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Oncolytic measles virus reprograms the tumor microenvironment in a vascularized mesothelioma-on-chip model

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Keywords: mesothelioma; microfluidic; tumor-on-chip; oncolytic virus; measles virus; endothelial; fibroblast; tumor microenvironment; scRNA-seq

Abstract

Background: Pleural mesothelioma (PM) is a rare and aggressive cancer primarily caused by asbestos exposure. Although dual immune checkpoint inhibition (anti-PD-1/anti-CTLA-4) is now approved as first-line therapy, clinical benefit is limited to a small subset of patients, necessitating the need for alternative strategies. Oncolytic viruses represent a promising therapeutic approach as they selectively infect and lyse tumor cells and display immunogenic properties. In PM, we showed that the oncolytic activity of measles virus (MV) is primarily dependent on alterations in the type I interferon (IFN-I) pathway. However, to date no *in vitro* models have yet been developed to mimic a sophisticated tumor microenvironment (TME), and accurately evaluate MV efficacy in PM.

Methods: We designed a humanized 3D “vascularized mesothelioma-on-chip” (VMOC) model using microfluidic chips that mimic a TME. VMOC are generated using primary endothelial cells, lung fibroblasts and patient-derived PM cells. We characterized the functionality of our model by confocal imaging, perfusion assay and single-cell RNA sequencing. The impact of MV on the VMOC was assessed by bulk transcriptomics, multiplex ELISA and deconvolution strategies, and compared to clinical data in patients with PM.

Results: We first proved VMOC functionality with an appropriate endothelial barrier integrity, perfusability, and vascular responsiveness to external stimuli. We demonstrated significant heterogeneity in the VMOC, with the presence of different mesenchymal, cancer and endothelial subpopulations. Interestingly, the addition of PM cells to the VMOC reduced both the IFN-I pathway in cells from the TME, and the abundance of a novel endothelial cell subset characterized by an IFN-I signaling signature. Following MV administration via the endothelial network, we detected PM cell infection and death, as well as strong activation of the IFN-I pathway with the production of multiple inflammatory mediators. Interestingly, the

25 proteomic and transcriptomic inflammatory response induced by MV in VMOCs was similar
26 to that induced in PM patients treated with an oncolytic virus.

27 **Conclusions:** The VMOC model we developed enables *in vitro* investigation of MV infection
28 and TME reprogramming, paving the way for a deeper understanding of the TME role in OV
29 responses and the development of novel strategies to improve clinical efficacy.

Background

Pleural mesothelioma (PM) is a rare, aggressive malignancy primarily caused by asbestos exposure that arises from the mesothelial cells of the pleura. For the past 20 years, the standard first-line regimen for PM has consisted of platinum plus pemetrexed, with a median overall survival (mOS) of ~12 months. In 2020, combined anti-PD-1/anti-CTLA-4 immune checkpoint inhibition was approved as first-line therapy for unresectable PM; however, durable responses remain limited to only a subset of patients (1). Despite extensive preclinical effort, most candidate therapies that demonstrated promising efficacy in preclinical models have failed to translate into meaningful clinical benefit (2,3). These challenges not only underscore the need for alternative therapeutic strategies but also for better preclinical models.

Among the various innovative therapeutic approaches under investigation, oncolytic viruses are of particular interest because they can selectively infect and lyse cancer cells while sparing healthy tissue, and reprogram the immunosuppressive tumor microenvironment (TME) toward an immunologically active state. The attenuated Schwarz strain of measles virus (MV), an enveloped single-stranded RNA paramyxovirus, has been investigated as an oncolytic agent in PM. MV preferentially uses CD46 for cell entry (4,5), which is frequently overexpressed on PM and other tumor cells to evade complement-mediated lysis (6,7), whereas wild-type strains rely on CD150/SLAM and Nectin 4 (8,9). However, receptor usage alone does not account for oncolytic selectivity. Previous studies from our team have demonstrated that permissiveness of PM cells to MV replication is primarily driven by alterations in the innate antiviral type I interferon (IFN-I) pathway, particularly homozygous deletions of all IFN-I genes (6,10). In addition, infection of PM cells by MV has been shown to improve immunogenicity by inducing dendritic cell maturation, enabling cross-priming of

tumor-associated antigen to T cells, and promoting antigen spreading (11–13). In immunocompetent mouse tumor models, MV treatment has been associated with increased intratumoral T cell infiltration, reduced tumor growth and prolonged survival (14,15).

Clinical evaluations of MV using the Edmonston-Zagreb strain have been performed on multiple cancers, including ovarian cancer, cutaneous T-cell lymphoma, multiple myeloma, and glioblastoma, which have reported acceptable safety profiles (16). However, clinical responses have been heterogeneous so far. This heterogeneity points to context-specific determinants of MV efficacy, including the complexity of the tumor ecosystem. This hypothesis is supported by recent work from our team showing that monocytes/macrophages produce IFN-I in response to MV infection, which reduces viral replication in neighboring virus-sensitive PM cells through paracrine activation of the interferon- α/β receptor (IFNAR) signaling pathway (17). In lung cancer, we also showed by single cell RNA sequencing (scRNA-seq) that a distinct subset of endothelial cells (ECs) displays a strong IFN signature (18). Although many PM cells harbor deletions of IFN-I genes and cannot produce IFN-I themselves, some remain responsive to exogenous IFN-I. Therefore, integrating relevant components from the TME in the evaluation of MV oncolytic activity is essential.

Rodents are not naturally permissive to MV infection, as they lack a functional equivalent of the human CD46 receptor and require genetic engineering, including CD46-transgenic or IFNAR-knockout, mice models to become permissive (19,20). Nevertheless, these modifications do not fully reproduce the physiological context of human infection, hence limiting their ability to model MV infection. On the other hand, the use of fresh patient tissues is limited due to the rarity of mesothelioma and the small proportion of patients eligible for surgery. In recent years, three-dimensional *in vitro* cancer models have emerged as promising approaches to study the TME, offering an alternative to conventional experimental systems.

They provide spatial control over cellular organization and enable physiologically relevant cell-cell and cell-matrix interactions. Models incorporating a functional endothelium compartment and other stromal components have already been used to study mechanisms of viral delivery and infection (21–24). Thus, the objective of this study was to develop a TME-relevant vascularized pleural mesothelioma-on-chip (VMOC) model using a three-channel chip, comprising endothelial-lined parental vessels in the two lateral media channels connected to a perfusable microvascular network (MVN) embedded in fibrin gel in the central channel. The parental and MVN compartments were optimized and characterized independently before combining them in a unified system with a PM cell line and primary human lung fibroblasts (hLFs) to recapitulate key tumor–stromal interactions. Cellular heterogeneity in the VMOC model was characterized using scRNA-seq. Different methods to establish stable MV infection were evaluated, and administration via the parental vessel was selected to mimic intravenous injection. The consequences of MV infection on tumor cell death as well as on the inflammatory and antiviral responses were evaluated in the model by bulk transcriptomic and deconvolution analyses. Finally, the clinical relevance of the inflammatory response observed in the preclinical VMOC model was compared to that of patients with PM treated with an OV.

Methods

Cell Culture

Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords obtained from consenting donors at the CHU maternity (Nantes, France) as previously

described (25). After collection, they were flushed with 20 mL of phosphate-buffer saline (PBS) to remove residual blood. The bottom of the cord was clamped with a sterile clip. Ten mL of digestion mix containing 0.2% collagenase type I (Life Technologies, cat. no. 17100-17), 2 mM CaCl₂, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (Gibco, cat. no. 15140) in 0.9% NaCl was injected from the top of the cord and incubated at 37°C. After incubation, the umbilical cord was unclipped, and the digestion mix was collected. The vein was rinsed with M199 medium to collect residual cells. The cell suspension was centrifuged at 300 x g for 5 minutes. The supernatant was discarded, and cells were cultured in M199 medium supplemented with 200 IU/mL penicillin and 0.2 mg/mL streptomycin. T75 tissue culture flasks pre-coated with 0.1% gelatin (Sigma, cat. no. G9391-100G) were used for all HUVEC culture. Cultures were maintained at 37 °C with 5% CO₂. After the first passage, the cells were cultured in EBM2 (PromoCell, cat. no. C-22211), supplemented with the provided supplement mix (PromoCell, cat. no. C-39216), hereafter referred to as supplemented EBM2. The cells were used from passage 2 to 6. For live microscopy experiments, HUVECs were further transduced with lentiviral vectors expressing the red fluorescent protein mRuby2.

Human Lung Fibroblast (hLFs)

Healthy lung tissue biopsies were obtained from consenting patients undergoing surgical resection for non-small cell lung carcinoma at Nantes University hospital (France) (DC-2017-2987). According to a previously described protocol from our group (25), tissue samples were rinsed with PBS and minced into fragments <1mm³. Samples were digested with 5 mL of digestion medium containing Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/l glucose) supplemented with 200 IU/mL penicillin, 0.2 mg/mL streptomycin, 0.1% collagenase type I (Life Technologies, cat. no. 17100-17), 0.1 % collagenase type II (Life Technologies, cat. No.

17101-015), and 2.5 U/mL dispase (Life Technologies, cat. no. 17105-041). Digestion was carried out at 37 °C with manual agitation every 5 minutes. After 30 minutes of digestion, 10 mL of cold PBS solution containing 0.1 % bovine serum albumin (Clearline, cat. no. 509387) was added. The suspension was then filtered through a 100 µm cell strainer (Biologix, cat. no. 15-1100). Cells were centrifugated at 300 x g for 5 minutes and resuspended in a 1:1 mixture of supplemented EBM2 and DMEM. Cells were cultured in 0.1% gelatin coated culture plates. After 24 hours, non-adherent cells were removed, and the medium was replaced with EBM2. Once cells reached confluence, a CD31 selection (CD31-targeted magnetic cell sorting, MACS) was carried out to remove the endothelial cells and isolate fibroblast from the negative population. The CD31-negative (fibroblastic) population was characterized by flow cytometry and cultured in DMEM (Gibco) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 10% heat-inactivated fetal calf serum (Biosera), hereafter referred to as supplemented DMEM. Cell cultures were maintained at 37 °C in a 5% CO₂ humidified incubator. The cells were used from passage 2 to 9.

Pleural mesothelioma cells

The pleural mesothelioma (PM) cell line Meso13 was established in our laboratory from the pleural effusion of a PM patient (26). It is part of a certified biocollection (Ministère de l'Enseignement Supérieur et de la Recherche, DC-2017-2987; CNIL, 1657097) and has been characterized for its mutational status using targeted sequencing (27). Meso13 PM cells were transduced with lentiviral vector (pLenti6.1) encoding the Blue Fluorescent Protein (Meso13-BFP) or Nano-Luciferase (Meso13-luc) for live imaging and viability assays, respectively. Cells were cultured in RPMI-1640 medium (Gibco) supplemented with 2 mM L-glutamine,

100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 10% heat-inactivated fetal calf serum (Biosera). Cell culture was maintained at 37 °C with 5 % CO₂ in a humidified incubator.

Microfluidic chip

The idenTX microfluidic chip (AIM Biotech, cat. no. DAX-1) and its holder (AIM Biotech, cat. no. HOL-1) were used for all experiments (Figure 1A). The device is manufactured in a standard microscope slide format (approximately 75 × 25 mm) and comprises three independent systems each constituted of three parallel microchannels: a central gel channel (~1.3 mm wide) flanked by two lateral media channels (~0.5 mm wide each). All channels are approximately 10.5 mm in length and 0.25 mm in height. The central channel is separated from the lateral channels by arrays of trapezoidal micropillars (0.05 mm wide, ~0.10 mm spacing) that retain the hydrogel within the central gel channel. Each independent system contains six inlets: four inlets connected to reservoirs feeding the two lateral media channels (two inlets per lateral channel) and two inlets connected to the central gel channel without reservoirs.

Fibrin hydrogel preparation

For all experiments, fibrin hydrogels were prepared using fibrinogen (6 mg/mL; Sigma-Aldrich, cat. no. 9001-32-5) dissolved in PBS. Fibrinogen solutions were aliquoted, stored at –80 °C and thawed as needed. Thrombin (Sigma-Aldrich, cat. no. 9002-04-4) was initially reconstituted in PBS to a stock concentration of 50 U/mL, aliquoted, and stored at –80 °C. Prior to gel preparation, thrombin was thawed and diluted to a final concentration of 4 U/mL in EBM-2 supplemented with 200 IU/mL penicillin and 0.2 mg/mL streptomycin. Equal

volumes of fibrinogen and thrombin solutions were mixed immediately before use, and subsequent steps were performed without delay to prevent premature gelation.

Generation of parental vessel in the two lateral channels

To generate a complete 3D endothelial lining within each lateral media channel, 10 μL of fibrin hydrogel (1:1 mixture of fibrinogen and thrombin) was injected into the central channel of idenTX chips (AIM Biotech, cat. no. DAX-1) and allowed to polymerize at 37 °C for 15 minutes. Both lateral channels were then coated with 0.1 mg/mL type I collagen (Corning, cat. no. 354236) for 15 minutes and rinsed once with supplemented EBM2 medium. HUVECs were suspended at 3×10^6 cells/mL in supplemented EBM2, and 12 μL was injected into the left inlet of the top lateral media channel. The chip was kept vertically (width of the chip 90° relative to the culture surface) for 1 minute, allowing cells to settle against the gel-channel interface through gravity. The chip was then placed face-down in the incubator for 10 minutes to promote cell adhesion. The remaining surfaces (side wall opposite the gel, top wall, and bottom wall) were sequentially coated using additional 12 μL injections with defined chip orientations. For the side wall opposite the gel, a second 12 μL injection was performed, followed by vertical orientation in the opposite direction for 1 minute. For the top wall, another 12 μL injection was performed, and the chip was incubated face-down for 10 minutes to seed the upper surface. Finally for the bottom wall, a 12 μL injection was followed by a 10 minutes face-up incubation to seed the lower surface. This multi-orientation seeding strategy ensures that all four internal surfaces of each media channel are uniformly lined with HUVECs. The same procedure was repeated for the opposite lateral media channel. After the final seeding steps, the chip was incubated for 15 minutes at 37 °C. Then, 50 μL of supplemented EBM2 was added to each media reservoir. Chips were maintained at 37 °C with daily medium

changes until HUVECs established a continuous, confluent 3D monolayered tube called the parental vessel.

All medium changes were performed by aspirating the entire medium volume from the reservoir wells connected to the two lateral media channels of the chip and immediately refilling with fresh supplemented EBM-2 (50 μ L in the right reservoir and 70 μ L in the left reservoir of each lateral channel). After medium change, all reservoirs were emptied again and refilled with 50 μ L of medium. An additional 20 μ L was added to the top-left reservoir of the upper lateral media channel to promote gravity-driven flow across the channels. Care was taken to avoid introducing air bubbles at the inlets of the lateral channels. Medium was replaced every 24 hours for all chip cultures unless otherwise specified.

Generation of microvascular network connected to the parental vessels

Microvascular network (MVN) was generated based on a previously published protocol from Wan *et al.*, which describes two approaches for seeding cells into the central gel channel (one-step and two-step protocol) (28). Both methods were used in this study for different purposes.

To optimize cell concentrations for our final model, the one-step method was used (Supplementary Information 1). For the two-step method seeding strategy, 10 μ L of highly concentrated HUVECs suspension (15×10^6 cells/mL) was first introduced into the central gel channel through the left inlet and aspirated to leave a residual coating in between the microposts. A second injection of 10 μ L containing HUVECs (10×10^6 cells/mL), hLFs (1×10^6 cells/mL), and Meso13 cells (0.25×10^6 cells/mL), unless otherwise specified, was introduced through the right inlet of the central gel channel to seed the middle region without

218 disrupting the residual layer coating. The chip was incubated at 37 °C for 15 minutes to allow
219 matrix polymerization.

220 To establish lateral connections between the MVN that will form in the central channel and
221 the lateral media channels, parental vessels were generated in both lateral media channels
222 using the multi-orientation seeding protocol described above, which can be applied directly
223 here as the central channel was already filled with polymerized gel. Chips were maintained at
224 37 °C with 5 % CO₂ and daily medium changes. MVN was formed, stabilized and connected to
225 the parental vessels by day 5 post-seeding, at which point the vascularized mesothelioma-on-
226 chip (VMOC) model was set for subsequent experiments.

227

228 **MVN perfusion and permeability**

229 To assess MVN perfusability in the central gel channel, medium was aspirated from the media
230 reservoir of the two lateral channels and replaced with fresh supplemented EBM-2 (50 µL per
231 well). The chip was placed on the stage of a Nikon A1 HD25/A1R HD25 confocal microscope,
232 and the imaging plane was set using a 10× objective focused on the central gel channel
233 containing the MVN. Twenty µL of 70 kDa FITC-dextran solution was added to the left media
234 reservoir of the upper lateral channel. After a delay of 5–10 seconds, 20 µL of FITC-dextran
235 was added to the left reservoir of the lower lateral channel. The Z-position was adjusted based
236 on the FITC fluorescence signal to ensure optimal focal depth. Time-lapse imaging was
237 initiated immediately after FITC-dextran loading, and images were acquired in the FITC
238 channel every 15 seconds for 40 minutes.

239 Permeability measurement of the parental vessel was performed on fluorescence images of
240 70 kDa FITC–dextran diffusion in the chip. Donor voxel in the parental vessel and receiver
241 voxel in gel are used for fluorescence intensity quantification. Voxel size = 50 x 50 x 250 µm.

The area between voxels defines the diffusion path across the barrier. To calculate the permeability coefficient (Pd, cm/s), we used the following equation as previously described (29).

$$Pd = \frac{1}{t_2 - t_1} \times \frac{I_{receiver}^{t_2} - I_{receiver}^{t_1}}{I_{donor}^{t_1} - I_{receiver}^{t_1}} \times \frac{V_{receiver}}{A_{barrier}}$$

Fixed parameters for the equation were: t_1 (time point 1) = 60 s, t_2 (time point 2) = 300 s, $V_{receiver}$ (receiver voxel volume) = 6.25×10^{-7} cm³, $A_{barrier}$ (barrier area between donor and receiver) = 1.25×10^{-4} cm², $V_{receiver}/A_{barrier}$ = 0.005 cm. $I_{donor}^{t_1}$, $I_{donor}^{t_2}$, $I_{receiver}^{t_1}$ and $I_{receiver}^{t_2}$ represent fluorescence intensities measured in donor and receiver voxels at t_1 and t_2 .

Immunofluorescence staining and confocal imaging

Cell fixation was carried out by perfusing 4% paraformaldehyde (EM grade, cat. no. GF720170-1010) into the lateral media channels for 1 hour. Permeabilization was done with 0.1% Triton X-100 for 5 minutes. Chips were blocked with 4% Bovine serum albumin (BSA) at 4°C overnight and washed with PBS. Chips were stained with Alexa Fluor 488 mouse anti-human CD31 (1:500, BioLegend, cat. no. 303110), Alexa Fluor 488 mouse anti-human ZO-1 (1:100, Invitrogen, cat. no. 339188), or rabbit anti-human VE-cadherin (1:1000, Invitrogen, cat. no. PA5-19612). For the unconjugated VE-cadherin antibody, a secondary Alexa Fluor 488 goat anti-rabbit IgG antibody (1:2000, Life Technologies, cat. no. A11034) was added the following day. DRAQ5 (1:5000, Thermo Fisher Scientific, cat. no. 62254) was used as a nuclear stain in all conditions. All conjugated, primary and secondary antibodies were diluted in 2% BSA. After a final set of PBS washes, chips were ready for imaging.

Fluorescence images were captured using a Nikon A1 HD25/A1R HD25 confocal microscope with 4×, 10×, or 20× objectives and the NIS-Elements software (Nikon, RRID:SCR_014329). Z-stacks were acquired using step sizes of 30 μm for the 4× objective, 3 μm for the 10× objective, and 1.5 μm for the 20× objective. Images were processed and reconstructed using NIS-Elements (Nikon), Fiji (ImageJ) and Imaris (Bitplane) software. The NIS Denoise.ai module was used for noise reduction to improve visual clarity. All image quantifications were done on raw images. Hollow structures within the MVN were considered as lumen and their diameter was measured using Fiji after orthogonal projection of Z-stacked confocal microscopy.

Cell collection from the chip

Cells were collected separately from the central gel channel and the two lateral media channels. Lateral media channels were first washed with PBS, followed by the injection of 50μL of TrypLE Select (Gibco, cat. no. 12563-029) into the left inlet of each lateral channel and incubation at 37°C for 15 minutes. After incubation, gentle up-and-down pipetting through the left inlet of both channels was performed to facilitate cell detachment. Detached cells were collected and neutralized with RPMI medium supplemented with 10% FBS. This collection step was repeated once more to maximize recovery. For recovery of cells in the central gel channel, 50μL of Liberase™ (50 μg/mL; Roche, cat. no. 05401119001) was injected into the left inlet of both lateral channels and incubated for 15 minutes at 37 °C to digest the fibrin gel. Released cells were collected through the media inlets. All collected cells were kept at 37°C prior to subsequent use.

Single cell RNA sequencing of the VMOC and analysis

287 After 5 days of culture, cells from the central gel channel and lateral channel (with or without
288 mesothelioma cells) were collected as described above. Single-cell suspensions were
289 prepared in supplemented EBM2 by mixing cells from the central channel and lateral channels
290 at a 9:1 ratio. 10,000 cells per sample were used to generate single-cell droplet libraries with
291 Chromium NEXT GEM Single Cell 3' Reagent kit v3.1, 10X Chromium Single Cell Controller (10X
292 Genomics, Pleasanton, CA, USA). Library size was determined by Agilent TapeStation assays
293 and the concentration by Qubit Flex (Invitrogen). Libraries were pooled and sequenced on
294 NovaSeq 6000 Sequencing System (Illumina), providing a read depth of >40,000 read pairs per
295 cell according to manufacturer's instructions. Four samples were sequenced in two batches,
296 each batch being composed of one chip with mesothelioma cells and one chip without.

