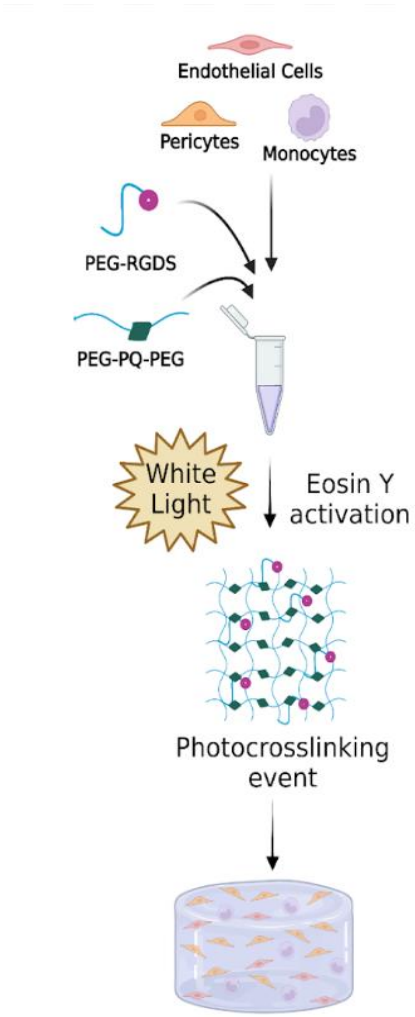
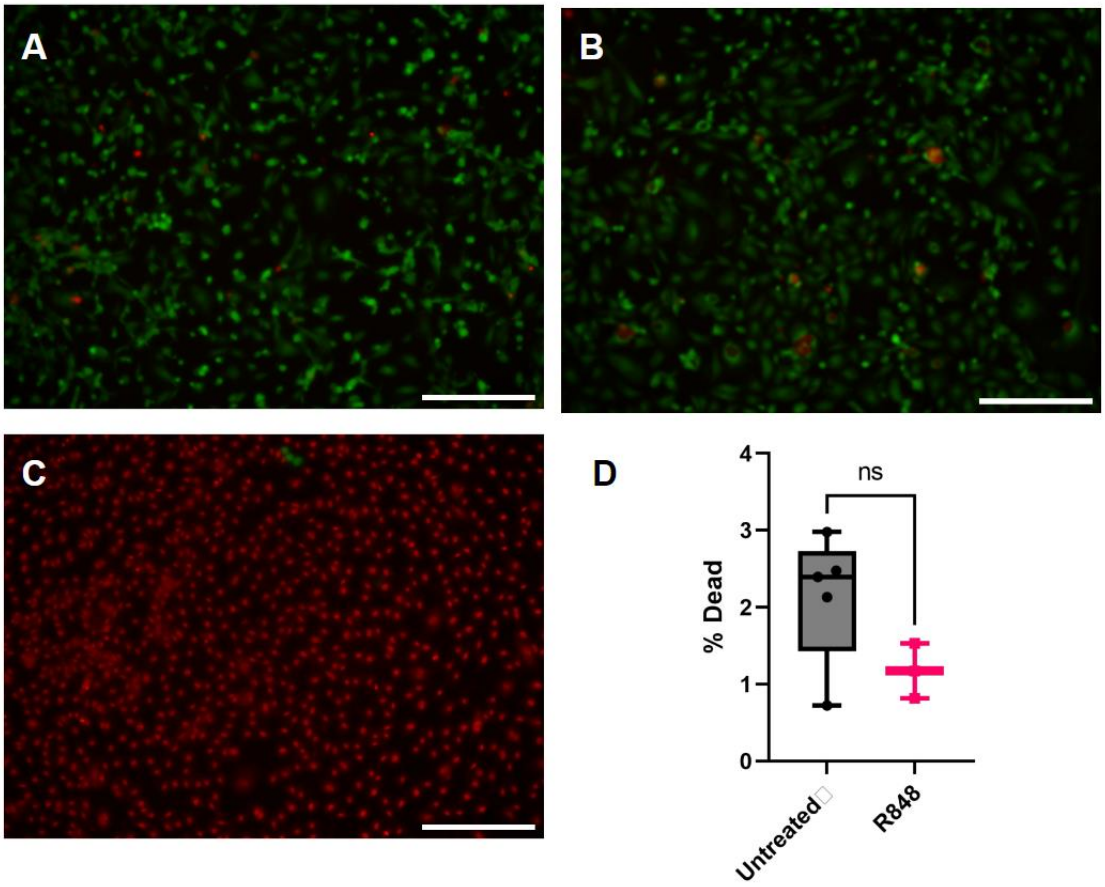


Supplement 1. Schematic demonstrating components and photocrosslinking event of our functionalized PEG hydrogel platform.



