maximum projection of the acquisition period (Supplemental Movie 10) and the spatio-temporal maps (STMS). The aberrant expressions of GCaMP6f in cells that demonstrated the typical cobblestone morphology of LECs or the circumferential LMCs that exhibited Ca²⁺ flashes and diastolic Ca²⁺ transients (**Figure 9D,E** [↗](#) green arrows) prior to contraction were not included in the *cKitCreER^{T2}*-GCaMP6f analysis. Of 39 *cKitCreER^{T2}*-GCaMP6f cells analyzed, only 1 *cKitCreER^{T2}*-GCaMP6f cell exhibited a spontaneous Ca²⁺ transient during the recording period (**Figure 9B,C** [↗](#) Cell 7). However, the Ca²⁺ transient in that cell did not align temporally with the “Ca²⁺ flash” of the LMC with incidental GCaMP6f expression (**Figure 9C,D** [↗](#)). Despite the lack of Ca²⁺ transients under the baseline conditions throughout the IALV contraction cycle, many *cKitCreER^{T2}*-GCaMP6f cells exhibited a robust and prolonged Ca²⁺ event in response to stimulation with the mast cell activator compound 48-80 (**Figure 9F, G, H** [↗](#)). Notably, the Ca²⁺ events in the ovoid cells elicited by administration of compound 48-80 did not acutely alter the LMC Ca²⁺ activity (**Figure 9I,J** [↗](#)). Like *cKitCreER^{T2}*-GCaMP6f, the majority of *PDGFRα*-GCaMP6f expressing cells also largely lacked Ca²⁺ transients and resulted in incidental LMC GCaMP6f expression (**Figure 10B** [↗](#), Supplemental Movie 11). Some cells exhibited high basal Ca²⁺ levels (**Figure 10A,D** [↗](#)) sustained throughout the recording, but without oscillations (**Figure 10B,C** [↗](#)). In contrast, spurious GCaMP6f expression in a circumferentially oriented LMC displayed Ca²⁺ flashes associated with contraction (**Figure 10B,C** [↗](#)). Of the 21 *PDGFRα*-GCaMP6f cells assessed, only 3 exhibited Ca²⁺ transients that were singular events with limited spatial spread within the 20 sec imaging period (**Figure 10E,F** [↗](#)). The lack of either global or consistent Ca²⁺ transients within either *cKitCreER^{T2}*-GCaMP6f or *PdgfraCreERTM*-GCaMP6f IALVs was in stark contrast to Ca²⁺ imaging of *Myh11CreER^{T2}*-GCaMP6f IALVs. *Myh11CreER^{T2}* drove GCaMP6f expression in the circumferential LMCs (**Figure 11A** [↗](#)), which had global and nearly synchronous Ca²⁺ flashes in 100% of the analyzed cells (**Figure 11B, C** [↗](#)). Additionally, non-synchronous stochastic and localized Ca²⁺ transients during diastole were commonly observed in the LMCs (**Figure 11D, E** [↗](#), Supplemental Movie 12). Many LMCs exhibited Ca²⁺ transients during each diastolic period while other LMCs displayed few Ca²⁺ transients or lacked diastolic Ca²⁺ transients during the recording period (**Figure 11B** [↗](#)). In aggregate, of the 39 *cKitCreER^{T2}*-GCaMP6f cells only 1 displayed a Ca²⁺ transient during recording, 3 of 21 *PdgfraCreERTM*-GCaMP6f cells, while 20 of 43 LMCs displayed at least one diastolic transient apart from 43 of 43 LMCs with global flashes.

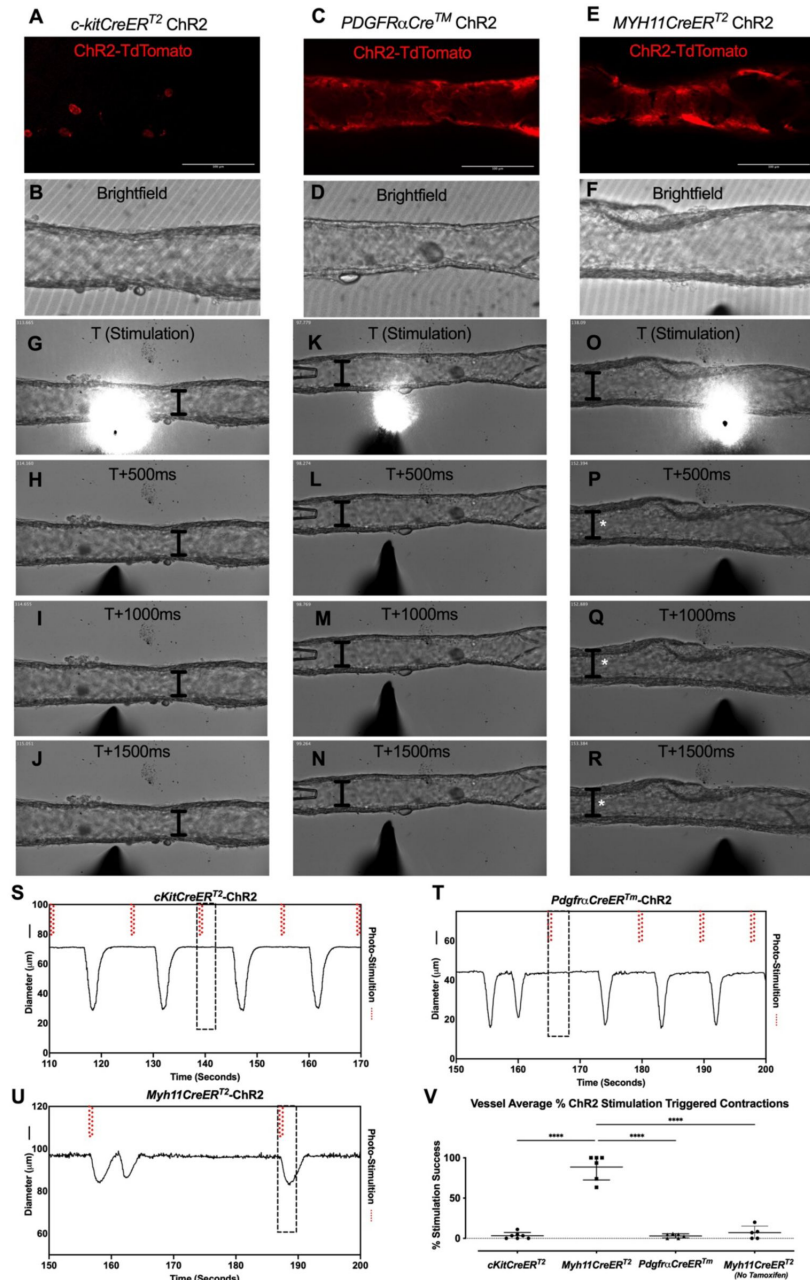


Figure 8

ChR2-Mediated Depolarization Only in LMCs Triggers Contraction

Representative max projections of tdTomato-ChR2 signal in popliteal cLVs isolated from *cKitCreER^{T2}*-ChR2-tdTomato (A), *PdgfraCreERTM*-ChR2-tdTomato (C), and *Myh11CreER^{T2}*-ChR2-tdTomato (E) with their corresponding brightfield image (B, D, F) respectively. Time-lapse brightfield images every 0.5 s starting at stimulation t=0 for *cKitCreER^{T2}*-ChR2-tdTomato (G-J), *PdgfraCreERTM*-ChR2-tdTomato (K-N), and *Myh11CreER^{T2}*-ChR2-tdTomato (O-R). The I bar denotes the inner diameter at t=0 over time and white asterisks denote the contraction. Representative diameter trace for the popliteal cLV demonstrate spontaneous contractions with the dotted boxes indicating the optical stimulation event in the respective brightfield images of the time lapse images. Isolated cLVs from *cKitCreER^{T2}*-ChR2-tdTomato (S), *PdgfraCreERTM*-ChR2-tdTomato (T), and *Myh11CreER^{T2}*-ChR2-tdTomato (U) were stimulated with light pulses (red dashed lines) and the summation of contraction triggering for each genotype (V). Mean and SEM are shown, **** denotes $p < 0.0001$. Contraction recorded from at least 6 popliteal cLVs from 3 mice per genotype.

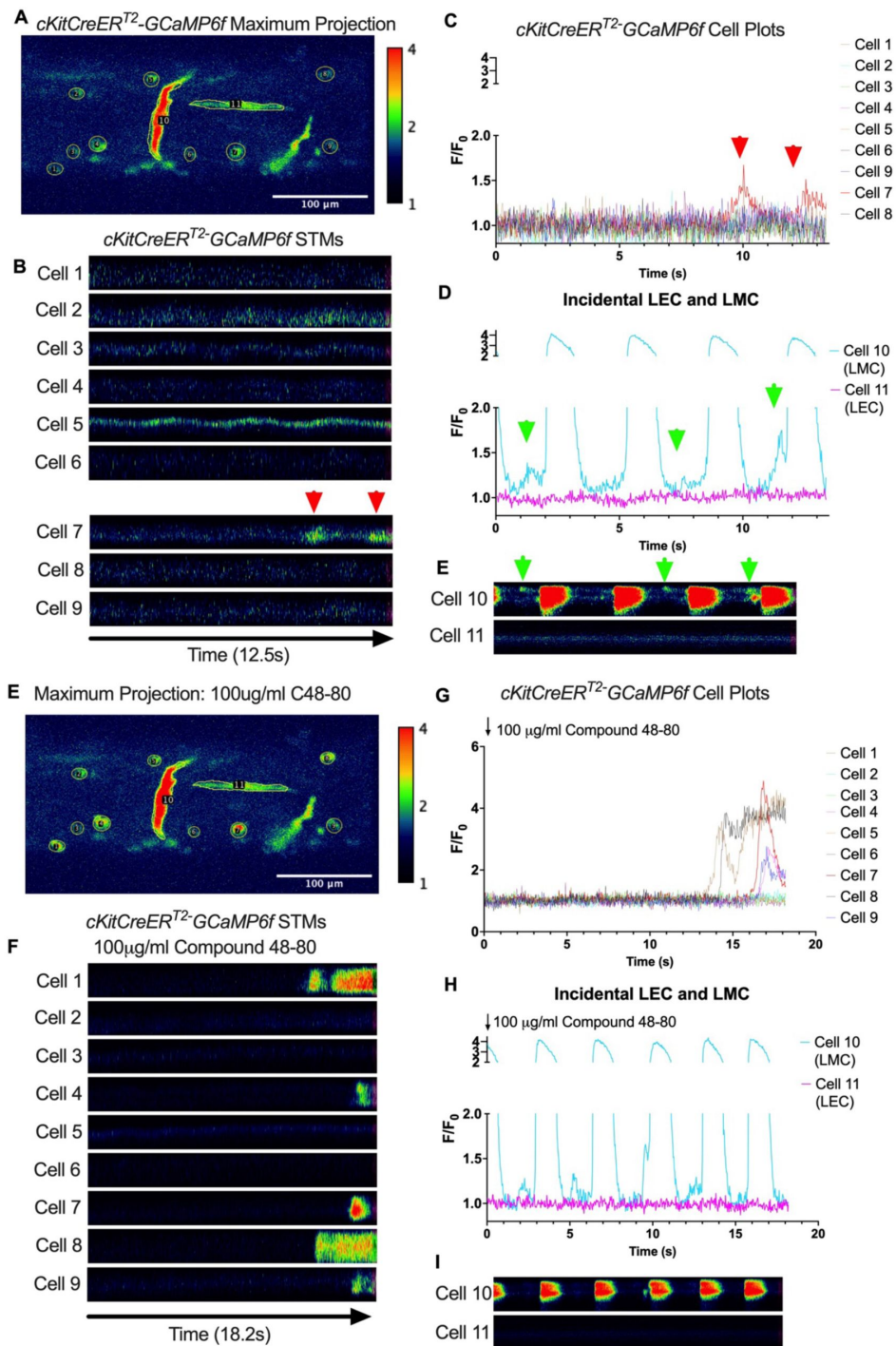


Figure 9

cKitCreERT2 Drives GCaMP6f Expression Primarily in Mast Cells in Mouse IALVs

Representative max projection of GCaMP6f signal over time in an IALV isolated from a *cKitCreERT2*-GCaMP6f mouse with ROI indicated around individual cells, primarily large ovoid cells, but also including a circumferential LMC (Cell10) and a horizontal LEC (Cell 11). Of cells 1-9, only cell 7 had any Ca^{2+} activity (red arrows) during the recording time as indicated by the STMs from each ROI (B) and their normalized F/F_0 plots in (C). In contrast, the LMC in ROI 10 had both rhythmic global Ca^{2+} events (D) that spanned the cell axis (vertical axis) in the STM (E) in addition to localized Ca^{2+} events intervening the time between global events (green arrows). Representative max projection of GCaMP6f signal over time after stimulation with C48-80 (F) with many large ovoid cells displaying long lasting global Ca^{2+} events (G, H) while not immediately affecting the LMC Ca^{2+} dynamics (I, J).

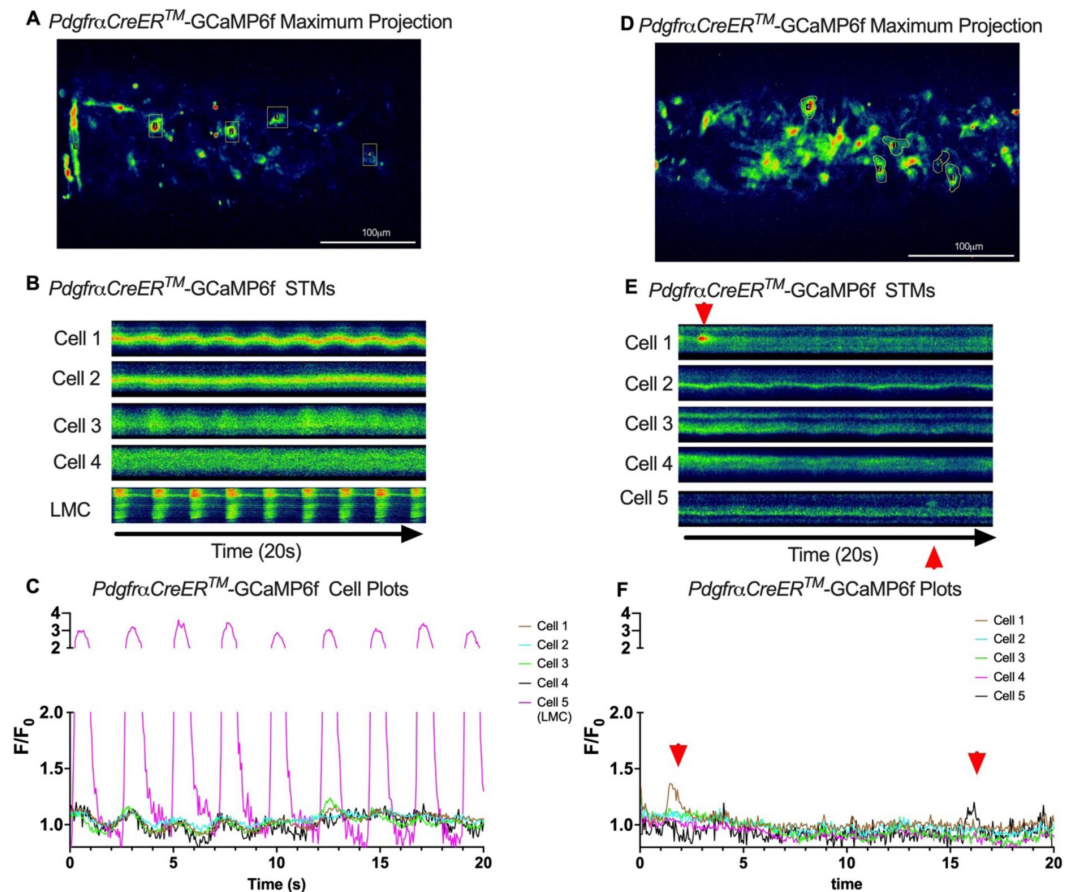


Figure 10

Lack of coordinated Ca^{2+} Activity Across Contraction Cycle in PDGFR α Cells

Representative max projections of GCaMP6f signal over time in an IALVs isolated from *PdgfraCreERTM*-GCaMP6f mice (A, D). ROIs were made around cells and GCaMP6f recorded over time to generate the corresponding STMs (B, E) for each cell and plots (C, F) respectively. Once again, incidental recombination occurred in a LMC which displayed rhythmic Ca^{2+} flashes (C) while the slight undulation in the other cells is due to movement artifact (B). Red arrows indicate the limited local Ca^{2+} activity observed in two cells from a *PdgfraCreERTM*-GCaMP6f IALV.

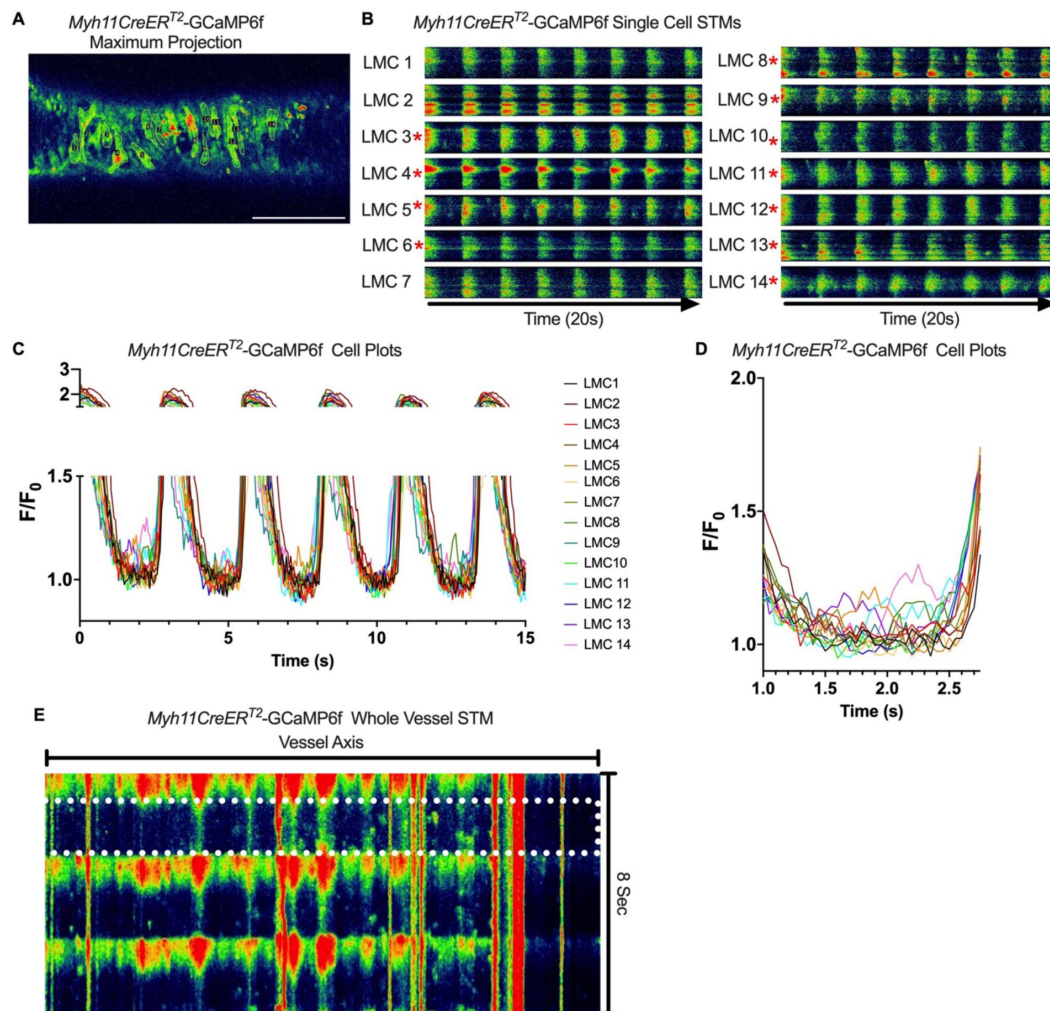


Figure 11

Heterogeneous Diastolic Ca²⁺ Transient Activity in LMCs

Representative max projections of GfCaMP6f signal over time in an IALVs isolated from *Myh11CreER^{T2}-GCaMP6f* mice (A). LMCs were outlined with ROIs to assess GfCaMP6f signal over time. Rhythmic global flashes (B) were entrained across all the LMCs in the FOV (C) with many cells exhibiting diastolic Ca²⁺ release events. Cells exhibiting at least one diastolic Ca²⁺ event, within the context of our focal plane constraints, over the recorded time were denoted by the red asterisks. The plot in (D) magnifies the first diastolic period, seconds 1-3 of C to assist in visualizing the lack of coordination of the diastolic events. (D) Max projection of the pseudo-linescan analysis across the axis of the vessel to highlight diastolic Ca²⁺ transients in all cells in the field of view and their lack of coordination across the cells (x-axis). The white dotted box shows the first diastolic period plotted in (D).

