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Micro-engineered perfusable 3D vasculature for cardiovascular diseases

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Vessel geometries in microengineered *in vitro* vascular models are important to recapitulate pathophysiological microenvironment for the study of flow-induced endothelial dysfunction and inflammation in cardiovascular diseases. Herein, we present a simple and novel extracellular matrix (ECM) hydrogel patterning method to create perfusable vascularized microchannels of different geometries based on the concept of capillary burst valve (CBV). No surface modification is necessary and the method is suitable for different ECM types including collagen, matrigel and fibrin. We first created collagen-patterned, endothelialized microchannels to study barrier permeability and neutrophil transendothelial migration, followed by the development of a biomimetic 3D endothelial-smooth muscle cells (EC-SMC) vascular model. We observed a significant decrease in barrier permeability in the co-culture model during inflammation, which indicates the importance of perivascular cells in ECM remodeling. Finally, we engineered collagen-patterned constricted vascular microchannels to mimic stenosis in atherosclerosis. Whole blood was perfused (1–10 dyne cm⁻²) into the microdevices and distinct platelets and leukocytes adherence patterns were observed due to increased shear stresses at the constriction, and additional convective flow through the collagen. Taken together, the developed hydrogel patterning technique enables formation of unique pathophysiological architectures in organ-on-chip microsystems for real-time study of hemodynamics and cellular interactions in cardiovascular diseases.

Introduction

Endothelium is a significant part of the human vasculature for bio-transport and regulating angiogenesis, inflammation and wound healing in health¹ and diseases such as cancer² and cardiovascular diseases.³ While *in vitro* and *in vivo* vascular models have been developed to study vessel integrity,^{4, 5} angiogenesis,⁶ leukocyte transendothelial migration (TEM)⁷ and cancer metastasis,⁸ *in vitro* models (mostly 2D) do not accurately emulate the intricacies of the 3D microenvironment found *in vivo*.^{9, 10} and animal models are complex due to limited control over the physical and biological parameters. 3D tissue engineered blood vessels or vascular grafts^{11, 12} provide a multifactorial environment which are critical for the endothelial cells (EC) functioning and vessel development, but they often fail to reproduce atherogenic features including blood perfusion and vessel geometry, which can affect endothelial functions. As flow disturbance and endothelial

dysfunction at the early stage can manifest into cardiovascular complications,¹³ it is important to develop perfusion-based vascular models with pathological vessel geometries to better understand disease pathophysiology.

Microfluidics is an attractive alternative to develop *in vitro* microvascular models¹⁴ with established microfabrication methods and integration of flow systems. A widely used method for microfluidic cell culture model is extracellular matrix (ECM) patterning using micropillars based on the concept of capillary burst valves (CBV).¹⁵ During ECM hydrogel loading, the micropillars cause a change in contact angle in the XY plane, thereby confining the ECM between the array of pillars due to surface tension.¹⁶ This method has been widely used to create ECM partitions to study cell-cell interactions and endothelial functions in cancer metastasis, angiogenesis and inflammation.^{17–20} However, a major design limitation is the inability to form complex channel geometries which are vital in cardiovascular complications. Moreover, the presence of micropillars leads to a discontinuous EC-ECM interface, and vascular cells will experience differential biomechanical cues arising from flow-induced shear stress and substrate stiffness. Other microfluidics approaches have been developed to address these issues, but are limited by laborious fabrication,^{21, 22} geometrical constraints,^{23–26} and lack of physiologically relevant perfusion.^{27–29}

Herein we introduce a novel ECM patterning technique to create biomimetic 3D vascular microchannels of different