297 Gene expression matrices were generated using Cell Ranger (10X Genomics, version 2.1.1)
298 with the GRCh38 build of the human reference genome, and further processed using RStudio
299 (version 2023.12.1+402). We used the following quality control steps: genes expressed by <3
300 cells were not considered, cells expressing <400 genes (low quality) or >10,000 genes, <500
301 genes or >80,000 unique molecular identifiers (UMIs), or >15% of UMIs derived from the
302 mitochondrial genome were removed. From the mesenchymal and endothelial clusters,
303 doublets were detected and labelled using the DoubletFinder package (version 2.0.6).
304 Doublets represented ~0.5% of filtered cells and were evenly distributed among the different
305 cell types from all samples. Notably, doublet annotation incorrectly identified the entire
306 tumor cluster as a doublet, probably due to the active nature of this cell type compared to
307 the other cells from the VMOC. Therefore, this doublet annotation was not considered for
308 quality filtering of the tumor cells. Since enzymatic and mechanical dissociations were
309 described as eventually inducing a specific transcriptomic response, we checked if a specific
310 cell cluster/subcluster was enriched for the dissociation signature (30). Visualization,

clustering, marker gene identification, and gene set enrichment analysis (GSEA) were performed using the SeuratV5 package. Signature enrichment scores were calculated using the UCell R package (version 2.12.0) using the Mann-Whitney U statistic. Cell trajectory analysis was performed using the Monocle3 R package (version 1.4.26). CellChat was performed using default parameters on the full CellChat database (version 2.2.0) by predicting interactions between all subclusters composing the VMOC. Gene Set Variation Analysis (GSVA) was performed with the GSVA R package (version 2.2.1) on the top 50 marker genes from each endothelial and mesenchymal subcluster. Deconvolution was achieved on endothelial and mesenchymal subclusters with the MuSiC R package (version 1.0.0) using common genes expressed between scRNA-seq and bulk 3'RNA-seq datasets generated in this study. Cluster similarities between VMOC and PM patients (GSE190597) scRNA-seq datasets was assessed using Cluster-Map R package on the marker gene signatures (min.pct = 0.25, logfc.threshold = 0.25, edge_cutoff = 0.05).

MV-GFP infection of vascularized mesothelioma-on-chip

The live-attenuated Schwarz strain of measles viruses expressing the green fluorescent protein (MV-GFP) was produced as previously described (31). Viral titer was determined using the TCID₅₀ method. TCID₅₀ quantifies the infectious dose of a virus by determining the dilution at which 50% of cell cultures show infection.

For virus infection, the VMOCs were washed with supplemented EBM2 and then each media reservoir was filled with 50 µL of the same medium. MV-GFP was introduced by preparing a viral suspension containing 10⁶ TCID₅₀ (50% tissue culture infectious dose) in a total volume of 40 µL supplemented EBM-2, which was added to the left reservoir of the upper lateral channel. After 6 hours of incubation at 37°C, the virus solution was aspirated and the lateral

channels were washed twice with fresh supplemented EBM2 medium. Medium was replaced every 24h. Images were acquired 48 h post-infection using a Nikon A1 HD25/A1R HD25 confocal microscope.

Luciferase release assay to quantify MV-induced PM cell death in VMOC

2,500 Meso13-luc cells were embedded in the 2nd layer of the central gel channel using the two-step method. Chips were infected with MV-GFP on day 5, as described above. Supernatants were collected at 24, 48, and 72 hours post-infection, followed by a medium renewal, and stored at -80°C. For each time point, 50 µL of medium was transferred to a 96-well plate and mixed with 50 µL of Nano-Glo[®] Luciferase Assay substrate (Promega, cat. no. N1120). Luminescence was measured using a Fluoroskan plate reader (ThermoFisher Scientific). Each time point was normalized to the uninfected condition.

Chemokine detection by Legendplex multiplex assay

Supernatants from infected and non-infected VMOCs were collected 48 hours after MV-GFP infection for cytokine measurements. LEGENDplex[™] Human Inflammation Panel 1 and LEGENDplex[™] Human Immune Checkpoint Panel 1 were used to measure IL-4, IL-2, IP-10, IL-1 β , TNF- α , MCP-1, IL-17A, IL-6, IL-10, IFN- γ , IL-12p70, IL-8, CD25 (IL-2R α), 4-1BB, sCD27, B7.2 (CD86), TGF- β 1, CTLA-4, PD-L1, PD-L2, PD-1, Tim-3, LAG-3, and Galectin-9 secretion.

3' RNA-sequencing analysis of the VMOC

RNA was extracted using the Nucleospin[®] RNA plus kit (Macherey-Nagel, cat. no. 740990.50). The mRNA quality was analyzed using the Agilent 20100 Bioanalyzer (Agilent) in RNA Nanochips (Agilent). The 3' RNA sequencing profiling was performed by the GenoBird

platform (IRSUN, Nantes, France) using a NovaSeq 6000 (Illumina) and NovaSeq 6000 SP Reagent Kit 100 cycles (Illumina) according to the manufacturer's protocol. The full procedure is described in a study by Charpentier *et al.* (32). The raw sequence reads were filtered based on quality using FastQC. Adapted sequences were trimmed from the raw sequence reads using Cutadapt. Reads were then aligned to the reference genome (Hg38) using BWA. Differential gene expression, principal component analysis (PCA) and gene set enrichment analysis (GSEA) were determined using Biomex software version 1.0-5 (33).

Meta-analysis of PM patients treated with an oncolytic virus

We reanalyzed nanostring RNA profiling from patients with PM, treated or not with the oncolytic virus Olvi-vec (GSE223395). We excluded patients "Pat_2_Post" and "Pat_12_Post", and "Pat_2_Pre" from the analysis as they were annotated as two NSCLC (1 squamous cell carcinoma and adenocarcinoma) and 1 triple negative breast cancer, respectively (34). PCA and differential gene expression and GSEA were performed on normalized raw data using Biomex software version 1.0-5 (33). Data normalization was performed with standard normalization methods as defined in Biomex for micro array dataset. Three groups of patients (untreated, treated with inflammatory response, or treated without inflammatory response) were determined using unsupervised hierarchical clustering and PCA. Immune cell proportions were estimated with CIBERSORTx (35) using the custom 'Impute Cell Fractions' with LM22 as reference signature matrix, and the curated GSE223395 expression data as mixture file. We expressed immune cell subsets as relative percentages for each patient condition. A meta-analysis using product-rank and Fisher method was performed to determine top differentially regulated genes between the VMOC at 24h or 48h post MV

infection and untreated VMOC, and untreated and Olvi-vec treated PM patients with inflammatory response.

Statistical data

All data were expressed as mean $\pm$ SEM, and statistical analyses were performed with GraphPad Prism V.8 (GraphPad Software, Boston, Massachusetts, USA). Statistical tests and significant differences for each figure were mentioned in the legends.

Results

Development and characterization of parental blood vessels from the microfluidic chip

As in many solid malignancies, the pre-existing primary vasculature in PM is hijacked to ensure blood supply to the tumor mass and enable metastatic dissemination. Neighboring and newly formed tumor-associated secondary vessels exhibit irregular morphology, increased permeability, and heterogeneous perfusion (36,37). To recapitulate these two distinct vessel types in our vascularized mesothelioma-on-chip (VMOC) model, we used the idenTX AIM-Biotech microfluidic chip (Figure 1A), which consists of a central gel channel flanked by two lateral media channels. Trapezoidal micropillars separate the channels, while the gaps between micropillars allow molecular exchange and endothelial connections across channels. To reduce experimental complexity and ensure controlled optimization, parental (lateral) and central vessels were optimized and characterized separately, after which both were generated within the same chip to form the complete VMOC.

403 To generate the primary vascular compartment, i.e., the parental vessel, the central
404 channel was first filled with fibrin gel to prevent endothelial cells (ECs, hereinafter referred to
405 as HUVECs, Human Umbilical Vein ECs) seeded in the lateral channel from spreading into the
406 central channel. Because parental vessel formation requires multiple injections through the
407 lateral channels (Figure 1B), the extracellular matrix (ECM) in the central channel needs to
408 remain mechanically stable throughout the process. Fibrin was selected over collagen
409 because it is less deformable under repeated injections (29) and maintains strong adhesion
410 to the micropillars. The polymerization process was optimized to ensure a stable and
411 reproducible gel. A fibrinogen-thrombin mixture (6 mg/mL and 4 U/mL, respectively) was
412 selected for all experiments, injected into the central channel and allowed to polymerize at
413 37°C for 15 minutes. This incubation period was the most effective, as an extended duration
414 resulted in gel contraction or the formation of air bubbles between the micropillars. After
415 fibrin polymerization, lateral channels were coated with collagen to promote cell adhesion,
416 and HUVECs were injected with sequential 90° rotations to ensure uniform cell attachment
417 along all four walls of both media channels (Figure 1B). After 4 days, confocal microscopy
418 showed a continuous monolayer of CD31+ HUVECs all around the inner surface of the lateral
419 channel (plane view) and the presence of a hollow lumen (cross-sectional view) (Figure 1C-
420 D). Immunofluorescence labeling for VE-cadherin and ZO-1 demonstrated the presence of
421 adherens and tight junctions, respectively (Figure 1E and 1F). This resulting tube-like structure
422 is referred to as the parental vessel. In order to evaluate its response to external stimuli, the
423 barrier functionality was evaluated using a 70 kDa FITC-dextran vascular permeability assay
424 (Figure S1A). A permeability coefficient (Pd) was calculated using quantitative analysis of FITC-
425 dextran diffusion, with lower values reflecting higher barrier properties. Pd was equal to 1.40
426 $\times 10^{-5} \pm 1.30 \times 10^{-6}$ cm/s without HUVECs, whereas Pd diminished to $8.10 \times 10^{-6} \pm 4.00 \times 10^{-6}$

cm/s in the presence of parental vessel (Figure S1A, panel i-ii)). To evaluate the responsiveness of parental vessels to external stimuli, we used Angiopoietin-1 (Ang-1). Ang-1 is a cytokine that promotes maturation and stabilization of blood vessels by stabilizing endothelial junctions through various mechanisms, thereby reducing vascular permeability (38–40). In a previous vasculature-on-chip model, Ang-1 led to a dose-dependent decrease in permeability to FITC-dextran and nanoparticles (41). Consistently, in our model, treatment of parental vessels with 100 ng/mL Ang-1 for 24 hours resulted in a reduction in Pd ($3.60 \times 10^{-6} \pm 4.00 \times 10^{-7}$ cm/s) compared to the untreated condition ($8.10 \times 10^{-6} \pm 4.00 \times 10^{-6}$ cm/s) (Figure S1A, panel iii). The second external stimulus evaluated was the application of a flow of culture medium at 5 μ L/min (a velocity of ~ 670 μ m/s) for 24 hours, which induced an alignment of the HUVEC in the direction of flow (Figure S1B). This velocity corresponds to the physiological range of capillary blood flow (~ 500 – $1,500$ μ m/second) (42–44). Overall, these results demonstrated that parental vessels form a functional endothelial barrier capable of responding to both molecular and mechanical signals.

Formation of a vascularized TME connected to parental vessels

Tumor-associated vasculature is highly heterogeneous, with irregular branching and architecture, so we aimed to recreate this complex vascular network as our secondary endothelial compartment (37). A one-step seeding approach was first evaluated to establish the secondary endothelial compartment by introducing a HUVEC–fibrin mixture into the central channel on day 0 (Figure S2A and Supplemental information 1). Previous studies have shown that HUVECs suspended in fibrin gel undergo vasculogenic organization resulting into a microvascular network (MVN) (28,45–47). Primary HUVECs expressing mRuby2 were injected into the central channel, allowing visualisation of endothelial connections and

network formation over time. Because the lateral channels serve as the primary source of culture medium for the chip, perfusion of the MVN requires direct endothelial connectivity across the micropillar gaps between the MVN and the parental vessels, in the central and lateral channels, respectively. These endothelial connections, designated as bridging vessels, span the micropillar gaps and form the sole fluidic connection between the parental vessels and the MVN (Figure S2A). Increasing HUVEC seeding density in the central channel increased the vessel lumen diameter of the MVN (Figure S2B-D; Supplemental information 2). At low density (80,000 HUVECs per central channel), the number of perfusable bridging vessels was low due to insufficient HUVEC deposition between the micropillar gaps. Increasing density (80,000–150,000 HUVECs per central channel) heightened the number of perfusable bridging vessels and improved connectivity with the lateral channels (Figure S2E). However, high densities (120,000–150,000 HUVECs per central channel) produced larger vessels and reduced MVN complexity (Figure S2B). Overall, this one-step seeding approach did not allow simultaneous formation of a MVN with interconnected vessel structures and sufficient perfusable bridging vessels, which could mirror tumor vessels.

To overcome this limitation, we implemented a two-step seeding strategy adapted from Wan *et al.*, 2022 (28) (Figure S3A). This approach allows independent control of the region between micropillars and the central channel. Briefly, two sequential injections of HUVECs in fibrin-thrombin gel were performed. The first with a high concentration of HUVECs to fill the spaces between the micropillars and the second with a lower HUVEC concentration to fill the middle area of the central channel. Once polymerization was complete, parental vessels were established in both lateral channels, enabling the parental vessels to act as the primary vasculature connected to bridging vessels and supplying medium to the fibrin-embedded MVN in the central channel. By day 5, the MVN was stable and perfusable (Figure S3B-panel

i). Having established a perfusable and branched secondary MVN that was connected to the parental vessels, we next introduced stromal cells. Fibroblasts are major components of the TME (48–50) and also play an important function in the structure and formation of vessels (51,52). Thus, to better reflect the stromal context of PM tumors, we first incorporated primary human lung fibroblasts (hLFs) into the central channel along with the MVN. To do this, we first evaluated the impact of hLFs on the formation of MVN by co-seeding HUVECs with hLFs in the second layer of the two-step method at different HUVECs/hLF ratio (H/F ratio) (Figure S3A-B). Fibroblasts were excluded from the first gel layer to prevent narrowing of the bridging vessels, as previously reported (45). We found that a H/F ratio of 10:1 represents an ideal condition to achieve a branched, well-formed, and interconnected MVN (Figure S3B, panel ii). FITC-dextran perfusion demonstrated that the H/F ratio of 10:1 yield in higher number of open bridging vessels with a high prevalence of small vessels (50 μ m of diameter) and the absence of large vessels (>300 μ m) (Figure S3B-D). Considering these results, the H/F ratio of 10:1 was used for all the subsequent experiments. The evolution of the two-step model was followed over a period of 10 days (Figure S4A). On day 0, HUVECs and hLFs were evenly distributed as single cell suspension in the fibrin gel. By day 2, connections between cells appeared. On day 4, network-like structures were visible, though still exhibiting parts with active sprouting and anastomosis. By day 5 and 6, the networks appeared well connected with smoother structures that remained stable up to day 10. To complete the characterization of the MVN, we performed CD31, VE-cadherin and ZO-1 labeling and confirmed the endothelium integrity and presence of adherens and tight junctions (Figure S4B).

Finally, to introduce the tumor compartment of the VMOC, we implemented the two-step method and added the Meso13 PM cell line to the mix of HUVECs and hLFs (H/F/Meso13 ratio = 10:1:0.25) in the second fibrin gel layer introduced into the central channel (Figure 2A). The

addition of PM cells did not modify the structure of the MVN, and by day 5, a stable MVN was formed, bridging vessels were preserved, and sufficient cancer cells were present for subsequent experiments (Figure 2B). Immunostaining and confocal microscopy of VE-cadherin showed the presence of adherens junctions in the MVN connected to the parental vessels (Figure 2B). Orthogonal projection of confocal microscopy data obtained after Z-acquisition confirmed the presence of round vessels with a hollow lumen in the MVN (Figure 2C). As in Figure S4B, in the presence of PM cells, we observed the presence of CD31 and ZO-1 positive cells corresponding to adherens and tight junction markers, respectively (Figure 2D).

To examine the 3D organization of the MVN and its spatial relationship with Meso13 PM cells, we generated a 3D representation of the MVN with VE-cadherin staining (Figure 3A, panel i), a magnification of an area of the MVN (Figure 3A, panel ii) and a transverse section of the MVN showing a hollow circular lumen (Figure 3A, panel iii). Meso13 cells were distributed throughout the fibrin matrix and were frequently located adjacent to the vessel structures organized into highly branched, complex networks (Figure 3A, panel i and iii and Movie 1). To demonstrate functionality and interconnection between the MVN and parental vessels, a perfusion test using 1 μ m green fluorescent microbeads was performed on day 5 of culture. After addition of microbeads to the media reservoir of the upper lateral channel, 3D reconstructions of confocal microscopy images confirmed that the microbeads were localized within the MVN lumens (Figure 3B). Time-lapse experiment showed that beads travelled from one parental vessel through the MVN to the opposite parental vessel, demonstrating continuous perfusion and vessel connections between vascular compartments of the lateral and central channels (Figure 3C, Movie 2). Perfusion of 70 kDa FITC-dextran through the MVN

further confirmed interconnected and perfusable vessels spanning the central gel region (Figure 3D).

Evaluation of cellular heterogeneity in the VMOC model

A recurrent question in the development of tumor models *in vitro* is the extent of cellular heterogeneity. Indeed, it is now well established that cells from the TME of PM exist under different subpopulations harboring distinct properties (53,54). Seemingly, the VMOC clearly shows a heterogeneity, with different vessel organization in parental vessels and MVN. To further evaluate this heterogeneity, we performed single-cell RNA-sequencing (scRNA-seq) analysis on VMOCs with or without Meso13 cells after 5 days of culture. After quality filtering, doublet removal and integration, we obtained 37,215 high-quality cells that grouped into three distinct clusters (Figure 4A). Annotated on the basis of their top 50 marker genes, we retrieved the three cell types included in the VMOC, namely cancer cells, fibroblast-like (mesenchymal) and ECs, with a homogeneous distribution across the 4 samples sequenced (Figure 4A-B; Figure S5A; Table S1). Considering the initial cell ratio used in the VMOC (H/F/Meso13 ratio = 10:1:0.25), the cancer cell subset was constituted of 318 cells that fell into three subclusters with a similar relative proportion across samples (Figure 4C; Figure S5B-C). As expected, no cancer cells were detected in the samples devoid of Meso13 cells (Figure S5A). Gene set enrichment analysis (GSEA) showed enrichment in specific transcriptomic signatures including stress and inflammatory responses, oxidative phosphorylation (OXPHOS) and cell proliferation (Figure 4C; Figure S5D-F; Table S1). Among Meso13 subpopulations, stress/inflammatory and OXPHOS subsets presented the highest gene signature for IFN- α response suggesting that PM cell subpopulations could present different level of sensitivity to MV (Figure S5G) (6,10). Interestingly, cell-ligand prediction analysis identified the TGF- β

546 signaling as the main signal arising from cancer cell subsets toward ECs and fibroblasts. Since
547 TGF- β is a cytokine involved in tumor progression and also described as an inhibitor of IFN-I
548 signalling pathway controlling cell infection by OV_s (55), our data suggest an important
549 communication network with TGF- β and potential consequences on cell infection (Figure
550 S6A).

551 Thereafter, we sought to investigate how cancer cells impact on the vascular and
552 mesenchymal compartments at the single cell level. The 18,151 ECs obtained post-filtering
553 segregated into 5 distinct subclusters with a similar distribution across samples (Figure 4D;
554 Figure S7A-B). We could first identify two populations previously described in scRNA-seq
555 datasets from cultured ECs (18,56), namely proliferative ECs expressing key proliferation
556 markers (*MKI67*, *TOP2A*, *BIRC5*) and genes related to chromosomal segregation, and a
557 mesenchymal transitory population expressing mesenchymal markers (*TAGLN*, *CDH2*, *ESM1*)
558 but devoid of cell proliferation traits (Figure 4D-E; Figure S7C-E; Table S1). Interestingly, a
559 subcluster of ECs expressed markers associated with artery specification (*HEY1*, *EFNB2*,
560 *CLDN10*) (57) that was absent in scRNA-seq datasets from static 2D culture (18,56). Hence,
561 this suggests that a vascular specification program may occur in our VMOC, in response to 3D
562 architecture, stretch or passive flux diffusion. Consistently, CellChat analysis revealed a strong
563 NOTCH signaling network between endothelial subpopulations, with multiple ligand–receptor
564 interactions, including DLL4–NOTCH1, supporting its known role in arterial fate specification
565 (Figure S6B) (58–60). The last two endothelial subclusters expressed high levels of genes
566 related to pro-inflammatory cytokines and response to them (*CCL2*, *CXCR4*, *ICAM1*, *LYVE1*,
567 *EGR1-3*, *NUPR1*) (61,62), but also key angiogenic markers (*KDR*, *PGF*, *DLL4*) (Figure 4D-E; Table
568 S1). Interestingly, among the inflammatory subclusters, one subset coined “EC interferon”
569 presented a marked signature in interferon signaling (with key interferon-stimulated genes

(ISGs): *IFIT1-3*, *IFIT5*, *IFI6*, *IFI44L*, *MX1-2*, etc.), and its abundance was reduced by 95% when PM cells were added into the VMOC (Figure 4D-E; Figure S7B-G; Table S1). In addition, a decrease in genes associated with IFN- α /- γ responses in the whole EC population was also observed following the addition of PM cells (Figure 4F; Figure S7H).