Pressure Dependency of Subcellular Ca^{2+} Transients in LMCs

We next sought to test whether diastolic Ca^{2+} transients were pressure-dependent, given that cLVs exhibit pressure dependent chronotropy (Zawieja et al., 2019). GCaMP6f expressing LMCs were studied at intraluminal pressures of 0.5–5 cmH_2O in the presence of nifedipine, which blocks the Ca^{2+} flashes but not local Ca^{2+} transients (Figure 12A). As intra-luminal pressure was increased, there was a marked increase in the occurrence of Ca^{2+} transients (Figure 12B, Supplemental Movies 13–15). We converted these calcium transients into particles (PTCLs) for further analysis as previously described. We generated activity maps of Ca^{2+} PTCL activity (Figure 12C) and determined PTCL area (Figure 12D) and frequency at each pressure (Figure 12E). The maps show that as pressure increased, the activity of PTCLs across the vessel also increased (as evident by the increase in PTCL area activation). Across 11 experiments, the area of the field of view activated by PTCLs/frame increased from $73.2 \pm 17.7 \mu\text{M}^2/\text{frame}$ at 0.5 cmH_2O to $108.6 \pm 20.5 \mu\text{M}^2/\text{frame}$ at 2 cmH_2O and further enhanced to $139.2 \pm 26.9 \mu\text{M}^2/\text{frame}$ at 5 cmH_2O (Figure 12F). The number of PTCLs per frame also increased with pressure, from 2.9 ± 0.4 at 0.5 cmH_2O to 4.1 ± 0.5 and 5.2 ± 0.6 PTCL/frame at 2 and 5 cmH_2O respectively (Figure 12G).

Contraction Frequency is Set by the Diastolic Depolarization Rate

To assess how pressure regulates LMC membrane potential we first recorded membrane potential in cells exhibiting action potentials (APs) using a microelectrode filled with biocytin-AF488 to label the cell. In each case ($n=3$ IALVs) the labeled cell were LMCs wrapping circumferential around the vessel (Figure 13A, B) and as these recordings were made over the course of many minutes the neighboring circumferential LMCs also exhibited fluorescence, albeit weaker in intensity, as expected (Figure 13A). In all the recorded cells exhibiting APs, we noted a diastolic depolarization preceding the sharp upstroke achieved once threshold was met at each pressure (Figure 13C). The AP frequency and rate of the diastolic depolarization increased with pressure (Figure 13D, E). Linear regression of a plot of each AP frequency and diastolic depolarization rate at each pressure demonstrated a tight association between the two parameters. However, we did not observe a significant effect of pressure on minimum membrane potential (Figure 13G), threshold potential (Figure 13H), the AP upstroke (Figure 13I), AP peak potential (Figure 13J), plateau potential (Figure 13K), or the time spent over threshold (Figure 13L).

Discussion

The identification of the cellular origin and signaling mechanisms underlying cLV pacemaking will reveal novel targets for pharmacological intervention in treating lymphedema and the associated lymphatic contractile dysfunction. In this study we tested proposed pacemaker cell types based on 3 parameters: 1) that the pacemaker cells are located along the entire length of the cLV, to accommodate spontaneous contractions and coordinated electrical conduction despite progressive shortening of cLVs; 2) that depolarization of the pacemaker cell can drive a coordinated and propagated contraction of the vessel; and 3) that the presence of Ca^{2+} transients precedes or coincides with contraction, as commonly observed in other pacemaker cells. We used confocal microscopy and a combination of immunofluorescence and fluorescent reporters under the control of various inducible Cres to identify and target both muscle and non-muscle cells, previously labeled as ICLCs, that coexpress the markers CD34 and PDGFR α . Our cell characterizations were supplemented by scRNAseq analysis of isolated and cleaned mouse IALVs which supported our finding of 3 major cell types including LECs, LMCs, and AdvCs each of which could be further sub-clustered into transcriptionally unique populations. From our initial fluorescence imaging studies, a role for intrinsic pacemaking by LMCs (Van Helden, 1993; von der Weid et al., 2008), or by a novel population of CD34 $^{+}$ lymphatic ICLC (McCloskey et al.,

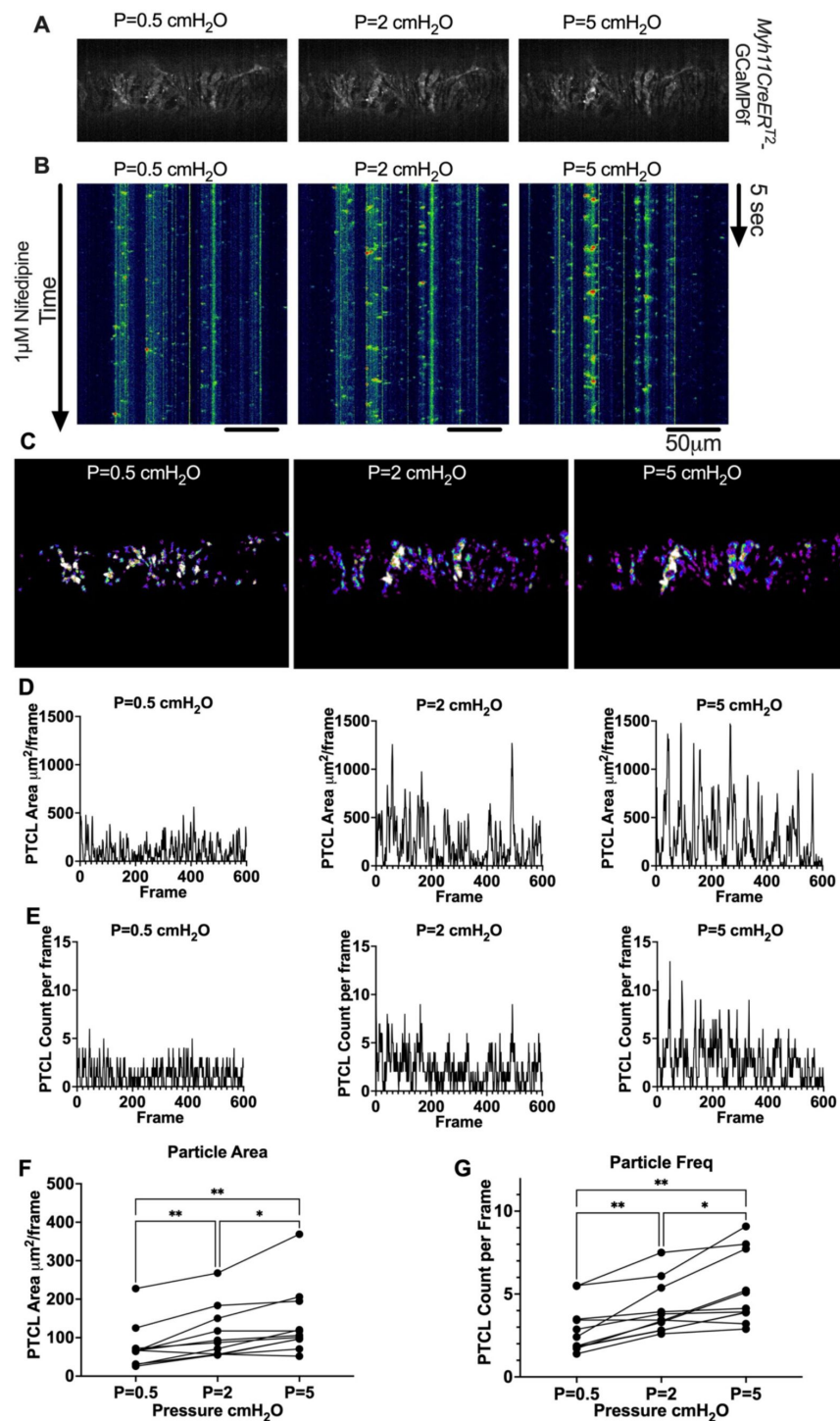


Figure 12

Pressure Dependency of Mouse LMC Diastolic Ca²⁺ Transients

Representative max projection of GCaMP6f signal over 20 s in an IALVs isolated from *Myh11CreER^{T2}-GCaMP6f* mice in the presence of the L-type blocker nifedipine (1μM) (A) pressurized to 0.5 cmH₂O, 2 cmH₂O, 5 cmH₂O. The local diastolic Ca²⁺ transients persist in the presence of nifedipine and increase with increasing pressure as demonstrated in the whole vessel STMs (B). Particle occurrence maps highlight the Ca²⁺ activity in each LMC as pressure is raised (C). Representative particle analysis plots for particle area (D) and particle counts/frame (E). Summary files for particle area (F) and count /frame (G). * Denotes p<0.05, Mean and SEM shown with n=12 separate IALVs from 8 MYH11-CreER^{T2}-GCaMP6f

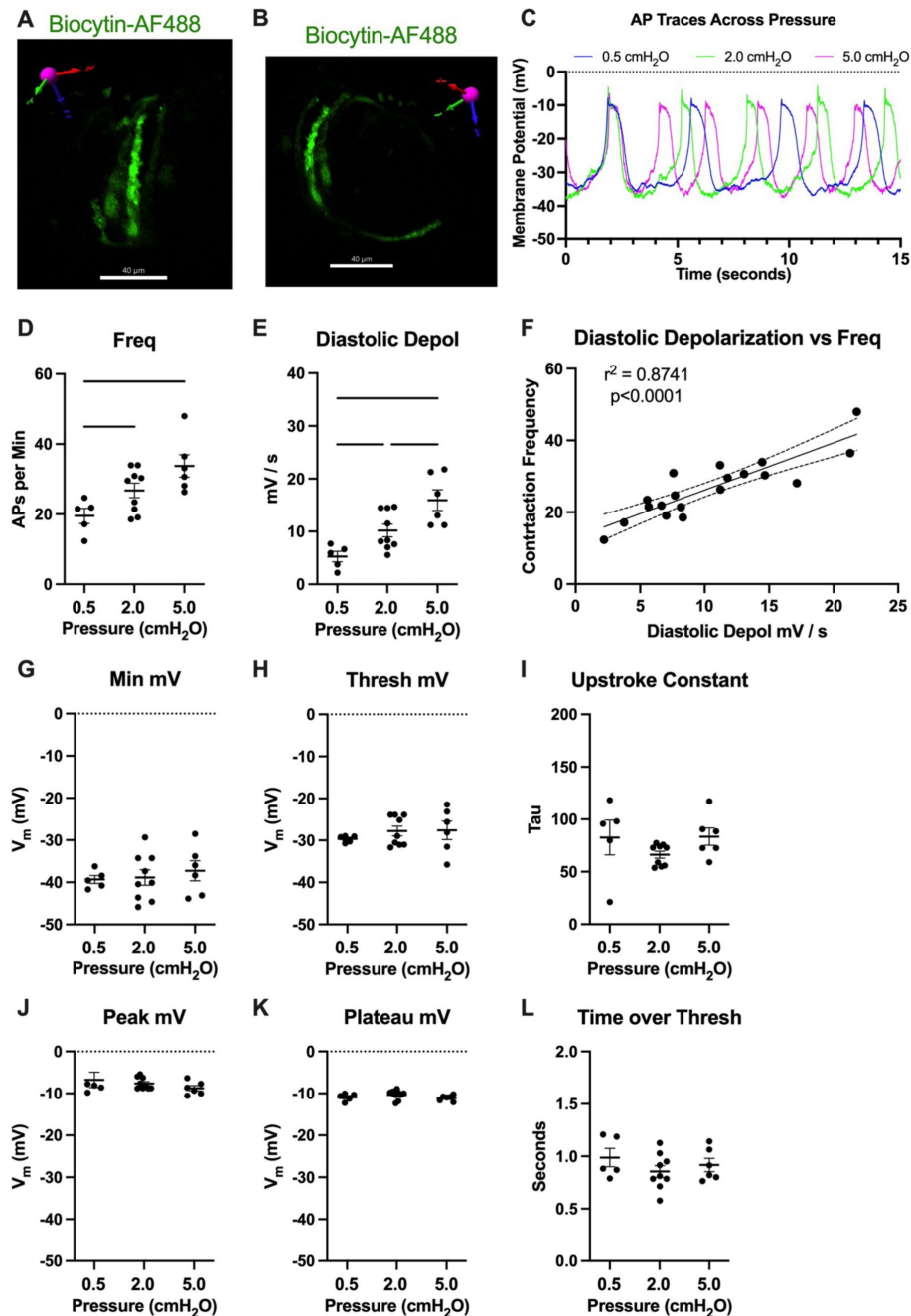


Figure 13

Pressure Dependent Diastolic Depolarization in LMCs

Intracellular recordings of LMC action potentials (AP) were confirmed by loading (greater than 10 minutes) the impaling electrode with 1M KCl 100ug/ml AF488-Biocytin while recording APs followed by imaging on a spinning disk confocal microscope. 3D reconstruction of the z-stack confirmed the circumferential pattern of the impaled LMC that was strongly labeled by AF488-Biocytin (A, B), which also labeled neighboring LMCs, likely through gap junctions as AF488-Biocytin is <1kDa. In a separate set of experiments APs were recorded at 3 different pressures, 0.5 cmH₂O, 2 cmH₂O, and 5cmH₂O. We plotted the representative recordings from 1 cell at each pressure (C). AP frequency was significantly increased with pressure (D) as was the diastolic depolarization rate. Plotting the AP frequency and diastolic depolarization rate from all recordings at each pressure (F) highlights the significant effect diastolic depolarization rate has on the AP frequency. Minimum membrane potential (G), threshold membrane potential of AP initiation (H), upstroke constant (I), peak membrane potential (J), plateau membrane potential (K), and time over threshold (L) are also reported, although not significant.

2002 [\[1\]](#); Briggs Boedtkjer et al., 2013 [\[2\]](#)), also referred to as telocytes, were further examined and found to co-express *Pdgfra*. We utilized *PdgfraCreERTM* to further test whether these cells exhibited pacemaker capabilities. However, these *Pdgfra*⁺ cells had minimal Ca²⁺ activity despite ongoing contractions and optogenetic stimulation of ChR2 in these cells failed to drive a spontaneous contraction. In contrast, photo-stimulation of LMCs expressing ChR2 elicited robust, propagated contractions with similar characteristics and propagation to spontaneous contractions in the same vessels. Furthermore, Ca²⁺ imaging in LMCs revealed diastolic Ca²⁺ transients in diastole that increased in frequency and spatial spread as pressure was elevated. We also demonstrated that the primary component of the AP driving the frequency change with pressure is the diastolic depolarization, which we have previously reported to be dependent on Ano1 (Zawieja et al., 2019 [\[3\]](#)) and IP3R1 (Zawieja et al., 2023 [\[4\]](#)). Notably, we recently reported that diastolic Ca²⁺ transients are abrogated in IALVs from *Myh11CreER^{T2}-Itpr1* inducible knockout mice, supporting a IP3R1-Ano1 axis as the pressure dependent pacemaker mechanism in LMCs. These results, in addition to the recent findings using targeted deletion of Cx45 (Castorena-Gonzalez et al., 2018b [\[5\]](#)) or Cav1.2 (To et al., 2020 [\[6\]](#); Davis et al., 2022 [\[7\]](#)) in lymphatic muscle support the model of LMCs as the intrinsic pacemaker as has been previously proposed (Van Helden, 1993 [\[8\]](#); Van Helden et al., 1996 [\[9\]](#); Van Helden and Zhao, 2000 [\[10\]](#)).