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geometries without micropillars. Using a 2-layered polydimethylsiloxane (PDMS) device comprising of an ECM loading base (bottom) and the microchannel design (top), the ECM gel introduced at the loading chamber stops at the interface defined by the microchannel design due to CBV at the sudden channel expansion in the Z-axis (Figure. 1). We first patterned perfusable endothelialized microchannels with different features like curvature, stenosis and bifurcations using type I collagen. A functional endothelial barrier was formed along the collagen wall which exhibited size-selective diffusive permeability and increased permeability during vascular inflammation. We further characterized the convective barrier permeability under flow and showed an increase in 2 μm bead penetration through the inflamed endothelial monolayer. To study leukocyte recruitment, chemoattractant N-Formylmethionine-leucyl-phenylalanine (fMLP) was added into the ECM to induce neutrophil transendothelial migration. We next created a 3D co-culture blood vessel model by patterning vascular microchannels in collagen laden with aortic smooth muscle cells (SMC), and demonstrated enhanced vascular integrity due to cell-cell interactions and ECM remodeling by the perivascular cells. Finally, we engineered biomimetic atherosclerosis-on-a-chip devices with different channel constrictions to mimic stenosis. Under whole blood perfusion, unique localization patterns of platelets and leukocytes on healthy and inflamed endothelium were observed due to shear-modulated interactions and secondary convective flow through the ECM constrictions. Taken together, these results clearly highlight the significance of our method to create perfusable, functional microvasculature models to study hemodynamics and cellular interactions in cardiovascular diseases.

Experimental Section

Device fabrication

The two-layered polydimethylsiloxane (PDMS, Dow Corning) blood vessel on-a-chip model was fabricated using standard photolithography and soft lithography procedures. Briefly, PDMS prepolymer was mixed with the curing agent in a 10:1 ratio (w/w) and poured on a patterned silicon wafer template. The mixture was cured in an oven at 80 $^{\circ}\text{C}$ for 2 hr and peeled carefully from the wafer. Inlet holes (1.5 mm) were defined using a biopsy puncher on the top layer. The alignment of the PDMS layers resulted in the formation of a differential height (channel expansion), with the channel intersection having a combined height of 300 μm .

Device preparation

Collagen Type I (rat tail, 2.5 mg/mL, Corning) was prepared by following the procedure used by Shin *et al.*¹⁸ The gel was introduced into the bottom ECM chamber and allowed to polymerize for 30 min at 37 $^{\circ}\text{C}$. To facilitate cell adhesion, the top microchannel was functionalized with fibronectin (50 $\mu\text{g}/\text{mL}$) for 15 minutes prior cell loading. Other ECM types such as fibrin gel (Baxter) and matrigel (BD Bioscience) were loaded

using the same method as described. To develop a co-culture model, SMC were suspended in the collagen gel at a concentration of 1×10^6 cells/mL before loading.

Monolayer permeability

Human Umbilical Vein Endothelial Cells (HUVECs) were grown for 24 hr to confluency and tested for barrier permeability using 40 kDa and 10 kDa dextran conjugated with fluorescein isothiocyanate (FITC, Sigma Aldrich). Fluorescence images were taken before and after 1 hr incubation with the FITC-dextran. The effective permeability was calculated from concentration difference across the EC barrier over time (Equation 1), using Fick's law as discussed by Huxley *et al.*³⁰

$$E.P = \frac{W}{T} \times \frac{I_{60} - I_0}{I_0 - I_b} \quad (1)$$

Where I_b is the background intensity at the start, I_0 is mean fluorescence intensity of a specific portion at the start, I_{60} is the mean fluorescence intensity at the same portion after 1 hr, W is the width of the channel in centimetres and T is total time in seconds. Effective permeability studies under inflammation were performed by treating HUVECs inside the channel with 10 ng/mL of tumor necrosis factor alpha (TNF- α , Peprotech) for 12 hr prior to dextran introduction. Convective barrier permeability was studied by perfusing 2 μm fluorescent polystyrene beads (Bangs Laboratories) for 15 min at a wall shear stress of 3 dynecm^{-2} using a syringe pump (Chemyx Inc.).