Supplement 2. TLR7/8 activation does not affect cell viability in a 3D hydrogel environment. Live/dead stained hydrogels with encapsulated HUVECs and monocytes: A) No treatment, B) R848 treatment, C) Ethanol-treated "dead" control. D) Box-and-whisker plot compares percent dead cells between untreated and R848-treated conditions with $p<0.05$ considered significant. Each point represents average counts per gel, with n=5 gels in the untreated and n=3 gels in the treated conditions after excluding gels with total counts <350. Red = Dead. Green = Alive. Scale bar = 300 µm.

Supplement 3. Methodology for SLE hydrogel model experimentation.

Participant recruitment

This study was approved by the University of Florida Institutional Review Board with reference number 202001085, and all human participants gave informed consent to participate. SLE patients were self-identified Black/African American or White/European American females between 18-60 years old and were recruited from the University of Florida nephrology clinic. All participants were diagnosed with Class III, IV, or V lupus nephritis. IRB requirements for this study ensured that SLE patients met the classification of SLE quiescence according to the modified Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) score [1].

A score of zero meant that the patient was within clinical quiescence and therefore did not require a shift in treatment or present with new symptoms of lupus activity relative to previous assessment. Patients on antimalarial drugs, low-dose steroids (equivalent to prednisolone ≤7.5 mg/day or other corticosteroid in an equivalent dose) or immunosuppressive drugs during the maintenance phase of their lupus were also included. Healthy controls were self-identified Black/African American or White/European American females between 18-60 years old, with no active medical diagnoses besides hypertension or hypercholesterolemia and no prescription medications except birth control.

Cell culture

Human umbilical vein endothelial cells (HUVECs; Lonza C2519A) were cultured in EGM-2 media (Lonza CC-3162). Human brain vascular pericytes (ScienCell 1200) were cultured in pericyte media (ScienCell 1201). HUVECs and pericytes were used between passages 4 and 6. Pericytes were used in subsequent experiments to support microvascular maturation of the endothelial cells.[2,3].

Monocyte preparation

Primary human monocytes were used as the model myeloid cells for all experiments. Peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainer collection tubes (Fisher) and peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll gradient with centrifugation. Briefly, 30 mL blood was diluted 1:1 in calcium- and magnesium-free phosphate buffered saline (PBS; Fisher Scientific) and slowly layered over 15 mL Ficoll-Paque PLUS media (Fisher) before centrifuging for 30 minutes at 400x g with the brake off. The PBMC band was collected and washed twice in fresh PBS with centrifugation for 10 minutes at 300xg. Monocytes were purified from fresh PBMCs by magnetic-activated cell sorting. Briefly, cells were suspended in PBS pH 7.2 with 0.5% bovine serum albumin (Fisher Scientific) and 2mM EDTA (Sigma Aldrich). Cells were then processed using a pan-monocyte isolation kit (Miltenyi Biotec) according to the manufacturer's directions. Monocytes were placed directly in the hydrogel platform.

PEG hydrogel formation and cell encapsulation

PEG hydrogels with cell-adhesive components and components subject to cell-mediated degradation were made according to established protocols [4–7]. The cell-adhesive peptide RGDS (Fisher) was conjugated to monomers of acrylate-PEG-succinimidyl valerate (acryl-PEG-SVA; Laysan Bio) at a 1.2:1 molar ratio by amine substitution to form the polymer that will be abbreviated PEG-RGDS. Similarly, the matrix-metalloprotease-degradable peptide GGGPQGIWGQGK (PQ; Genscript USA Inc) was conjugated to monomers of acryl-PEG-SVA at 1:2 ratio to form degradable polymers that will be abbreviated as PEG-PQ-PEG. The peptide-conjugated polymers were dissolved in HEPES buffer with the photoinitiator eosin-y (Fisher) combined with N-vinyl pyrrolidone (NVP; Fisher) for a final concentration of 1.5% triethanolamine (Fisher), 10 μM eosin-y, 0.35% NVP, 4% PEG-PQ-PEG, and 3.5 mM PEG-RGDS.