Pacemaking in Smooth Muscle

In many smooth muscle organs, regulation of a coordinated contraction is a complex and multicellular phenomenon. Multiple cell types integrate physical and biological information into electrical activity to be transmitted to the force-producing smooth muscle cells, sometimes across great distances relative to cell size, to regulate Ca²⁺ influx by voltage dependent Ca²⁺ channels required for contraction. The intestine is one such documented tissue in which cKit⁺ ICCs and interstitial PDGFRα⁺ cells form an electrical syncytium to regulate intestinal motility (Sanders et al., 1999 [\[11\]](#); Sanders et al., 2014 [\[12\]](#)). The pacemaking function of intestinal ICCs relies heavily on Ano1, a Ca²⁺ activated Cl⁻ channel, which is required for slow wave activity in the ICCs. Both cKit and Ano1 can be used as a marker for ICCs in the intestine (Hwang et al., 2009 [\[13\]](#); Cobine et al., 2017 [\[14\]](#); Malysz et al., 2017 [\[15\]](#)), cKit⁺ and vimentin⁺ ICLCs have been observed in sheep lymphatic vessels (McCloskey et al., 2002 [\[16\]](#)), yet these cell populations did not form gap junctions with the smooth muscle to form electrical connections (Briggs Boedtkjer et al., 2013 [\[2\]](#)) as occurs in the intestinal ICCs. Our cKit staining and *CkitCreER^{T2}-ROSA26mTmG* reporter studies on mouse IALVs revealed a sparse population of large ovoid cells previously classified as mast cells (Chatterjee and Gashev, 2012 [\[17\]](#); Zawieja et al., 2019 [\[3\]](#)). Their identity as mast cells was further supported by a sustained global Ca²⁺ event after stimulation with the mast cell degranulating agent compound 48-80. However, both vimentin and CD34 showed robust staining throughout the mouse lymphatic vessel wall. Vimentin stained LECs, as well as non-muscle stellate shaped cells, with many co-expressing CD34, and other smaller circular cells some of which were cKit⁺ as well and some whose morphology was similar to that of the macrophage staining profile of the GFP⁺ cells in IALVs from MacGreen mice, consistent with previous reports of macrophage staining in cLVs (Bridenbaugh et al., 2013b [\[18\]](#); Chakraborty et al., 2015 [\[19\]](#); Zawieja et al., 2016 [\[20\]](#)). While vimentin⁺ cells had a perinuclear staining profile, CD34 demarcated the cell membrane and was useful for assessing the morphology of these cells. Of particular interest, the vimentin⁺CD34⁺ cells densely populated the length of the mouse IALV, with a majority displaying a flattened stellate morphology characterized by the classic rounded oak leaf appearance, although some displayed fine dendrite extensions. Contrasting with the previous findings in the human thoracic duct (Briggs Boedtkjer et al., 2013 [\[2\]](#)), we did not observe a significant population of CD34⁺ cells with a bipolar morphology oriented axially along the vessel. However, z-stack reconstructions of sections of the mouse IALV that included the secondary valves revealed interstitial CD34⁺PDGFRα⁺ cells that resembled the bipolar cells with multiple axon-like extensions throughout the endothelial leaflets similar to interstitial cells that were reported in in collecting vessel valves (Leak and Burke, 1968 [\[21\]](#)) and lymphovenous valves (Geng et al., 2016 [\[22\]](#)). While these cells have not been frequently described in the peripheral cLV valves, we observed these cells in each of the valve regions imaged in addition to labeling them with other Cre drivers, including *NG2Cre-ROSA26mTmG* and

Pdgfr β CreER^{T2}-ROSA26mTmG (data not shown). Whether these cells regulate leaflet extracellular matrix deposition or lymphatic valve integrity is unknown, but a role as a critical pacemaker can be excluded as vessel segments without valves display normal contractile behavior (Van Helden, 1993 [↗](#); Gashev et al., 2002 [↗](#)). Instead, the majority of the CD34⁺PDGFR α ⁺ cells were found in the adventitia with 2-3 layers overtop the LMCs and were consistently observed in high density along the IALV. Some CD34⁺PDGFR α ⁺ cells or their extensions were present between the lymphatic endothelial and muscle layers as had been previously reported with electron microscopy of human lymphatic vessels (Briggs Boedtkjer et al., 2013 [↗](#)). Thus while some of these AdvCs may be contained within the extracellular matrix in the immediate vicinity of the lymphatic vessel, and thus collected during dissection, many others are intimately dispersed within the vessel wall.

PDGFR α ⁺CD34⁺ Cells are Not Involved in cLV Pacemaking Under Physiological Conditions

Co-expression of CD34 and PDGFR α has recently been ascribed as a delineating feature of telocytes, although PDGFR α routinely labels fibroblasts and specific interstitial cells in the GI tract involved in purinergic neurotransmission (Kurahashi et al., 2011 [↗](#); Kurahashi et al., 2013 [↗](#); Clayton et al., 2022 [↗](#)), and CD34 expression has been observed in multipotent cell populations of various origins (Sidney et al., 2014 [↗](#)). In the intestinal villi, PDGFR α ⁺ fibroblasts appear to be the progenitors of the smooth muscle fibers associated with the lacteal, the lymphatic capillary in the villi (Sanketi et al., 2024 [↗](#)). Of course, mesenchymal stromal cells (Andrzejewska et al., 2019 [↗](#)) and fibroblasts (Muhl et al., 2020 [↗](#); Buechler et al., 2021 [↗](#); Forte et al., 2022 [↗](#)), and it remains controversial to what extent telocytes are distinct from or are components/subtypes of either cell type (Clayton et al., 2022 [↗](#)), are not monolithic in their expression patterns displaying both organ directed transcriptional patterns as well as intra-organ heterogeneity (Lendahl et al., 2022 [↗](#)) as readily demonstrated by recent single cell RNA sequencing studies that provided immense detail about the subtypes and activation spectrum within these cells and their plasticity (Luo et al., 2022a [↗](#)). We were able to distinguish up to 10 subclusters of AdvCs, the majority of which expressed or co-expressed CD34 and PDGFR α . These cells were consistently negative for smooth muscle markers such as *Des*, *Cnn1*, *Acta2*, *Myh11* or the pericyte marker *Mcam*. However, PDGFR β expression was noted in our scRNAseq and sorted *PdgfraCreERTM-ROSA26mTmG* cells, in addition to the staining of LMCs. PDGFR β protein expression was confirmed with variable immunofluorescence staining amongst the PDGFR α stained cells as well as LMCs. The *Pdgfr β CreER^{T2}-ROSA26mTmG* mice had only modest recombination in both the LMC and PDGFR α ⁺ cell population, but potentially highlighted a myofibroblast-like cell subpopulation, cells that might lie on the spectrum of differentiation from lymphatic muscle and PDGFR α ⁺ cells, or perhaps a cell with pacemaker activity as PDGFR β is widely used as a pericyte marker and some pericytes display pacemaker activity (Hashitani et al., 2015 [↗](#)). Adding to this intrigue, the *PdgfraCreERTM* sorted cells expressed transcripts for *Cacna1c*, the voltage-gated L-type Ca²⁺ channel critical for lymphatic contractions (Zawieja et al., 2018a [↗](#); To et al., 2020 [↗](#)); *Ano1*, the ion channel underlying pressure dependent chronotropy (Mohanakumar et al., 2018 [↗](#); Zawieja et al., 2019 [↗](#)); and *Cx45*, the primary connexin mediating electrical conduction in mouse lymphatic collecting vessels (Castorena-Gonzalez et al., 2018b [↗](#); Hald et al., 2018 [↗](#)). Expression of these genes in certain sub-populations of the AdvCs was also apparent in our scRNAseq analysis. Thus, the presence of those gene transcripts does not appear to be due to muscle cell contamination or incidental recombination in LMCs as we did not detect LMC markers in the RT-PCR profiling of the sorted PDGFR α ⁺ cells nor were GFP-expressing cells with an LMC morphology observed in imaging of *PdgfraCreERTM-ROSA26mTmG* vessels. Critically, however, deletion of *Cav1.2*, *Cx45*, or *Ano1* through *PdgfraCreERTM*-mediated recombination neither recapitulated the previous phenotypes achieved with *Myh11CreER^{T2}* (Castorena-Gonzalez et al., 2018b [↗](#); Zawieja et al., 2019 [↗](#); To et al., 2020 [↗](#); Davis et al., 2022 [↗](#)) nor significantly affected pacemaking in mouse popliteal cLVs. This is in stark contrast to the complete lack of contractions observed in *Myh11CreER^{T2}-Cav1.2^{fl/fl}* vessels (To et al., 2020 [↗](#)) or the vessels from vascular muscle specific *Itga8CreER^{T2}-Cav1.2^{fl/fl}* mice (Davis et al., 2022 [↗](#); Warthi et al., 2022 [↗](#)), and the significant loss in pressure-induced chronotropic

modulation of pacemaker function in IALVs with *Myh11CreER^{T2}*-mediated deletion of *Ano1* that we have previously reported (Zawieja et al., 2019). While a sub-population of $CD34^{+}PDGFR\alpha^{+}$ cells may share expression of critical pacemaker genes identified in the LMCs, they do not appear to be involved in cLV pacemaking or contractile function under physiological states. Instead, $CD34^{+}PDGFR\alpha^{+}$ cells co-stained significantly with $Sca1^{+}$, suggesting they may be primed to act as resident multipotent cells (Song et al., 2020; Kimura et al., 2021). To this point, the *PdgfraCreERTM* FACS purified cells also expressed markers associated with “stemness” such as *CD34*, *Klf4*, *Gli1*, *CD29*, *CD105*, *CD44*, and *Vimentin*, in addition to *Sca1*, and it is likely that the *PdgfraCreERTM* population includes various distinct subpopulations (Jolly et al., 2022) expressing these markers. These cells may play a role in rebuilding the lymphatic collecting vessel vasculature following collecting vessel damage or lymph node resection and further studies are required to assess their functional multipotency.

SR Ca^{2+} Cycling in Pacemaking

The use of the mouse model, in addition to the simplicity of the vessel architecture, provided the use of genetic tools that previously had been instrumental in identifying the $cKit^{+}$ ICC as the pacemaker cells of the GI tract (Ward et al., 1994; Huizinga et al., 1995; Torihashi et al., 1995). Through the use of the respective *PdgfraCreERTM* and *Myh11CreER^{T2}* models, we were able to specifically image Ca^{2+} in each cell type in pressurized, contracting vessels. Pacemaking initiating cells have an inherently unstable membrane potential, oftentimes utilizing the oscillatory nature of Ca^{2+} release from the sarcoendoplasmic reticulum coupled to Ca^{2+} sensitive electrogenic exchangers and ion channels to drive depolarization (Van Helden, 1993; Hashitani et al., 2015; Baker et al., 2021b; Sanders et al., 2022). One such example is the pacemaker ICC in the gastric corpus which exhibits abundant Ca^{2+} transients that couple to *Ano1*-mediated chloride currents in both the intervening period between slow waves as well as the plateau phase of the slow wave (Baker et al., 2021a), although such activity is not characteristic of all pacemaker ICC types. The identification of a Ca^{2+} activated chloride current in LMCs (Van Helden, 1993; Toland et al., 2000) and its correspondence with subcellular Ca^{2+} transients (Van Helden, 1993; Ferrusi et al., 2004; von der Weid et al., 2008) led Van Helden to postulate that LMCs had an intrinsic pacemaking capability (Van Helden, 1993; Van Helden et al., 1996). We have previously reported that mouse LMCs in pressurized vessels routinely display subcellular Ca^{2+} release events that reflect the kinetics and characteristics of Ca^{2+} puffs and waves in addition to the coordinated global Ca^{2+} flash associated with Ca^{2+} influx during an AP (Castorena-Gonzalez et al., 2018b; Zawieja et al., 2018a; Zawieja et al., 2019). Here we confirmed the consistent presence of subcellular Ca^{2+} transients only in LMCs with GCaMP6f driven by *Myh11CreER^{T2}* but not in the cells with GCaMP6f driven by *PdgfraCreERTM*. Critically, we also demonstrated that the Ca^{2+} transients increased in both frequency and spatial spread as pressure was elevated in the vessel, as would be expected to account for the pressure dependent chronotropy observed in lymphatic collecting vessels. This underscores the recent finding that the genetic deletion of *Ano1* in the LMCs dramatically reduced contraction frequency and abolished pressure-dependent chronotropy in those vessels (Zawieja et al., 2019). This phenotype was largely replicated with a similar reduction in frequency and loss of pressure dependent chronotropy in our recent study utilizing *Myh11CreER^{T2}* to drive deletion of *IP3R1* from LMCs (Zawieja et al., 2023) in which these diastolic Ca^{2+} transients were absent. This fits with the central role of *IP3R* and subcellular Ca^{2+} release as critical components of intrinsic LMC pacemaking (Van Helden et al., 1996; von der Weid et al., 2008). In addition to the transcriptional heterogeneity identified by scRNASeq, we also noted heterogeneity in the propensity of LMCs to display diastolic Ca^{2+} transients under control conditions or the sustained Ca^{2+} oscillations that occur in the presence of nifedipine. We did not detect a significant difference in the expression of *Itpr1*, the gene encoding the *IP3R1*, across our LMCs subclusters. However, the LMC cluster “0” had significantly increased expression of *Itprid2* which encodes the Kras-induced actin-interacting protein (KRAP). KRAP has recently been implicated in *IP3R1* immobilization and licensing and was required for *IP3R1*-mediated Ca^{2+}

puffs (Thillaiappan et al., 2021 [↗](#); Atakpa-Adaji et al., 2024 [↗](#)). Whether the higher expression of KRAP results in a greater probability of these LMCs to display IP3R1-dependent Ca^{2+} oscillations in LMCs requires further investigation.

The membrane potential recordings we made in this work suggest that the regulation of pressure dependent chronotropy is through modulation of the diastolic depolarization rate in LMCs, as previously demonstrated in rat mesenteric lymphatic vessels (Zawieja et al., 2018b [↗](#)). The appearance of the diastolic depolarization may depend on the method of stretch employed as it is not always observed in preparations using a wire myograph (von der Weid et al., 2014 [↗](#)). Notably, in this study *PdgfraCreERTM* mediated deletion of *Ano1* had no effect on contractile parameters. The lack of Ca^{2+} transients in PDGFR α^+ cells across any stage of the lymphatic contraction cycle diminishes any expected role for this cell type to perform as the pacemaker for the mouse IALV. In the pacemaker ICCs, slow wave activity is dependent on calcium transient clusters. Instead pressure-dependent Ca^{2+} mobilization from the SR, through IP3R1 (Zawieja et al., 2023 [↗](#)), appears to set the basis for LMC pacemaking as previously proposed (Van Helden, 1991 [↗](#); von der Weid et al., 2008 [↗](#)). However, the mechanisms driving IP3R1 activation and Ca^{2+} oscillations remain to be fully addressed.

A pacemaker cell would be expected to be electrically coupled to the LMC layer to permit the nearly synchronous conduction velocity of the contraction wave (Zawieja et al., 1993 [↗](#); Castorena-Gonzalez et al., 2018b [↗](#); Hald et al., 2018 [↗](#)) and to transmit depolarization into coupled LMCs to activate the voltage dependent Ca^{2+} channels that are responsible for lymphatic muscle APs. Connexins are the molecular constituents of gap junctions and, as stated above, we detected Cx45 expression in the *PdgfraCreERTM* sorted cells. However, we did not detect any impairment in pacemaking, nor were contraction conduction speed deficits or multiple pacemakers noted in the *PdgfraCreERTM*-Cx45fl/fl popliteal cLVs, in contrast to the development of multiple pacemaker sites and the lack of entrainment that characterize cLVs from *Myh11CreER^{T2}*-Cx45fl/fl mice (Castorena-Gonzalez et al., 2018b [↗](#)). Admittedly, we did not perform an exhaustive assessment of the connexin expression profile of the CD34⁺PDGFR α^+ cells, and Cx45 may not be the dominant connexin expressed in the CD34⁺PDGFR α^+ cells, or heterotypic connexons could exist (Koval et al., 2014 [↗](#)). However, electron microscopy studies of the putative ICLC in the human thoracic duct did not detect any gap junctions, although peg-and-socket connections were observed (Briggs Boedtkjer et al., 2013 [↗](#)). We utilized optogenetics to directly depolarize the specific cell populations in both the *PdgfraCreERTM* and *Myh11CreER^{T2}* mouse models in an attempt to drive contractions. Local photo-stimulation of the PDGFR α cells failed to initiate contraction while the stimulation of *Myh11CreER^{T2}* recombined cells resulted in contractions that were indistinguishable from the spontaneously occurring contractions. These results give functional credence to the lack of hetero-cellular coupling via gap junctions that was previously reported (Briggs Boedtkjer et al., 2013 [↗](#)). Just as critically, they also highlight the regenerative nature of the lymphatic muscle AP as local depolarization was sufficient to drive a coordinated contraction along the vessel and that a single or few LMCs reaching threshold for AP initiation are sufficient to drive the conducted activity observed at the tissue level.

Conclusions

Our present findings lend further support to the hypothesis that the LMCs are intrinsic pacemakers (van Helden et al., 2006 [↗](#); Mitsui and Hashitani, 2020 [↗](#)) and mouse cLVs do not require an ICC-like cell network to drive propagated contractions. These findings also underscore the significance of lymphatic muscle Ca^{2+} handling as the driver of lymphatic pacemaking, which can be compromised in disease states leading to impaired lymphatic contractile activity (Stolarz et al., 2019 [↗](#); Lee et al., 2020 [↗](#); Van et al., 2021 [↗](#)). Further studies delineating the specific SR Ca^{2+} release and influx pathways, and the contributions of Ca^{2+} sensitive ion channels are required to develop sophisticated in silico models and identify potential therapeutic targets to rescue lymphatic pacemaking in lymphedema patients (Olszewski, 2002 [↗](#), 2008 [↗](#)).

Limitations

One fundamental assumption underlying our conclusions is that there is a conserved pacemaking pathway and cell type regulating lymphatic collecting vessel contractions across species, specifically pertaining to the capability of lymphatic muscle to maintain pacemaking and coordination despite changes in tissue complexity and cLV wall thickness. It is worth noting that lymphatic collecting vessels in mice have similar pressure-dependent chronotropy and contraction conduction velocity as recorded in rats and human vessels (Castorena-Gonzalez et al., 2018b [DOI](#)). These similarities exist despite the fact that mouse lymphatic collecting vessels are typically encircled by a single layer of lymphatic muscle while larger species may have multiple layers of LMCs in the wall. It is possible that vessels with multiple layers of LMCs need a network of ICLC to coordinate their activity. The simplicity in the makeup of the mouse cLV and the use of cell targeting Cre models provide great control over experimental variables, but other cell types may provide coordination of LMC pacemaking in other species where the lymphatic cLV walls are larger and thicker and contain multiple muscle cell layers. Our scRNAseq analysis also is likely biased using ROSA^{mTmG} mice with FACS purification to remove debris and concentrate specific cell types from these pooled small vessels. Larger and more complex cells, with attributes that can be ascribed to ICCs, are more likely to be lost in this methodology (e.g., depending on the FACS gating parameters) and this procedure can also elicit a stress response in the transcriptome of the analyzed cells. However, we also did not observe long and complex cells, aside from the circumferential LMCs, in our immunofluorescence and recombination reporter imaging experiments. Immediate and early gene expression motifs driven by a stress response may be a component of the differences in sub-clusters that were identified. Future scRNAseq or snRNAseq studies with deeper sequencing will be required to ensure the full transcriptomic heterogeneity is accounted under different cellular stress conditions.

Our data demonstrate that limited staining of a few cell markers alone is insufficient to identify discrete cell populations in the mouse cLVs. Additionally, mRNA expression does not equal protein translation nor guarantee specific function as we did not readily detect endothelial CD34 with immunofluorescence despite detecting transcript; additionally, *Pdgfra*Cre^{ER}TM mediated deletion of *Ano1*, *Cx45*, or *Cav1.2* had no effect on cLV pacemaking. Hence, further experimentation is also required to fully characterize expression of multipotent cell markers and function of CD34⁺PDGFR α ⁺Sca1⁺ cells invested within the mouse cLVs, although this was beyond the scope of this study assessing pacemaker identity. Tangentially, another limitation of our approach pertains to the specificity and recombination efficiency of inducible Cre recombinase models, which can be a notable confounding variable (Chakraborty et al., 2019 [DOI](#)). We observed that our inducible Cre models led to a degree of nonspecific recombination within the mouse cLV, with the GCaMP6f and ChR2 particularly susceptible to recombination compared to the ROSA26mT/mG reporter. Recombination in multiple cell types was expected with the constitutive Cre models we employed (*Ng2*Cre and *Pdgfra*Cre), as vascular and lymphatic muscle precursor cells can transiently express *Nestin*, *Pdgfra*, and *Ng2* (Hill et al., 2015 [DOI](#); Castorena-Gonzalez et al., 2018b [DOI](#); Kenney et al., 2020 [DOI](#)). We also observed that *Pdgfr β* Cre^{ER}^{T2} drove recombination in a sub population of LMCs and PDGFR α ⁺ cells. These appeared to be two distinct populations that only share expression for PDGFR β based on our scRNAseq dataset but may exist along a continuum of differentiation. PDGFR β -PDGFR β signaling is critical for normal mural cell recruitment to both the blood and lymphatic vasculature (Gaengel et al., 2009 [DOI](#); Wang et al., 2017 [DOI](#)) and proliferating vascular smooth muscle cells and pericytes have both been documented to express PDGFR β (Andrae et al., 2008 [DOI](#); Pitulescu and Adams, 2014 [DOI](#)). Ideally, novel Cre or combinatorial Cre models that specifically target LMCs or sub populations of LMCs may be developed to further tease out the functional role of these cells.