Leukocyte transendothelial migration assay

Neutrophils were isolated from whole blood using Dean flow fractionation³¹ and stained with Hoechst 33342 (Life Technologies) before introduction into the cell channel. N-Formylmethionine-leucyl-phenylalanine, fMLP (500 nM, Sigma Aldrich), a potent chemoattractant, was used to perform chemotaxis. To generate a chemoattractant gradient in the ECM, a 100 μL drop of fMLP was placed at each of the inlet ports of the ECM chamber and allowed to diffuse towards the cell channel through the ECM. FITC dye was added to the chemoattractant to visualize the chemical gradient. Fluorescence images were taken before and after a 4 hr incubation using an inverted fluorescence microscope (Nikon Eclipse Ti, Japan).

Atherosclerosis-on-a-chip

To study atherosclerosis, the two-layer PDMS device consisted of an ECM chamber with dimensions of 10 mm (w) $\times$ 10 mm (l) $\times$ 0.1 mm (h) and a microchannel with dimensions of 1 mm (w) $\times$ 25 mm (l) $\times$ 0.05 mm (h). FITC-conjugated 2 μm beads were used to characterize the flow disturbance created by a 50% or 80% occlusion in the microchannel design. All perfusion studies were performed at inlet wall shear stresses of 1 dynecm^{-2} or 10 dynecm^{-2} . To determine the impact of flow disturbance on leukocytes and platelets adhesion, whole blood was used. The blood was diluted by 3 times with phosphate buffered saline (PBS) to maintain the device integrity at higher flow rates, and stained with rhodamine 6G (Sigma Aldrich) to visualize leukocytes and platelets.

Study approval

Written informed consent was obtained for all subjects during recruitment. All protocols were approved by the institutional review board of Nanyang Technological University (IRB-2014-04-27), in compliance with the Human Biomedical Research Act (Ministry of Health, Singapore). For fingerprick blood sampling, blood was obtained from healthy donors using a disposable lancet (Roche Diagnostics Corp.) and collected in EDTA tubes (BD Microtainer). For blood sampling by venipuncture, ~3 mL of blood was collected into sodium citrate vacutainer (BD Biosciences).

Statistical analysis

All numerical data were expressed as mean $\pm$ standard deviation (SD) unless specified otherwise. Mann-Whitney test was used to assess the statistical significance between two groups and $P < 0.05$ was considered as significant difference.

Refer to experimental section in SI for information on cell culture, device characterization and immunostaining.

Results

Formation of vascular microchannels using CBV

To generate CBV in the Z-axis (height), we developed a two layered PDMS device consisting of a top microchannel design and a bottom ECM loading chamber (Figure 1A). ECM gel introduced at the loading chamber stops at the interface defined by the microchannel design due to a sudden channel expansion along the height (Figure S1). By varying microchannel designs and ECM chamber with different gel port configurations, we successfully patterned microchannels with bifurcations and curvatures which are otherwise difficult to achieve using existing microfluidics methods (Figure S2). We further tested different ECM hydrogels including collagen, matrigel and fibrin and observed similar patterning efficacy without requiring surface modification, illustrating the robustness and versatility of our ECM patterning technique (Figure S3). After the collagen gel had polymerized, HUVECs were seeded into the microchannel and cultured for 24 to 72 hr to form a confluent monolayer (Figure 1B, C). Confocal imaging showed the growth of an endothelial barrier along the ECM sidewalls (Figure 1D, E). We next perfused FITC conjugated 2 μ m beads at physiological wall shear stresses ($1 - 10 \text{ dyne cm}^{-2}$), and did not observe significant ECM breakages or shrinkages, further indicating its potential for subsequent perfusion-based studies (Figure 1F).