In the VMOC model, the fibroblastic compartment was abundantly represented with 18,746 high-quality cells, distributed in four different subclusters (Figure 4G; Figure S8A-B). The larger fibroblast subcluster expressed genes and gene sets associated with cell proliferation, chromatin remodeling and nucleosome formation (*PCLAF*, *HMGB2*, *H2AFZ*) but lacked expression of the cancer-associated fibroblast (CAF) marker FSP1 (*S100A4*) (Figure 4H). On the contrary, two fibroblast subclusters expressed CAF markers, characteristic of myofibroblasts (myCAF) (63) and ECM-myCAF (*FAP*⁺ *ANTXR1*⁺ *SDC1*⁺ *LAMP*⁻) (Figure 4H; Table S1). Mirroring the effect seen in ECs, the addition of PM cells in the VMOC induced a sharp diminution in gene sets associated with IFN- α /- γ responses and TNF- α signaling in the fibroblast subsets (Figure 4I and S8C). Intriguingly, a fibroblast subcluster coexpressed endothelial (*CDH5*, *PECAM1*, *VWF*, *CLDN5*) and mesenchymal markers, with key transcription factors involved during the endothelial-to-mesenchymal transition (EndMT) including *ZEB1*, *ERG* and *FLI1* (Figure 4J; Table S1) (64). Monocle cell trajectory analysis revealed that this mesenchymal cluster (dubbed Fibro EndMT) may originate from an ultimate differentiation of ECs into fibroblasts via EndMT, a process relevant in fibrotic and inflammatory diseases, and cancer (64–66) (Figure 4K; Figure S8D-E). Finally, we observed similarities between the different EC and fibroblast subtypes identified in the VMOC and tumor biopsies from patients with PM (Figure S9) (54). This emphasizes the potential therapeutic relevance of our VMOC model.

Establishment of MV infection and assessment of oncolytic activity in the VMOC

We have previously shown that human PM cell lines intrinsically deficient in IFN-I production and sensitive to MV infection (such as Meso13), still retain functional IFNAR signaling and can be protected by other cells from the TME through paracrine production of IFN-I (17). To better understand the mechanisms of sensitivity/resistance to MV, models integrating both tumor cells and cells from the TME are necessary. We studied replication, spread, and oncolytic activity of the live-attenuated, vaccinal Schwarz strain of MV coding for GFP (MV-GFP) in the VMOC. We evaluated multiple delivery strategies and viral doses to define the best conditions for studying the MV mechanisms of action. MV added directly to the central channel during gel loading (MOI 1 and 10) (Figure S10A) or pre-infection of Meso13 cells (MOI 1) (Figure S10B) resulted in low PM cell infection, with GFP mainly detected in fibroblasts.

To mimic the intravenous administration of oncolytic virus in cancer patients, we then assessed intravascular administration by introducing MV through the media inlet of the lateral channel containing the established parental vessel after VMOC maturation (day 5) (Figure 5A). Several combinations of viral concentration and exposure duration were tested to identify the optimal conditions for PM cell infection. Mature VMOCs were first exposed to 0.2×10^6 , 0.5×10^6 , or 1×10^6 viral particles for 24 hours. At 48 hours post infection, 0.2×10^6 viral particles produced minimal infection but retained vascular integrity (Figure S11A). In contrast, 0.5×10^6 and 1×10^6 viral particles produced numerous detectable GFP signals but caused pronounced disruptions and complete collapse of the MVN (Figure S10A) as already observed with other viruses in *in vitro* vascularized models (21,22). We then tested a high-dose, short-exposure strategy with 1×10^6 or 2×10^6 MV-GFP particles that were added in a parental vessel for 6 hours and then removed (Figure 5A). Viral replication, as indicated by GFP signal, was detectable as early as 24 hours post-infection with 1×10^6 MV (Figure S11B). 48 hours after

administration, 1×10^6 MV particles in a parental vessel led to the infection of numerous PM cells and also of some fibroblasts (Figure 5B). With 2×10^6 MV particles, the number of infected PM cells increased as well as the number of infected fibroblasts and an excessive alteration of the MVN was observed (Figure S10C). Therefore, the condition of 1×10^6 viral particles and 6 hours of incubation was selected as the optimal condition for establishing efficient PM cell infection.

In order to specifically measure the death of PM cells, we used Meso13 cells expressing Nano-Luciferase in the VMOC model. Detection of luciferase activity in the culture supernatant is proportional to the extent of PM cell death. While basal Nano-Luciferase activity in control conditions remained stable over time, MV-GFP administration increased Nano-Luciferase activity at 48 hours post infection, indicating virus-induced lysis of PM cells (Figure 5C). These data demonstrate that MV induced measurable and significant lysis of PM cells in the VMOC.

MV infection induces inflammatory response in the VMOC

MV infection is well documented to trigger robust innate immune activation, including induction of ISGs, as well as production of inflammatory chemokines and cytokines (67). To study the consequences of MV infection on the VMOC, we performed a 3' RNA-seq analysis on the whole VMOC chips at 24 and 48 hours post-infection. MV infection induced extensive transcriptional modifications in the VMOC, with 197 genes upregulated and 2 downregulated at 24 hours, and 210 upregulated and 25 downregulated at 48 hours ($p_{adj} < 0.01$) (Figure 6A; Table S2). The majority of the deregulated genes are common between the two time-points ($n=157$) (Figure S12A; Table S2). Pathway enrichment analysis revealed strong activation of innate immune and inflammatory pathways, including type I and type II interferon responses

and inflammatory signaling at both time points (Figure 6B and S12B). Indeed, MV triggered a strong inflammatory transcriptional program by inducing the expression of numerous genes coding for chemokines and cytokines (Figure 6C; Table S2). As expected, ISGs were strongly upregulated at both time points (Figure 6C; Table S2). Canonical activation markers, commonly expressed in ECs upon inflammation, were also upregulated after MV infection (*SELL*, *SELE*, *VCAM1*, and *ICAM1*) (Figure 6C; Table S2). Hence suggesting that the vascular compartment may be impacted by MV, thereby fueling the inflammatory response. Genes described as hallmarks of response, resistance and toxicity to immune checkpoint blockade were also affected following MV infection, with an induction of genes involved in antigen presentation (*HLA-A*, *HLA-B*, *HLA-C* and *B2M*), but also in immune response inhibition (*CD274* encoding for PD-L1, *CD47*, and *HLA-E*) (Figure 6D). Cytokine secretion profiles were validated and measured in VMOC culture supernatants 48 hours post-infection using multiplex ELISA (Figure 6E and S13). MV infection induced significant release of numerous cytokines compared to the non-infected controls, including some of them already observed at the gene level (*CXCL8* (IL-8), *LGALS9* (Galectin-9), *CXCL10* (IP-10) and *IL6*) (Figure 6E). IP-10 showed the strongest induction (>1,000-fold change), followed by significant increases in IL-6, IL-8 and Galectin-9 indicating activation of both IFN-responsive and inflammatory pathways. Notably, TNF- α , IFN- γ , IL-1 β , IL-2 and IL-4 were also significantly induced, albeit expressed at low levels. In contrast, IL-12p70 and MCP-1 remained unchanged, possibly due to their already high basal expression in control condition, particularly for MCP-1 (Figure S13).

To integrate bulk transcriptomic data with our description of the VMOC heterogeneity, we applied deconvolution and gene set variation analysis (GSVA) approaches to infer how MV impacts the endothelial and fibroblast subpopulations identified by scRNA-seq (Figure 4). The proportions of proliferative EC, inflammatory EC (Figure S14A) and EndMT fibroblast subtypes

were estimated to increase at 48h post MV infection (Figure S13B), while this tendency was inverted in proliferative fibroblasts (Figure S14B). GSVA from the top 50 marker genes showed increased pathway activity scores from the EC interferon in all conditions with MV infection (Figure S14C-D). Altogether these results show that MV deeply remodels the TME through the induction of inflammation and interferon-related signaling in ECs and in cells from the TME.

MV infection in the VMOC yields in similar pro-inflammatory response to that observed in PM patients treated with oncolytic virus

We took advantage of transcriptomic data from a phase I clinical trial comparing PM tumors before and after intrapleural administration with the oncolytic vaccinia virus Olvi-vec (NCT01766739)(34). Upon data reanalysis, unsupervised hierarchical clustering and principal component analysis (PCA) stratified PM patients in three groups (Figure 7A; Figure S15A). Differential analyses identified a first group of patients treated with Olvi-vec harboring a pronounced inflammatory response including expression of genes related to macrophage (*HLA-DQA1, HLA-DQB1, FCER2, IDO1, CCCL19*), cytotoxic T/NK cell (*CD3D, CD3E, CD8B, PRF1, GZMB, GNLY*) and B cell (*CD79A, CD79B, CD19*) infiltration and recruitment (Figure 7B; Figure S15B-C; Table S3). This group of patients, hence dubbed treated inflammatory (“T inflam”), appears different from treated patients without this inflammatory signature (“T no-inflam”) and who clustered with the non-treated arm (Figure 7A; Figure S15A). Upon estimating immune cell type abundance using CIBERSORTx (35), we confirmed an enrichment in B and CD4 T naive cells, activated NK cells, and M1-like macrophage in the ‘T inflam’ compared to ‘T no-inflam’ or untreated PM patients (Figure 7C). We next thought to compare Olvi-vec treated inflammatory patients to the VMOC models upon stimulation with another oncolytic virus (after 24 or 48 hours). Venn diagram based on differentially expressed genes across

models and conditions identified 55 commonly deregulated genes ($p_{adj} < 0.01$) (Figure 7D) related to IL-6 JAK/STAT3, IFN-I and TNF- α signaling pathways (Figure 7E). A product rank meta-analysis further confirmed that key marker genes found up-regulated in the VMOC model were also induced in Olvi-vec 'T inflam' patients (*CXCL10*, *CXCL11*, *IFNB1*, *SELL* and *CD274*/PD-L1) (Figure 7F). Finally, comparing cytokines present in the pleural fluids and sera of PM patients treated with Olvi-vec (34) show a similar pattern to those arising from the VMOC as IFN- γ , TNF- α , IL-1 β , IP-10 and IL-8 were always upregulated after oncolytic virus therapy (Figure 7G). Taken together, our data highlight the clinical relevance of the VMOC model for modelling responses to oncolytic viruses.

Discussion

Much work has focused on elucidating the mechanisms underlying tumor-selective replication of the vaccinal Schwarz strain of measles virus (MV) in pleural mesothelioma (PM) and other cancers. In contrast, the mechanisms governing viral interactions with the tumor microenvironment (TME) and the physical and biological barriers that influence viral activity remain less characterized. A better understanding of viral dissemination within the TME may contribute to improved therapeutic efficacy. In this work, we examined MV oncolytic activity and its impact on the TME using virus intravascular delivery in a vascularized mesothelioma-on-chip (VMOC) model. We first optimized the experimental conditions to obtain an endothelial compartment consisting of parental vessels connected to a microvascular network (MVN). We showed the importance of the methodological procedure and of the endothelial cell (EC)/fibroblast ratio to obtain an optimal number of bridging vessels for an optimal circulation of molecules and particles through the vascular network. A thorough

713 characterization of this compartment demonstrated the presence of adherens and tight
714 junctions between ECs in both the parental vessel and MVN, as well as the formation of a
715 complex network with numerous branches in the MVN and the absence of leaks. Parental
716 vessels also exhibited functional responses, including modulation of permeability in response
717 to Angiopoietin-1 and elongation under flow. Analysis of cellular heterogeneity using scRNA-
718 seq revealed subpopulations of different cell types that constitute VMOC, which bear similar
719 transcriptomic signatures to those found in PM tumors. Interestingly, we observed that the
720 addition of PM cells in VMOC impacted endothelial and fibroblast cell heterogeneity from the
721 TME, the main consequence being a decrease of the basal type I interferon pathway.
722 Administration of MV into the endothelial compartment via the parental vessel led to the
723 infection and death of PM cells but also to fibroblasts infection. Transcriptomic analysis
724 reveals a remodeling of the TME with a strong induction of ISGs, of inflammatory markers, of
725 EC activation, coupled to secretion of multiple pro-inflammatory cytokines, and a
726 modification of the overall immunogenicity.

727 Fibroblasts are major components of the TME, where they can be remodeled into CAFs
728 and thus contribute to tumor progression, resistance to therapies, and angiogenesis.
729 Moreover, fibroblasts have been widely used *in vitro* to promote microvascular network
730 formation and stability through their pro-angiogenic signaling (45,51,68,69). Fibroblasts have
731 also been reported to support the formation of a normal vasculature (70). Here, we observed
732 that the ratio of ECs to hLFs was critical for the formation of functional and perfusable MVN.
733 Low fibroblast density resulted in poorly organized networks with larger vessel diameters,
734 whereas high fibroblast density led to the formation of smaller vessels with limited
735 connectivity to parental vessels. This effect of lung fibroblasts on endothelial network
736 architecture was already observed in a previous endothelial–fibroblast co-culture model, in

which increased fibroblast density was directly proportional to reduced vessel diameter (28). To confirm the quality of the endothelial network, we evaluated the permeability of the endothelial barrier to 70 kDa FITC-dextran. The permeability coefficient (Pd) measured for parental vessels was consistent with what is described in literature (41,71). Unfortunately, the complexity of the MVN did not allow an accurate measurement of Pd; however, the presence of adherence and tight junctions as well as the absence of leaks (diffusion of 70 kDa dextran), criteria already used in other studies (72,73) strongly support the quality of the network.

CAFs exist as different sub-populations with pro- or anti-tumor properties (74). The scRNASeq analysis revealed that different sub-population of hLFs were present in VMOC with some of them displaying transcriptomic signatures close to the previously described myCAFs and particularly ECM-myCAF populations (75). Addition of PM cells did not drastically impact fibroblasts heterogeneity; however, a decrease of ISG expression was observed that could explain their infection by MV in VMOC. Indeed, in 2D monoculture conditions, hLF infection was very poor (Figure S16) due to a functional type I interferon response, as we previously observed with another type of human lung fibroblasts, CCD-19Lu (6). A reduction in basal antiviral responses in CAFs was already described and attributed to tumor-derived TGF- β signaling (55). PM cells are known to produce TGF- β (76,77), and in our model, all PM cell states showed the highest outgoing TGF- β signaling activity, indicating that they are the main source of TGF- β signals toward endothelial and fibroblast populations (Figure S7). Thus, as for fibroblasts, a decrease in the IFN-I pathway transcriptomic signature in ECs was also observed after the addition of PM cells in VMOC. Moreover, at the level of the subpopulations, addition of PM cells led to the disappearance of the EC-interferon subpopulation. However, the consequence for EC infection is less evident. One explanation could be that PM cells are in

closer proximity to fibroblasts than to ECs in VMOC, which may also result in higher IFN-I pathway inhibition in fibroblasts. A modification of EC activation was already observed in 2D co-culture with lung cancer cells, in complex 3D models or at the level of the subpopulation, but not in the context of PM (25,65,78). These consequences for ECs and fibroblasts could be different depending on the PM cells used, as it was already shown with lung cancer cells (25,65). These data highlight the importance of complex co-culture models for studying oncolytic virus activity, as they enable the integration of intercellular signaling that influence viral susceptibility.

As expected, an induction of the expression of genes coding for type I interferon, *IFNB1*, and of a high number of ISGs was obtained after MV infection, including genes coding for chemotactic mediators (CXCL10, CXCL11), viral RNA sensors (DDX58, DHX58, IFIH1), translation inhibitors (IFIT1/2/3/5), RNA-degradation enzymes (OAS1/2/3, OASL), and restriction factors (Mx1/2, BST2, RSAD2, IFI6/27). This ISG induction is consistent with our previous observations in monocultures of PM cells (10) and in co-culture systems with myeloid cells (17). The mechanisms are well-described and involve innate immune sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) that detect viral RNA in cells (79). In addition, a strong induction of inflammation was obtained after MV infection, with notably a dramatic increase in CXCL10/IP-10, a chemokine involved in the recruitment of immune cells in the TME. Induction of IP-10 is frequently observed after virus infection and has also been reported in complex co-culture models (21,22). This increase in IP-10 is at least partly due to infected PM cells, as we have already demonstrated (10,17,80). An activation of endothelial cells was also observed, with the induction of the expression of genes coding for proteins involved in the adherence and rolling of circulating immune cells. Similar transcriptional signatures have been reported in an MVN-

on-chip system exposed to SARS-CoV-2 (21). In addition, genes coding for proteins involved in antigen presentation were also induced in the VMOC. Altogether, these effects should contribute to the recruitment of immune cells and promote an anti-tumor immune response. In support of this, a recent vascularized skin-on-chip model showed that IL-8 induction following herpes simplex virus (HSV) infection drove neutrophil migration from the vascular channel toward infected keratinocytes, while IL-8 neutralization reduced vascular adhesion and transendothelial migration (81). Given that IL-8 is strongly induced after MV infection in the VMOC model, recruitment of neutrophils could be expected if they are introduced into our vascular compartment. However, an increase in the expression of genes coding for proteins involved in the inhibition of immune responses, such as *CD274* (PD-L1), *CD47*, and *HLA-E*, were also observed. This suggests that combination with immune checkpoint inhibitors (ICIs) may improve the anti-tumor immune effects of MV. Anti-PD-1 blocking antibodies are particularly relevant in this context, as they already constitute the first line therapy in PM in combination with an anti-CTLA-4 (1). Moreover, activation of RIG-I and/or MDA5 has been reported to improve ICIs efficiency, including anti-PD-1, thus reinforcing this hypothesis (82,83).

As mentioned above, though limited infection was observed, the endothelial compartment in the VMOC was affected by MV and led to the induction of genes coding for proteins involved in immune cell recruitment. However, an alteration of vessels was also observed, in particular in parental vessels and with the condition 2×10^6 viral particles (Figure S10C). Vascular network-on-chip models infected with H1N1 or SARS-CoV-2 had already shown structural disruption despite low levels of infection (21,22). In the latter study, the authors reported that paracrine type I IFN signaling from infected organoids co-cultured with the MVN mediated endothelial disruption without direct infection, which may explain our observation.

Indeed, a strong activation of the type I interferon pathway was observed after MV infection in the VMOC, in addition to severe inflammation, which together could be comparable to a cytokine storm as proposed by the authors using SARS-CoV-2 or H1N1 viruses (21,22).

Recently, vascularized models of PM-on-chip were used to assess the response to CAR-T cell using patient-derived PM samples (84). Building on this approach, the VMOC model could potentially be adapted at a translational level to evaluate therapeutic responses and combination strategies. In addition, VMOC could be useful to decipher resistance mechanisms to therapy and/or the contribution of the cells from the TME in the response to treatment, given its tunable cellular composition. The VMOC could also be interesting for the study of intercellular communication within the TME in a controlled and more integrated setting. This is supported by our observation that the incorporation of PM cells into the MVN modified cellular heterogeneity and transcriptional profiles.

In conclusion, the VMOC model provides a platform to study the response to oncolytic viruses across multiple components of the TME, including tumor, vascular, and stromal compartments. The consequences of virus infection in the model are consistent with known antiviral and inflammatory responses to MV and other viruses. This model may therefore serve as a useful platform for investigating resistance mechanisms and evaluating novel therapeutic combinations, including oncolytic viruses, to improve antitumor efficacy.

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1159

1160 **Author contributions**

1161 NR, JF, MW, and HP performed experiments; OH and BVS analyzed vessel permeability; ED,
1162 MD, and NR performed scRNA-Seq experiments; LT performed scRNA-Seq analysis; LT and IC
1163 provided a strong expertise on endothelial cell to analyze and interpret the data; NB, FT and
1164 JFF provided a strong expertise on oncolytic viruses to design experiments, analyze and
1165 interpret the data; NR, DF, NB, JFF, IC, LT, BVS and CB interpreted the study and provided
1166 critical review; CB, LT and BVS conceived, designed, supervised, analyzed, interpreted the
1167 study. NR, BVS, LT and CB wrote the manuscript. All authors reviewed and approved the
1168 manuscript.

1169

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1173

1174 **Data availability**

1175 The datasets used and/or analyzed during the current study are available from the
1176 corresponding authors on reasonable request. scRNA-seq and 3'RNA-seq datasets generated

1177 in this study are available for reviewing purposes at the following URL: <https://uncloud.univ->
1178 [nantes.fr/index.php/s/GEMcYYZjwinDNH3](https://uncloud.univ-nantes.fr/index.php/s/GEMcYYZjwinDNH3). Datasets will be deposited on Zenodo once
1179 accepted for publication. For any additional inquiries or requests, interested parties are
1180 encouraged to contact the corresponding author.

1181

1182 **Declarations**

1183 **Ethics approval and consent to participate**

1184 Meso13 cancer cell line was established from the pleural fluid of a mesothelioma patient.
1185 Meso13 cell line belongs to a biocollection of samples, from patients from Nantes University
1186 Hospital, collected in accordance with the standards established by the Declaration of
1187 Helsinki. Recruited patients had received no prior anticancer therapies and gave signed
1188 informed consent. All the collected samples and the associated clinical information were
1189 registered in a database (DC-2017-2987) validated by the French Ministry of Research. Our
1190 study was approved by a local independent ethical committee (CPP Ouest-IV-Nantes).

1191

1192 **Consent for publication**

1193 Not applicable.

1194

1195 **Competing interests**

1196 The authors declare no competing interests.

Figure legends

Figure. 1: Characterization of parental vessels (PVs) generated in lateral media channels. A)

Schematic diagram of the idenTX microfluidic chip from AIM Biotech. The device comprises three parallel microchannels. The central gel channel is flanked by two lateral media channels. All channels are approximately 10.5 mm long and 0.25 mm high. The central channel is separated from the lateral media channels by arrays of trapezoidal micropillars. The chip contains six inlets: four inlets connected to reservoirs feeding the two lateral media channels (two per channel) and two inlets connected to the central gel channel without reservoirs. **B)** Schematic of the procedure used to generate PVs in the microfluidic chip. The central chamber was filled with fibrin gel. The two lateral channels were coated with collagen and sequentially seeded four times with 10 μ L of HUVECs (3×10^6 cells/mL). PVs were generated within 4 days. **C)** Brightfield microscopy image of PVs formed in the lateral channel with fibrin gel in the central channel. **D-F)** Representative confocal microscopy images showing in lateral channel planar and cross-sectional views of HUVECs stained for CD31 (**D**), VE-cadherin (**E**) or ZO-1 (**F**). Purple: Nuclei.