Materials and Methods

Mice

Wild-type (WT) male mice (25-35 g) on the C57BL/6J background, ROSA26mT/mG reporter (Muzumdar et al., 2007 [\[1\]](#)) (Strain#007676), transgenic *PdgfraCre* (Strain#013148), CSFR1-EGFP (MacGreen) (Sasmono et al., 2003 [\[2\]](#)) (Strain#018549), genetically encoded Ca²⁺ sensor GCaMP6f (Chen et al., 2013 [\[3\]](#)) (Strain#028865), transgenic *PdgfraCreERTM* (Kang et al., 2010 [\[4\]](#)) (Strain#018280), NG2-Cre (Strain #:008533) (Zhu et al., 2008 [\[5\]](#)), and Chr2 /tdTomato fusion mice (Madisen et al., 2012 [\[6\]](#)) (Strain#012567) were purchased from The Jackson Laboratory (Bar Harbor, MA, USA). *PdgfrβCreER^{T2}* (Gerl et al., 2015 [\[7\]](#)) mice were a gift from Ralf Adams (Mac Planck Institute) and kindly provided by Lorin Olson (Oklahoma Medical Research Foundation) and are currently available at Jax (Strain#029684). The *Myh11CreER^{T2}* mice (Wirth et al., 2008 [\[8\]](#)) were a gift from Stefan Offermanns, Max-Planck-Institut für Herz- und Lungenforschung, Bad Nauheim, Germany, and are currently available at Jax (Strain #019079, Y-Linked). c-KitCreER^{T2} mice (Heger et al., 2014 [\[9\]](#)) were a gift from Dieter Saur (Technical University of Munich). Prox1-eGFP mice (Choi et al., 2011 [\[10\]](#)) were a gift from Young-Kwon Hong (University of Southern California). For genotyping, we isolated genomic DNA from mouse tail clips using the HotSHOT method (Truett et al., 2000 [\[11\]](#)). Specific mouse genotypes were confirmed via PCR using 2x PCR Super Master Polymerase Mix (Catalog # B46019, Bimake, Houston, TX) performed as specified by the provider. Mice used for this study were 3-8 months of age. All animal protocols were approved by the University of Missouri Animal Care and Use Committee and conformed to the US Public Health Service policy for the humane care and use of laboratory animals (PHS Policy, 1996).

iCre Tamoxifen Induction

Mice harboring *PdgfraCreERTM*, *PdgfrβCreER^{T2}*, *Myh11CreER^{T2}*, and *cKitCreER^{T2}* were crossed with ROSA26mT/mG mice to generate *PdgfraCreERTM*-ROSA26mT/mG, *PdgfrβCreER^{T2}*-ROSA26mT/mG, *Myh11CreER^{T2}*-ROSA26mT/mG, and *cKitCreER^{T2}*-ROSA26mT/mG mice, respectively. The resulting iCre-ROSA26mT/mG mice were induced with tamoxifen 2-4 weeks after weaning. Tamoxifen induction was performed via consecutive 100 µL i.p. injections of tamoxifen ranging from 1 to 5 days at concentrations ranging from 0.2 -10 mg/mL in safflower oil, using a titrated induction protocol to determine the extent of recombination in specific cell populations. We used our maximal induction protocol, 100 µL of tamoxifen at 10 mg/mL over 5 consecutive days, for *cKitCreER^{T2}*-GCaMP6f, *Myh11CreER^{T2}*-GCaMP6f, and *PdgfraCreERTM*-GCaMP6f mice. Due to the paucity of recombined cells in the *cKitCreER^{T2}*-ROSA26mT/mG reporter mice, we used our maximal tamoxifen induction protocol for *cKitCreER^{T2}*-Chr2 mice as this still resulted in the ability to excite single recombined cells. *Myh11CreER^{T2}*-Chr2/tdTomato mice were induced with one 100 µL i.p. injection of tamoxifen at 0.2 mg/mL while *PdgfraCreERTM*-Chr2/tdTomato were induced with 1 injection at 0.4 mg/mL tamoxifen to get mosaic induction sufficient for single cell stimulation. All mice, regardless of induction duration, were given at least 2 weeks to recover following tamoxifen injection.

Lymphatic Vessel Isolation

We utilized both popliteal and inguinal-axillary lymphatic collecting vessels (IALVs) in this study, which were isolated as described previously (Zawieja et al., 2018a [\[12\]](#)). In brief, mice were anaesthetized with a cocktail of 100/10 mg/mL ketamine/xylazine and shaved along the flank or the legs for IALVs and popliteal cLVs respectively. The IALV (also referred to as the flank cLV) is located adjacent to the thoracoepigastric vein and connects the inguinal and axillary lymph nodes. A cut was made along the dorsal midline and the skin retracted and pinned out to reveal the thoracoepigastric vascular bed. The thoracoepigastric vascular bed and connected perivascular adipose containing the IALVs vessel was dissected out and pinned onto a Sylgard coated dish in

Krebs buffer. Popliteal lymphatic vessels were exposed through a superficial incision in the leg, removed and transferred to the Krebs-albumin filled dissection chamber. After removal, the vessel was carefully cleaned of adipocytes and excess matrix using fine forceps and scissors through micro-dissection. For immunofluorescence, sections containing 2-3 valves were isolated, while smaller IALV sections consisting of 1-2 valves were isolated for GCaMP6f Ca^{2+} imaging. Similarly, popliteal cLVs were isolated (Castorena-Gonzalez et al., 2018a [↗](#)) following an incision along the skin overlying the saphenous vein for contractile function analysis and for ChR2 optogenetic depolarization experiments.

Lymphatic Vessel Isobaric Function

PdgfraCreERTM mice were crossed with *Ano1^{fl/fl}*, *Cx45^{fl/fl}*, and *Cav1.2^{fl/fl}* mice to generate *PdgfraCreERTM-Ano1^{fl/fl}*, *PdgfraCreERTM-Cx45^{fl/fl}*, and *PdgfraCreERTM-Cav1.2^{fl/fl}* mice. These mice and their respective fl/fl controls were injected with tamoxifen as described above for 5 days and given two weeks to recover. The popliteal vessels were isolated, cleaned, and prepared for isobaric contractile tests as previously reported (Davis et al., 2023 [↗](#)). Once equilibrated, inner diameter was tracked over a physiological pressure range (stepped from 3 to 2, 1, 0.5, 3, 5, 8, and 10 cmH₂O) with 2min of recording at each pressure. Following the pressure step protocol the vessels were equilibrated in with Ca^{2+} -free Krebs buffer (3mM EGTA) and diameter at each pressure recorded under passive conditions (DMAX). The contractile parameters end diastolic diameter (EDD), end systolic diameter (ESD), and contraction frequency (FREQ) were recorded with a custom LabVIEW program and the following contractile parameters assessed:

1. Contraction Amplitude (AMP) = EDD-ESD
2. Normalized Contraction Amplitude = ((EDD-ESD)/DMAX) × 100
3. Ejection Fraction (EF) = (EDD²-ESD²)/EDD²
4. Fractional Pump Flow (FPF) = EF × FREQ
5. Tone = ((DMAX-EDD)/DMAX) × 100

Methylene Blue Staining

Isolated IALVs sections were transferred into a Krebs-BSA buffer filled 3-mL observation chamber, with a cover slip bottom, and cannulated onto two glass micropipettes (30-80 μm, outer diameter) held in place by pipette holders on a Burg-style V-track mounting system. The pipette holders were attached to a 3-way valve stop cock with polyethylene tubing filled with Krebs-BSA buffer. Vessels were pressurized to approximately 5 cmH₂O by raising the 3-way valve and the vessels were stretched to remove any slack. For methylene blue staining, IALVs from wild type C57Bl6 mice were stained with 50 μM methylene blue in Krebs-BSA buffer for two hours at room temperature and covered in foil to limit light-induced phototoxicity. After the staining period, the vessel chambers were washed three times with Ca^{2+} free PSS to remove methylene blue. Brightfield images and manual Z-stack videos were collected on an inverted Leica DMi1 4X or 20X air objective, or a Leica DMi8 with a 25X water objective or an inverted DMi8 using a Leica Flexacam C1 color camera for image acquisition. Some Methylene blue images were also collected using a color Nikon DS-Fi3 camera. The collected z-stacks were analyzed using Image J and the “Stack Focuser” plugin (<https://imagej.nih.gov/ij/plugins/stack-focuser.html> [↗](#)). To accentuate the methylene blue stained cells, the color image stack was split into red, green, and blue channel stacks. The blue channel stack was then divided by the green channel stack using the “Image Calculator” function. The resulting 32-bit image was then converted into 16-bit image to permit the use of the Stack Focuser plugin with the ‘n kernel value’ set to 11.

Fluorescence Confocal Imaging

IALVs vessels from each respective iCre-ROSA26mT/mG mouse were prepared in a similar manner (excluding the addition of methylene blue). We performed confocal imaging to acquire z-stacks of 7-10 overlapping regions of interests to allow for manual stitching, with 1 μ M z-steps (20X) or 0.5 μ M steps at 40X. We imaged through to the midpoint of the vessel except when imaging the valve interstitial cells, in which case the entire vessel was imaged. Max projections were made using FIJI. Following live imaging, the vessels were pressurized to 5 cmH₂O and fixed with 4% paraformaldehyde for 30 min at room temperature. IALVs were then washed with PBS containing 0.1% Triton X-100 (PBST) 3 times and blocked for a minimum of 2 hr with Blockaid® (B-10710, ThermoFisher Scientific). IALVs were then stained with the corresponding primary antibodies in BlockAid® Solution: anti-smooth muscle actin (SMA) 1:500 (Sigma, A2547), anti-GFP 1:200 (ThermoFisher, A11122), anti-cKit 1:100 (Cell Signaling, 3074), anti-Vimentin 1:100 (ThermoFisher, OMA1-06001), anti-desmin 1:200 (Invitrogen, PA5-16705), anti-GFP 1:200 (Abcam, ab13970, anti-CD34 1:200 (Invitrogen, 14-0341-82), anti-PDGFR α 1:200 (R&D Systems, AF1062), anti-PDGFR β 1:200 (eBiosciences, 14-1402-82), anti-calponin 1:500 (Abcam, AB46794), anti-MYH11 1:500 (Abcam, AB124679), anti-Sca1 1:200 (Biolegend, 108101). IALVs were then washed in PBS and incubated overnight with the corresponding donkey secondary antibodies (ThermoFisher®) at 1:200. After a final wash, IALVs were re-cannulated and pressurized for imaging using the aforementioned spinning disk confocal and Hamamatsu Orca Flash4 camera using a 20X air objective (Olympus UplanApo, 0.75) or 40X (Olympus UApo A340, 1.15) water objective. Images were taken as described above, and the resulting stacks were turned into a max projection using FIJI. Colocalization analysis of the max projections of CD34 and PDGFR α was performed using the BIOP JACoP colocalization plugin (Bolte and Cordelières, 2006 [DOI](#)) with both Pearson's and Mander's coefficients reported.

LMC Dissociation and FACS Collection-

IALVs vessels *PdgfraCreERTM*-ROSA26mT/mG, *Pdgfr β CreER^{T2}*-ROSA26mT/mG, *Myh11CreER^{T2}*-ROSA26mT/mG, Macgreen, and *Prox1-eGFP* mice were dissected and cleaned of excess adventitia and adipose tissue in Krebs buffer. Isolated vessels were then transferred into a low Ca²⁺ PSS solution supplemented with 0.1 mg/mL bovine serum albumin (BSA, Amersham Life Science, Arlington Heights, IL). Primary LMCs were collected by enzymatic dissociation of IALVs. The dissected vessels were cleaned in room temperature Krebs-BSA buffer and then transferred into a 1-mL tube of low-Ca²⁺ PSS on ice, washed, and equilibrated for 10 min. Vessels were then digested in low-Ca²⁺ PSS with 26 U/mL papain (Sigma, St. Louis, MO) and 1 mg/mL dithioerythritol for 30 min at 37°C with gentle agitation every few minutes. This solution was then decanted and replaced with low-Ca²⁺ PSS with containing 1.95 collagenase H (U/mL, Sigma), 1.8 mg/mL collagenase F (Sigma), and 1mg/mL elastase (Worthington LS00635) and incubated for 3 - 5 min at 37°C. The mixture was then spun down at 1000 rpm for 4 min, the digestion buffer removed, and replaced with low-Ca²⁺ PS. This process was repeated twice to remove residual digestion buffer. The vessel was then triturated with a fire-polished Pasteur pipette to dissociate the cells into a single cell suspension, passed through a Falcon cap strainer (35 μ m), and resuspended in ice-cold low-Ca²⁺ PSS for sorting. For iCre-ROSA26mT/mG mice, GFP⁺RFP⁻ cells or GFP⁺ cells from Macgreen and *Prox1-eGFP* mice were then FACS purified straight into RNA isolation buffer for RT-PCR analysis. FACS was performed with a Beckman-Coulter MoFlo XDP instrument using an excitation laser (488 m) and emission filter (530/40 m). Sorting was performed using 70- μ m nozzle at a sheath pressure of 45 p.s.i. and sort rate of 100 events/s and with an efficiency of >90%. To maximize cell yield, we isolated both the left and right full-length IALVs vessels from 2 mice for digestions and subsequent FACS collection. For *Myh11CreER^{T2}*-ROSA26mT/mG and *PdgfraCreERTM*-ROSA26mT/mG, the yield averaged 1000-2000 cells per mouse. For *Prox1-eGFP* mice, LEC yield was typically 1500-2000 cells per mouse.

RT-PCR Profiling of FACS Purified Cells

Total RNA was extracted from FACS purified GFP⁺ cells from the isolated IALVs vessels using the Arcturus PicoPure RNA isolation kit (ThermoFisher Scientific, Waltham, MA) per the listed instructions. Prior to elution in 20 µl of water, on-column DNase digestion (Qiagen, Valencia, CA) was performed to ensure removal of genomic DNA contaminants. RNA was converted into cDNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA) using oligo (dT) and random hexamer priming following the manufacturer's protocol. Each RT reaction used approximately 50-100 cells worth of RNA based on the sorted cells count number. Our PCR reaction mixture contained first-strand cDNA as the template, 2 mM MgCl₂, 0.25 µM primers, 0.2 mM deoxynucleotide triphosphates; and GoTaq® Flexi DNA polymerase (Promega, Madison, WI). The PCR program comprised an initial denaturation step at 95°C for four min; followed by 35 repetitions of the following cycle: denaturation (94° C, 30 s), annealing (58° C, 30 s), and extension (72° C, 30 s). This was followed by a final elongation step for 5 min at 72° C. PCR amplification products were separated on a 2% agarose gel by electrophoresis, stained with SYBR-Safe (Thermo Fisher Scientific, Waltham, MA), and visualized by UV trans-illumination. All primers were designed to amplify an intron-spanning region. Endpoint RT-PCR Primer sequences, amplicon size, accession numbers, and source are listed in [Table 1](#).

scRNASeq Analysis of Mouse IALVs

For scRNASeq analyses of isolated IALVs we used a total of 10 *Rosa26mTmG* mice, without Cre and without tamoxifen treatment, with equivalent representation of sex (5 males and 5 females). Full length IALVs from both the left and right side of each *ROSA26mTmG* mouse were isolated and cleaned of excessive matrix and adipose tissue. Isolated vessels were digested into single cell suspensions as described above and the cells were kept on ice following single cell suspension until all the tissues had been processed. Cells from all vessels were combined and sorted for tdTomato expression to remove debris and concentrate the cells for downstream single cell 3' RNA-Seq libraries creation with 10x Genomics Chromium Chip and Chromium Next GEM Single Cell 3' RNA-Seq reagents. Samples were sequenced with the NovaSeq 6000 S4-PE100 flow cell.

Mus musculus genome GRCm39 and annotation GTF (v106) from Ensembl (https://useast.ensembl.org/Mus_musculus/Info/Index) were used to build the reference index and the reads were processed using Cell Ranger (v7.0.1; Zheng et al., 2017) with default parameters. The quality control and filtering steps were performed using R (v4.2.1; <https://www.r-project.org/>). Ambient RNA was removed from the Cell Ranger output with SoupX (Young and Behjati, 2020). Doublet score for each cell was estimated using scDBIFinder (v1.12.0; Germain et al., 2021). Non-expressed genes (sum zero across all samples) and low-quality cells (>10% mitochondrial genes, < 500 genes, < 1,000 UMIs per cell and doublet score <0.5) were removed with custom R scripts. Cells passing filtering were normalized/scaled (SCTransformation), dimensionally reduced (t-distributed stochastic neighbor embedding (t-SNE) and uniform manifold approximation and projection (UMAP)) clustered, and hierarchically analyzed with Seurat (Hao et al., 2021; Hao et al., 2024) with default parameters. Marker gene expression profile on cell clusters and gene co-expression was visualized using Seurat and ShinyCell R application (Ouyang et al., 2021). The full scRNAseq raw dataset has been uploaded to the NIH GEO under the accession number GSE277843. Differential gene expression within subclusters of LECs, LMCs, and AdvCs was performed using Seurat's "Find Markers" function and with a minimum of either 40% or 50% cell expression and log fold change minimum of 1. In the volcano plot for LEC subcluster 8 differential gene expression, listed genes were had a cutoff of a log fold change of 2 or -2 to be displayed on the plot.