Figure 1: Flexible 3D extracellular matrix (ECM) patterning using microfluidics: (A) Schematic illustration of the microfluidic devices for ECM loading channels and microchannel designs. The ECM loading channel (bottom) and microchannel design (top) devices are aligned together and ECM hydrogel (with coloured dye) is loaded into the ECM chamber ports. (Scale bar: 1 mm) **(B)** Overview of the device fabrication process. The hydrogel loaded into the ECM chamber will stop at the boundaries of the microchannel design due to CBV principle, and hence form a patterned cell culture channel with ECM sidewalls. **(C)** (Top) Fluorescence images of FITC-laden collagen gel for different patterned channel geometries. (Bottom) Brightfield images of corresponding endothelialized (HUVECs) channels. **(D)** Brightfield and fluorescence images of a planar section (dotted line) of the HUVECs monolayer inside the channel. **(E)** Confocal image showing a continuous 3D HUVEC monolayer along the collagen sidewall. (Scale bar: 100 μ m) **(F)** Representative fluorescent stacked image illustrating perfusion of 2 μ m fluorescent polystyrene beads in a Y-channel. (Scale bar: 500 μ m)

Characterization of ECM patterning using CBV

ECM hydrogel injected into the bottom chamber flows into the channel due to pressure differences and the dynamic contact angles of the solution (Θ_T with the top surface and Θ_B with the bottom surface, $\Theta_T \approx \Theta_B$) are greater than the critical advancing contact angle (Θ_A)¹⁶. When the advancing meniscus encounters the channel expansion at the top due to the additional presence of the microchannel design layer, the dynamic contact angle with the top wall decreases from Θ_T to $\Theta_T - 90^\circ$ and the flow stops immediately when $\Theta_T - 90^\circ < \Theta_A$. The meniscus remains pinned at the top until a larger driving force is applied, to increase Θ_T and burst the meniscus ($\Theta_T > \Theta_A$).

To characterize the patterning resolution of CBV on channel width, we manually pipetted FITC-laden collagen gel into the ECM ports of microchannels of varying widths (1000 μm , 500 μm , 200 μm and 100 μm). For larger channel widths of 200 – 1000 μm , microchannels were patterned with high reproducibility due to the larger gap size between both ECM sides. However, patterning microchannels of 100 μm width was more challenging as the collagen gel would bulge outwards along the bottom wall from either or both sides and completely seal the microchannel, resulting in ECM patterning failure. (Figure 2A). Given the difficulties in controlling the injection pressure during manual pipetting, the lower limit of the CBV method was determined as 100 μm . To study the effect of channel height of the microchannel design (top layer), we carefully sliced the aligned 2-layered PDMS devices along the channel width (x-direction), and bonded the channel cross-section on a cover slip. The device configuration enables us to load the gel into the bottom ECM chamber and observe the

position of hydrogel from the side view (Figure 2B). As expected, we observed successful CBV effects for all heights (25 μm , 50 μm and 150 μm) tested, further confirming that the width of the microchannel design is the most critical design parameter for ECM patterning using CBV. In our subsequent studies, we fixed the dimensions of the top microchannel design (500 $\mu\text{m} \times 150 \mu\text{m}$), unless specified otherwise.

Figure 2: Characterization of CBV effect on ECM patterning: (A) Fluorescence images of microchannels patterned using FITC laden collagen gel. Cross section schematics indicate corresponding channel dimensions. (B) Cross sectional view of FITC-laden collagen gel with different microchannel heights. (C) Fluorescence images of FITC-laden collagen gel inside sectioned chips (x-x') illustrating increase in top contact angle (θ_T , purple line) while collagen remained pinned at the top edge with higher loading pressure. Inset images (1, 2 and 3) highlight the pinning effect of the collagen at the top edge with increasing loading pressure. Plots indicating strong linearity of θ_T and pressure drop with maximum gel distance (D) which is measured at the centre of the collagen bulge, and the gel distance along the bottom surface (d). (Scale bar: 200 μm)

To quantify the CBV effects in our device, we defined 2 parameters, namely the maximum gel distance (D) which is

measured at the centre of the collagen bulge (from the edge of the top microchannel), and the gel distance along the bottom surface (d). As shown in Figure 2C, the collagen gel bulged further along the bottom surface with increasing pressure while remaining tightly pinned at the top due to CBV effect. A strong linearity (0.87 – 0.97) was observed when we plotted the bulging distance (D and d) against θ_T and pressure drop, which was consistent with the Young-Laplace equation that describes the pressure drop as a function of curvature between 2 static fluids (air and collagen in our model).¹⁶ Noteworthy, the maximum D observed was $\sim 50 \mu\text{m}$, which corresponds to a minimum channel width of $\sim 100 \mu\text{m}$ and was similar to our prior experimental observations.

