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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.[1] 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.[2] 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.[3–5] We functionalised our PEG platform by conjugating a cell-adhesive peptide, RGDS, and a cell-mediated degradable component peptide, GGGPQGIWGQGK (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.[6] TLR7/8 are proposed to be key players in the modulation of pathological microvasculature and aberrant monocyte activity in SLE.[6] 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

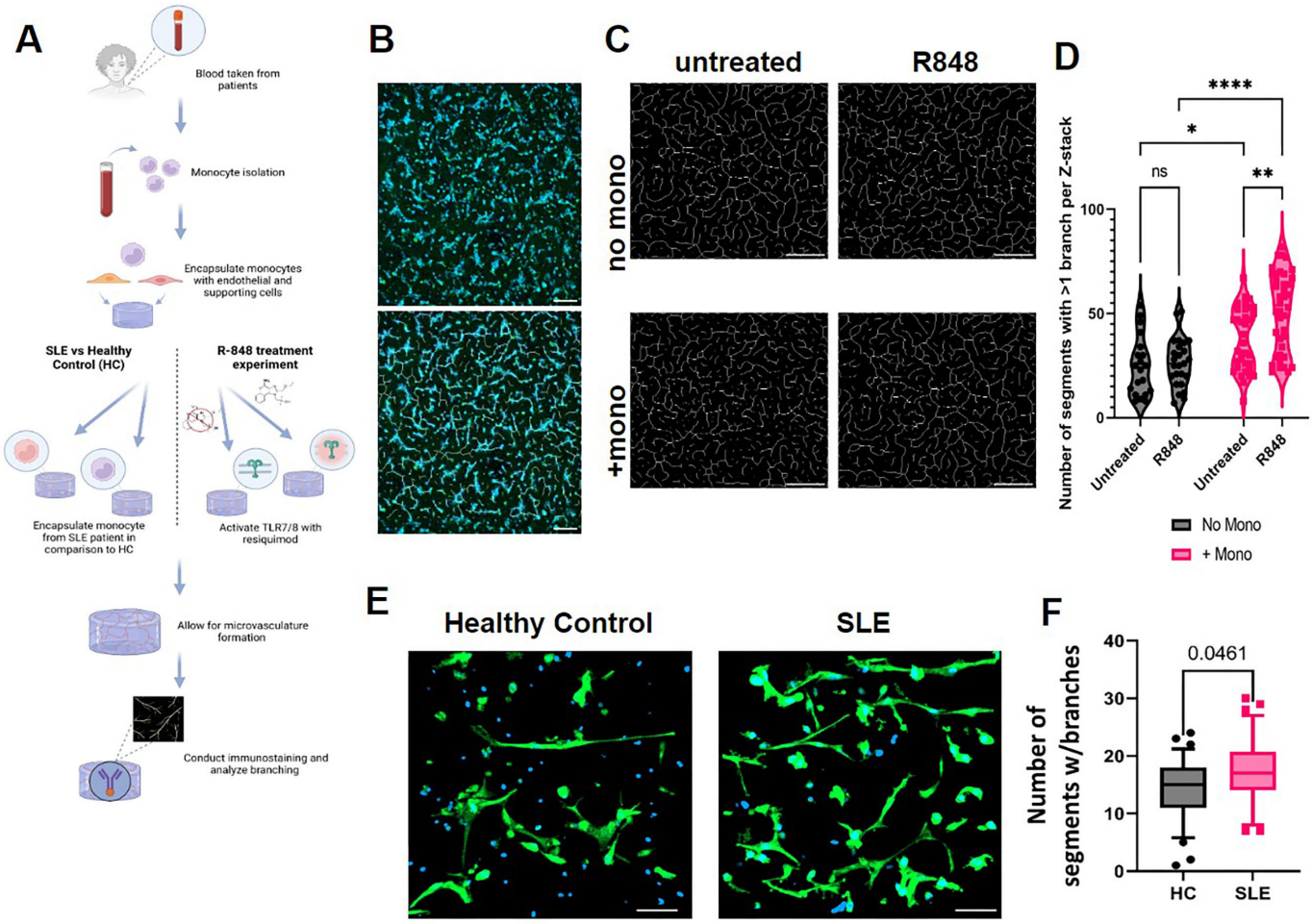

Figure 1 (A–F) Preclinical model of monocyte-mediated microvessel structural changes in systemic lupus erythematosus (SLE). (A) Schematic demonstrating proof of concept of an in vitro model of SLE pathological microvessel formation under monocyte modulation. (B–D) R-848 treatment experiment with tri-culture of monocytes, pericytes and endothelial cells demonstrates significantly more branching segments with R-848 treatment in a three-dimensional hydrogel system. Scale bar=100 µm. (B) Representative flattened Z-stack confocal image (top) and representative skeletal overlay (bottom) of stained endothelial marker CD31 (green) and nuclei marker DAPI (blue). (C) Representative skeletonised images for branch point analysis of R-848 treated and untreated hydrogels with or without monocytes. (D) The number of skeletonised segments with at least one branch point was analysed for each Z-stack and the results were compared among the different groups with *p≤0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001. Each dot in the violin plot represents the number of segments with >1 branch in a single Z-stack, with five non-overlapping images per gel. Statistical analysis was conducted through a two-way analysis of variance with Šídák's multiple comparisons test. Monocytes derived from patients with SLE (n=7) (E, right) demonstrate greater number of microvessel segments compared with healthy controls (n=3) (E, left) when co-cultured in a three-dimensional environment. Scale bars=50 µm. Green=CD31; blue=DAPI. (F) Comparison of numbers of branching endothelial segments among different groups. Statistical analysis performed through a Kruskal-Wallis test with Dunn's post-hoc multiple comparisons test. TLR, Toll-like receptor.

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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