Gene	Strand	Accession #	Sequence (5'-3')	Size	Exon	Source
<i>Prox1</i>	s	NM_008937	GTA AGA CAT CAC CGC GTG C	218	1	NIH Primer Tool
	as		TCA TGG TCA GGC ATC ACT GG		2	
<i>CD11b</i> (<i>Itgam</i>)	s	NM_008401	ATG GAC GCT GAT GGC AAT ACC	203	13	MGH Primer Bank ID 668048a1
	as		TCC CCA TTC ACG TCT CCC A		14	
<i>Pdgfra</i>	s	NM_011058	AGA GTT ACA CGT TTG AGC TGT C	252	8	MGH Primer Bank 26349287a1
	as		GTC CCT CCA CGG TAC TCC T		10	
<i>Myh11</i>	s	NM_013607	AAG CTG CGG CTA GAG GTC A	238	33	MGH Primer Bank ID 7305295a1
	as		CCC TCC CTT TGA TGG CTG AG		34	
<i>CD117</i> (<i>cKit</i>)	s	NM_021099	CGC CTG CCG AAA TGT ATG ACG	162	21	(Drumm et al., 2018)
	as		GGT TCT CTG GGT TGG GGT TGC		23	
<i>Pdgfrβ</i>	s	NM_008809	AGC TAC ATG GCC CCT TAT GA	367	16	(Basciani et al., 2004)
	as		GGA TCC CAA AAG ACC AGA CA		19	
<i>CD144</i> (<i>VE-cadherin</i>)	s	NM_009868	CTT CCT TAC TGC CCT CAT TGT	313	3	IDT Primer Quest
	as		CTG TTT CTC TCG GTC CAA GTT		5	
<i>Nos3</i> (<i>eNOS</i>)	s	NM_008713	ctg cca cct gat cct aac ttg	143	22	IDT Real time primer tool
	as		cag cca aac acc aaa gtc atg		23	
<i>Acta2</i> (<i>Smooth Muscle Actin</i>)	s	NM_007392	GAG CTA CGA ACT GCC TGA C	129	7	IDT TaqMan Mm.PT.58.16320644
	as		CTG TTA TAG GTG GTT TCG TGG A		8	

Table 1

Primer list for RT-PCR

<i>CaV 1.2 exon1b</i>	s	NM_001159533	ATG GTC AAT GAA AAC ACG AGG ATG		1	(Cheng et al., 2007)
	as		GGA ACT GAC GGT AGA GAT GGT TGC	234	2	
<i>CD34</i>	as	NM_001111059	GGT ACA GGA GAA TGC AGG TC	119	2	IDT Mm.PT.58.8626728
	s	NM_133654	CGT GGT AGC AGA AGT CAA GT		1	
<i>Cspg4 (Ng2)</i>	as	NM_139001	CTT CAC GAT CAC CAT CCT TCC	132	5	IDT Mm.PT.58.29461721
	s		CCC GAA TCA TTG TCT GTT CCC		6	
-	-	-	-	-	-	
<i>Vimentin</i>	s	NM_011701	CTG TAC GAG GAG GAG ATG CG	249	1	(Li et al., 2016)
	as		AAT TTC TTC CTG CAA GGA TT		3	
<i>Desmin</i>	s	NM_010043	GTG GAT GCA GCC ACT CTA GC	218	3	MGH Primer Bank ID 33563250a1
	as		TTA GCC GCG ATG GTC TCA TAC		4	
<i>CD146 (Mcam)</i>	s	NM_023061	CCC AAA CTG GTG TGC GTC TT	220	1	MGH Primer Bank 10566955a1
	as		GGA AAA TCA GTA TCT GCC TCT CC		3	
<i>KLF4</i>	s	NM_010637	ATT AAT GAG GCA GCC ACC TG	400	1	(Majesky et al., 2017)
	as		GGA AGA CGA GGA TGA AGC TG		3	
<i>Ly6a (Sca1)</i>	s	NM_001271416	CTC TGA GGA TGG ACA CTT CT	400	2	(Majesky et al., 2017)
	as		GGT CTG CAG GAG GAC TGA GC		4	
<i>Gli1</i>	s	NM_01029	ATC ACC TGT TGG GGA TGC TGG AT	316	8	(Kramann et al., 2015)
	as		CGT GAA TAG GAC TTC CGA CAG		10	
<i>CD29 (Itgb1)</i>	s	NM_010578	TCG ATC CTG TGA CCC ATT GC	170	14	NIH Primer Tool

Table 1 (continued)

	as		AAC AAT TCC AGC AAC CAC GC		15	
<i>CD105</i> (<i>Endoglin</i>)	s	NM_007932	TGA GCG TGT CTC CAT TGA CC	416	11	NIH Primer Tool
	as		GGG GCC ACG TGT GTG AGA A		15	
<i>CD44</i>	as	NM_009851	CAC CAT TTC CTG AGA CTT GCT	148	19	IDT Mm.PT.58.12084136
	s		TCT GAT TCT TGC CGT CTG C		18	
<i>CD31</i> (<i>Pecam1</i>)	s	NM_008816	CTG CCA GTC CGA AAA TGG AAC	218	7	MGH Primer Bank ID 6679273a1
	as		CTT CAT CCA CTG GGG CTA TC		8	
<i>GJC1</i> (<i>Connexin</i> 45)	s	NM_008122	GGT AAC AGG AGT TCT GGT GAA	140	2	IDT Mm.PT.58.8383900
	as		TCG AAA GAC AAT CAG CAC AGT		3	
<i>Anoctamin 1</i> (<i>TMEM16A</i>)	s	NM_178642	ggc att tgt cat tgt ctt cca g	141	25	IDT Real time primer tool
	as		tcc tca cgc ata aac agc tc		26	
<i>CD45</i> (<i>Ptprc</i>)	s	NM_001111316	ATG CAT CCA TCC TCG TCC AC	225	29	NIH Primer Tool
	as		TGA CTT GTC CAT TCT GGG CG		31	
MGH Harvard Primer Bank (Wang and Seed, 2003; Spandidos et al., 2008; Spandidos et al., 2010)						

Table 1 (continued)

Ex vivo Ca^{2+} imaging with the genetically encoded GCaMP6f Indicator

cKitCreER^{T2}, *Myh11CreER^{T2}*, and *PdgfraCreERTM* mice were crossed with GCaMP6f mice in a similar manner as described for ROSA26mT/mG. *cKitCreER^{T2}*-GCaMP6f, *PdgfraCreERTM*-GCaMP6f, and *Myh11CreER^{T2}*-GCaMP6f were induced with tamoxifen (10 mg/mL) for 5 consecutive days by i.p. injection. IALVs isolated from *cKitCreER^{T2}*-GCaMP6f, *PdgfraCreERTM*-GCaMP6f, and *Myh11CreER^{T2}*-GCaMP6f were cannulated as described above. The cannulated vessel, with micropipette holders, observation chamber and V-track mounting system, was transferred to the stage of the spinning disk confocal with a Prime95B sCMOS camera (Photometrics), a Cascade II EMCCD (Photometrics), or an Ixon888 EMCCD camera (Andor) for Ca^{2+} imaging (Castorena-Gonzalez et al., 2018b [\[link\]](#)). Pressures for the input and output cannula were connected to a T-junction which was set briefly to 8 cmH₂O and the vessel lengthened to remove axial slack. A peristaltic pump maintained constant perfusion of the observation chamber with Krebs buffer at a rate of 0.5 mL/min while the vessel equilibrated at 37°C for 30-60 min with pressures set to 3 cmH₂O. Spontaneous contractions were allowed to stabilize over a period of 30 min and then were blunted with 2 μM wortmannin to limit movement associated with contractions during Ca^{2+} imaging. A Windows-based computer was used to digitize the pressure transducer signals and video image of the vessel from a firewire camera at 30-40 Hz (Davis et al., 2012 [\[link\]](#)). A custom-written LabVIEW program (National Instruments; Austin, TX) detected the inner diameter of the vessel from the video (Davis et al., 2011 [\[link\]](#)). Once contractions were <5 μm in amplitude, Ca^{2+} recordings were made at 20FPS for 20-40 s.

Ca^{2+} Imaging and Analysis in IALVs Over the Contraction Cycle

Background noise was determined by using the histogram feature of FIJI in a rectangle in a region of the field of view without sample. This value was subtracted from the entire field of view. In some cases, the vessel movement due to contraction was offset with video stabilization with the FIJI plugin Image Stabilizer. A max projection was used to create non-overlapping ROIs of GCaMP6f⁺ cells for each iCre-GCaMP6f IALV. From these cell ROIs, the “reslice z” function was used to create pseudo-linescan STMs, which were divided by their baseline values to obtain F/F_0 values for each individual cell. At least 3 cells, except in the case of 1 *cKitCreER^{T2}*-GCaMP6f IALV, in which only two cells were observed, were analyzed in this manner for each vessel segment. Max projections of the image stack were then used to create non-overlapping cell masks of 3-5 muscle cells per field of view of one vessel. Ca^{2+} traces for those cells contained 5-10 contraction cycles and Ca^{2+} transients and were characterized for peak intensity (expressed as a baseline-referenced ratio, F/F_0), frequency, and duration in seconds.

Analysis of Subcellular Ca^{2+} Transients in *Myh11CreER^{T2}*-GCaMP6f IALVs

For *Myh11CreER^{T2}*-We performed Ca^{2+} imaging as above in the presence of 1 μM nifedipine to stop the “ Ca^{2+} flashes” associated with APs (Zawieja et al., 2018a [\[link\]](#)) and focus on the subcellular activity at 3 different experimental pressures of 0.5, 2, and 5 cmH₂O. For this protocol, we used a particle analysis approach to analyze all Ca^{2+} transients in the field of view. Ca^{2+} transients in intact vessels were quantified by particle analysis as previously described (Drumm et al., 2017 [\[link\]](#); Drumm et al., 2019 [\[link\]](#)). Movies of Ca^{2+} transients in intact vessels were imported into custom built Volumetry software (version G8d) and background subtracted. Movies were smoothed using a Gaussian filter: 1.5 x 1.5 mM, StdDev 1.0). Raw Ca^{2+} transients were converted to Ca^{2+} particles (PTCLs) using a flood-fill algorithm as previously described (Drumm et al., 2017 [\[link\]](#); Drumm et al., 2019 [\[link\]](#)). PTCLs <10 μm^2 were rejected to facilitate the removal of noise and then the total PTCL area and PTCL count could be tabulated for each recording.

Membrane Potential Recordings in IALVs

Mouse IALVs were isolated and cleaned as described above. IALVs were pressurized in our isobaric myography apparatus and allowed to equilibrate to ensure typical contractile activity was evident. A bolus of wortmannin at 2 μM was then applied to the bath to blunt contraction amplitude below 5 microns. Intracellular recordings of lymphatic muscle were made with microelectrodes (250–300 M Ω) filled with 1 M KCl and an SEC-05x amplifier (NPI) connected to a Grass S48 stimulator, viewed with a Tektronix TDS3052 digital oscilloscope. Membrane potential and diameter were simultaneously recorded using a custom Labview program. Membrane potential and APS were allowed to stabilize and then pressure was slowly raised from 0.5 cmH₂O to 2 cmH₂O and then 5cmH₂O. In some cases, the electrode dislodged due to the intrinsic contractions of the vessel or wall displacement as pressure was modulated. In these situations, we attempted to re-impale the cell or one of the neighboring cells. Only vessels in which a recording with a minimum of 3 stable APs was successfully made at 2 of the 3 experimental pressures were used for subsequent analysis.

We also confirmed LMC impalement using microelectrode filled with 1 M KCl and (100 $\mu\text{g}/\text{ml}$) Biocytin-AF488 (A12924, ThermoFisher) to label impaled cells that displayed APs, over a 10-minute recording period. Following the impalement and loading with Biocytin-AF488 the vessel was transferred to our imaging apparatus for confocal imaging and 3D reconstruction using the Andor Dragonfly 200 and IMARIS. Image stacks were taken with a 25x water objective at 0.5-micron intervals throughout the diameter of the vessel.

Light Activation of ChR2 to stimulate Popliteal Collecting Lymphatic Vessel Contractions

As the IALV has a nearly continuous contractile cycle, we utilized the popliteal vessel for its much slower contraction frequency in the experiments testing our ability to trigger a propagated contraction upon stimulation of the enforced expression of ChR2. Popliteal vessels were isolated from *cKitCreER^{T2}-ChR2/tdTomato*, *PdgfraCreERTM-ChR2/tdTomato*, or *Myh11CreER^{T2}-ChR2/tdTomato* mice as previously described (Scallan and Davis, 2013 [DOI](#)), although we intentionally retained some connective tissue and adipose tissue to ensure we had a sufficient population of recombined cells to test in the adventitia layer of the vessel. Contractions were allowed to stabilize over a 30-min equilibration period with pressure set to 3 cmH₂O. If basal contraction frequency was too high, we applied pinacidil to the bath in 100 nM increments, without exceeding 600 nM, to further slow contraction frequency to around 6 contractions per minute. Pinacidil at sub 1 μM doses can slow contraction frequency without causing overt hyperpolarization of membrane potential (Davis et al., 2020 [DOI](#)). Supplemental 100 nM doses of pinacidil were applied throughout the experiment to maintain a spontaneous contraction frequency below 6 per minute to allow ample diastolic time for ChR2 stimulation. Throughout this protocol the popliteal was allowed to contract spontaneously to ensure we had not overly inhibited APs by the pacemaking cells with pinacidil. Occasionally, spontaneous contractions occurred just prior to light-evoked contractions, resulting in a potential false positive, so we performed multiple stimulations over a period of 5 - 10 min, typically waiting at least 3 s after any spontaneous contraction before stimulating. Care was made to align the light fiber in such a way that only part of the vessel would be directly illuminated and so target cells of interest would be directly activated by 473 nm light using a Laser diode (Doric LD Fiber Light Source, Quebec, Canada), through an optical probe with a 10- μm tip (Doric, OPT_200_0.22.010). To further limit the excitation field, the optical probe was coated with black acrylic paint using an eyelash brush so that the uncoated opening was $\sim$ 2-3 μm . With the probe positioned within 5 μm of one side of the vessel wall, the spread of light covered an area $\sim$ 10-100 μm wide on the back side of the vessel (depending on the diode amplitude setting). Light pulses, 200 ms in length, were triggered by a Grass S9 stimulator (Harvard Apparatus, Holliston, MA) connected to the external TTL input of the laser diode. Pulse amplitude was adjusted between 40-90 mA using the Laser Diode Module Driver

(Doric). A contraction was considered to be triggered if it occurred within 50ms of stimulation. We performed photo-stimulation from 2-4 sites within each vessel, with 6-14 stimulations per site. If a photo-stimulation was triggered incidentally after the initiation of a “spontaneous contraction” it was discarded from the analysis. For *Myh11CreER^{T2}*-ChR2-tdTomato 6 vessels from 3 separate mice were tested. For *PdgfraCreERTM*-ChR2-tdTomato 6 vessels from 4 separate mice were tested with a max of two vessels per mouse. For *cKitCreER^{T2}*-ChR2-tdTomato 7 vessels from 3 separate mice were assessed. Diameter was recorded to align photo-activation with the contraction cycle in a custom Labview program.

Solutions and Chemicals

Krebs buffer was composed of (in mM): 146.9 NaCl, 4.7 KCl, 2 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄•H₂O, 3 NaHCO₃, 1.5 NaHEPES, and 5 d-glucose (pH = 7.4 at 37°C). Krebs-BSA buffer was prepared with the addition of 0.5% (w/v) bovine serum albumin (BSA) while Krebs Ca²⁺-free replaced CaCl₂ with 3mM EGTA. Tamoxifen was dissolved to 10mg/ml in a Safflower Oil-Ethanol (95%-5% v/v) solution with rocking agitation, separated into aliquots, and stored at -20 °C. Wortmannin was dissolved in DMSO to a stock solution of 1 mM. Pinacidil was dissolved in DMSO to a stock concentration of 1 μM. Nifedipine was dissolved in DMSO to a stock concentration of 1 mM. All chemicals were obtained from Sigma (St. Louis, MO), except for BSA (US Biochemicals; Cleveland, OH), MgSO₄ and NaHEPES (Fisher Scientific; Pittsburgh, PA).

Statistical Tests

Statistical differences in the isobaric contractile tests for popliteal cLVs isolated from *PdgfraCreERTM*-*Ano1^{fl/fl}*, *PdgfraCreERTM*-*Cx45^{fl/fl}*, and *PdgfraCreERTM*-*Cav1.2^{fl/fl}* mice over the various contractile parameters were assessed via 1) repeated measures two-way ANOVAs with Sidak's multiple comparison tests performed using Prism9 (Graphpad). Data are plotted as mean ± SEM and significance determined at p < 0.05 and 0.10 > p > 0.05 were reported. Data from cLVs in which a negative tone value was recorded at any pressure, which typically indicated incomplete passivation or bubbles in the cannula, were not included in the tone analysis. Vessels that failed to contract at a given pressure had no value recorded for ejection fraction or normalized amplitude and REML mixed effects model was used in place of repeated measures 2-way ANOVA. We used a categorical Chi-squared statistical test for the experiments assessing our ability to trigger a contraction with activation of ChR2+ cells. Ca²⁺ particle area and frequency was compared using 1-way ANOVA with Tukey's post-hoc test. Significance was determined at a p value of < 0.05. A mixed effects analysis with Tukey's multiple comparison post-hoc test was used to compare AP parameters across pressure using Prism9 (Graphpad).

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Author contributions

S.D. Zawieja, J.A. Castorena-Gonzalez and M. J. Davis conceived and designed the experiments. S.D. Zawieja, S.E. Broyhill, J.A. Castorena-Gonzalez, H.J. Kim, B. Drumm, M. Li, C.E. Norton, and M.J. Davis performed the studies, collected, and analyzed and interpreted the data. S.D. Zawieja and

M.J. Davis drafted the manuscript and all authors participated in critically revising the manuscript for intellectual concepts and content. All authors have approved the final draft of the manuscript, and their contributions qualify them as authors.

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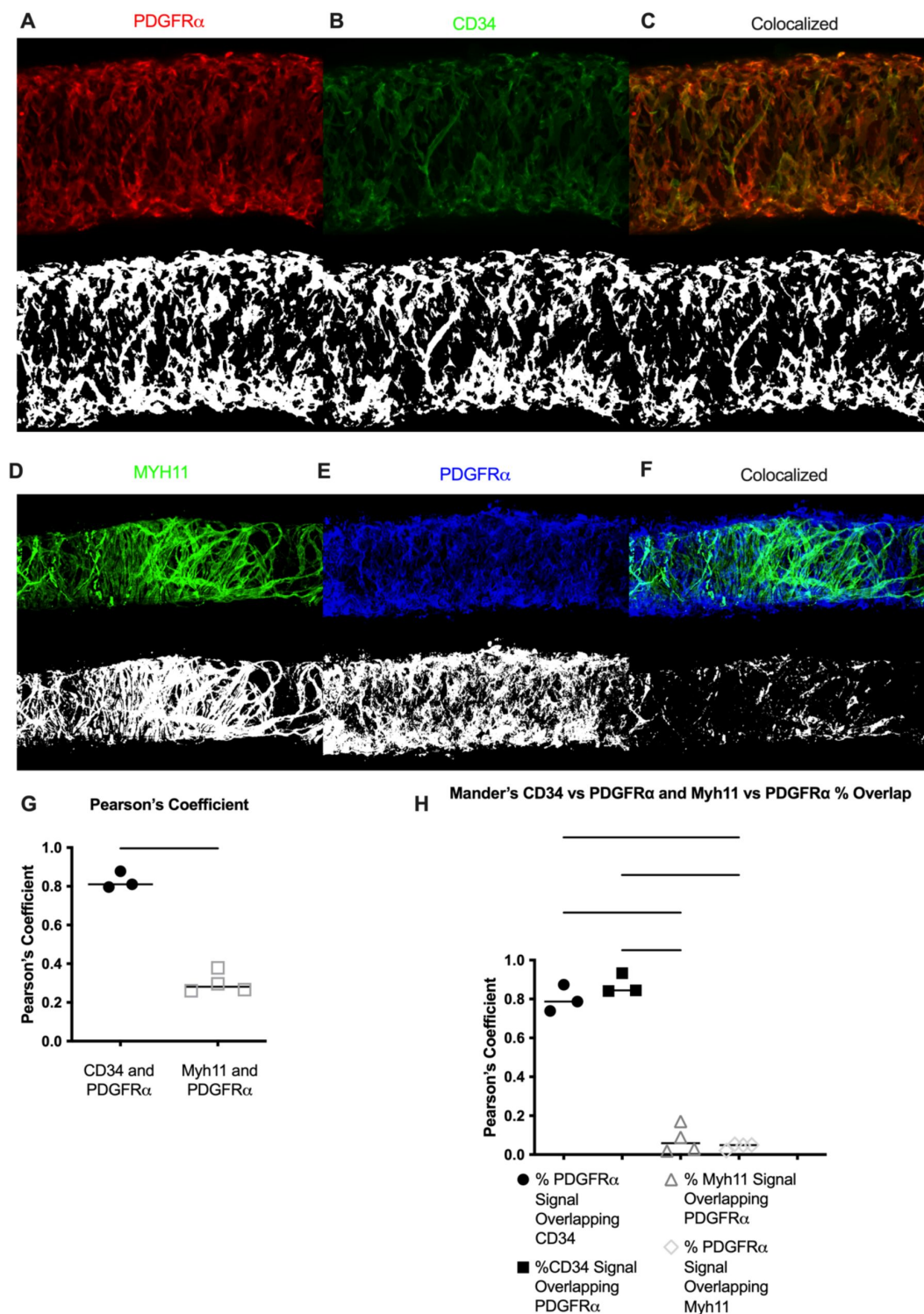
Data availability

Single-cell mRNA sequencing data generated to support this study have been deposited in NCBI GEO under accession number GEO: GSE277843. The authors declare that all other data supporting the findings of this study are available within the paper, its supplementary information files, and the uploaded scRNAseq dataset.