Functional characterization of endothelial cell (EC) barrier

After the formation of a confluent EC monolayer (HUVECs) in the collagen-patterned microchannel, FITC-conjugated dextran (40 kDa, 10 μM) was loaded into the channel, which formed a diffusion based gradient into the ECM sidewalls after 1 hr under static conditions (Figure 3A). The change in fluorescence intensity was then used to calculate the effective permeability (see experimental section). The barrier functionalities in our vascular model were well-preserved as it exhibited size-selective diffusive permeability ($6.93 \times 10^{-6} \text{ cm/s}$ for 10 kDa dextran vs. $3.014 \times 10^{-6} \text{ cm/s}$ for 40 kDa dextran, $P < 0.01$) and the values obtained were similar with previous studies (Figure 3B).^{22, 23, 28} A significant modulation of barrier permeability ($3.014 \times 10^{-6} \text{ cm/s}$ for healthy EC vs. $7.9 \times 10^{-6} \text{ cm/s}$ for inflamed EC, $P < 0.01$) was also observed when treated with pro-inflammatory cytokine tumor necrosis factor α (TNF α), indicating its physiological relevance in modeling vascular inflammation (Figure 3C).

Figure 3: Characterization of endothelial (HUVECs) barrier function: (A) Fluorescence intensity linescan and image of the microchannel loaded with FITC-conjugated Dextran (40 kDa). Blue region indicates channel position. (B) Selective barrier permeability of the endothelial monolayer using FITC-conjugated dextran molecules of different molecular weights (10 kDa and 40 kDa) ($n=4$). (C) Significant increase in endothelial barrier permeability when treated with TNF α ($n=4$). (D) Representative images illustrating convective barrier permeability under flow. 2 μm beads penetrated further into the collagen gel for TNF α -treated endothelial cells (EC) as compared to healthy EC. (E) Plot indicating 2 μm beads penetration into collagen gel. Shaded line (at 100 μm) corresponds to position of EC barrier. (F) Characterization of full width at half maximum

(FWHM) of the fluorescent spectrum for healthy and TNF α -treated EC (n=4). Data are presented as mean $\pm$ s.d. (**p<0.01, *** p<0.001).

Next, we assessed the EC convective barrier permeability under flow by perfusing 2 μ m fluorescent beads into the channel (3 dyne cm^{-2}). As expected, the microbeads accumulated on the healthy EC due to intact barrier while a significant number of beads penetrated further into the ECM for TNF α -treated EC, indicating a leakier barrier (Figure 3D). This can be further quantified by measuring the full width at half maximum (FWHM) of the fluorescent spectrum between healthy and TNF α -treated EC (Figure 3E). ~2-fold increase in convective barrier permeability was observed in TNF α -treated EC as compared to healthy EC, which was similar to the static diffusive permeability assay (Figure 3F). Taken together, these results highlight the potential of our platform to assess barrier functions in both static and perfusion conditions.

Neutrophil transendothelial migration

Figure 4: Neutrophil chemotaxis and endothelial-smooth muscle cells (EC-SMC) co-culture: (A) Chemoattractant (N-Formylmethionine-leucyl-phenylalanine (fMLP), 500 nM) is introduced at one side of the collagen gel to create a concentration gradient for neutrophil migration. Brightfield image illustrates neutrophil migration across the EC barrier (HUVECs) into the collagen gel after 2 hr. (B) Cell count of the migrated neutrophils in the presence and absence of fMLP (n=4). (C) Confocal images of the endothelialized (EC) microchannel surrounded by aortic smooth muscle cells (SMC). Inset shows a magnified section of the EC-SMC boundary and its corresponding Z-stacked image to illustrate 3D arrangement of different cell types. (D) Comparison of EC barrier permeability with and without SMC in healthy (unshaded) and TNF α -treated (solid) conditions (n=4). Data are presented as mean $\pm$ s.d. (*p<0.05, *** p<0.001)