For R-848 studies, monocytes, HUVECs, and pericytes were suspended in the polymer solution at a 2:5:2 ratio with a final cell concentration of 324,000 cells per 5 μL gel, according to protocols previously established in our lab [6,7]. For SLE vs healthy control studies, monocytes, HUVECs, and pericytes were suspended in the polymer solution at a 12:5:2 ratio with a final cell

concentration of 114,000 cells per 5 μL gel. An extra set of control gels with or without R-848 treatment was also included in this group. Following suspension in polymer solution, 5 μL aliquots were transferred to a poly-dimethyl siloxane (PDMS) slab between two PDMS spacers of 380 μm thickness. A methacrylated coverslip was set on top of the 5 μL droplet, supported by the spacers. The assembly was exposed to white light for 60 seconds to allow free radical formation by the photoinitiator, which caused the polymer solution to gel with the three cell types suspended throughout its thickness. The coverslip with the newly formed hydrogel was then transferred hydrogel-side up to a 24-well plate, and 1 mL of EGM-2 media was added. Hydrogels made using monocytes from different donors were made on different days. A summary of the platform can be visualized in the schematic (**Supplement 1**).

It is of important note that different ratios of monocytes, HUVECs, and pericytes were used between experimental workflows. The 2:5:2 ratio, used for the R848 studies, was a slight modification from previous published work in our lab [6]. The 12:5:2 ratio, used for SLE vs HC monocyte studies, was due to a protocol change when the lab first started working with patient samples, as these studies were conducted at a different time than the R848 studies. This results in a lower concentration of HUVECs which is consistent with other HUVEC encapsulations in the literature [8]. The ultimate concentration of monocytes was held consistent across conditions. The ratio differences allow for comparisons only between groups within individual studies and not between the studies themselves.

Live/dead imaging and image processing

Monocytes and HUVECs were co-encapsulated without pericytes as described above in a 2:5 ratio with a final concentration of 252,000 cells per 5 μL gel. Hydrogels were fed with 1 mL EGM-2 media per well and kept in culture overnight at 37ºC and 5% CO2. The next day, media was changed for fresh EGM-2 media with or without 1 μg/mL of R-848. Eight days after plating, cells were stained with calcein AM and ethidium homodimer-1 live/dead stain (Fisher) for 30 minutes. Prior to staining, one cell-laden hydrogel was treated for 5 minutes with 70% ethanol for a 'dead' control. Images of the hydrogels with live/dead stained cells were taken on a Keyence BZ-X800 microscope. The red (dead) and green (alive) channels were thresholded and particles with size greater than 144 μm^2 and circularity from 0.4-1.00 were counted using the "Analyze Particles" function in ImageJ v1.54b.

Confocal imaging and image processing

Hydrogels containing monocytes, HUVECs, and pericytes were fed with 1 mL per well of fresh EGM-2 media with or without 1 μg/mL of R-848 immediately after formation. Hydrogels were then kept in culture overnight at 37ºC and 5% CO2 before the media was changed. On the third day after cell encapsulation, media was removed from the wells containing the hydrogels and replaced with 10% buffered formalin phosphate to fix the cells encapsulated in the hydrogels for 40 minutes. Hydrogels were then rinsed three times with Tris-buffered saline (TBS) (Fisher Scientific) and stored in 0.2% sodium azide in TBS until ready to use. Once ready to use, the cells were permeabilized with 0.25% Triton-x (Sigma Aldrich) for 45 minutes, and then rinsed in TBS. Cells were blocked with 5% donkey serum (Fisher) in TBS overnight. After rinsing again in TBS, cells were exposed to rat anti-human CD31 primary antibody (Fisher) at a 1:200 dilution in 0.5% donkey serum for two days. Cells were rinsed in TBS with 0.02% Tween and then exposed to AlexaFluor 488 donkey anti-rat secondary antibody (Fisher) at a 1:200 dilution for two days. Cells were rinsed in TBS and exposed to 4′,6-diamidino-2-phenylindole (DAPI)

(Fisher) overnight. Cells were rinsed again in TBS before imaging and were kept in 0.2% sodium azide in TBS for storage.