Abbreviations

- Ano1: Anoctamin 1
- ChR2: channel rhodopsin
- cLV: collecting lymphatic vessel
- IALV: Inguinal Axillary Lymphatic Collecting Vessel
- ICC: Interstitial Cell of Cajal
- ICLC: Interstitial Cell of Cajal Like Cell
- LEC: Lymphatic Endothelial Cell
- LMC: Lymphatic Muscle Cell
- STDs: Spontaneous transient depolarizations
- STMs: Spatio-Temporal Maps

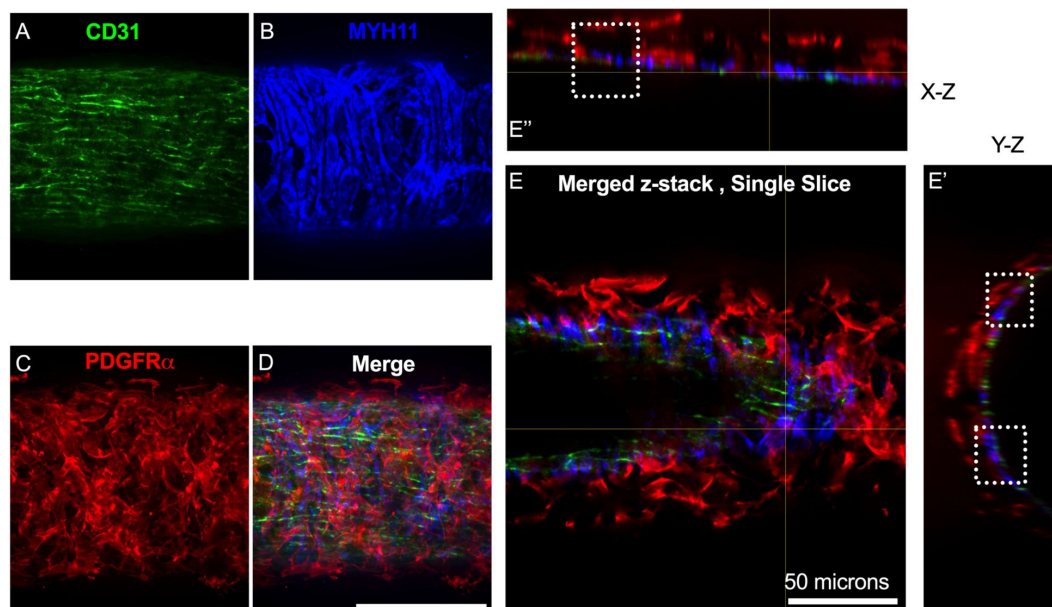
Supplemental Figures



SuppFigure 1

Colocalization of CD34 and PDGFR α

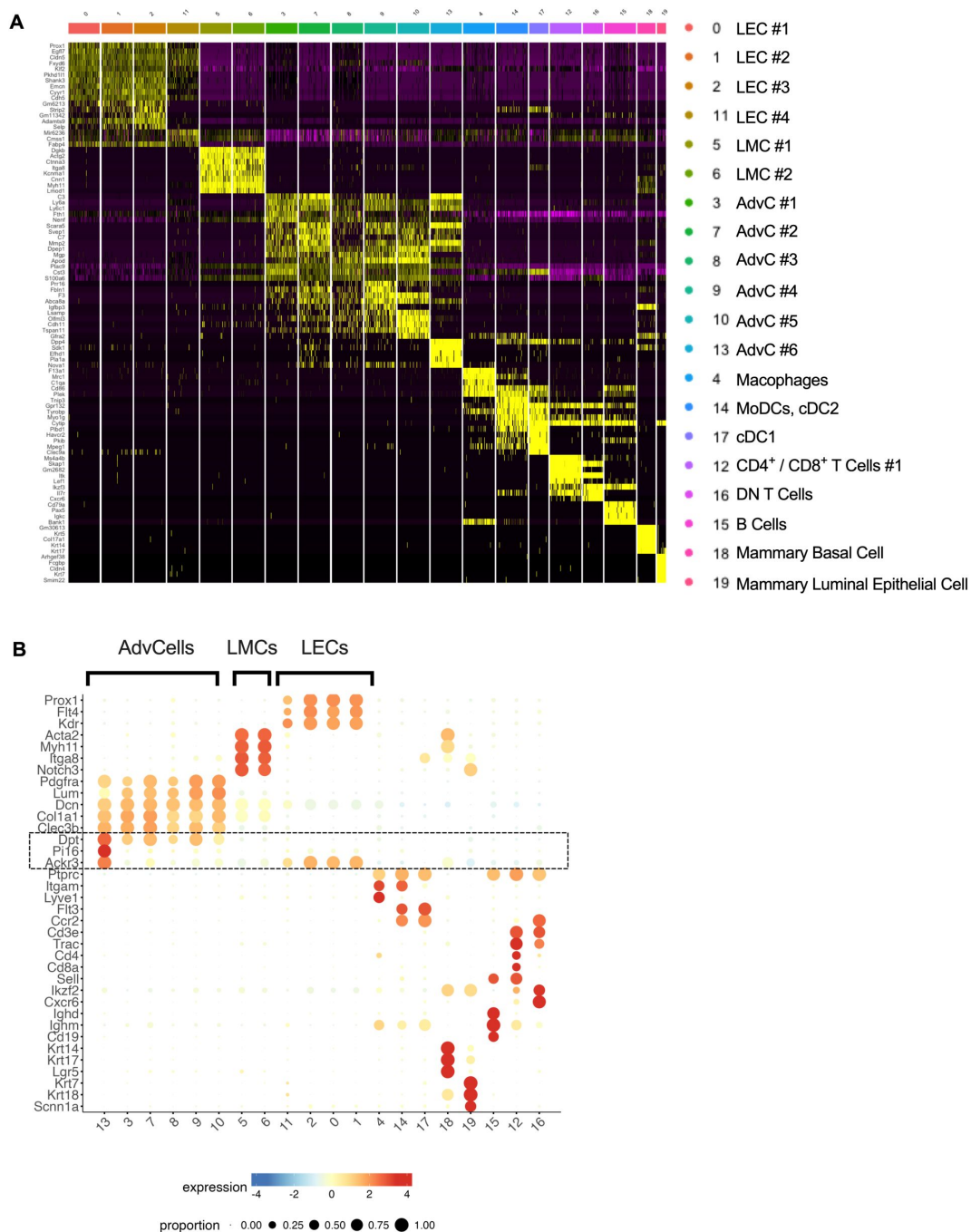
Representative max projections and their corresponding threshold adjusted image for colocalization analysis for PDGFR α (A), CD34 (B), and their colocalized signal (C) and for comparison we tested Myh11 (D) and PDGFR α (E) colocalization (F) using the FIJI BIOP-JACoP colocalization plugin on the z-stacks acquired by confocal microscopy. Pearson's coefficient (G) and Mander's coefficients (H) were calculated from n=3 separate stained IALVS, each from a separate mouse for CD34 and PDGFR α and n=4 for Myh11 and PDGFR α . Magnification for A-C 40X and 25x for D-F. Significant differences in colocalization below 0.05 are signified by the overhead lines.



SuppFigure 2

PDGFR α ⁺ Cells Reside Primarily in the Mouse Lymphatic Collecting Vessel Adventitia and Some in the Subendothelial Space

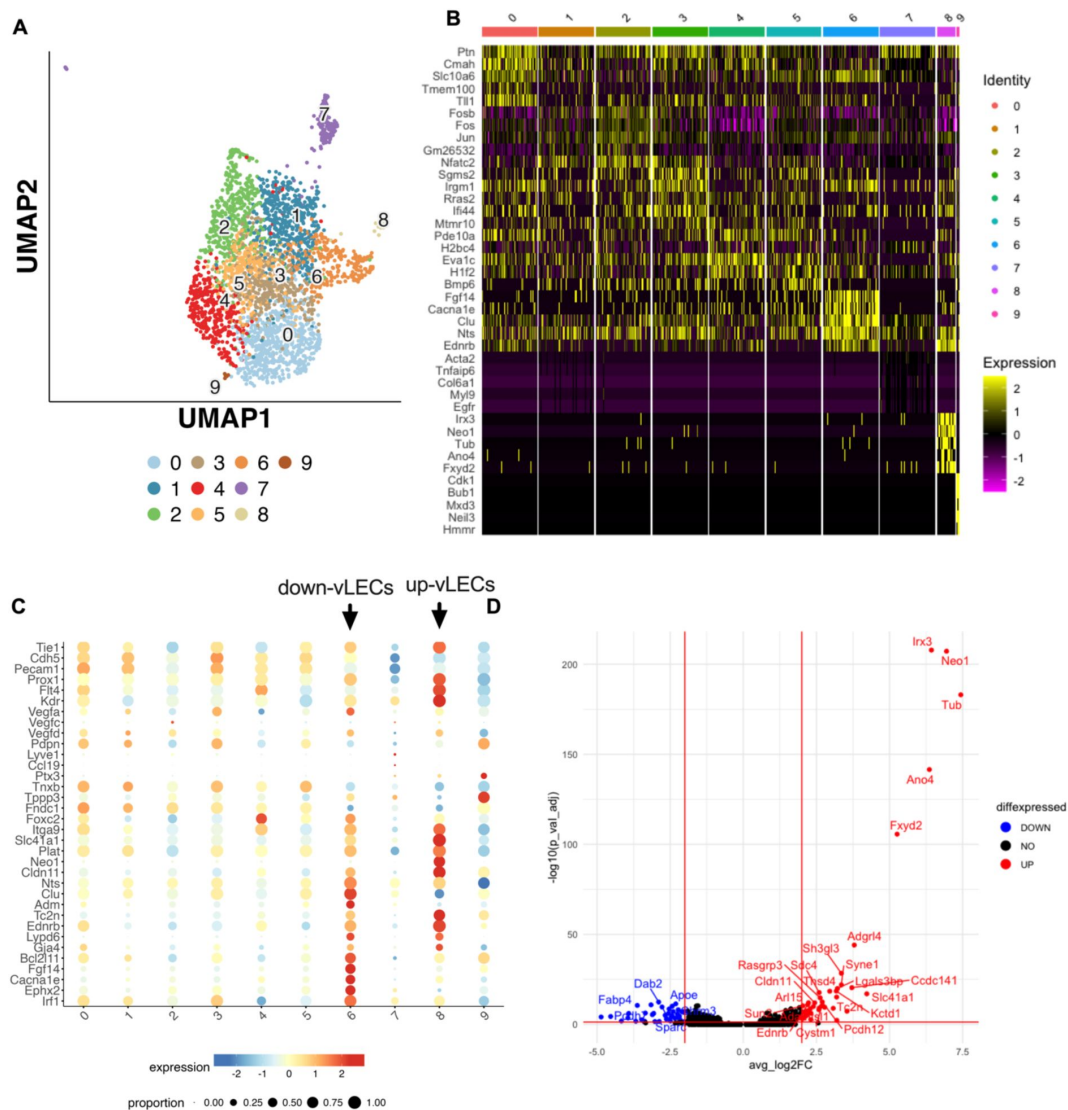
Max projection of confocal imaging of an IALV stained for LECs with CD31 (A), LMCs with MYH11(B), and for PDGFR α (C) with the corresponding merge file (D). Orthogonal views of the z-stack with (E) showing a single slice in the z stack and E' and E'' the orthogonal views. White dotted boxes outline locations where PDGFR α signal is observed between LMC and LEC layers. Scale bar is 100 μ m in (D) and 50 μ m in (E).



SuppFigure 3.

scRNASeq Analysis of the mouse IALV cell populations.

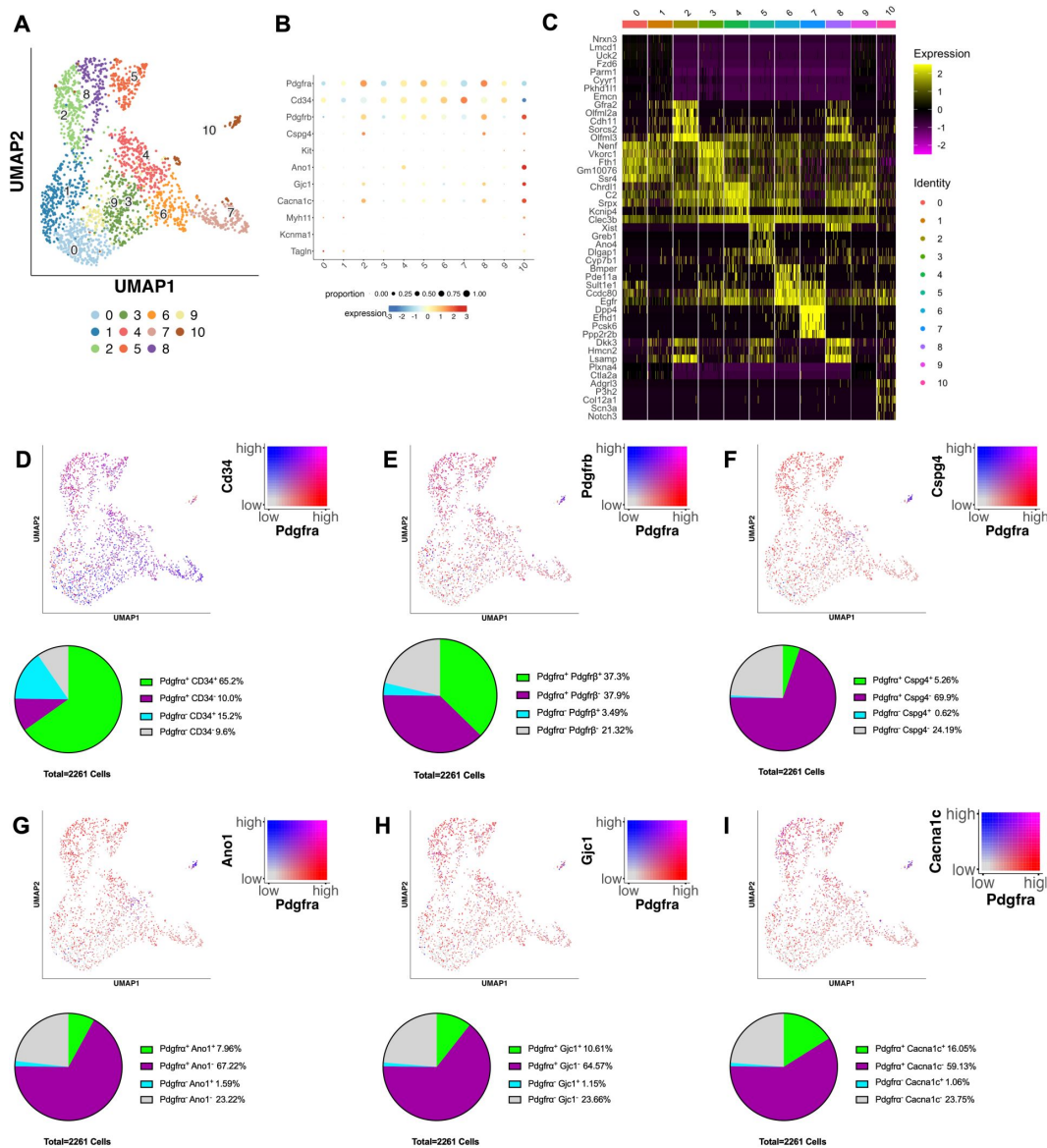
Heatmap of top 4-5 differentially expressed genes, based on p value, for each major cell cluster identified. LECs (Clusters 0,1,2, 11), LMCs (Cluster 5,6), and IALV adventitial cells (AdvC, 3,7,8,9,10,13) were comprised of multiple clusters. B) Bubble plot of common identification genes reveal that the previous reported LMC transcriptome markers *Dpt*, *Pi16*, and *Ackr3* are specific for a sub population of the Adv and not LMCs.



SuppFigure 4.

Subclusters of IALV LECs revealed by scRNAseq.

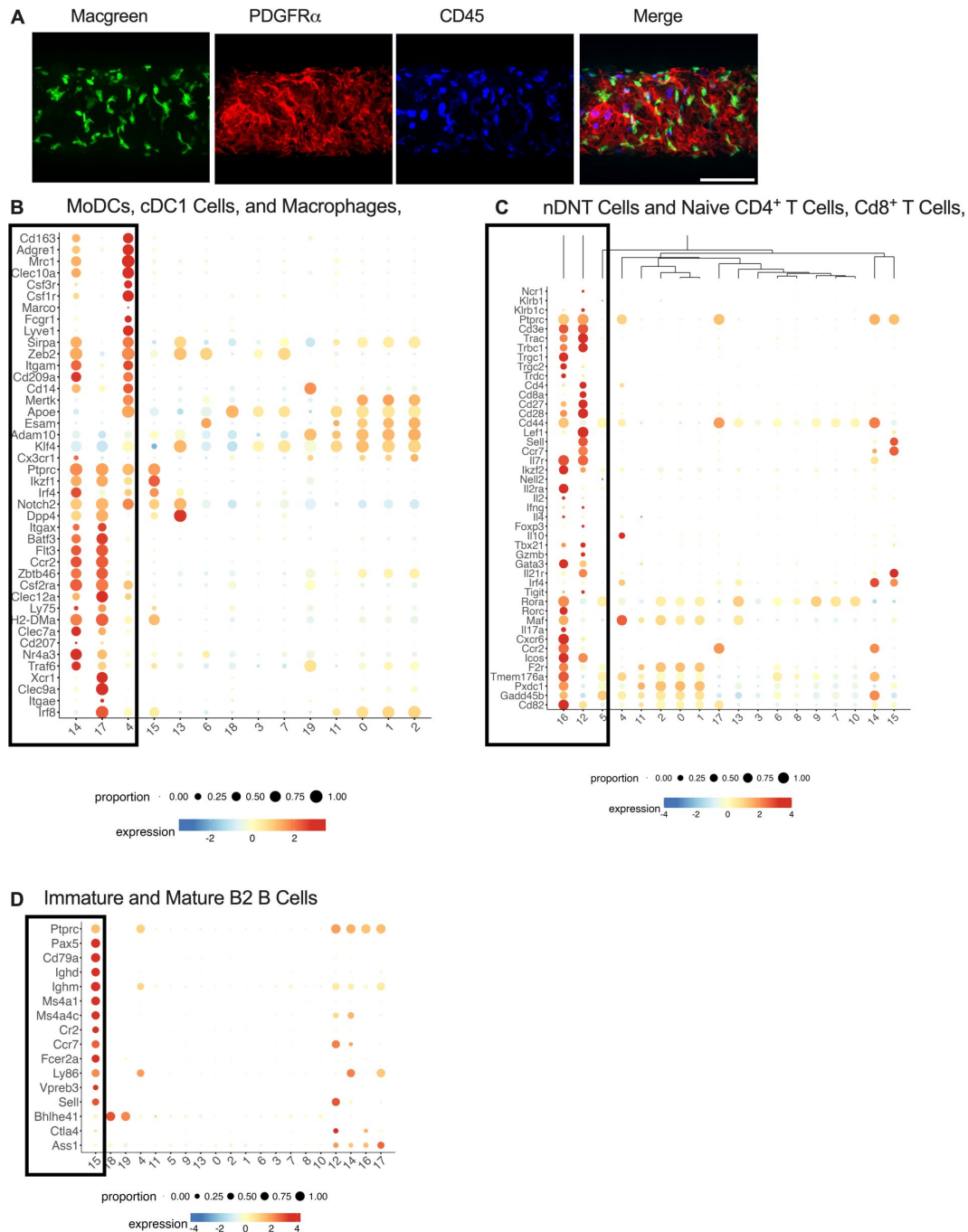
The LECs were further sub-clustered to reveal 10 putative LEC subclusters (0-9) as shown in the UMAP (A) and the top differentially expressed genes amongst those sub-clusters are provided in the adjacent heatmap (B). (C) Bubble plot showing sub-cluster 8 was significantly enriched for previously documented lymphatic endothelial cell up valve genes including *Itga9*, *Cldn11*, and *Neo1* and Cluster 6 had down valve gene signature including *Clu* and *Adm*. The top 30 differentially expressed genes in cluster 8, both positive and negative fold change regulated, are labeled in the volcano plot(D).