During infection or acute inflammation, leukocyte transendothelial migration (TEM) is an important innate immune response for neutrophils to migrate to the site of injuries based on chemical gradients, which can be studied using 3D microfluidic cell-based models.^{20, 32} Using our developed microvascular models, neutrophils purified using Dean Flow Fractionation³¹ were introduced into the endothelialized microchannel treated with TNF α to facilitate

neutrophil-endothelial interactions.³³ A potent bacterial peptide (fMLP, 500 μ M) was then loaded on one side of the ECM to establish a chemoattractant gradient. FITC solution (MW ~380 Da, similar to fMLP (438 Da)) was used to study the diffusion gradient which remained stable and linear for up to 4 hr (Figure S4). During this time, we observed persistent neutrophils migration across the endothelial monolayer (Figure 4A), which led to a significant increase in neutrophil migration into the gel with fMLP as compared to the non-fMLP side (Figure 4B).

Co-culture of perivascular niche in 3D biomimetic vascular model

A key advantage of ECM-based vascular model is the ability to introduce other perivascular cells in the microenvironment for 3D co-culture.²² As proof-of-concept, we engineered an endothelialized Y-microchannel surrounded by SMC in collagen, which are integral in angiogenesis and maintaining vascular stability.³⁴ Confocal imaging along the channel height indicated the formation of an EC monolayer lumen and 3D arrangement of SMC in the collagen after 3 days of culture (Figure 4C). We observed lower diffusive permeability in the co-culture model (8.61×10^{-6} cm/s for EC vs 5.21×10^{-6} cm/s for EC-SMC, P<0.001) during vascular inflammation, which was consistent with previous report,³⁵ possibly due to EC-SMC interactions and active ECM remodelling by the perivascular cells (Figure 4D). These results were further validated in a separate vascular model where we seeded SMC on only one side of the ECM, and observed lower EC barrier permeability in the SMC side as compared to the non-SMC side during inflammation (Figure S5). These results highlight the flexibility and importance of our method to develop a 3D microvasculature consisting of endothelial and perivascular cells to study endothelial barrier functions.

Engineered stenotic vascular channels to mimic atherosclerosis

Atherosclerosis is a major cause of cardiovascular diseases and is marked by the accumulation of cholesterol-containing low density lipoproteins (LDL) and its subsequent uptake by macrophages to form atherosclerotic lesions or plaques in the subendothelial space (intima).^{36, 37} This results in severe aneurysms or vessel occlusion which can cause blood flow abnormalities and shear stress disturbance on vascular cells.³⁸ By engineering collagen-patterned constrictions in our EC-SMC model, we successfully recreated the atherogenic microenvironment of the lesion (SMC in collagen), and the degree of constriction can be easily controlled by the microchannel design (top layer) (Figure 5A). To illustrate the importance of vascular microenvironment, we introduced FITC-conjugated 2 μ m beads into microchannels with PDMS or collagen-patterned constriction (80%) at an inlet wall shear stress of 10 dyne cm^{-2} , which is within the range of physiological wall shear stress (~10-70 dyne cm^{-2}) in the arterioles.³⁹ As shown in Figure 5B, beads flow profile was laminar and there were negligible beads binding to the PDMS constriction. In contrast, a significant number of beads accumulated at the proximal edge of the collagen bump and

Figure 5: Atherosclerosis-on-a-chip model: (A) Brightfield and confocal images of an endothelialized lumen surrounded by SMC in a microchannel with 50% collagen-patterned constriction. (B) (Top) Representative fluorescent images illustrating perfusion of FITC-conjugated 2 μm beads in microchannels with PDMS and collagen-patterned constriction (80%). Beads accumulated at the proximal edge (red arrows) of the collagen bump at an inlet wall shear stress of 10 dyne/cm². (Bottom) Composite image indicating beads accumulation at the EC barrier in an endothelialised channel. (C) Whole blood perfusion through a microfluidic chip with collagen-patterned constriction (80%). (D) Characterization of platelet (R6G-stained) binding at the proximal region of the 50% and 80% collagen constriction under whole blood perfusion at 10 dyne/cm². Representative images and fluorescent intensity plot indicating significant platelet accumulation (red arrows) in the 80% constricted microchannel.