Confocal images were taken on Zeiss LSM980 and LSM900 microscopes. 70 images were taken 1 μm apart. Five images were taken in non-overlapping regions of each gel. Images were processed in ImageJ. For R-848 studies, maximum intensity Z-projections of image stacks were taken to compress the 3-dimensional images to 2 dimensions, and then a vessel analysis package was used to convert the compressed images to binary images [28]. The binary images were inverted and skeletonized, and the skeletons were analyzed for branch length and number using the built-in skeletonize package of ImageJ.Further analysis of confocal images was performed using Imaris x64 v10.0.0. Z-stacks were manually thresholded based on visual CD31 positivity (metadata, raw images, and Imaris-rendered images available upon request). The resulting channel representing endothelial cells was converted to 3D surfaces. To ensure that downstream analyses included only multicellular endothelial structures and excluded any single cells, 3D surfaces were filtered to include only structures with size greater than 1060 μm^3 and sphericity less than 0.544. Included structures were analyzed based on total number present, as well as by their volume and sphericity.

For SLE vs healthy control studies, Z-stacks were processed in Imaris x64 v10.0.1. Each Z-stack was processed using one of six different algorithms that were designed to create "Surface" objects under different conditions of off-target staining. On occasions where none of the six algorithms produced a "Surface" that was representative of the structures within the z-stack, the best-fitting "Surface" was chosen and manually trimmed to remove nonspecific signal. The resulting "Surfaces" were used as masks to filter the original z-stacks. To ensure that downstream analyses included only multicellular endothelial structures and excluded any single cells, only structures with size greater than 1200 μm^3 were included. Masked z-stacks were then processed to skeletonized images as discussed previously, and numbers of branching segments were compared between conditions.

Statistics

For live/dead experiments, five untreated gels and six treated gels were imaged and compared to the single dead control. Images were taken in three field views for each gel and cell count per image was averaged for each gel. Gels with total cell counts per image (live + dead) less than 350 were excluded from analysis. The average counts of live or dead cells per gel were normalized to the average total cell counts per gel. Normalized average fractions of live or dead cells were compared between untreated and R-848-treated conditions using unpaired two-tailed Student's t-test.

For immunofluorescence experiments using R-848 hydrogels with monocytes, HUVECs, and pericytes had seven in the untreated group and eight in the R-848-treated group. Control hydrogels with HUVECs and pericytes but without monocytes had four in the untreated group and five in the R-848-treated group. Five non-overlapping immunofluorescence images were taken for each gel. A two-way ANOVA with Šídák's multiple comparisons test was used to compare numbers of endothelial segments with at least one branch, considering each Z-stack in a gel as a separate observation.

For immunofluorescence experiments comparing monocytes from SLE patients to those of healthy controls, 2-6 gels were imaged for each monocyte sample with 5 z-stacks taken per gel.

All observations were pooled for each experimental group of monocyte samples, and a normality test demonstrated a non-normal distribution in the numbers of branching skeletonized segments among the groups. For this reason, a Kruskal-Wallis test with Dunn's post-hoc multiple comparisons test was chosen to analyze the differences in branch numbers among the different groups, with each skeletonized image from each gel and donor considered as a separate observation.

All statistics were done using GraphPad Prism v9.3.0 (463).

	Healthy donors	SLE patients
African American Ancestry	0/3	5/7
European American Ancestry	3/3	2/7
Sample purchased from Charles River	1/3	n/a
Class of lupus nephritis		
Class IV	n/a	3/7
Class V	n/a	4/7
Current treatments		
Hydroxychloroquine	n/a	6/7
Mycophenolate	n/a	5/7

Supplement 4. Table summarizing patient clinical information for samples used in SLE vs HC experiment. This table discloses information regarding ethnicity, class of lupus nephritis, and hydroxychloroquine/mycophenolate treatment. A total of 7 SLE patients and 3 healthy donors were used for this study.

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