Figure 2: Development of tumor-associated microvascular network (MVN) connected to

parental vessels (PV) in the chip. A) Schematic of the procedure used to obtain the full model using a two-step method to form an MVN composed of HUVECs (ECs), human lung fibroblast (hLFs), and Meso13-BFP cells, connected to two PVs composed of ECs. The two-step method consists of injecting ECs into the first fibrin gel from the left inlet and immediately aspirating it, leaving a thin layer of cells trapped between the micropillars. A second fibrin gel layer is then seeded from the right inlet by adding a mixed suspension of ECs, hLFs, and Meso13-BFP cells. After complete polymerization of the gel, the PVs are formed by seeding ECs in the two

lateral channels. **B)** Confocal images showing (i) VE-cadherin immunostaining (grey) (ii) Meso13-BFP (red), and (iii) a merged image with nuclei (purple). (iv) Orthogonal view of the chip. Scale bar = 500 μm . **C)** Magnified representation of the MVN from panel B). Scale bar = 200 μm . **D)** Representative confocal microscopy images of the MVN after CD31 and ZO-1 labeling (green). Scale bar = 100 μm . Red: Meso13-BFP; purple: nuclei.

Figure 3: Characterization of the microvascular network (MVN) functionality by 3D reconstruction and perfusion. ECs-mRuby2, Meso13-BFP, and hLFs were used to constitute the VMOC. **A)** 3D Imaris reconstruction of the MVN from confocal imaging. (i) Maximum intensity projection showing the MVN. (ii) Planar and (iii) transversal views of the area defined by the yellow rectangle in (i). White: VE-cadherin; red: Meso13-BFP. Scale bar = 50 μm . **B)** Confocal microscopy images and orthogonal projections showing 1 μm green fluorescent polystyrene beads within a vessel of the MVN. (ii) Orthogonal projection of a 3D stack and (iii) 3D image reconstruction of the white square in (i) showing microbeads in the MVN. **C)** Diffusion of microbeads in the VMOC over time after injection into one parental vessel. Scale bar = 200 μm . **D)** Confocal microscopy images. Left panel: structure of the MVN in the central channel. Grey: HUVECs-mRuby2; red: Meso13-BFP. Right panel: diffusion of 70 kDa FITC-dextran (green).

Figure 4. Characterization of the VMOC by single-cell RNA sequencing. **A)** UMAP representation of cell types from the VMOC following scRNA-sequencing (n = 2), including pleural mesothelioma (PM) tumor cells, fibroblast-like (mesenchymal) cells and ECs, **B)** Feature plots of all cell types in VMOC with different marker gene signature, identifying ECs (*CDH5*, *CLDN5*), fibroblast-like/mesenchymal (*COL1A1*, *TIMP1*), and PM cells (*KRT8*, *MSLN*). **C)**

t-SNE projection of PM cells showing segregation into transcriptionally distinct states. **D)** UMAP projections of ECs from VMOC conditions with and without PM cells (Meso and No_Meso, respectively; n = 2). **E)** Dot plot showing the expression of selected marker genes across EC subsets. Dot size represents the percentage of cells expressing each gene, and color intensity indicates the average expression level. **F)** Enrichment plots of hallmark interferon- α / γ response gene sets in ECs from VMOC conditions with PM cells compared to conditions without PM cells. **G)** UMAP representation of fibroblasts from the VMOC showing clustering into distinct transcriptional states and **H)** marker genes for CAF subtypes (*S100A4*, *FAP*, *ANTXR1*, *SDC1*, and *LAMP5*). Scatter plot (bottom left) shows the relationship between *FAP* expression and the average expression of *ANTXR1* and *SDC1* for Fibro_ECM_myCAF subtype. **I)** Enrichment plots of hallmark interferon- α / γ response gene sets in fibroblasts from VMOC conditions with PM cells compared to conditions without PM cells. **J)** Feature plots of fibroblasts with marker genes for EC identity and endothelial-to-mesenchymal transition (EndMT). **K)** UMAP and pseudotime representation of EC and fibroblast populations. Cells are colored by annotated transcriptional states (left panel) and by pseudotime values (right panel), with the inferred trajectory overlaid.

Figure 5: MV administration in the microfluidic chip induces tumor cell infection and death.

A) Schematic of the MV infection protocol in the chip. 1×10^6 MV-GFP particles were injected into a PV on Day 5 of the MVN formation. After 6h, MV-GFP was removed, and analyses were performed at 24h, 48h and/or 72h post-infection. **B)** Representative confocal microscopy images of MV-GFP infection at 48h post-infection. Green: Infected cells; red: Meso13 BFP; yellow: Infected Meso13; grey: HUVEC. **C)** Luciferase release from Meso13-Luc cells after MV-

GFP infection. Supernatants were collected at 24 and 48 h post-infection and luciferase activity was measured. n=5, * $p<0.05$, Unpaired t -test (two-tailed).

Figure 6: MV infection induces modification of the TME at the transcriptomic and proteomic level. Chips were infected by administration of 1×10^6 MV particles in the lateral channel on day 5 of the MVN formation. mRNA of the whole chips was extracted at 24h and 48h post-infection and analyzed using 3'RNA-seq. **A)** Volcano plots of differentially expressed genes between 24h or 48h post MV-infection vs non treated (NT). Red: $p \leq 0.01$; Grey: $p > 0.01$. Dotted lines correspond to fold change equal to 2 or -2. **B)** GSEA Pathway enrichment analysis. **C)** Heatmaps of the expression of inflammatory-related genes and **D)** hallmark genes of response, resistance, and toxicity to immune checkpoint blockade. **E)** Heatmap of cytokine concentrations measured in VMOC samples collected at 48h post-infection using Legendplex Assay. Representations were obtained using log10 values. n=3, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Unpaired t -test (two-tailed).

Figure 7: Comparison of the effect of oncolytic virus in patients and in VMOC: A) Correlation heatmap of NanoString data obtained from pleural mesothelioma (PM) patients treated or not with Olvi-vec. 3 groups of patients were identified: non-treated (NT, n=4), Olvi-vec treated without inflammatory response induced (T no-inflam, n=6) and treated with an induced inflammatory response (T inflam, n=7). **B)** Volcano plots of differentially expressed genes between NT and T inflam groups of PM patients. Red: $p \leq 0.01$; Grey: $p > 0.01$ padj. Dotted lines correspond to fold change equal to 2 or -2. **C)** Estimation of relative immune cell subtype proportion from the in PM patients using CIBERSORTx. p value from Kruskal-Wallis, Benjamini & Hochberg FDR correction. **D)** Venn diagram representing the number of genes commonly

deregulated across VMOC at 24h and 48h after MV infection and T-inflam group of PM patients. **E)** GSEA Pathway enrichment analysis of the 55 congruent genes from D). **F)** Meta-analysis of induced-genes in VMOC at 24h or 48h post MV infection and in T inflam PM patients. **G)** Table of cytokines induced in VMOC 24h, pleural fluids (24h and 48h post Olvi-vec) and blood (72h post Olvi-vec). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Supplementary figure legends

Figure S1: Characterization of the permeability and response to stimuli of the parental vessels (PV). **A)** Permeability measurement of PV. (i) Representative fluorescence image of 70 kDa FITC–dextran diffusion in the chip. Donor voxel in PV and receiver voxel in gel (white dashed squares) indicate the regions used for fluorescence intensity quantification. Voxel size = $50 \times 50 \times 250 \mu\text{m}$. The area between voxels (orange line) defines the diffusion path across the barrier. (ii) Equation used to calculate the permeability coefficient (P_d , cm/s), as previously described by Tu *et al.* (29). Fixed parameters for the equation were: t_1 (time point 1) = 60 s, t_2 (time point 2) = 300 s, V_{receiver} (receiver voxel volume) = $6.25 \times 10^{-7} \text{ cm}^3$, A_{barrier} (barrier area between donor and receiver) = $1.25 \times 10^{-4} \text{ cm}^2$, $V_{\text{receiver}}/A_{\text{barrier}} = 0.005 \text{ cm}$. $I_{t1 \text{ donor}}$, $I_{t2 \text{ donor}}$, $I_{t1 \text{ receiver}}$ and $I_{t2 \text{ receiver}}$ represent fluorescence intensities measured in donor and receiver voxels at t_1 and t_2 . (iii) Permeability coefficient (P_d , cm/s) measured under three conditions: absence of PV (-PV), presence of PV (+PV) and PV with Ang-1 treatment for 24h (+PV+Ang-1) (R&D Systems, cat. no. 923-AN/CF). Bars represent mean $\pm$ standard deviation. $n = 2$ devices (-PV), $n = 3$ (+PV), and $n = 1$ (+PV + Ang-1). **B)** Response of PVs to the application of a flow of medium for 24h. Upper panels: Optic microscopy images; lower panel: Confocal microscopy images. Green, VE-cadherin; purple, nucleus.

Figure S2: Optimization of the microvascular network (MVN) by the one-step method. A)

Schematic of MVN formation in the microfluidic chip. **B)** Representative confocal microscopy images showing MVN obtained using 8, 10, 12 and 15×10^6 HUVECs/mL (upper panels) and after 70 kDa FITC-dextran injection in PVs. Grey, HUVECs mRuby; Green, 70 kDa FITC dextran. Scale bar = 200 μ m. **C)** Graph showing average lumen size of the microvessels in the MVN obtained with various HUVECs quantity. Horizontal bars represent mean $\pm$ SEM (n=2 chips, 3 ROIs of 1272×1272 μ m per chip). **D)** Lumen size distribution of microvessels in the MVN. Graph represents means $\pm$ SEM (n=2, 3 ROIs of 1272×1272 μ m for each chip). **E)** Percentage of open bridging vessels obtained with different quantities of HUVECs loaded in the central channel. Bars represent mean $\pm$ SEM. (n=2 chips, 1 ROI of 1767×8134 μ m per chip).

Figure S3: Two-step method for generating a perfusable microvascular network (MVN)

connected to parental vessels (PV). **A)** Schematic of the two-step method. The experimental procedure consists of injecting a first layer of HUVECs (15×10^6 cells/mL) to promote the formation of bridging vessels, followed by injection of a second layer of HUVECs (10×10^6 cells/mL) with human lung fibroblasts (hLFs; 1×10^6 cells) to induce MVN formation. Ultimately, parental vessels were generated in both lateral media channels using HUVECs (3×10^6 cells/mL). **B)** Representative confocal microscopy images showing MVNs generated using the two-step method (left panels) and after 70 kDa FITC-dextran injection into PVs (Right panels). Grey, HUVECs mRuby; Green, FITC dextran 70 kDa. Scale bar = 200 μ m. **C)** Graph showing the average microvessel lumen size obtained using the two-step method with varying hLF numbers. Graph represents means $\pm$ SEM. n=3 chips, 3 ROIs of 1272×1272 μ m for each chip. **p<0.01; *p<0.05. Unpaired t-test (two-tailed). **D)** Microvessel lumen size

distribution within the MVN obtained using the two-step method. Graph represents mean $\pm$ SEM. n=3 chips, 3 ROIs of 1272x1272 μ m for each chip.

Figure S4: Characterization of the microvascular network (MVN) obtained using the two-step method. A) Microscopy images showing the evolution of MVN formation over 10 days. Grey, HUVECs mRuby. Scale bar = 500 μ m. **B)** Representative confocal microscopy images of the MVN showing VE-cadherin, CD31, and ZO-1 immunostaining (green) on day 5. Purple, nucleus.

Figure S5: Transcriptional heterogeneity and functional characterization of pleural mesothelioma (PM) cells in the VMOC model. A) UMAP representation of all cells in the VMOC with (Meso, n=2) or without (No_Meso, n=2) PM cells showing overall clustering structure without cell type annotation. **B)** t-SNE projection of PM cells subtypes across two biological replicates (Meso_1 and Meso_2). **C)** Relative proportion of Stress, Proliferative, and OXPHOS cancer cell subtypes across the two biological replicates (Meso_1 and Meso_2). **D-F)** Gene set enrichment analysis across PM cell transcriptional states (Proliferative, OXPHOS, and Stress), showing normalized enrichment scores (NES), with each state compared against all other cancer cell states. **G)** Heatmap of interferon IFN- α response genes across PM cell subtypes.

Figure S6: Ligand-receptor interaction analysis in the VMOC model. A) TGF- β and **B)** Notch signaling-inferred cell-cell communication across cell populations. i) Chord diagram predicting cell-cell communication through the mentioned signaling pathways in all subclusters identified in the VMOC model. The inner bar colors represent the targets that receive signals

from the corresponding outer bar. ii) Heatmaps for selected signaling pathways. The top colored bar plot represents the sum of columns of values displayed in the heatmap. The right colored bar plot represents the sum of rows of values. The blue scale represents the interaction probability between each subcluster considered. iii) Relative contribution of each ligand–receptor pair to overall signaling activity.

Figure S7: Transcriptional heterogeneity and functional characterization of endothelial cells in the VMOC model. **A)** UMAP projection of endothelial cells (ECs) showing distinct transcriptional states, including EC_Interferon, EC_Artery, EC_Inflam, EC_Mesenchymal, and EC_Proliferative, in four VMOC, including with (n=2) and without PM (n=2). **B)** Relative proportion of EC states across VMOCs with PM cells (Meso_1, Meso_2) and without PM cells (No_Meso_1, and No_Meso_2), highlighting shifts in endothelial composition in the presence or absence of PM cells. **C-D)** Gene set enrichment analysis in the two EC states (EC_Proliferative and EC_Mesenchymal) under No_Meso conditions, with each EC cluster compared against all other EC clusters. **E)** Feature plots of EC population with marker genes for proliferative (*MKI67*, *TOP2A*) and activated EC states (*PTX3*, *ESM1*). **F)** Reactome pathway enrichment analysis of the EC_Interferon state, with pathways enriched relative to all other EC clusters. **H)** Heatmap of interferon-stimulated gene induced across all EC states under the Meso condition.

Figure S8: Fibroblast heterogeneity and trajectory analysis in the VMOC model. **A)** UMAP projection of fibroblast showing distinct transcriptional states, in four VMOC, including with (n=2) and without PM cells (n=2). **B)** Relative proportion of fibroblasts cell states across VMOCs with PM cells (Meso_1, Meso_2) and without PM cells (No_Meso_1, and

1387 No_Meso_2). **C)** Gene set analysis of fibroblast populations in the Meso condition relative to
1388 No_Meso. **D)** UMAP projections showing endothelial and fibroblast populations across Meso
1389 and No_Meso replicates. **E)** Integrated UMAP of all cells colored by annotated cell states.
1390 Pseudotime analysis showing inferred cellular progression across the integrated cell
1391 population.

1392

1393 **Figure S9: Evaluation of MV infection after administration in the central channel. A)** (i)
1394 UMAP projection of endothelial cells (ECs) subclusters identified in the VMOC model and
1395 biopsies from patients with PM (GSE190597). (ii) Dot plot showing the expression of selected
1396 marker genes across EC subsets. Dot size represents the percentage of cells expressing each
1397 gene, and color intensity indicates the average expression level. (iii) Dendrogram depicting
1398 similarities between VMOC and PM using top marker genes from each cell subcluster. **B)** (i)
1399 UMAP projection of fibroblast subclusters identified in the VMOC model and biopsies from
1400 patients with PM. (ii) Dendrogram depicting similarities between VMOC and PM using top
1401 marker genes from each cell subcluster.

1402

1403 **Figure S10: Evaluation of MV infection after administration in the central channel. A)**
1404 Representative confocal microscopy images of MV-GFP infection on Day 6 after
1405 administration of MV directly in the central channel at the time of gel loading on day 1 and at
1406 different multiplicity of infection (MOI). **B)** Representative confocal microscopy images of
1407 infection on Day 6. Meso13 cells were pre-infected with MV-GFP in 2D for 2 h at MOI 1 prior
1408 to incorporation into the central channel during gel loading on Day 1. Green: GFP expressing
1409 cells (infected cells); red: Meso13; yellow: infected Meso13; grey: HUVECs mRuby. Scale Bar
1410 = 500 μ m.

1411

1412 **Figure S11: Optimization of MV-GFP infection parameters (dose, exposure time) via the**
1413 **lateral channel in the VMOC. A)** Representative confocal images of VMOC exposed on Day 5
1414 for 24h to varying doses of MV-GFP. Images were acquired at 48 h post-infection. Scale bar is
1415 200µm. **B)** Representative image of the system at 24h after infection by 1×10^6 TCID₅₀ units of
1416 MV-GFP. **C)** Representative confocal images of MV-GFP infection at 2×10^6 TCID₅₀ units of MV-
1417 GFP with 24h exposure on Day 5 of chip culture. Green: MV-GFP–infected cells; red: Meso13;
1418 yellow: infected Meso13; grey: HUVECs. Scale bar is 100µm.

1419

1420 **Figure S12: Transcriptional effects of MV in VMOC. A)** Venn diagram representing the
1421 number of genes commonly and specifically deregulated ($p_{adj} < 0.01$) 24h and 48h after MV
1422 infection. **B)** Pathway enrichment analysis of genes commonly deregulated 24h and 48h after
1423 MV infection.

1424

1425 **Figure S13: Cytokine concentrations measured in culture supernatants of VMOC 48h after**
1426 **MV infection.** Cytokine concentrations were measured using MultiPlex ELISA. Graphics
1427 represent mean $\pm$ SEM of 3 independent experiments. *, $p < 0.05$; ***, $p < 0.001$. Unpaired t
1428 test (two-tailed).

1429

1430 **Figure S14: Deconvolution of endothelial cell (EC) and fibroblast cell states estimated from**
1431 **bulk 3' RNA-seq data following MV infection using single cell RNA-seq derived reference**
1432 **signatures. A-B)** Relative mean proportions and **C-D)** GSVA of A,C) EC and B,D) fibroblast
1433 states estimated across conditions (Ctrl and MV-infected; lateral vessel and microvascular
1434 network (MVN) conditions at 24h and 48h).

1435

1436 **Figure S15: MV infection induces modification of the TME at the transcriptomic level. A)**

1437 Principal component analysis (PCA) of Nanostring data obtained from patients treated or not
1438 with Olvi-vec. **B-C)** Volcano plots of differentially expressed genes between non-treated (NT)
1439 and treated with no induced-inflammation (T no-inf) (**B**) or T no-inf and treated with induced-
1440 inflammation (T-inf) (**C**). Red: $p \leq 0.01$; Grey: $p > 0.05$. Dotted lines correspond to fold change
1441 equal to 2 or -2.

1442

1443 **Figure S16: Infection of hLFs, HUVECs, and Meso-13 cells in monoculture by measles virus**

1444 **(MV).** Cells were seeded at 10,000 per well in 24-well plates. and infected the following day
1445 with MV-GFP (MOI = 1). Cells were maintained in culture for 72 h. Representative brightfield
1446 images and corresponding GFP fluorescence images are shown. GFP signal indicates MV-
1447 infected cells.

1448

1449 **Supplementary movie S1. Endothelial network from Parental vessel into the microvascular**

1450 **network of the VMOC.** ECs-mRuby2, Meso13-BFP, and hLFs were used to constitute the
1451 VMOC. Acquisition of the pictures was performed on day 5 of the formation of the VMOC
1452 using confocal microscopy. The movie was generated using Imaris software. White, MVN;
1453 Red, Meso13.

1454

1455 **Supplementary movie S2. Diffusion of 1 micrometer fluorescent microbeads from one**

1456 **Parental vessel through the microvascular network into the other Parental Vessel in the**
1457 **VMOC.** ECs-mRuby2, Meso13-BFP, and hLFs were used to constitute the VMOC. Fluorescent
1458 Microspheres were injected on day 5 of the formation of the VMOC. The movie was

1459 performed using confocal time-lapse microscopy. White, MVN; Red, Meso1; Green,
1460 microbeads.