was consistent with previous observations reporting localized regions of cell attachment at the proximal and distal edges.^{40, 41} We hypothesize that this is due to additional convective shear flow through the porous collagen constriction at the proximal edge which enhances cellular contact with the EC monolayer. Lastly, we also varied flow conditions in different collagen constrictions (50% and 80%) and did not observe beads accumulation for other conditions (Figure S6), indicating the importance of channel geometry and porous vascular microenvironment in *in vitro* blood vessel model.

Whole blood perfusion in stenotic collagen-patterned vascular channels

For blood perfusion studies, we used endothelialized (HUVECs) constricted microchannels (without SMC) as the goal was to assess the biophysical effects of blood flow and immune cells-EC interactions in collagen microenvironment. In our experiments, human whole blood was diluted 3 times with saline to match the ECM viscosity to prevent leakages into the collagen sidewalls (Figure 5C). Whole blood was first perfused into the collagen-patterned constricted microchannels in the absence of EC monolayer to eliminate cellular interactions with the vasculature. Similar to 2 μm beads, there were minimal platelets or immune cells adhesion upstream and downstream of the constriction at 10 dyne/cm². However, platelets (R6G stained) accumulated at the proximal edge of the collagen constriction with higher fluorescence intensities observed at the 80% constriction as compared to 50%

constriction region, confirming the role of shear stress in platelets build up during stenosis (Figure 5D).

Next, we perfused whole blood into endothelialized collagen patterned microchannels with 80% constriction. For a healthy endothelium, there was minimal leukocytes interactions with the EC monolayer at 1 dyne/cm². Platelets binding was locally increased at the channel constriction due to the higher shear flow which promotes shear-induced platelet activation⁴² and EC secretion of the von Willebrand factor (vWF) (Figure 6A).⁴³ For whole blood perfusion (1 dyne/cm²) on an inflamed endothelium (TNF-α treated), we observed significant leukocyte-EC interaction (rolling and adhesion) and was consistent with previous studies reporting upregulation of inflammatory-related endothelial adhesion markers which led to enhanced leukocyte recruitment (~1-4 dyne/cm²)³³ (Figure 6B). When we increased the blood perfusion to 10 dyne/cm², leukocyte adhesion was greatly reduced on the inflamed EC along the channel while there was a localized increase in platelets/leukocytes adhesion at the proximal and distal edges of the collagen constriction after 15 min of blood perfusion (Figure S7). Interestingly, there was also significant platelets build up at the proximal edge nearer to the constriction (Figure 6C), which was similar with 2 μm beads behaviour, and further suggests the presence of additional convective flow through the collagen sidewalls at higher shear conditions. Taken together, these results illustrate the application of our novel ECM-patterning technique to engineer atherogenic vessel geometries and microenvironment for studying hemodynamics and immune cells interactions at pathological shear stresses.

Figure 6: Endothelial-blood cell interactions during vascular inflammation: (A) Representative fluorescence image of healthy endothelium after 15 min of whole blood (R6G stained) perfusion at 1 dyne cm^{-2} . Magnified image of the proximal region (red box) of the constriction highlighting platelet adhesion (green arrows). (B) Representative fluorescence image of the inflamed endothelium (TNF- α treated) after 15 min of whole blood perfusion at 1 dyne cm^{-2} . Magnified image indicating platelets (green arrow) and leukocytes (blue arrows) adhesion. (C) Representative fluorescence image of the inflamed endothelium (TNF- α treated) after 15 min of whole blood perfusion at 10 dyne cm^{-2} . Red arrows indicate significant accumulation of platelets and leukocytes at the proximal edge of the occlusion. Magnified image highlighting platelets (green arrow) and leukocytes-platelets (red arrows).