SuppFigure 6.

Subclusters of IALV AdvCs revealed by scRNAseq.

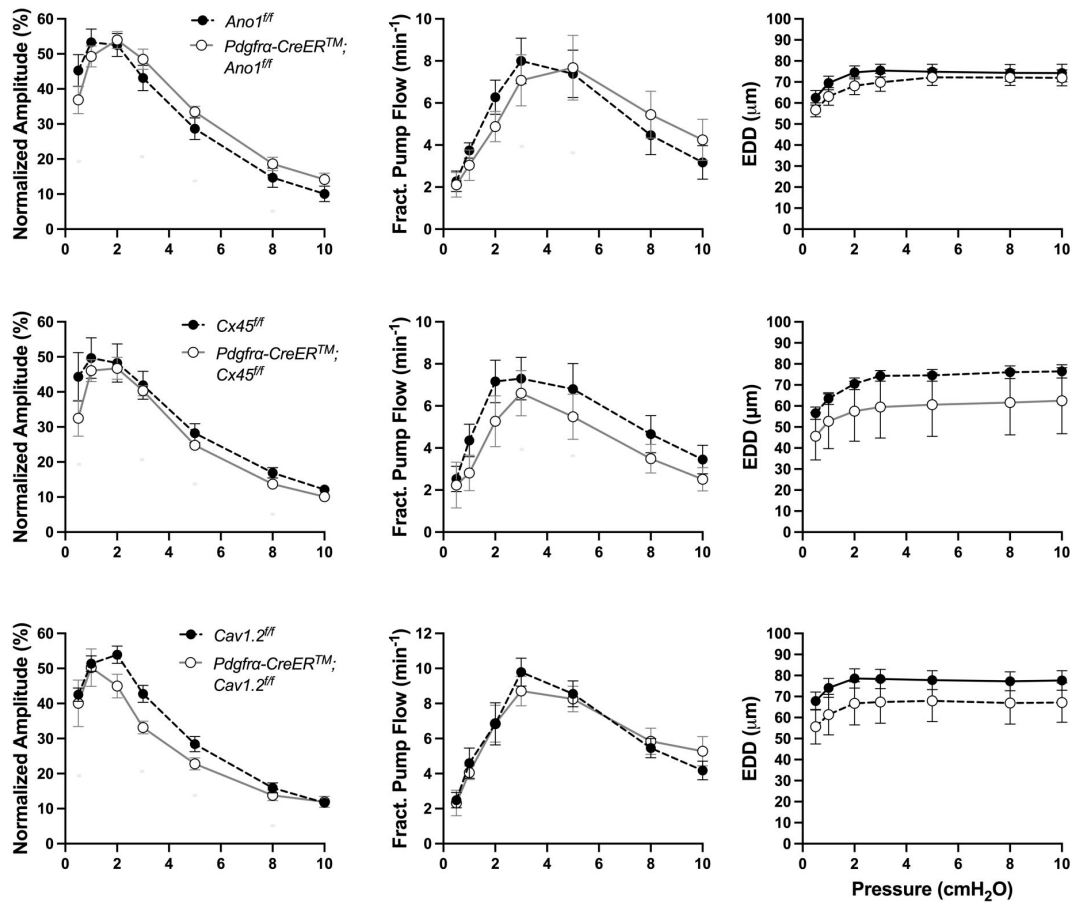
AdvCs also could be further subclustered into multiple populations as shown in the UMAP (A). Bubble plot of genes used as Cre drivers and genes associated with pacemaking revealed subcluster 10 had expression of *Ano1*, *Cx45*, and *Cacna1c* (*CaV1.2*) but with only minimal evidence of LMC contamination as indicated by muscle signature genes *Myh11*, *Kcnma1*, and *Tagln*. C) Heatmap of the top differentially expressed genes among each of the subclusters. We assessed co-expression of *Pdgfra* with CD34 (D) to confirm our immunofluorescence imaging (Sugg Figure 1 [1](#)), and assessed the co-expression of *Pdgfra* with the pericyte markers *Pdgfrb* (E) and *Cspg4* (F). We further assessed co-expression of *Pdgfra* the genes linked with contractile dysfunction *Ano1* (G), *Gjc1* (H), and *Cacna1c* (I) to ensure *Pdgfra*^{CreER} would target the AdvCs expressing these genes. The cyan colored slice of the pie chart indicates the minor population of cells expressing these genes that did not express *Pdgfra*.



SuppFigure 7.

Immune cell populations associated with the mouse IALV.

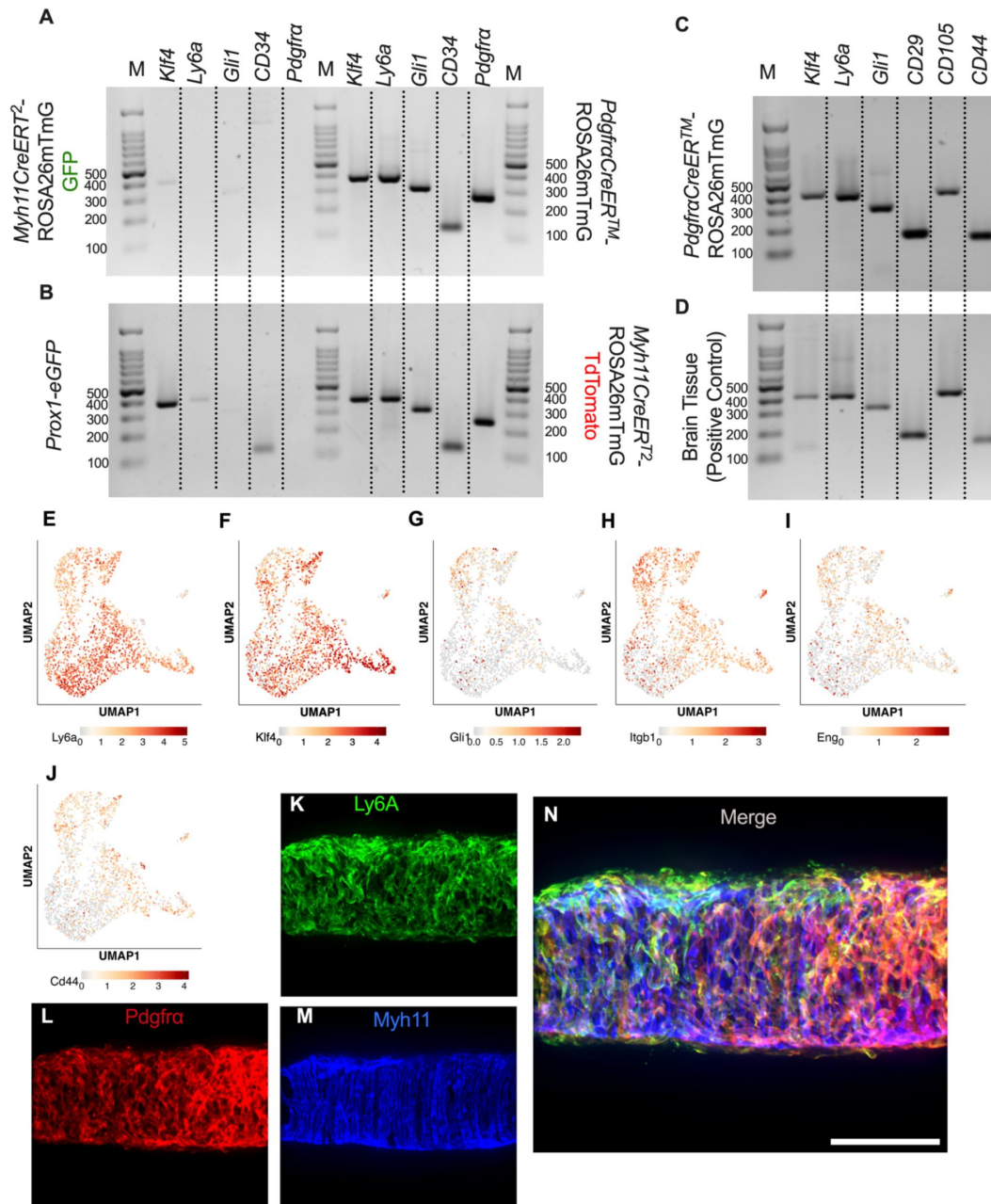
Lymphatic vessels are host to numerous immune cell populations, including monocyte, macrophage, and dendritic cell populations are revealed by immunofluorescent staining for eGFP in the “Macgreen” (Csf1r-eGFP) reporter mice (A). Staining for *Pdgfra* (B) demonstrates that AdvCs are distinct from the GFP⁺ cells nor do they stain for the hematopoietic marker *Ptprc* (CD45) (C, D). Bubble plot of our scRNASeq analysis of IALVs revealed macrophages (cluster 4), moDCs (cluster14) and cDC1 cells (17) based off identifying gene markers (B). C) Bubble plot of T-cell markers revealed multiple populations of T cells including naive double negative T-cells (Yang et al., 2021 [\[1\]](#)) and naive CD4⁺ and CD8⁺ T-cells. A bubble plot for B-cell markers showed that cluster 15 had an expression profile for immature and mature B2 B-cells (D)(Luo et al., 2022b [\[2\]](#)).



SuppFigure 8

Contractile indices from isobaric myography on cLVs from *PdgfraCreERTM* driven deletion of *Ano1*, *CX45*, and *Cav1.2*

Summary of the contractile parameters recorded from popliteal cLVs in *PdgfraCreERTM-Ano1^{fl/fl}*, *PdgfraCreERTM-Cx45^{fl/fl}* mice, *PdgfraCreERTM-Cav1.2^{fl/fl}* mice. No differences in normalized contraction amplitude (A, D, G), fractional pump flow (B, E, H), or end diastolic diameter (C, F, I) were observed. The contractile data from control *Cav1.2^{fl/fl}* vessels was previously published but was separated by sex (Davis et al., 2022) while they are combined here.



SuppFigure 9

PDGFRα AdvCs Include Multipotent Cell

Representative RT-PCR results profiling purified GFP⁺ cells purified from IALVs isolated from *PdgfraCreERTTM-ROSA26mTmG* via FACS. PDGFRα cells expressed the multipotent markers Klf4, Sca1, Gli1, CD29, CD105, and CD44 (A) with total brain cDNA serving as a positive control (B). Representative RT-PCR results showing lack of expression of some of these markers in the GFP⁺ cells purified from *Myh11CreERT²-ROSA26mTmG* (C) or *Prox1-eGFP* mice, in contrast to the RFP⁺ population from *Myh11CreERT²-ROSA26mTmG* mice (D). RT-PCRs were repeated at least 2 times from separate purified cells populations from different mice. Dot plots of only the AdvCs cluster highlights populations of cells that express genes associated with multipotency such as Ly6a (E), Klf4 (F), Gli1 (G), Itgb1 (H, CD29), Eng (I, CD105), CD44 (J). Expression of protein for Ly6a was confirmed with immunofluorescence. Representative max projections of IALVs stained for Sca1 (K), PDGFRα (L), Myh11 (M) and the corresponding merged file (N). Scale bar is 100 μm.

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Reviewer #1 (Public review):

Summary:

This manuscript explores the multiple cell types present in the wall of murine collecting lymphatic vessels with the goal of identifying cells that initiate the autonomous action potentials and contractions needed to drive lymphatic pumping. Through the use of genetic models to delete individual genes or detect cytosolic calcium in specific cell types, the authors convincingly determine that lymphatic muscle cells are the origin of the action potential that triggers lymphatic contraction.

Strengths:

The experiments are rigorously performed, the data justify the conclusions and the limitations of the study are appropriately discussed.

There is a need to identify therapeutic targets to improve lymphatic contraction and this work helps identify lymphatic muscle cells as potential cellular targets for intervention.

<https://doi.org/10.7554/eLife.90679.2.sa3>

Reviewer #2 (Public review):

Summary:

This is a well written manuscript describing studies directed at identifying the cell type responsible for pacemaking in murine collecting lymphatics. Using state-of-the-art approaches, the authors identified a number of different cell types in the wall of these lymphatics and then using targeted expression of Channel Rhodopsin and GCaMP, the authors convincingly demonstrate that only activation of lymphatic muscle cells produces coordinated lymphatic contraction and that only lymphatic muscle cells display pressure-dependent Ca^{2+} transients as would be expected of a pacemaker in these lymphatics.

Strengths:

The use of targeted expression of channel rhodopsin and GCaMP to test the hypothesis that lymphatic muscle cells serve as the pacemakers in musing lymphatic collecting vessels.

Weaknesses:

The only significant weakness was the lack of quantitative analysis of most of the imaging data shown in Figures 1-11. In particular, the colocalization analysis should be extended to show cells not expected to demonstrate colocalization as a negative control for the colocalization analysis that the authors present. These weaknesses have been resolved by

revision and addition of new and novel RNAseq data, additional colocalization data and membrane potential measurements.

<https://doi.org/10.7554/eLife.90679.2.sa2>

Reviewer #3 (Public review):

Summary:

Zawieja et al. aimed to identify the pacemaker cells in the lymphatic collecting vessels. Authors have used various Cre-based expression systems and optogenetic tools to identify these cells. Their findings suggest these cells are lymphatic muscle cells that drive the pacemaker activity in the lymphatic collecting vessels.

Strengths:

The authors have used multiple approaches to test their hypothesis. Some findings are presented as qualitative images, while some quantitative measurements are provided.

Weaknesses:

- More quantitative measurements.
- Possible mechanisms associated with the pacemaker activity.
- Membrane potential measurements.

Comments on revisions:

The authors have answered my comments with additional experiments, data and manuscript edits.

<https://doi.org/10.7554/eLife.90679.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

This manuscript explores the multiple cell types present in the wall of murine-collecting lymphatic vessels with the goal of identifying cells that initiate the autonomous action potentials and contractions needed to drive lymphatic pumping. Through the use of genetic models to delete individual genes or detect cytosolic calcium in specific cell types, the authors convincingly determine that lymphatic muscle cells are the origin of the action potential that triggers lymphatic contraction.

Strengths:

The experiments are rigorously performed, the data justify the conclusions, and the limitations of the study are appropriately discussed.

There is a need to identify therapeutic targets to improve lymphatic contraction and this work helps identify lymphatic muscle cells as potential cellular targets for intervention.

Weaknesses:

My only major comment would be that the manuscript provides a lot of rich information describing the cellular components of the muscular lymphatic vessel wall and that these data are not well represented by the title. The title (while currently accurate) could be tweaked to better represent all that is in this manuscript. Maybe something like

"Characterization/Interrogation of the cellular components of murine collecting lymphatic vessels reveals that lymphatic muscle cells are the innate pacemaker cells regulating lymphatic contractions" or "Discovery/Confirmation of lymphatic muscle cells as innate pacemaker cells of lymphatic contraction through characterization of the cellular components of murine collecting lymphatic vessels". Potentially a cartoon summary figure of the components that make up the collecting lymphatic vessel wall could also be included. In my opinion, these changes will make this manuscript of more interest to a broader group of scientists. I have a few additional comments for consideration to improve the clarity and enhance the discussion of this work.

We agree with the reviewer that our original manuscript, and our resubmission even more so with the addition of the scRNAseq data, provides a significant amount of information regarding the composition of the lymphatic collecting vessel wall. We have changed our title to match one suggestion of the reviewer: "Characterization of the cellular components of murine collecting lymphatic vessels reveals that lymphatic muscle cells are the innate pacemaker cells regulating lymphatic contractions".

Reviewer #2 (Public Review):

Summary:

This is a well-written manuscript describing studies directed at identifying the cell type responsible for pacemaking in murine-collecting lymphatics. Using state-of-the-art approaches, the authors identified a number of different cell types in the wall of these lymphatics and then using targeted expression of Channel Rhodopsin and GCaMP, the authors convincingly demonstrate that only activation of lymphatic muscle cells produces coordinated lymphatic contraction and that only lymphatic muscle cells display pressure-dependent Ca^{2+} transients as would be expected of a pacemaker in these lymphatics.

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The use of a targeted expression of channel rhodopsin and GCaMP to test the hypothesis that lymphatic muscle cells serve as the pacemakers in musing lymphatic collecting vessels.

Weaknesses:

The only significant weakness was the lack of quantitative analysis of most of the imaging data shown in Figures 1-11. In particular, the colocalization analysis should be extended to show cells not expected to demonstrate colocalization as a negative control for the colocalization analysis that the authors present.

We understand the reviewer's concern regarding the lack of a control for the colocalization analysis and that the colocalization analysis was limited to just one set of cell markers. We have now provided a colocalization analysis of Myh11 and PDGFR α , to serve as a co-localization negative control based on our RT-PCR and scRNASeq findings, which is incorporated into the current Supplemental figure 1. In regard to the staining pattern of other various marker combinations, the results were often quite clear with the

representative images that two separate cell populations were being stained such as the case with labeling endothelial cells with CD31, macrophage labeling with the MacGreen mice, or hematopoietic cells with CD45.