Figure 1

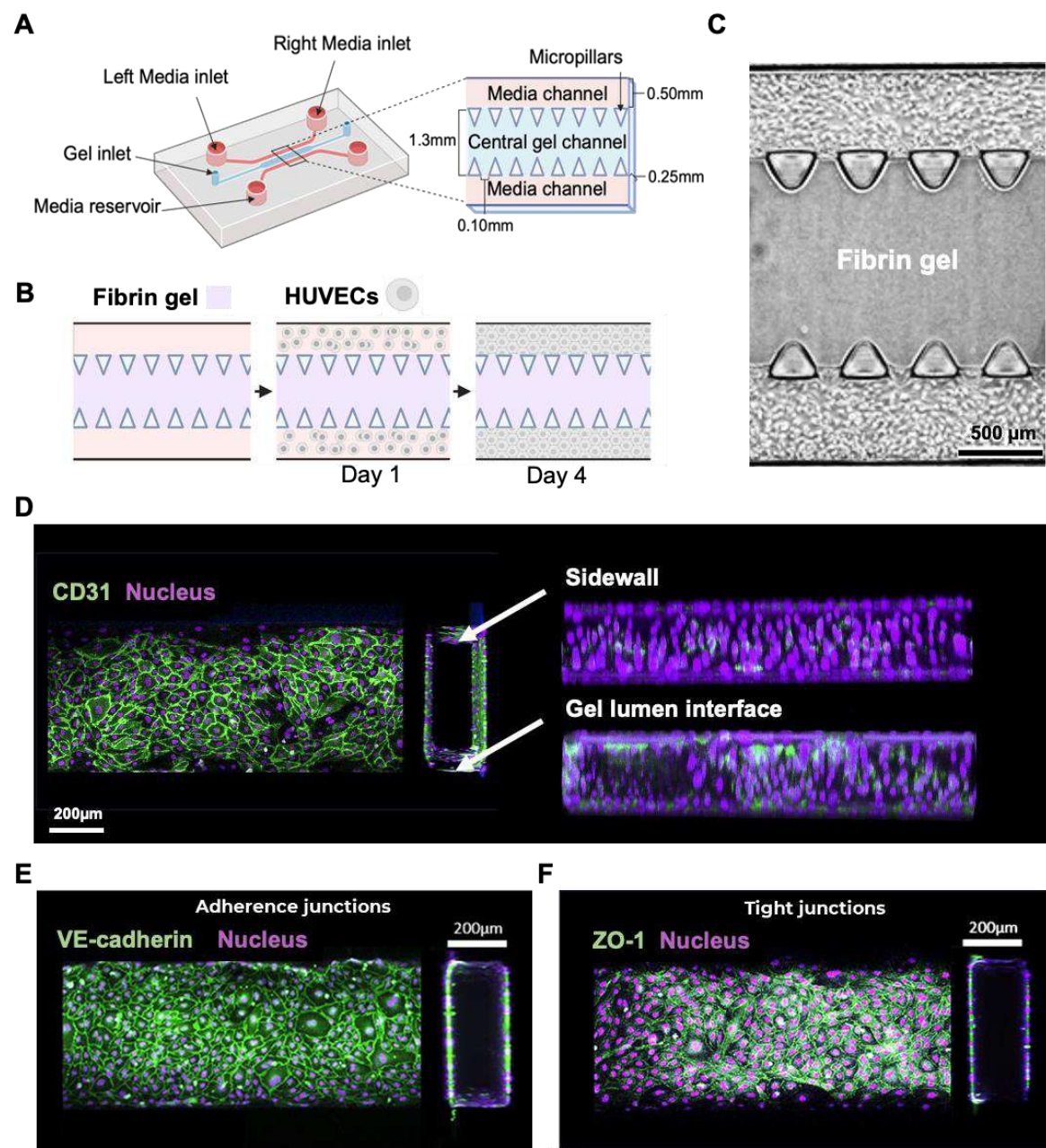
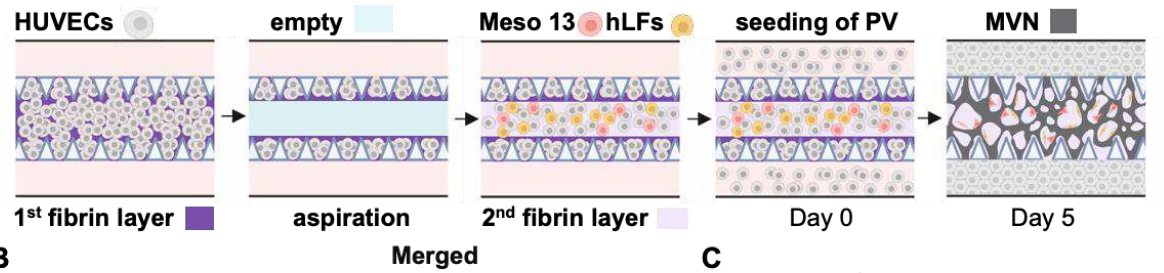
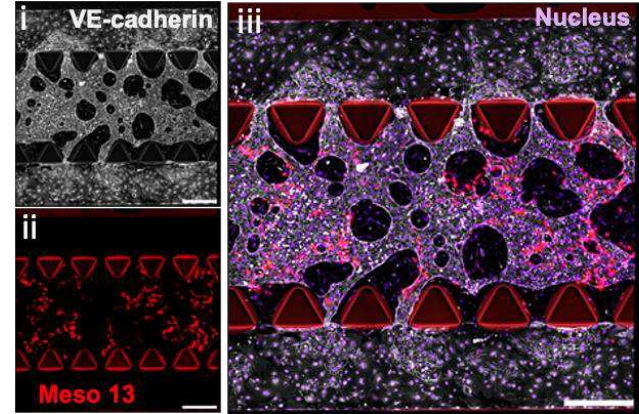


Figure 2

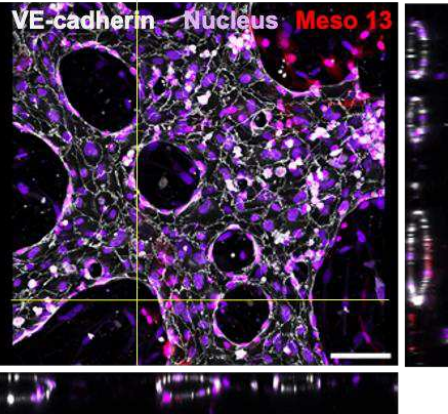
A Two-step method:



B



C



D

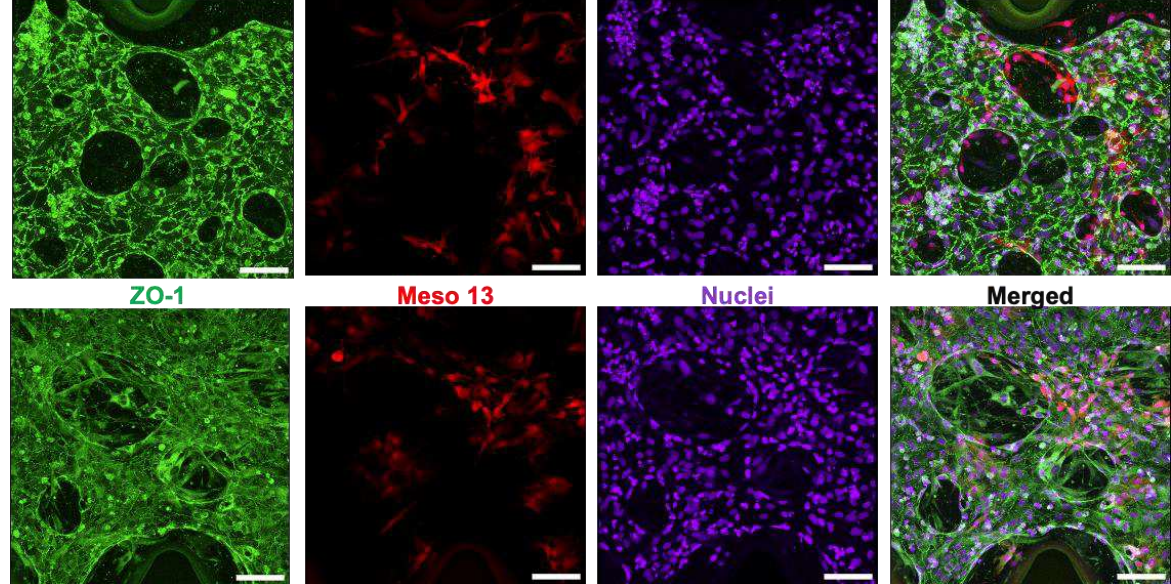
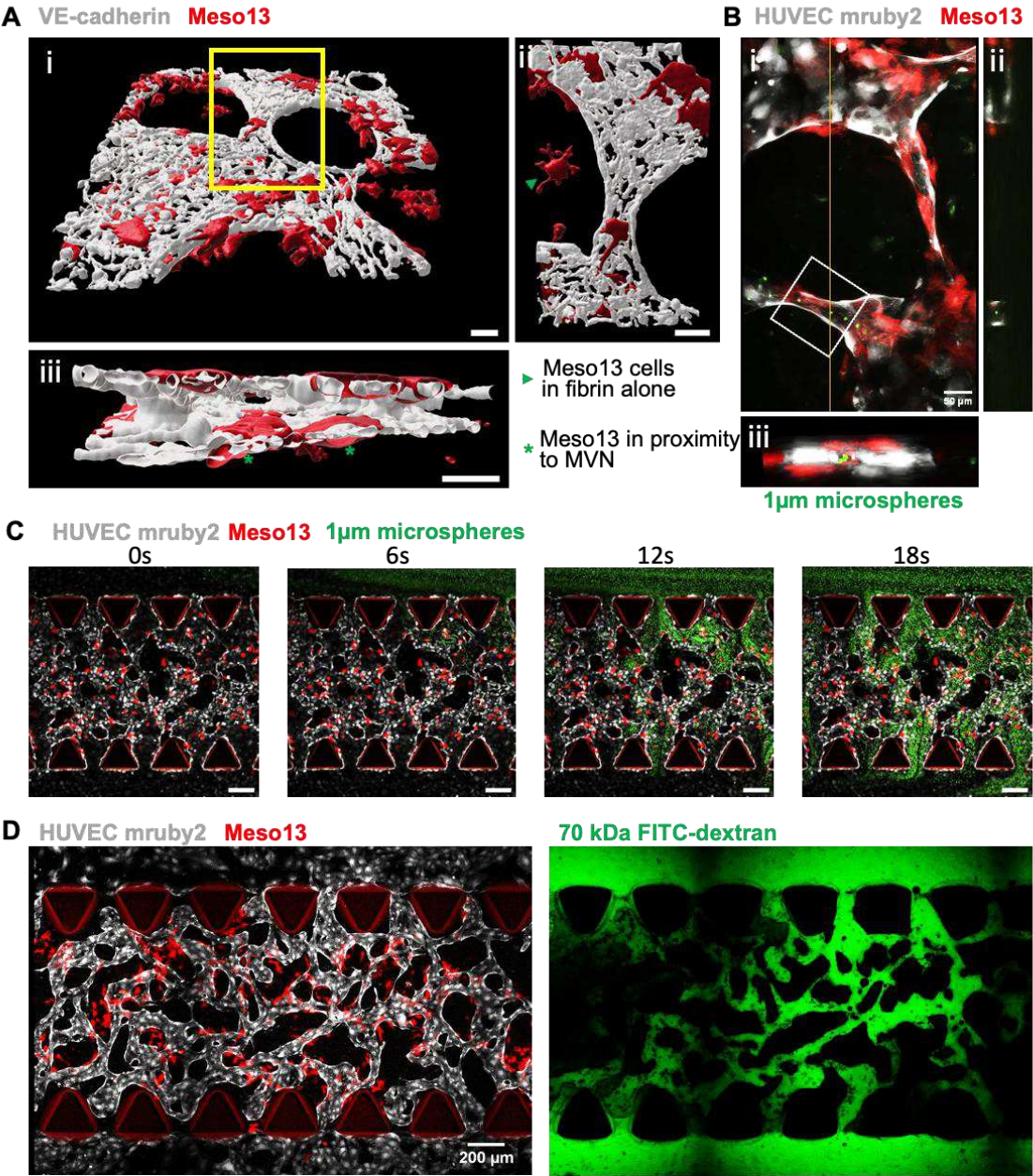


Figure 3



A

Cancer_Cell
Mesenchymal
EC

B

CDH5
COL1A1
KRT81
CLDN5
TIMP1
MSLN

C

CancerCell_Stress
CancerCell_OXPHOS
CancerCell_Proliferative

D

Meso_1
Meso_2
No_Meso_1
No_Meso_2

E

Percent Expr
Average Expr

F

Hallmark IFN- α response

G

Fibro_Proliferative
Fibro_ECM_myCAF
Fibro_myCAF
Fibro_EndMT

H

S100A4
FAP
ANTXR1
SDC1
LAMP5

I

Hallmark IFN- α response

J

VWF
ZEB1
ERG
FLI1

K

pseudotime

Figure 5

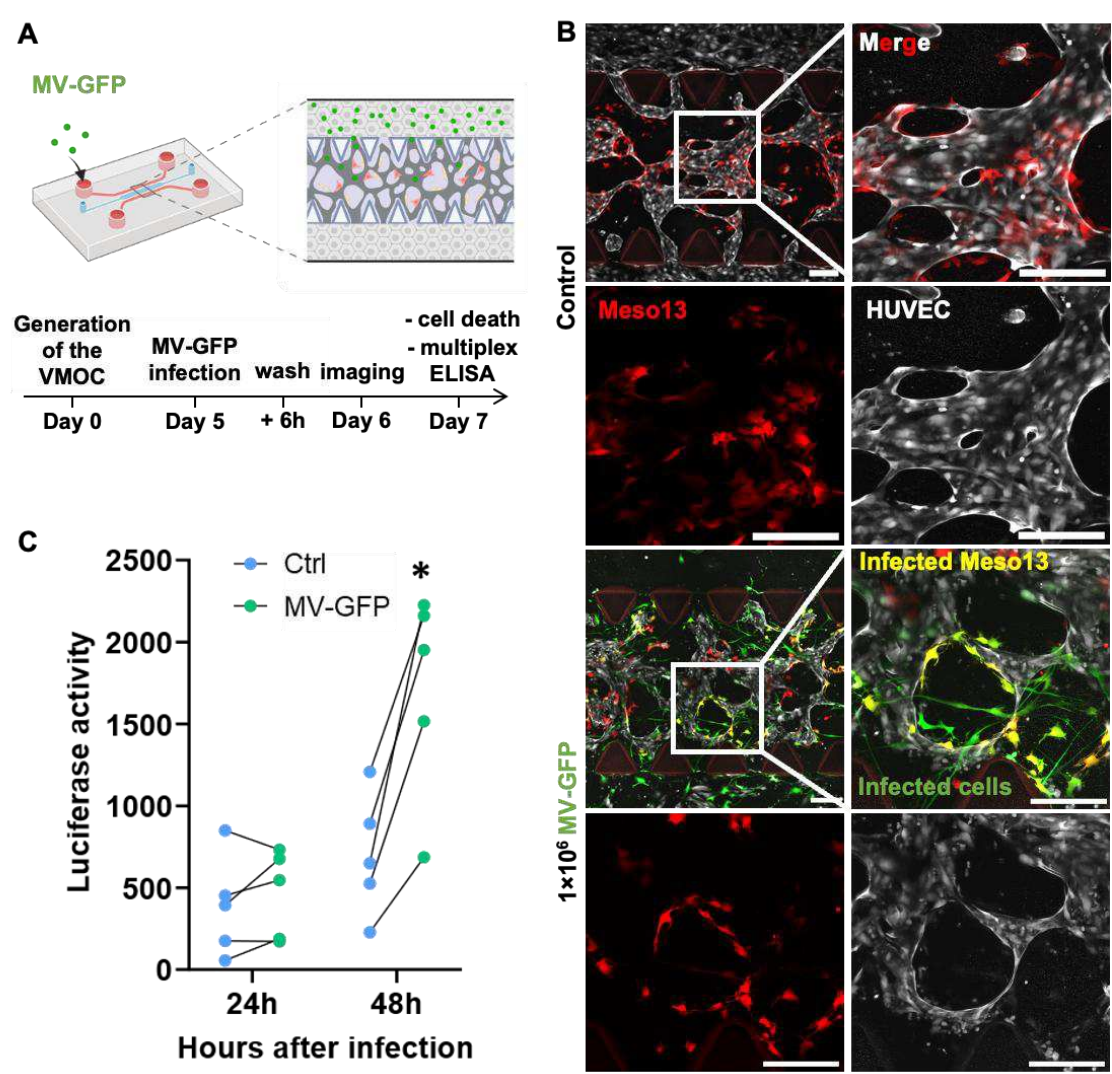
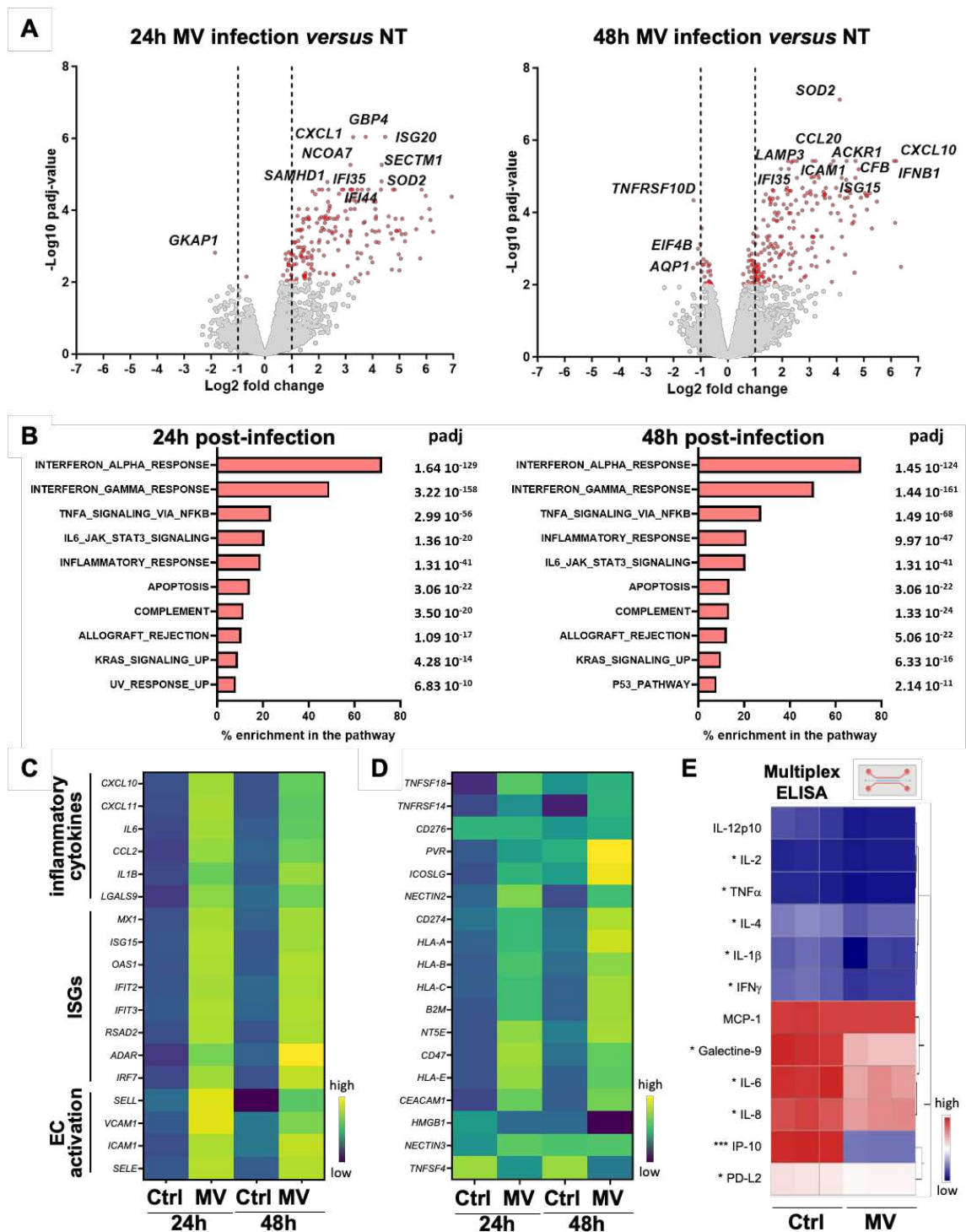


