Preclinical in vitro model of monocyte influence on microvessel structure in systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disease that dysregulates both the innate and the adaptive immune systems. Vasculitis is one of the hallmarks of SLE and has been named a leading cause of death for patients. The immune profiling of SLE vasculitis is theorised to involve monocyte modulation and recruitment of leucocytes into a given microvascular site. This in turn leads to fibrinoid changes in the vascular cell wall and inflammation. Aberrant monocyte activity has been found to result in recruitment of proinflammatory cytokines and proteolytic enzymes, resulting in atypical vasculature formation through internal and external membrane elasticity disturbances. While monocyte-microvascular interactions are critical to understanding vascular alterations in SLE, there are currently no preclinical models for the interrogation of microvascular phenotype associated with SLE.

Using a functionalised polyethylene glycol (PEG) hydrogel, we encapsulated endothelial cells, support cells and monocytes to create a preclinical model of SLE microvessel phenotype. PEG-based hydrogels are three-dimensional (3D) hydrophilic polymeric biomaterial complexes. Their high biocompatibility allows them to be good platforms for cell culture, and their high tunability has allowed for their manipulation to mimic complex biological systems, such as the extracellular matrix (ECM). PEG scaffolds functionalised with cell-adhesive peptides have demonstrated the capacity to facilitate microvessel formation. We functionalised our PEG platform by conjugating a cell-adhesive peptide, RGDS, and a cell-mediated degradable component peptide, GGGPQGI-WGQGK (online supplemental file 1). These components allow for necessary cellular adhesion as well as subsequent migration.

Toll-like receptor (TLR)7/8 are endogenous pattern recognition receptors expressed in monocytes, and aid in disposal of single-stranded viral and self-RNA. TLR7/8 are proposed to be key players in the modulation of pathological microvasculature and aberrant monocyte activity in SLE. We leveraged resiquimod (R-848), a TLR7/8 ligand, to elucidate the effects of monocyte TLR7/8 pathway activation on monocyte and endothelial cells’ interactions through microvessel formation in vitro. Additionally, we encapsulated SLE and healthy control (HC) primary monocytes to assess microvessel formation within our hydrogel platform.

We first evaluated cell viability within the hydrogel system with and without treatment with R-848 (online supplemental file 2). We then investigated microvessel formation in our preclinical model to quantify the interaction between monocytes and microvessels. Leveraging confocal imaging, we quantified branch points and segmentation of observed microvessels as a measure of aberrant microvasculature. We found that monocyte stimulation with TLR7/8 agonist and monocytes from patients with SLE did alter microvessel structure, specifically in the number of structures containing at least one branching point. Conditions tested within this work include monocytes isolated from HCs with/without R-848, and monocytes from patients with SLE. Differences between groups are in regard to either R-848 treatment or disease state; additional cells used to formulate microvessels (endothelial cells and pericytes) remained consistent throughout.

Monocytes were obtained from patient samples and isolated from peripheral blood mononuclear cells. For studies involving interrogation of TLR7/8, monocytes used were from HCs. In studies for visualising SLE in a hydrogel platform, both HCs and SLE donor monocytes were used. These monocytes were then co-encapsulated with human umbilical vein endothelial cells and pericytes into a
photocrosslinked polymer hydrogel composed of PEG and previously mentioned ECM peptides as established within past literature. These hydrogels were cultured in media with or without R-848. Hydrogels were stained with CD31, an endothelial marker, and DAPI, a nuclear marker. Confocal images of these immunostains were taken, made into binary images and skeletonised to facilitate analysis of microvessel structures. Maximum-intensity Z-stack projections were made to allow for tracing of endothelial structures and their branches (figure 1B,C). Figure 1A summarises this workflow and a more detailed methodology is described in online supplemental file 3.

Microvessel segments were analysed based on the number of segments per condition having at least one branch. Segments with only two endpoints and no branching points were excluded from analysis (figure 1D).

After treatment with R-848, the number of endothelial segments in each Z-stack having at least one branch point increased in the hydrogels including monocytes, but not in the hydrogels without monocytes (figure 1D). This suggests that activation of TLR7/8 in monocytes, and not off-target effects of R-848 on endothelial cells and supporting pericytes, is responsible for altered branching patterns. TLR activation in macrophages leads...
to an inflammatory, or M1, state, and this cell type has been shown previously to cause significant alterations in branching patterns of 3D-modelled vasculature. The presence of monocytes at baseline, regardless of treatment with R-848, also slightly increased the number of endothelial segments with branches (figure 1D).

Representative images of hydrogels made with monocytes from patients with SLE (n=7) versus HC donors (n=3) are shown in figure 1E. Figure 1F shows a significant increase in the number of segments with at least one branch in the hydrogels with monocytes from patients with SLE compared with those of hydrogels with monocytes from HC donors. These observed differences between SLE versus HCs are present even in the presence of certain medication treatments disclosed in online supplemental file 4. This increase suggests that the monocytes may be at least partially responsible for microvessel dysfunction seen in SLE. In addition, there was an observed increase in branched segments in the SLE condition (figure 1F), emphasising that monocyte modulation may play an important role in aberrant microvessel formation.

These results demonstrate a proof-of-concept preclinical model of monocyte-mediated altered microvessel formation as a result of TLR 7/8 targeting. This work also demonstrates that a hydrogel platform can uphold an SLE phenotype within an in vitro setting for benchtop investigations. Further investigation of this model will look towards confirming more attributes related to SLE microvasculature, such as cytokine recruitment. Given the complexity of SLE, much of the aetiology and disease progression is still unknown, and pinpointing causal effects has proven to be challenging. This model can aid in unpacking confounding factors of symptom onset and severity, such as the environment, genetics, hormonal deviations, viral exposure and ancestry.

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**Patient consent for publication**  Not required.

**Ethics approval**  This study involves human participants and was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Florida (protocol code 202001085, approved 29 May 2020). Participants gave informed consent to participate in the study before taking part.

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**REFERENCES**


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 37°C 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 μm² 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 37°C 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 Šidák’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).

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**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.

**Resources**

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