Discussion

Unlike animal models and tissue engineered blood vessels, *in vitro* microfluidic vascular models offer well controlled microenvironment including precise flow control, gradient generation and ability to integrate flow systems to recapitulate *in vivo* physiology and pathological responses.⁴⁴ In this work, we report a novel ECM patterning method based on CBV principle to engineer perfusable-functional vasculature microsystems of complex vessel geometries including curvatures, bifurcations and stenosis. Vessel growth by angiogenesis or vasculogenesis can form perfusable 3D vascular networks but these strategies have poor spatial control over vessel geometry and architecture.²¹ By eliminating the use of micropillars and implementing the CBV effects along the channel height (z-axis),¹⁵ we can form a continuous EC-ECM interface which is not only more physiologically relevant, but the absence of PDMS micropillars greatly reduces bubbles generation (between pillars) for study of barrier functions and cell-cell interactions during perfusion. We demonstrated the abilities of our collagen-patterned vascular models to reproduce key inflammatory-related features including increased diffusive and convective EC barrier permeability, neutrophil transmigration, as well as

development of a biomimetic 3D EC-SMC co-culture platform. As proof-of-concept for atherosclerosis-on-a-chip, we further developed constricted microchannels to study whole blood perfusion and immune cells-EC interactions during stenosis.

Vessel microenvironment comprises of porous semi-solid ECM (fibronectin, collagen, laminin etc.) to provide nutrients supply to perivascular cells and generation of spatio-temporal concentration gradients for immune responses.⁴⁵ In our model, we mimicked a bacterial infection by generating a stable fMLP gradient over extended time periods to induce neutrophil transendothelial migration. With the ability to selectively introduce chemicals in different ECM compartments, we can further establish complex diffusive-based concentration gradients to study various physiological processes such as angiogenesis and cancer metastasis.^{17, 19} Perivascular cells (SMC, fibroblasts) interacts constantly with EC and secrete ECM to maintain vascular integrity,³⁵ and are implicated in cancer⁴⁶ and atherosclerosis.⁴⁷ While we observed reduced vascular permeability during inflammation in our EC-SMC model, further studies are warranted to provide mechanistic insights into the crosstalk between EC and perivascular cells, as well as ECM remodeling. Nevertheless, these results elucidate the physiological relevance of our co-culture system to assess vascular integrity and can be extended to study SMC phenotypes in different diseases.

Atherosclerosis is a geometrically-focused disease with vessel bifurcations and curvatures being prone to lesion formation due to low shear stresses and blood flow disturbance. Our technique is ideal for atherosclerosis studies as it provides the geometric flexibility to create relevant vascular geometries and microenvironment for atherosclerotic plaque. Microfluidic stenosis models have been previously developed to study the flow effects around the constriction on platelet adhesion⁴⁸ and drug targeting.⁴⁹ However, these models are fabricated using PDMS and fail to mimic leakiness of the constriction itself. In our collagen-patterned constricted vascular channels, we clearly illustrated the importance of porous constrictions with additional convective flow at the proximal region of the collagen bump which resulted in increased beads and platelet accumulation at higher flow conditions (10 dyne cm^{-2}). These results provide supporting evidence that in addition to atherosclerotic lesion growth at the distal region due to the recirculation zone,⁴⁰ enhanced leukocyte and platelet adhesion at the proximal region, may also contribute to the overall atherosclerosis development.⁴¹

Conclusion

In summary, we have developed a novel ECM patterning technique to create functional, perfusable *in vitro* 3D blood vessel model of different atherogenic architectures and perivascular microenvironment. Besides simple and low-cost fabrication processes, this versatile method is suitable for different ECM hydrogels, and can be used to engineer 3D co-culture in complex organ on-a-chip models to study cardiovascular diseases or other endothelial dysfunction in general.

Conflicts of interest

There are no conflicts of interest to declare.

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