Figure 6



1466

Figure 7

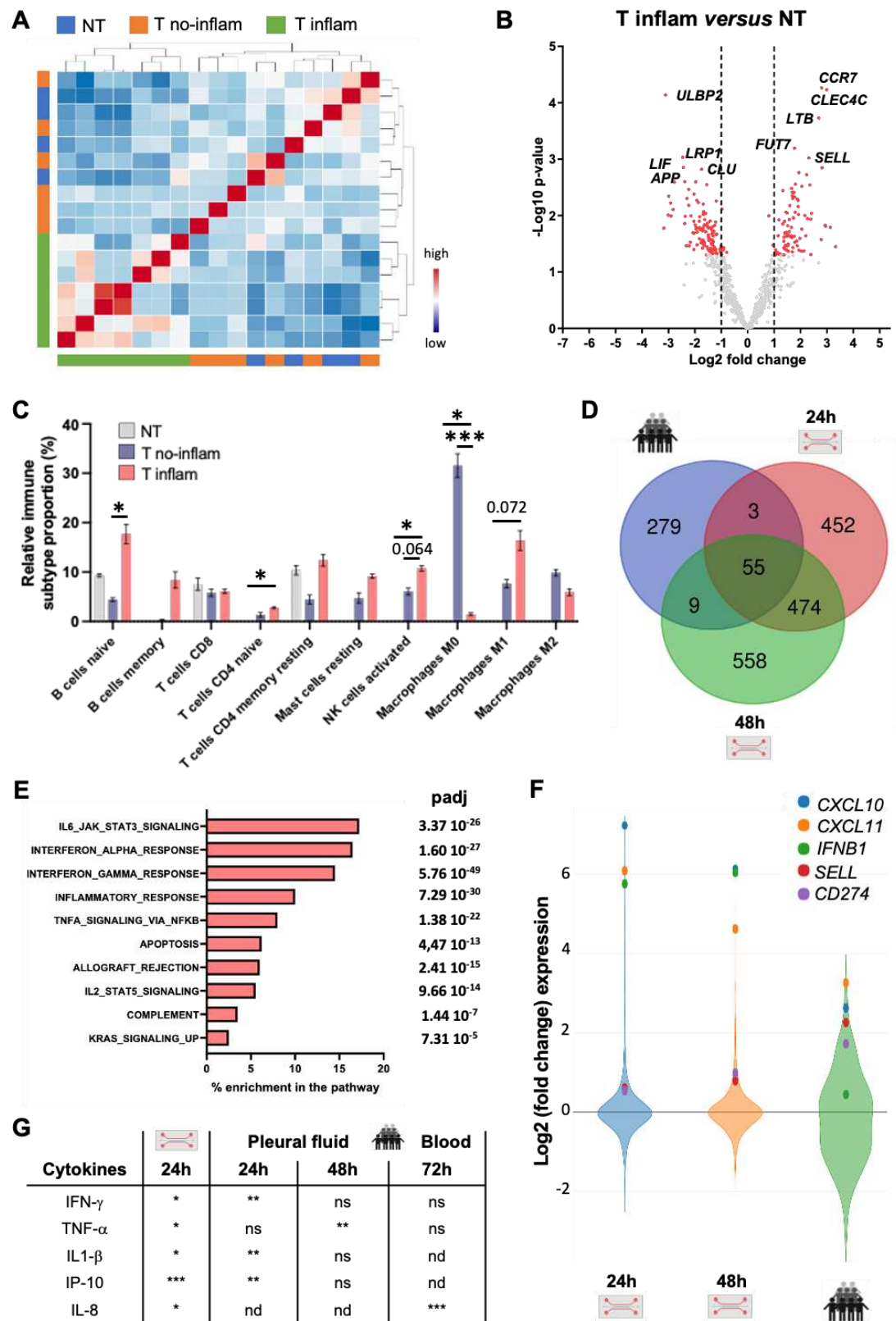


Figure S1

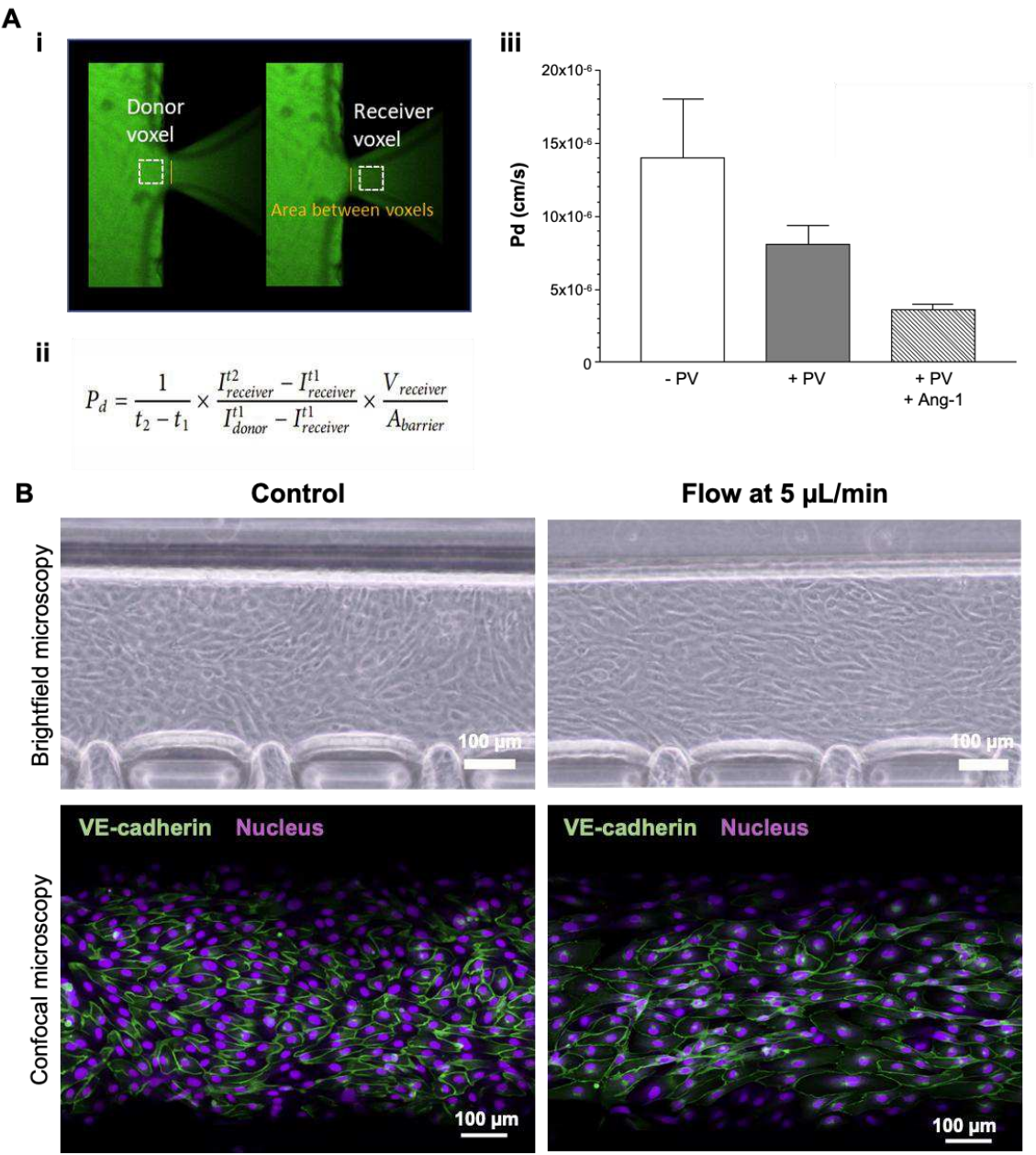
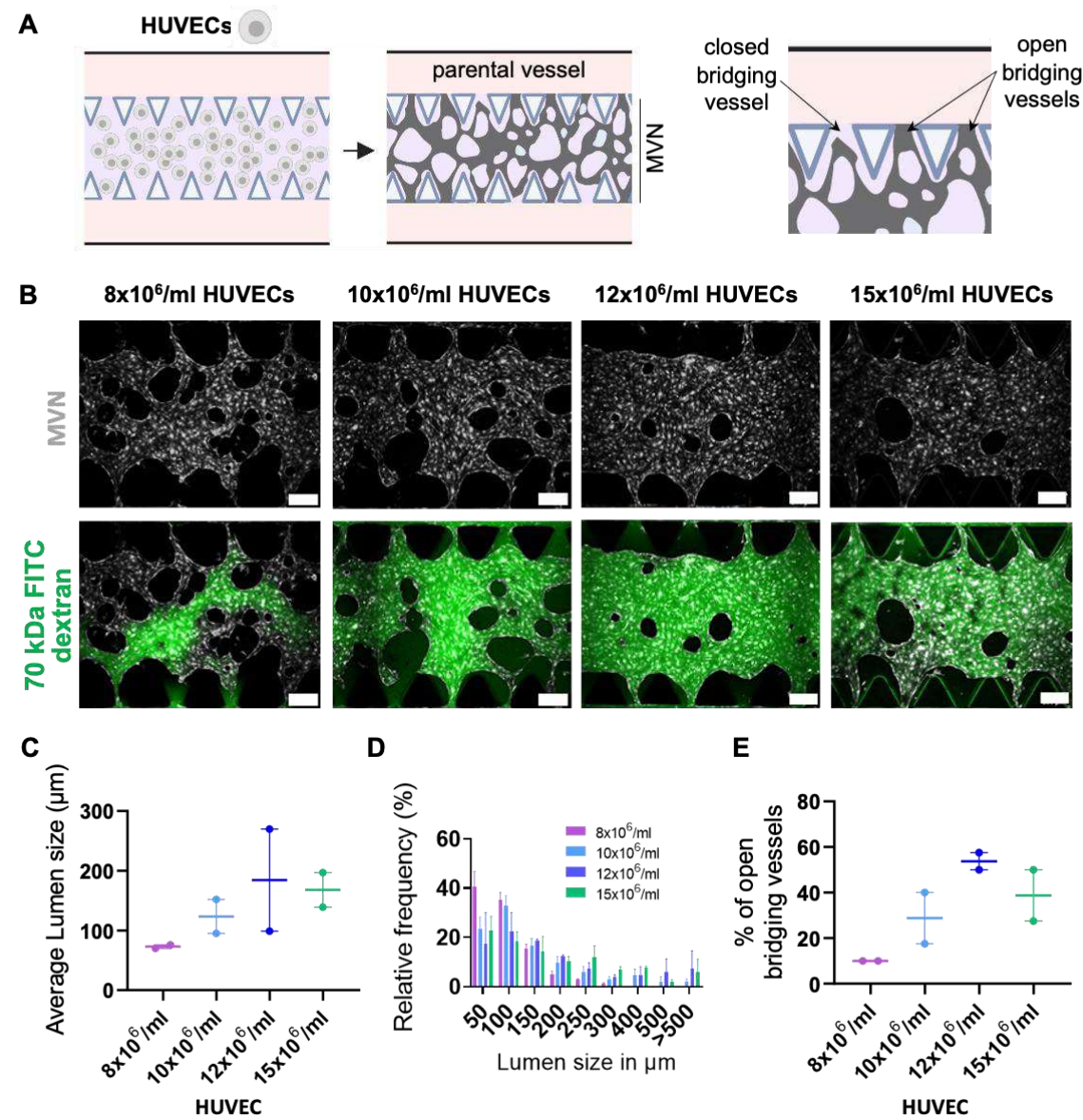


Figure S2



1469

Figure S3

A Two-step method to form the MVN:

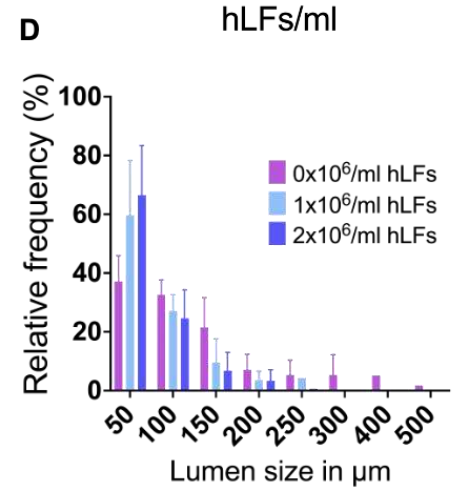
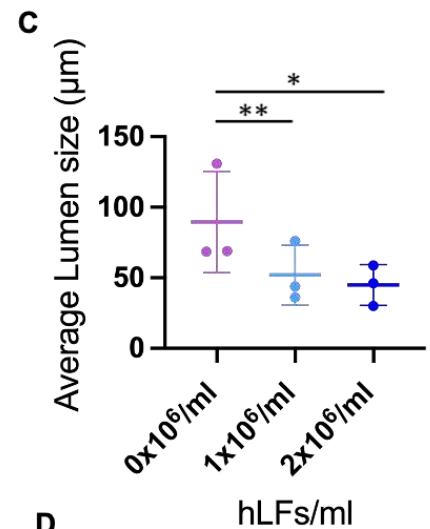
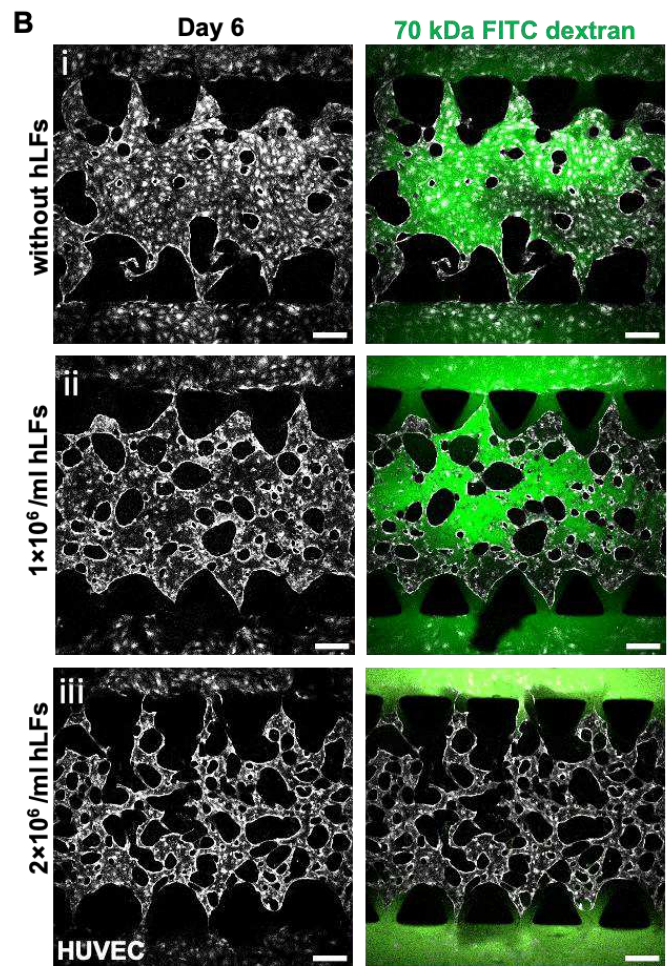
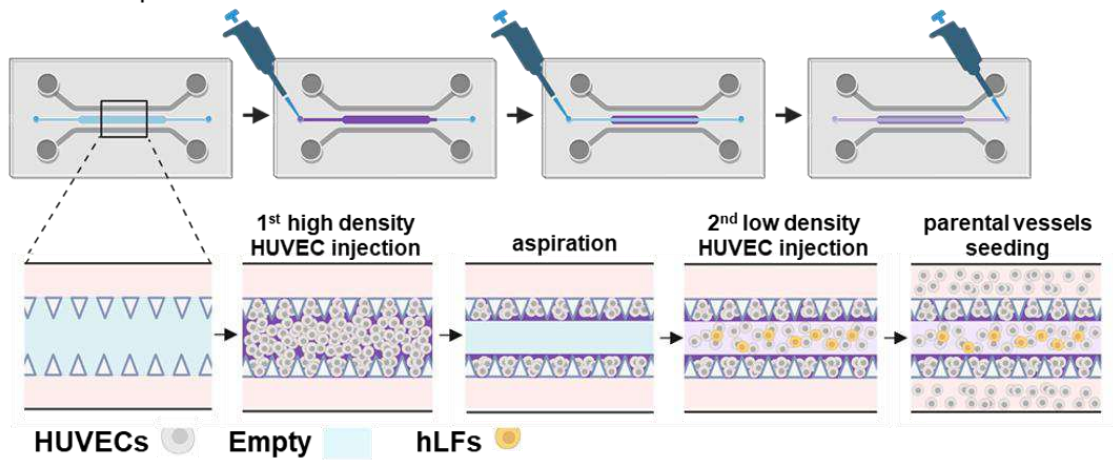


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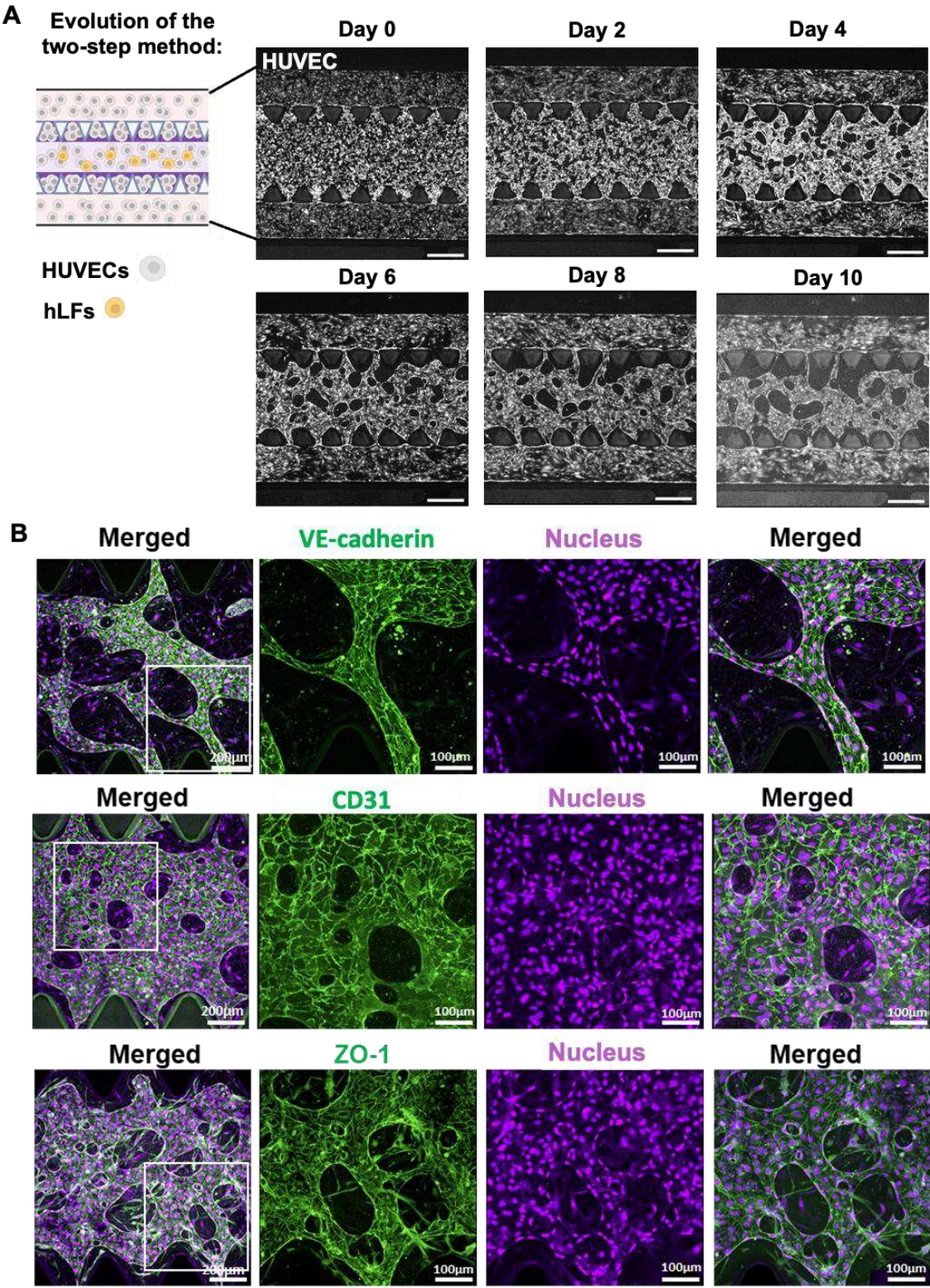
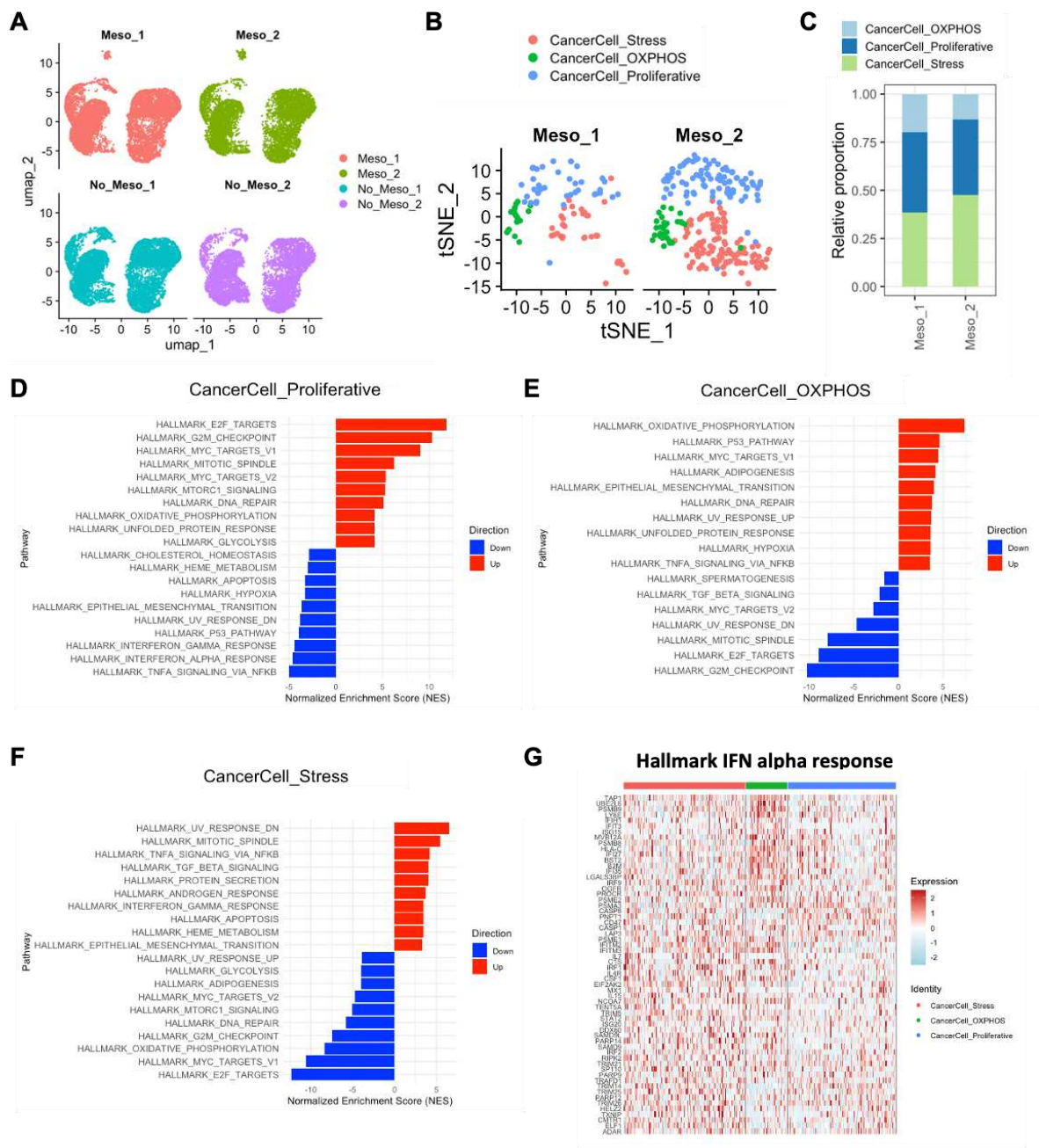