During our lengthy rebuttal process we completed a single cell RNA sequence analysis using our isolated and cleaned mouse inguinal axillary lymphatic collecting vessels to aid in our characterization of the vessel wall and to more thoroughly answer these questions regarding colocalization in arguably a robust manner. The generation of our scRNAseq dataset, derived from isolated and cleaned mouse inguinal axillary collecting vessels from 10 mice, 5 male and 5 females, allowed us to profile over 2200 of the adventitial fibroblast like cells (AdvCs) we had identified in our original submission. Using this dataset, we were able to confirm co-expression of Cd34 and Pdgfra in AdvCs and assess the co-expression of other genes of interest from our RT-PCR experiments and immunofluorescence experiments. This approach will also allow other lymphatic investigators to assess their genes of interest as our dataset is uploaded to the NIH Gene Omnibus and will be uploaded to the Broad Institute Single Cell Portal upon publication.

Here we show that the vast majority of non-muscle fibroblast like cells referred to as AdvCs were double positive for both CD34 and PDGFR α . We also show that the AdvCs that express commonly used pericyte markers Pdgfrb and Cspg4 also co-expressed Pdgfra. Critically, this data also shows that the AdvCs that express genes linked with lymphatic contractile dysfunction (Ano1, Gjc1 or connexin 45, and Cacna1c “Cav1.2”) co-express Pdgfra and would render these genes susceptible to Cre-mediated recombination using our Pdgfra-CreERTM model.

Reviewer #3 (Public Review):

Summary:

Zawieja et al. aimed to identify the pacemaker cells in the lymphatic collecting vessels. Authors have used various Cre-based expression systems and optogenetic tools to identify these cells. Their findings suggest these cells are lymphatic muscle cells that drive the pacemaker activity in the lymphatic collecting vessels.

Strengths:

The authors have used multiple approaches to test their hypothesis. Some findings are presented as qualitative images, while some quantitative measurements are provided.

Weaknesses:

- More quantitative measurements.*
- Possible mechanisms associated with the pacemaker activity.*
- Membrane potential measurements.*

We thank the reviewers for their concerns and have addressed them in the following manner.

- We added novel single cell RNA sequencing of isolated and cleaned inguinal axillary vessels from 10 mice (5 males and 5 females). This allowed us to quantify the number of AdvCs that coexpress CD34 and Pdgfra as well as the number of cells co-expressing Pdgfra and other markers.
- We have added a negative control with quantification for the co-localization analysis assessing Myh11 and Pdgfra. We have added a negative control with quantification for the

Chr2-photo stimulated contraction experiments using Myh11CreERT2-ChR2 mice that were not injected with tamoxifen.

- We also used Biocytin-AF488 in our intracellular Vm electrodes to map the specific cells in which we recorded action potentials and in neighboring cells since Biocytin-AF488 is under 1KDa and can pass through gap junctions. This approach independently labeled lymphatic muscle cells and their direct neighbors for 3 IALVs from 3 separate mice.

- We performed membrane potential recordings in isolated, pressurized (under isobaric conditions), and spontaneously contracting inguinal axillary lymphatic collecting vessels at different pressures.

- We also show that the pressure-frequency relationship is dependent on the slope of the diastolic depolarization as no other parameter was significantly altered in our study and the diastolic depolarization slope was highly correlated with contraction frequency.

We believe the addition of these novel data, controls, experiments, and quantifications have improved the manuscript in line with the reviewers' suggestions.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Lines 149-162: The authors rule out the methylene blue staining cells in the cLV wall as pacemakers because they don't form continuous longitudinal connections to drive propagation. Is it possible for a pacemaker cell to only initiate the contraction and then have the LMCs make the axial electrical connections to propagate the electrical wave? I am not trying to suggest the methylene blue cells are pacemakers, but I am not sure the lack of longitudinal (or radial) connectivity is sufficient evidence to rule out the possibility. This comment also is relevant to the 3 criteria for a pacemaker cell listed in the Discussion (Lines 413-417).

We agree with the reviewer's broader point that a pacemaker cell may not require direct contact with other 'pacemaker' cells within the tissue as long as they are still within the same electrical syncytium. However, we do expect a continuous presence of a pacemaker cell type throughout the vessel wall length to account for the persistence of spontaneous contractile behavior despite vessel length, and the ability for contraction initiation to shift (Akl et al 2011, Castorena et al 2018 and Castorena et al 2022) and the occurrence of spontaneous action potentials. In Dirk van Helden's seminal work in 1993 on lymphatic pacemaking, a major finding was that "SM of small lymphangions or that of short segments, cut from lymphangions of any length, behaved similarly". We have adjusted our phrase regarding the requirement of a contiguous network and instead suggest a continuous presence along the vessel network and integrated into the electrical syncytium.

Methylene blue is an alkaline stain that will stain acidic structures and historically methylene blue is noted to stain Interstitial cells of Cajal in the gastrointestinal tract which typically exist as network of cells (Huizinga et al 1993 and Berezin 1988). No such network was readily apparent in our methylene blue staining nor did the stained cells have a similar morphology to the ICCs of the gastrointestinal tract. Further, methylene blue staining is not limited to ICCs or pacemaker cells at large as it has been used to kill cancer cells. Within the small intestine methylene blue was noted to also stain macrophage like cells (Mikkelsen et al 1988), and we too draw parallels between the macrophage morphology observed with Macgreen mice and methylene-blue stained cells. The specific structure for the ICC affinity for methylene blue is not well described and while the innate cytotoxicity of methylene blue and light has been used to kill ICCs and impair slow wave generation, the lack of specificity of this

method leaves much to be desired. What is clear is that the ICC network highlighted by methylene blue in the gut is absent in lymphatic collecting vessels.

In Figure 15/Video12, is it possible that the cells that are showing intracellular Ca^{2+} in diastole are the cells that reach a threshold membrane potential that then trigger the rest of the LMCs? As the authors have shown heterogeneity in the LMCs surface markers, is it possible that the cells with Ca^{2+} activity during diastole are identifiable by a distinct molecular phenotype? Or is the thought that these cells are randomly active in diastole? Some discussion/speculation about this seems appropriate.

We are in agreement with the reviewer's conclusion that there is heterogeneity in the LMCs as it pertains to the calcium oscillations in diastole, either under normal buffer conditions or when L-type channels are inhibited with nifedipine. We also note significant heterogeneity in the gene expression noted within the four LMC subclusters (0-3), though we did not see significant differences in either in *Ip3R1* or *Ano1* expression. However, subcluster "0" had increased expression of *Itprid2*, also known as Kras-induced actin-interacting protein (KRAP) which is thought to tether, and thus immobilize, IP3 receptors to the actin cortex beneath the cell membrane. KRAP has been recently proposed to be a critical player in IP3 receptor "licensing" which allows IP3 receptors to release calcium (Vorontsova et al., 2022). However, whether similar requirement of IP3R licensing is necessitated in all cells or specifically in LMCs is unknown it is quite clear there are specific release sites within the cell and this topic is currently under further investigation for a separate manuscript. We would like to note that there is yet to be a clear consensus on whether IP3R licensing is required as much of these studies are performed in cultured cells and this mechanism has only recently been described.

Healthy lymphatic collecting vessels typically have a single pacemaker driving a coordinated propagated contraction in ex vivo isobaric myograph studies (Castorena-Gonzalez et al., 2018), which is typically at either end of the cannulated vessel. We believe that this is due to the lack of a bordering cell in one direction and allows charge to accumulate and voltage to reach threshold at these sites preferentially. We have tried to image calcium at the pacemaking pole of the vessel to observe the specific Ca^{2+} transients at these sites though invariably the act of imaging GCaMP6f results in the pacemaker activity initiating from the other pole of the vessel. It is our opinion that the fact that LMCs are heterogenous in their Ca^{2+} transients is a feature to the system as it permits a wider range of depolarization signals, and thus allows finer control of the pacing as different physical/pressure or signaling stimuli is encountered. Likely, the cells with the higher propensity of Ca^{2+} transients act as the contraction initiation site *in vivo*, though it must also be noted that the LMC density decreases around lymphatic valve sites. In fact, in guinea pig collecting vessels there are very few LMCs at the valves which can render them electrically uncoupled or poorly coupled (Van Helden, 1993). Thus, valve sites in which there is greater electrical resistance due to lower LMC-LMC coupling may allow for charge accumulation in the LMCs at the valve site, similar to the artificial condition achieved in our myograph preparations with two cut ends, and allow them to reach threshold first and drive coordination at the valve sites.

An additional description of what the PTCL analysis is meant to represent physiologically would be helpful for readers.

We have better described the conversion of the calcium signals into "particles" for analysis at first mention in the methods and results section and have included the requisite reference to this specific methodology in Line 429-30.

A description of how DMAX is experimentally determined is needed.

We have adjusted our methods section to describe DMAX in line 774-775.

“with Ca^{2+} -free Krebs buffer (3mM EGTA) and diameter at each pressure recorded under passive conditions (DMAX).”

I think the vessels referred to as popliteal lymphatic vessels are actually saphenous lymphatic vessels (afferent to the popliteal lymph node). Please clarify.

Indeed, some of the vessels used in this study are the afferents to the single popliteal node. They travel with the caudal branch of the saphenous vein, but have routinely been described as popliteal vessels, as opposed to saphenous lymphatic vessels, by the lymphatic field at large (Tilney 1971 PMID: PMC1270981, Liao 2015 PMID: 25512945). To move away from this nomenclature would likely add to confusion although we agree that the lymphatic field may need to improve or correct the vessel naming paradigm to match the vascular pairs they follow.

Reviewer #2 (Recommendations For The Authors):

Lines 214-215 - can you cite a reference for the observation that rhythmic contractions don't require the presence of valves?

We have added the reference. In Dr. Van Helden’s seminal work on the topic in 1993, “Vessel segments were then cut from selected small lymphangions (length 300-500 μm) by cutting at the valves.” Additionally, work by Dr Anatoliy Gashev utilized sections of lymphatic vessels that lacked valves to study orthograde and retrograde shear sensitivity (Gashev et al., 2002).

Lines 224-230 - It would have been nice to see colocalization analysis for all cell types so that "negative" results could be compared with the "positives" that you report. This would help bolster evidence of your ability to separate cell types.

We understand the reviewer’s sentiment and agree. We have now added a “negative control” colocalization staining and analysis for PDGFR α and Myh11 which has been added to the current SuppFigure 1. We stained 3 IALVs from 3 separate mice with PDGFR α and Myh11 and performed confocal microscopy. We ran the FIJI BIOP-JACOP colocalization plugin as before and observed very little colocalization of the two signals. Additionally, we have also added a coexpression assessment for CD34 and PDGFR α and other genes using our scRNAseq dataset.

line 293 - Should read "Cx45 in..."

This has been corrected.

“The expression of the genes critically involved in cLV function—Cav1.2, Ano1, and Cx45—in the *PdgfraCreERTM-ROSA26mTmG* purified cells and scRNAseq data prompted us to generate *PdgfraCreERTM-Ano1^{fl/fl}*, *PdgfraCreERTM-Cx45^{fl/fl}*, and *PdgfraCreERTM-Cav1.2^{fl/fl}* mice for contractile tests.”

lines 470-473 - A reference for this statement should be cited.

We have added the reference. In Dr. Van Helden’s seminal work on the topic in 1993, “Vessel segments were then cut from selected small lymphangions (length 300-500 μm) by cutting at the valves.” Additionally, work by Dr Anatoliy Gashev utilized sections of lymphatic vessels that lacked valves to study orthograde and retrograde shear sensitivity (Gashev et al., 2002).

Lines 483-487 - References should be cited for these statements.

We have narrowed and clarified this statement and supported it with the necessary citations.

“Of course, mesenchymal stromal cells (Andrzejewska et al., 2019) and fibroblasts (Muhl et al., 2020; Buechler et al., 2021; Forte et al., 2022) are present, and it remains controversial to what extent telocytes are distinct from or are components/subtypes of either cell type (Clayton et al., 2022). Telocytes are not monolithic in their expression patterns, displaying both organ directed transcriptional patterns as well as intra-organ heterogeneity (Lendahl et al., 2022) as readily demonstrated by recent single cell RNA sequencing studies that provided immense detail about the subtypes and activation spectrum within these cells and their plasticity (Luo et al., 2022).”

| *Lines 584-585 - Missing a reference citation.*

Thank you for catching this error, the correct citation was for Boedtkjer et al 2013 and is now properly cited.

| *Line 638 - "these this" should read "this"*

Thank you for catching this error. This particular sentence was removed in light of the addition of the scRNAseq data.

Reviewer #3 (Recommendations For The Authors):

This manuscript from Zawieja et al. explored an interesting hypothesis about the pacemaker cells in lymphatic collecting vessels. Many aspects of lymphatic collecting vessels are still under investigation; hence this work provides timely knowledge about the lymphatic muscle cells as a pacemaker. Although it is an important topic of the investigation, the data provided do not support the overall goal of the manuscript. Many figures (Figure 1-5) provide quantitative estimation and the description provided in the results section might only be useful for a restricted audience, but not to the broader audience. Some of the figures are very condensed with multiple imaging panels and it is hard to follow the differences in qualitative analysis. Overall, this manuscript can be improved by more streamlined description/writing and figure arrangements (some of the figures/panels can be moved to the supplementary figures).

We disagree with the notion that the original data provided did not support the goal of the manuscript- to identify and test putative pacemaker cell types. Nonetheless we believe we have also added ample novel data to the manuscript, including membrane potential recordings and scRNAseq to highlight and to add further support to our conclusion that the pacemaker cell is an LMC. We believe the scRNAseq data will also greatly enhance the appeal of the manuscript to a broader audience and have renamed the manuscript in line with the wealth of data we have collected on the components of the vessel wall as we tested for putative pacemaker cells.

As requested, we have moved many figures to the supplement to allow readers to focus more on the more critical experiments.

A few other points that need to be addressed:

(1) Authors used immunofluorescence-based differences in various cell types in the collecting vessels. Initially, they chose ICLC, pericytes, and lymphatic muscle cells. But then they started following adventitial cells and endothelial cells. It is not clear from the description, why these other cells could be possibly involved in the pacemaker activity. It will be easier to follow if authors provide a graphical abstract or summary figure about their hypothesis and what is known from their and others' work.

We would like to clarify that we used the endothelial cells as controls to ensure what we observed via immunofluorescence and FACs RT-PCR were a separate cell type from either lymphatic muscle or lymphatic endothelial cells on the vessel wall. Staining for the endothelium also allowed us to assess where these PDGFR α +CD34+ cells reside in the vessel wall. We started with a wide range of markers that are conventionally used for targeting specific cell types, but as expected those markers are not always 100% specific. Specifically, we focused on CD34, Kit, and Vimentin as those were the markers for the non-muscle cells observed in the lymphatic collecting vessel wall previously. What we found was that CD34 and PDGFR α labeled the same cell type. As there was not a CD34Cre mouse available at the time we instead utilized the inducible PDGFR α CreERTM. We are unsure how well an abstract figure will condense the conclusions from the experiments listed here but if absolutely required for publication we can attempt to highlight the representative cell populations identified on the vessel wall.

(2) Authors used many acronyms in the manuscript without defining them (when they appeared for the first time). Please follow the convention.

We have checked the manuscript and made several corrections regarding the use of abbreviations.

(3) How specific PDGFR-alpha as a marker of the pericytes? It can also label the mesenchymal cells. Why did the author choose PDGFR-alpha over beta for their Cre-based expression approach?

We tried to assess if there were a pericyte like cell present in or along the wall using PDGFRbeta (Pdgfr β). Pdgfr β is commonly used to identify pericytes (Winkler et al., 2010), while in contrast Pdgfra is a known fibroblast marker (Lendahl et al., 2022). Pdgfr β CreERT2 resulted in recombination in both LMCs and AdvCs, preventing it from being a discriminating marker for our study where as Myh11CreERT2 and PDGFR α CreERTM were specific at least to cell type based on our FACSS-RT-PCR and staining. As you can tell from the scRNAseq data in Figure 5, there was no cell cluster that Pdgfr β was specific for in contrast to PDGFR α and Myh11. In Figure 6 we show the expression of another commonly used pericyte marker NG2 (Cspg4) in our scRNAseq dataset which was observed in both LMCs and AdvCs as well. Lastly, MCAM (Figure 6) can also be a marker for pericytes though we see only expression in the LMCs and LECs for this marker. Notably, almost all of the AdvCs express PDGFR α rendering the PDGFR α CreERTM a powerful tool to study this population of cells on the vessel wall including those that were PDGFR α +Cspg4+ or PDGFR α + Pdgfr β +

We were reliant on PDGFR α CreERTM as that was the only available PDGFR α Cre model at the time. Note we used Pdgfr β CreERT2 and Ng2Cre in our study but found that both Cre models recombined both LMCs and AdvCs.

(4) Please include appropriate references for all the labeling markers (PDGFR-alpha, beta, and myc11 etc.) that are used in this manuscript.

We have added multiple references to the manuscript to support the use of these common cell “specific” markers as of course each marker is limited in some capacity to fully or specifically label a single population of cells (Muhl et al., 2020).

(5) One of the criteria for the pacemaker cells is depolarization-induced propagated contractions. Authors have used optogenetics-induced depolarization to test this phenomenon. Please include negative controls for these experiments.

We have now added negative controls to this experiment which were non-induced (no tamoxifen) Myh11CreER^{T2}-Chr2 popliteal vessels. This data has been added to the Figure 8.

(6) What are the resting membrane potentials of Lymphatic muscle cells? The authors should provide some details about this in the manuscript.

We agree with the reviewer and have added membrane potential recordings (Figure 13) at different pressures and filled our recording electrode with the cell labeling molecule BiocytinAF488 to highlight the action potential exhibiting cells, which were the LMCs. Lymphatic resting membrane potential is dynamic in pressurized vessels, which appears to be a critical difference in this approach as compared to pinned out vessels or those on wire myographs likely due to improper stretch or damage to the vessel wall. In mesenteric lymphatic vessels isolated from rats the minimum membrane potential achieved during repolarization ranges from -45 to 50mV typically while IALVs from mice are typically around -40mV, though IALVs have a notably higher contraction frequency. Critically, we have also added novel membrane potential recordings to this manuscript in IALVs at different pressures and show that the diastolic depolarization rate is the critical factor driving the pressure-dependent frequency.

(7) In the discussion, the authors discussed SR Ca²⁺ cycling in Pacemaking, but the relevant data are not included in this manuscript, but a manuscript from JGP (in revision) is cross-referenced.

As discussed above, we have recently published our work where studied IALVs from Myh11CreERT2-Ip3R1fl/fl (Ip3r1ismKO) and Myh11CreERT2-Ip3R1fl/fl-Ip3r2fl/fl-Ip3r3fl/fl mice (Zawieja et al., 2023). Deletion of Ip3r1 from LMCs recapitulated the dramatic reduction in frequency we previously published in Myh11CreERT2-Ano1fl/fl mice and the loss of pressure dependent chronotropy. Furthermore, in this manuscript we also showed that the diastolic calcium transients are nearly completely lost in IALVs from Myh11CreERT2-Ip3R1fl/fl knockout mice. There was no difference in the contractile function between IALVs from single Ip3r1 knockout and the triple Ip3r1-3 knockout mice suggesting that it is Ip3r1 that is required for the diastolic calcium oscillations. Further, in the presence of 1uM nifedipine there were still no calcium oscillations in the Myh11CreERT2-Ip3r1fl/fl LMCs. These findings provide further support for our interpretation that the pacemaking is of myogenic origin.

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