Figure S5



1472

Figure S6

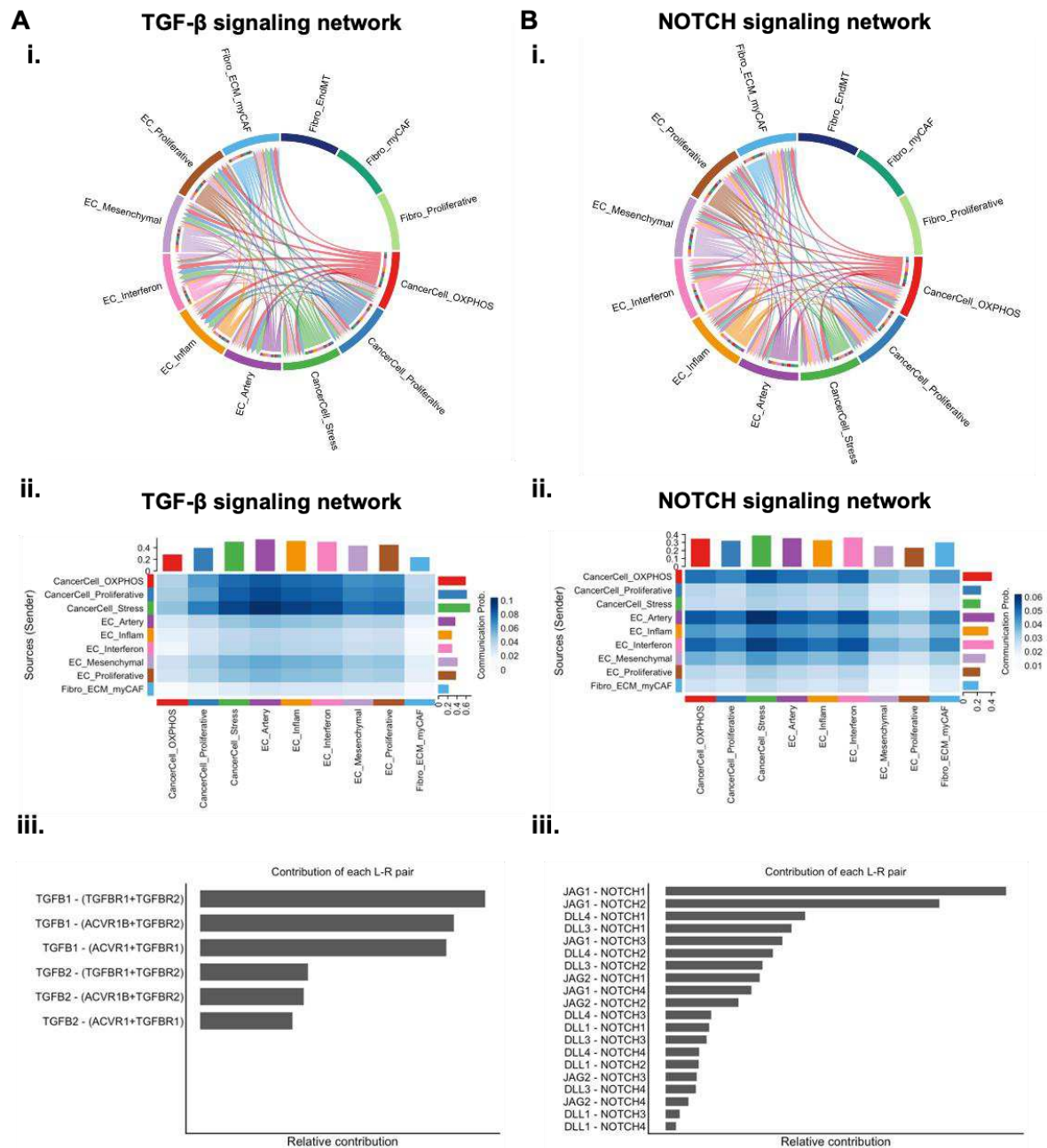
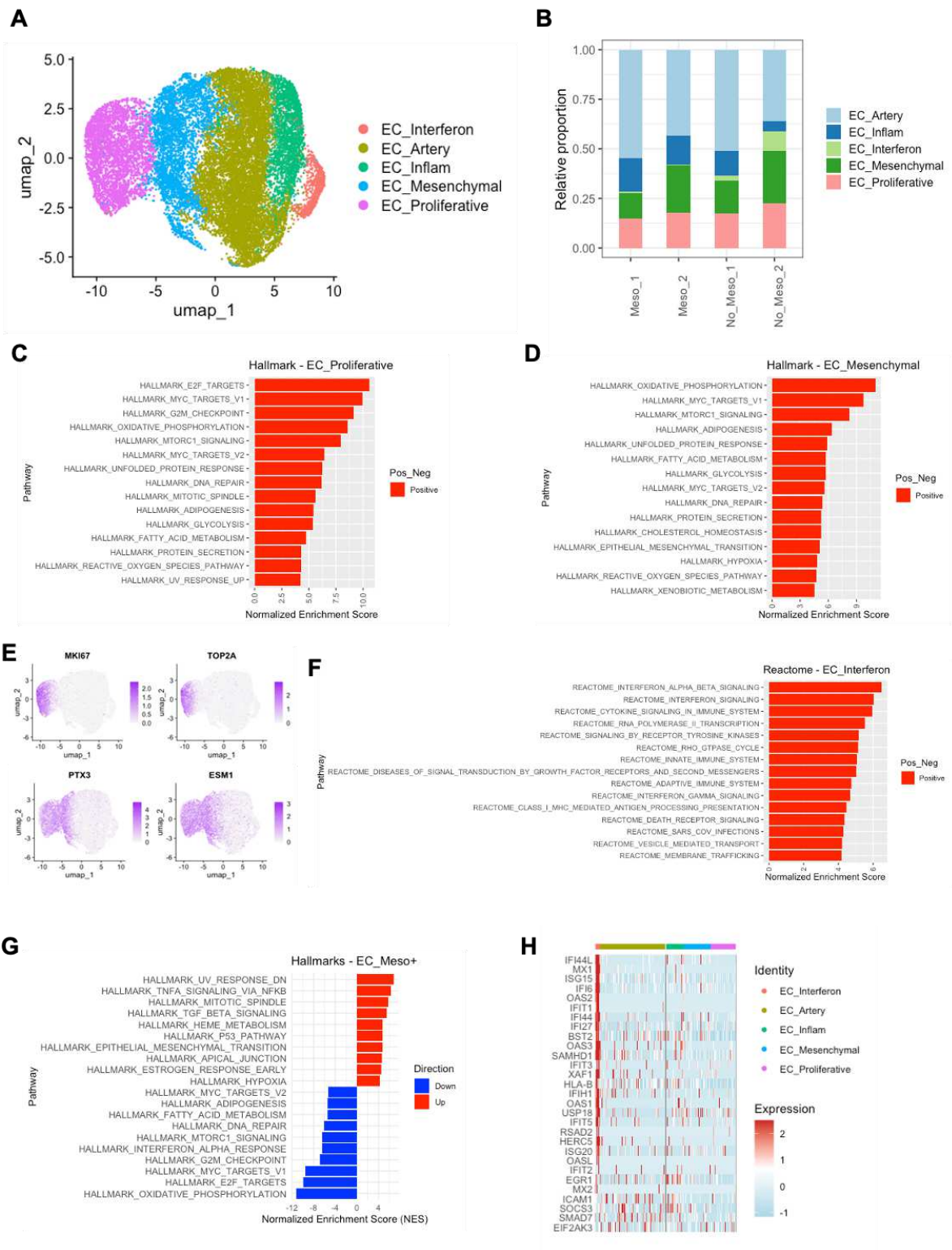
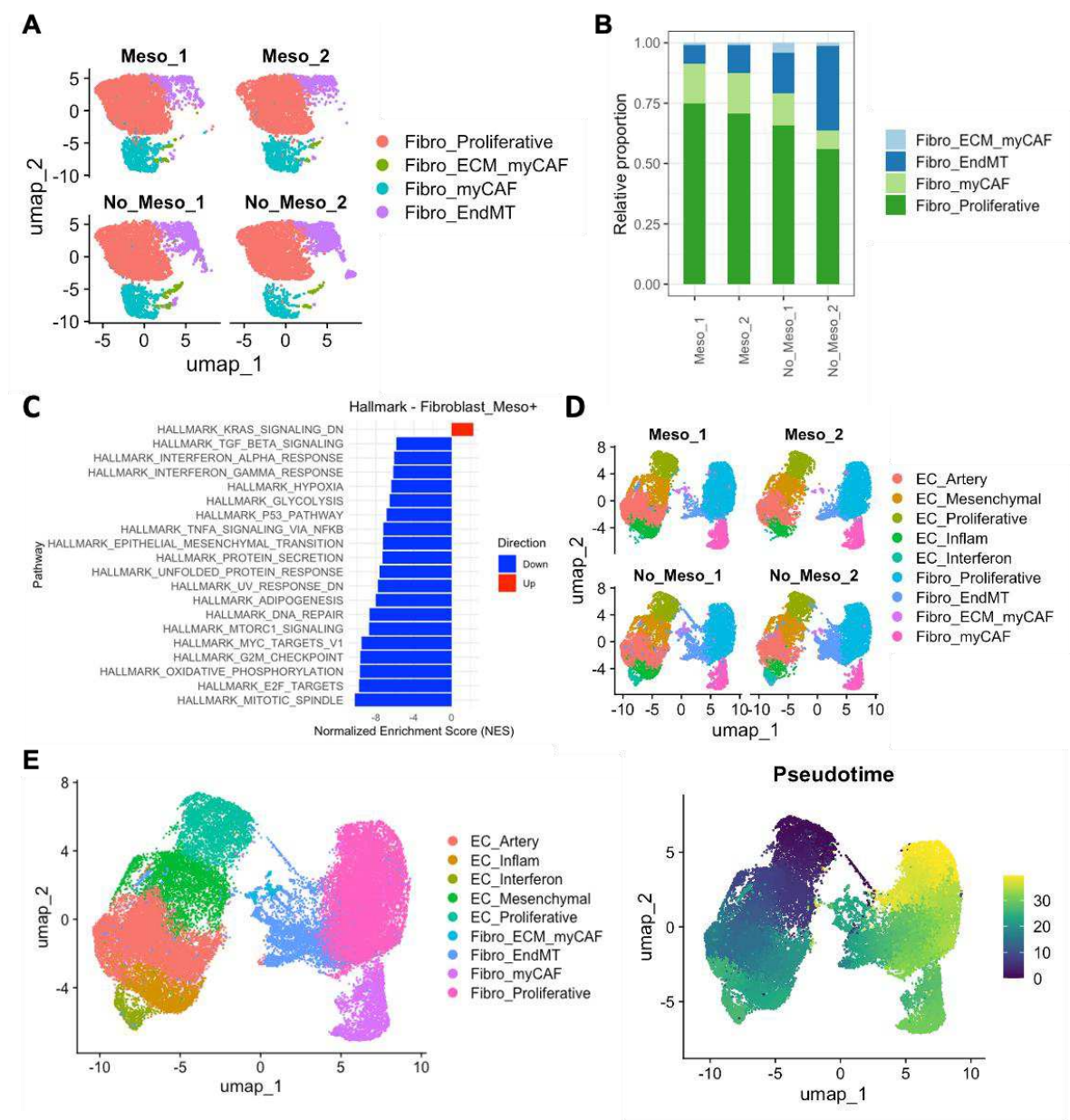


Figure S7



1474

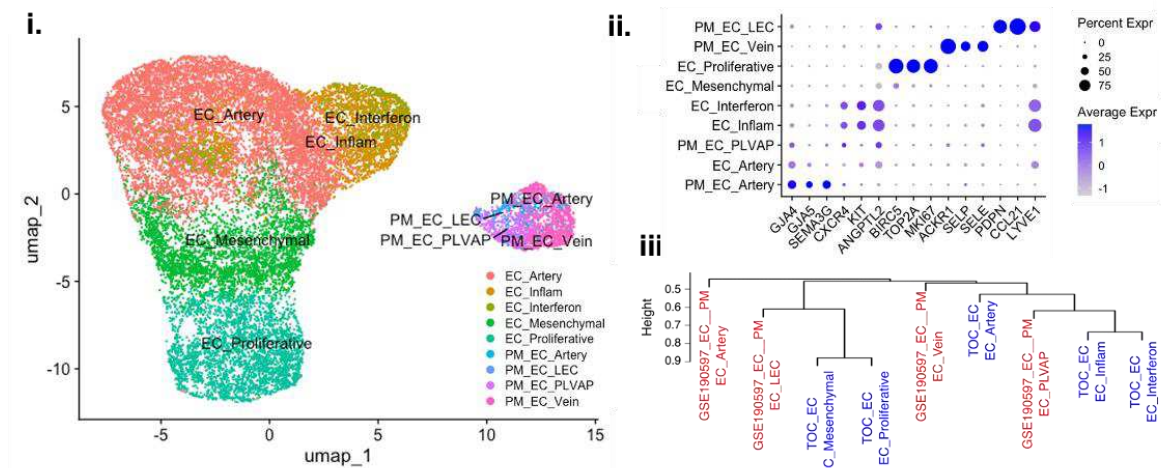
Figure S8



1475

Figure S9

A Similarities between ECs from VMOC & patients with PM



B Similarities between fibroblasts from VMOC & patients with PM

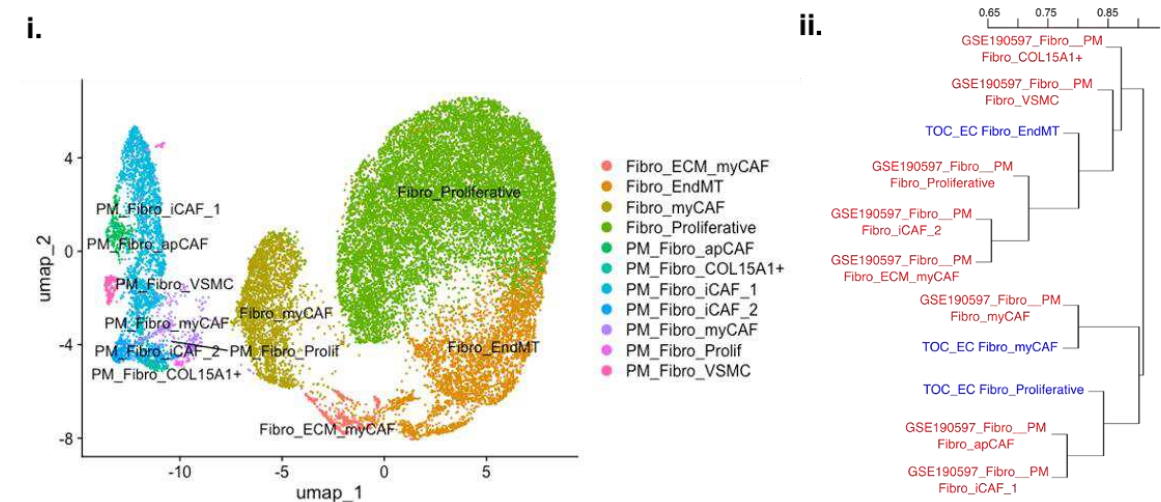


Figure S10

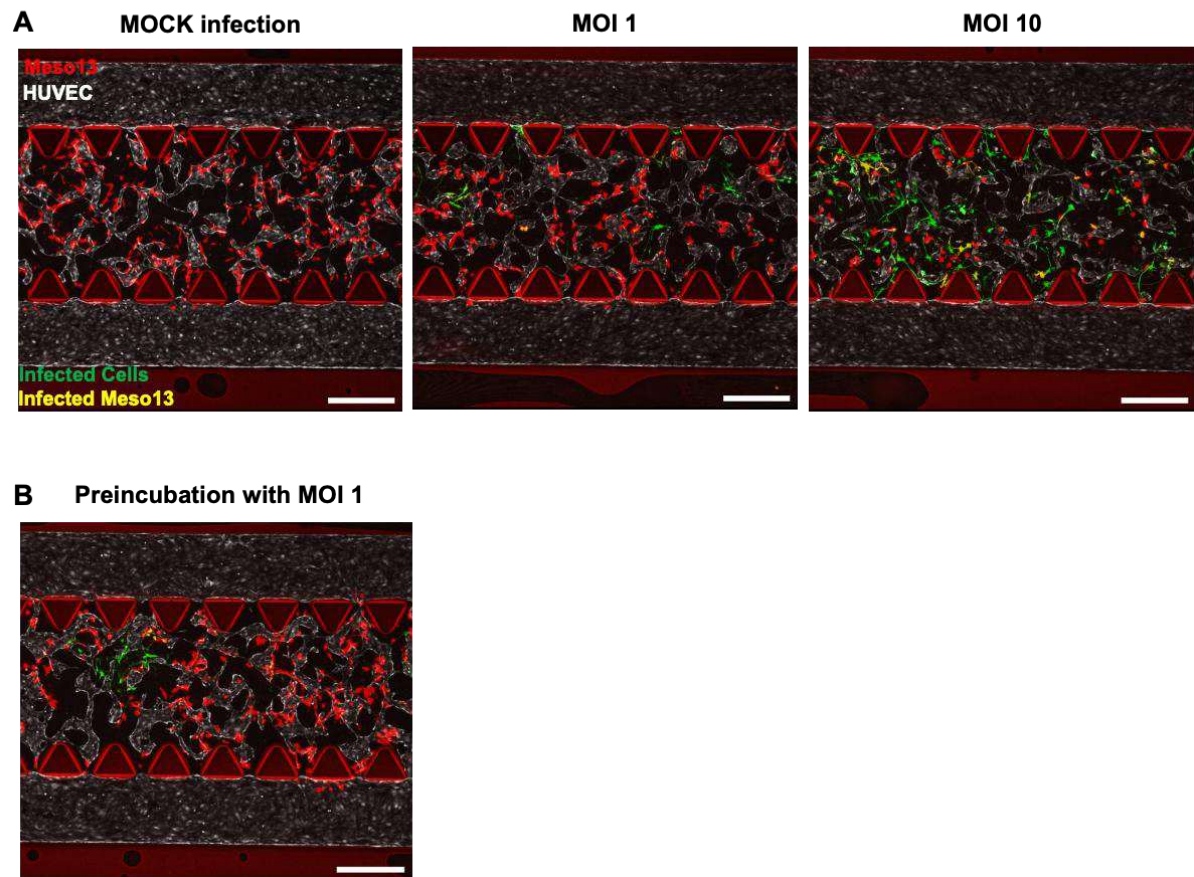


Figure S11

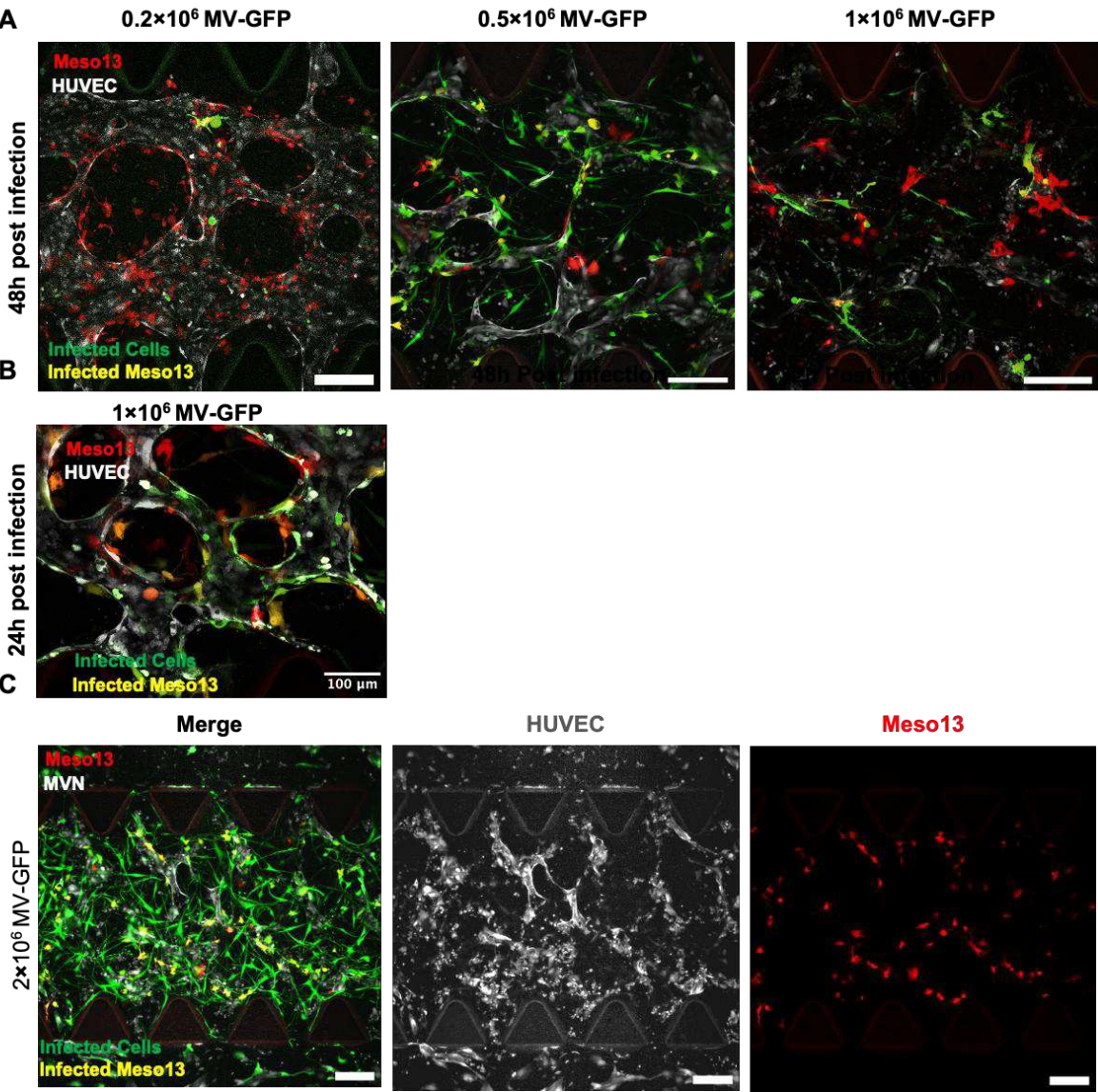
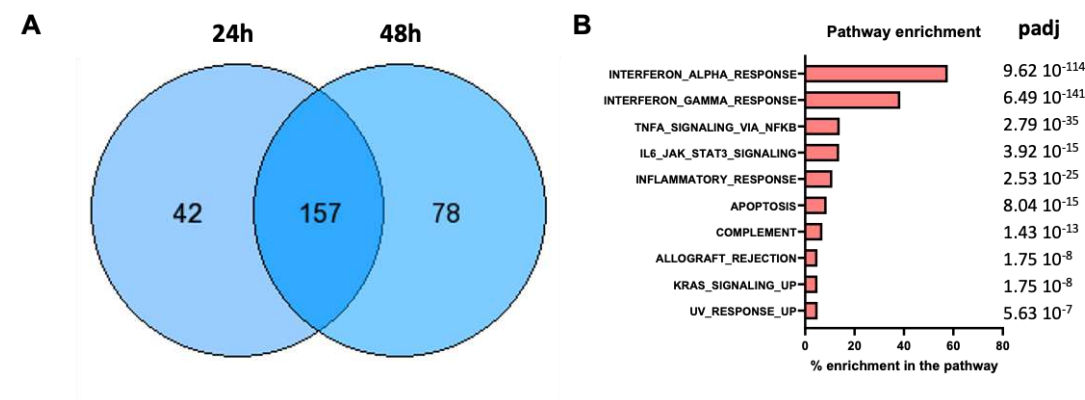
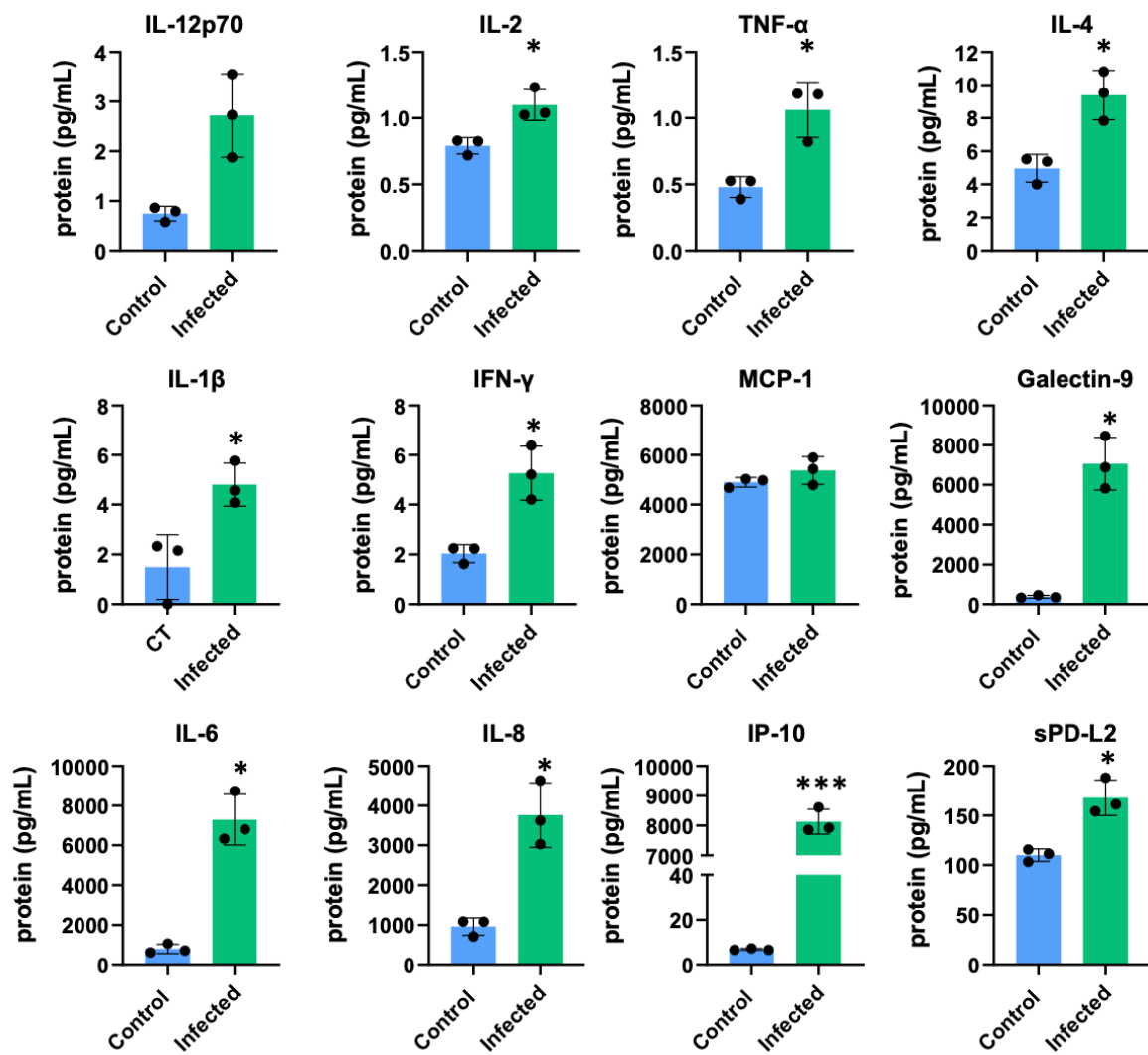


Figure S12



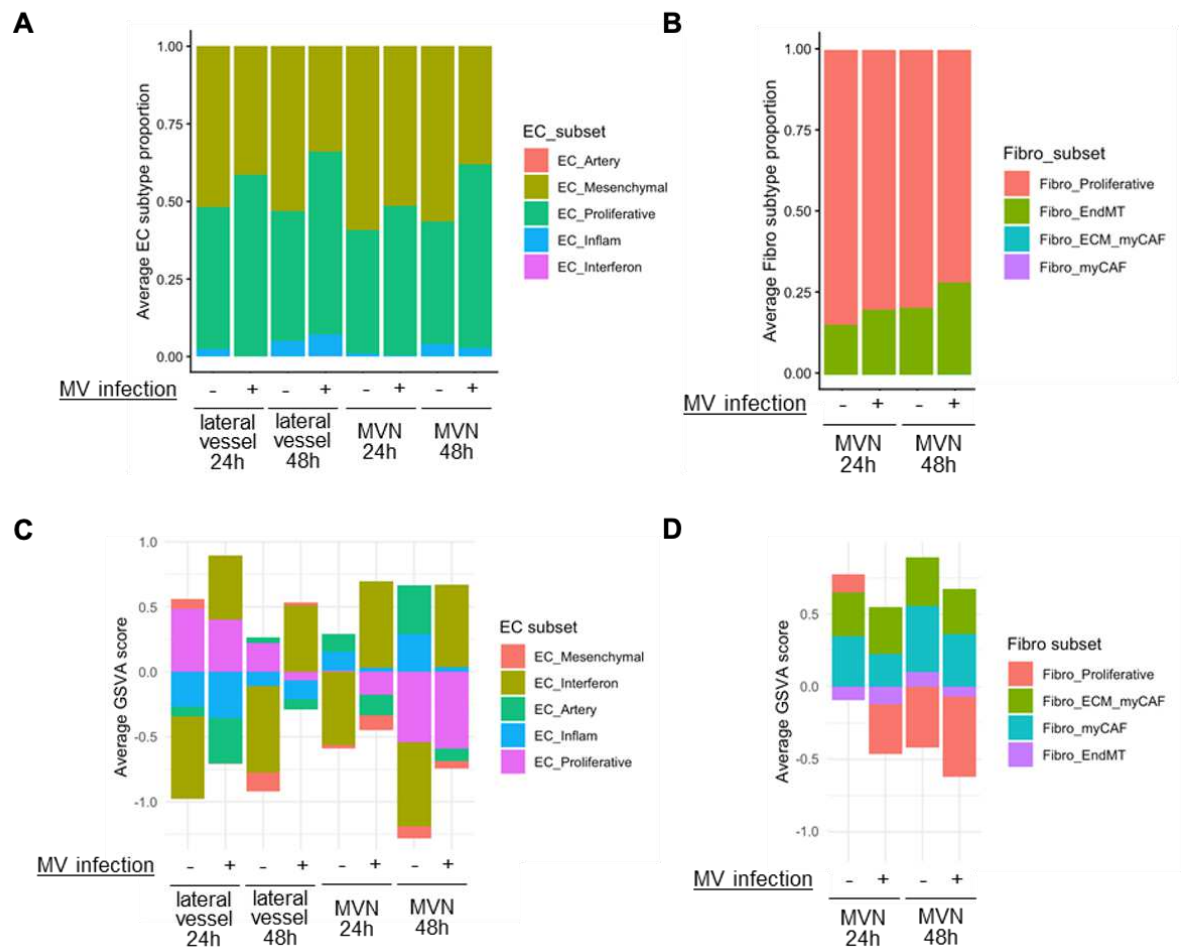
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Figure S13



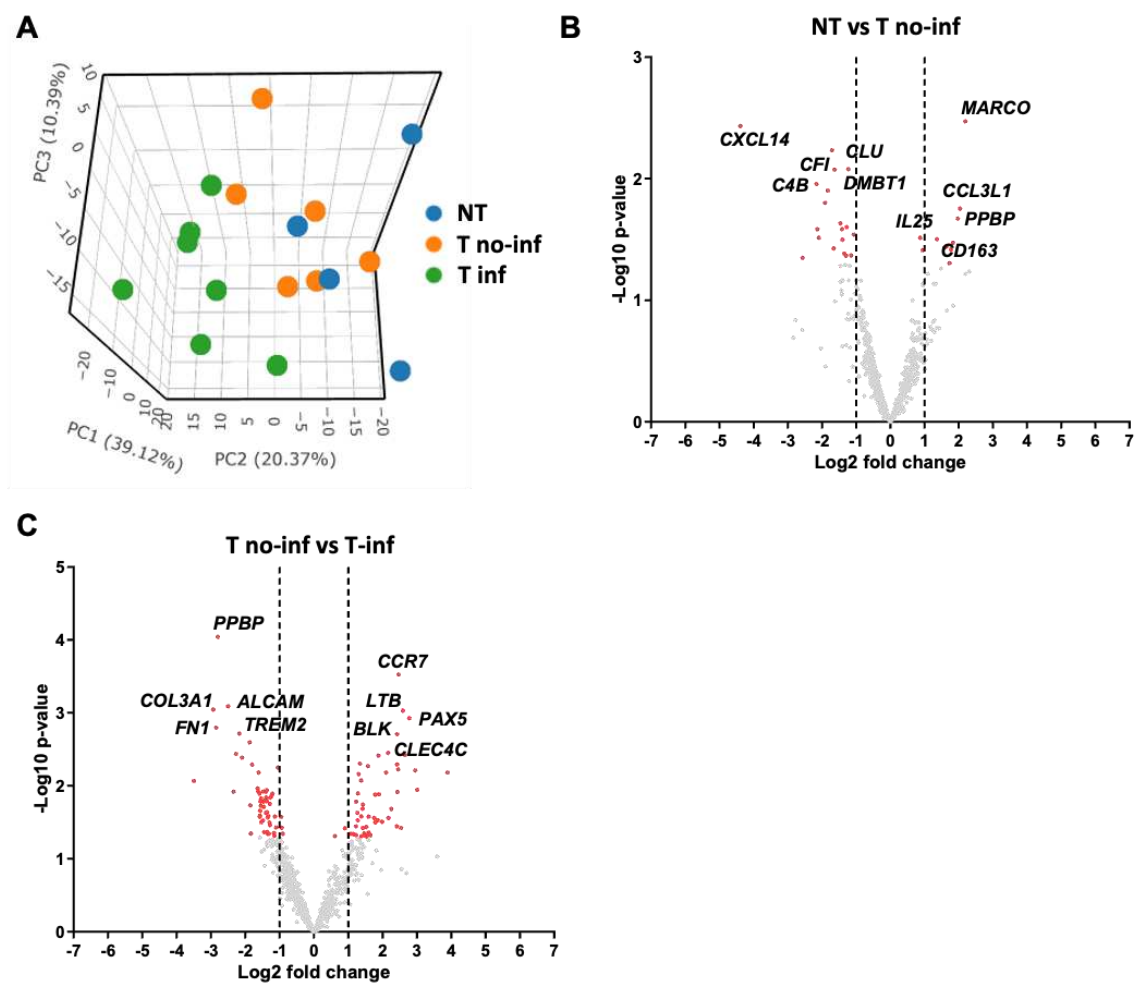
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Figure S14



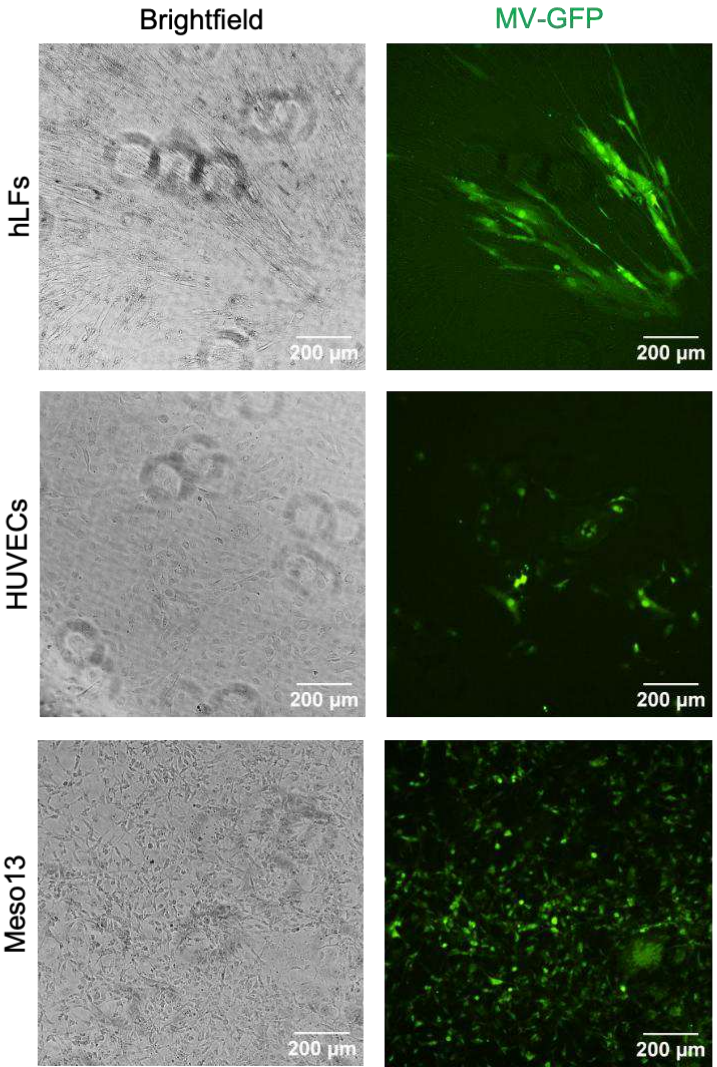
1481

Figure S15



1482

Figure S16



1483

Supplemental information

Supplemental information 1: Generation of Microvascular network connected to the

Parental Vessels using one-step method. HUVECs and hLFs were suspended in EBM2 medium containing thrombin (4 U/mL). Five μ L of the cell suspension and 5 μ L of fibrinogen (6 mg/mL) were mixed, and 10 μ L of the resulting hydrogel-cell mixture was immediately injected into the central gel channel. The chip was incubated at 37 °C for approximately 15 minutes to allow polymerization, followed by the addition of 50 μ L of supplemented EBM2 into all the media reservoirs of the two lateral media channels.

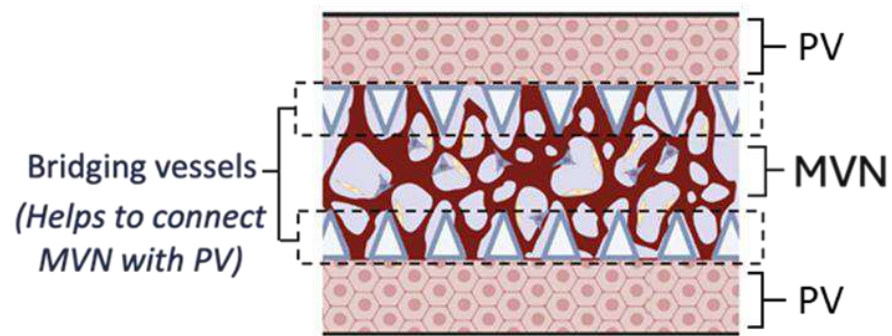
Results: Modulating HUVEC seeding density produced opposing results between vessel size and perfusability. First, higher lumen size was observed with the increase in HUVEC seeding densities (from 50 μ m (40%) to 300 μ m (1%) with 80,000 cells per CC and from 50 μ m (20%) to > 500 μ m (5%) 150,000 cells per CC (Figure S2B and S2C). Secondly, lower densities (e.g., 80,000 HUVECs per CC) produced highly branched but partially perfusable networks, while increasing density (e.g., 100,000, 120,000 or 150,000 HUVECs per CC) improved perfusability but generated progressively larger, less branched vessels (Figure S2B and S2D). Enhanced perfusability at higher densities was associated with increased formation of open, perfusable bridging vessels and greater connectivity between the central and lateral channels, as confirmed by 70 kDa FITC–dextran perfusion (Figure S2B, S2E and S3). However, the same high HUVEC densities (120,000 and 150,000 cells per CC) produced oversized, unbranched structures in the central gel, resulting in an oversimplified MVN. These findings demonstrated that the HUVEC density required to generate a branched MVN was incompatible with the density required to form perfusable bridging vessels and achieve connection with the lateral channels.

1508 MV infection in central channel: First, MV was added directly to the central channel (CC)
1509 at the time of gel loading to mimic intratumoral administration. Based on our previous work
1510 (PMID: 31945495), we used multiplicity of infections (MOI) of 1 and 10 equivalent to the
1511 number of Meso13 loaded. This led to a poor infection of PM cells even with the higher dose,
1512 with most GFP-positive cells corresponding to infected fibroblasts (Figure S9A). The pre-
1513 infection of Meso13 cells for 2 h at MOI 1 prior to their gel loading in the CC did not improve
1514 infection efficiency, and again primarily resulted in infection of fibroblasts (Figure S9B).

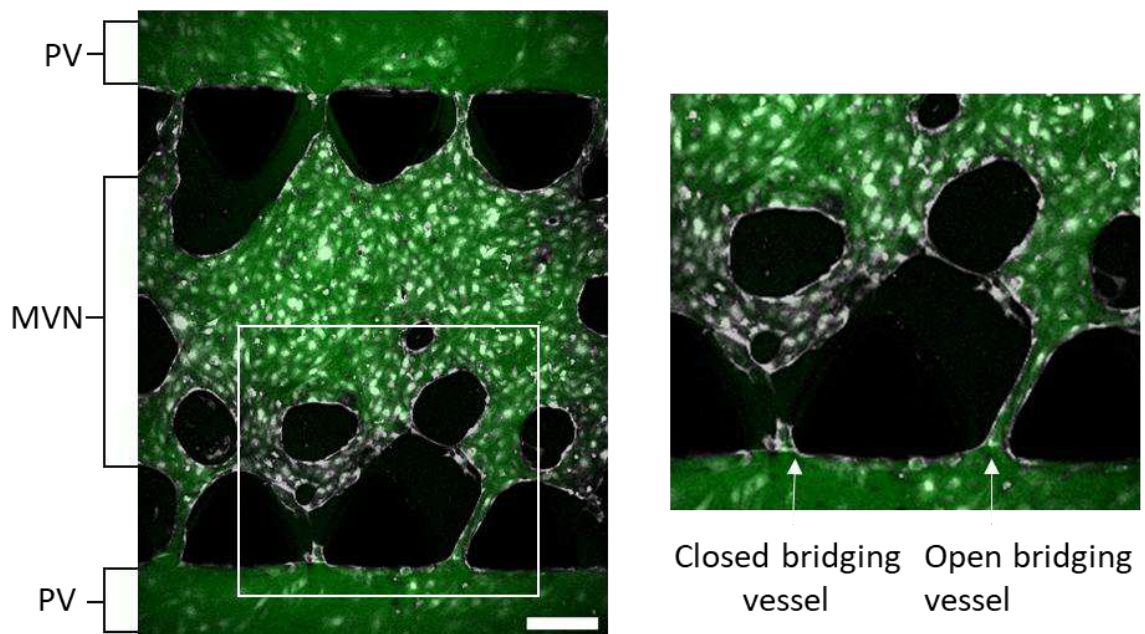
1515

1516 **Supplemental information 2. Methods to measure open bridging vessels and size of lumen**
1517 **in MVN. A)** Schematic representation of the VMOC indicating the position of bridging vessels.
1518 **B)** Representative confocal microscopy image showing the difference between open and
1519 closed bridging vessels. Grey, HUVECs RFP; Green, 70 kDa FITC dextran. Scale bar = 200 μm .
1520 **C)** Image processing for lumen size measurement, as previously described by Wan *et al.*
1521 (PMID: 35373502). (i) A representative ROI ($1272 \times 1272 \mu\text{m}$) from one chip was used for
1522 lumen size quantification. (ii) Lumen diameters in the MVN were measured using the Fiji
1523 distance measurement tool. Three ROIs were analyzed per chip, and the mean value was
1524 calculated for each chip. Scale bar = 100 μm .

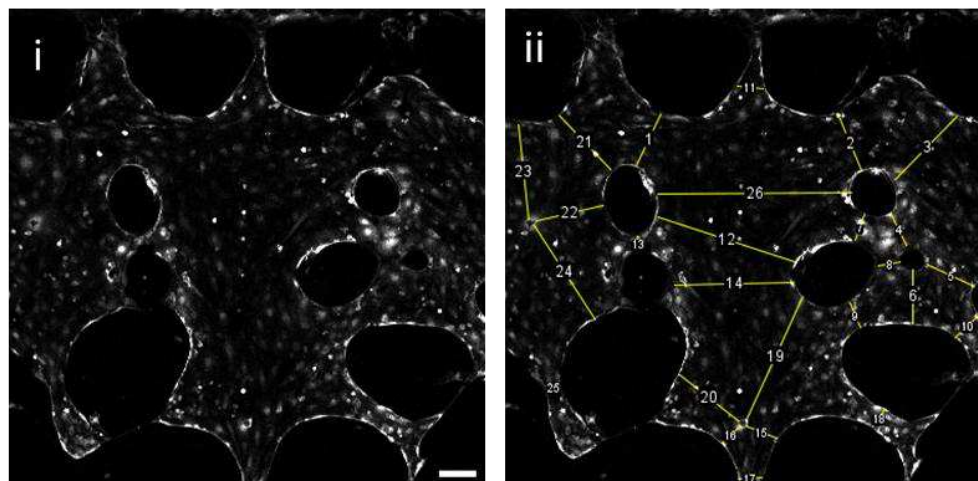
A



B



C



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.xls](#)
- [TableS2.xlsx](#)
- [TableS3.xlsx](#)
- [MovieS1.mp4](#)
- [MovieS2.mp4](#)