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Engineered human vascular models to study endothelial dysfunction in infectious disease

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FULL TECHNICAL REPORT

Engineered human vascular models to study endothelial dysfunction in infectious disease

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Abstract

Vascular involvement plays a pivotal role in the pathogenesis of numerous emerging viral threats, with climate changes amplifying the likelihood of these diseases evolving into pandemics. Establishing an infectable and physiologically relevant human in vitro model holds immense importance in comprehending the pathogenesis of these threats and devising effective countermeasures. Although *in vivo* models provide valuable insight into disease, their application is restricted by interspecies variations, particularly concerning pathogens exclusive to humans. Moreover, animal models often fail to replicate comorbidities prevalent in patient populations, thus limiting our understanding of disease behavior in humans. In this study, we have devised a *testing platform aimed at exploring pathogen interactions with innate immunity within the vasculature*, encompassing both healthy and dysfunctional endothelial conditions.

Background and Research Objectives

The pathogenesis of numerous emerging viral threats is intricately linked to vascular involvement. Pathogens that affect endothelial cells lining blood vessels have profound systemic consequences, leading to severe complications such as thrombosis and vascular leakage. [1] While the innate immune response serves as a critical defense against these infections, it can also contribute to endothelial damage[2], as seen in diseases like COVID-19 where "cytokine storms" or massive release of inflammatory mediators are associated with mortality[3]. The absence of therapies to restore endothelial homeostasis in infectious diseases necessitates new approaches that promote pathogen clearance while minimizing inflammation and collateral tissue damage.

Addressing the perilous consequences of vascular infections in clinical settings demands innovative approaches that can foster pathogen elimination while minimizing unnecessary inflammation and collateral tissue damage. The creation of such methodologies necessitates an understanding of intricate interplays among the endothelium, invading vasculotropic pathogens, and the innate immune system. Currently, there is no existing human model system that enables direct visualization of host-pathogen interactions in 3 dimensions (3D*)*. While *in vivo* models offer insights into disease pathogenesis, their utility for human-restricted pathogens is curtailed by interspecies disparities. Furthermore, animal models often inadequately mimic the comorbidities observed in patient populations, leading to a deficient comprehension of disease dynamics in humans. Establishing a platform to examine pathogen interactions with innate immunity in the vasculature would expedite the development of novel therapeutics for emerging vasculotropic pathogens like SARS-CoV-2 and pathogens anticipated to proliferate due to climate changes.

Our group has recently developed a human biomimetic model incorporating complex vascular structure, cell- extracellular matrix interactions, lumen structure and fluid flow which demonstrates an *in vivo* like antithrombotic surface.[4-6] We have demonstrated the ability to flow cells through this system at arterial pressures and flow rates. Unlike older 2D *in vitro* cultures or pseudo-3D transwell models, our models offer complex microenvironments akin to those that cells encounter *in vivo*. While our systems have demonstrated usefulness in monitoring *in vitro* thrombosis[5] or attachment of circulating tumor cells[4,6], adapting the model for investigating infectious disease of the vasculature is still needed to demonstrate the utility of platform for studying emerging threats.

In this work we aimed to establish the first human *in vitro* model to interrogate the interactions of pathogens, the innate immune system and endothelium that causes vasculopathy. The two objectives of this work were to (1) modify our model to include diseased states/comorbidities and (2) incorporate immune components (i.e., monocytes). *With our novel disease-immune model we can characterize how introduction of a pathogen gives rise to vascular dysfunction*.

Scientific Approach and Accomplishments

Developing vascular models of comorbidities that affect vascular health

Comorbidities that influence vascular health are often linked to poorer prognosis following pathogen infection.[7] Certainly, in the SARS-CoV-2 pandemic, the presence of one or more cardiovascular comorbidities is associated with heightened disease severity. In this work, we extended our existing vascular model to encompass a diseased state resulting in endothelial dysfunction. To create a model of vascular dysfunction, we exposed human brain microvascular endothelial cells to various concentrations of TNF alpha (**Fig. 1**), a multifunctional proinflammatory cytokine. TNF alpha is produced following activation of the nuclear factor kappa B (NF-κB) inflammatory pathway, often in response to stress or infection. At a dose of 0.5ng/mL of TNF alpha, increased vascular adhesion molecule-1 (VCAM-1, an inflammatory mediator) expression was observed. For subsequent studies to create inflammatory diseased states the dose of 0.5 ng/mL was used.

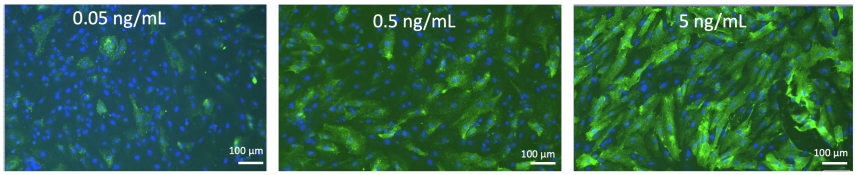

***Figure 1**. TNF alpha dosing experiments. TNF alpha treatment increased the level of VCAM-1 expression in endothelial cells*

We previously found that SARS-CoV-2 does not productively infect cells (21-FS-050). To explore the influence of comorbidities on vascular health after exposure to a pathogen, endothelial cells in well plates were treated with virus competent VSV pseudovirus at 10MOI with or without 1ng/ml TNF alpha and effluent collected for multiplex immunoassay panels for inflammation. We observed increased median nuclear area, increased NF-κB signaling and decreased cell proliferation, despite lack of productive infection. We were further interested in quantifying the endothelium glycocalyx layer, a protective luminal layer of membrane-bound proteoglycans and glycoproteins, in response to diseased states. The main components of the endothelial glycocalyx are proteoglycans, like

syndecans, rich in glycosaminoglycan side chains, such as heparan sulfate. Working with our collaborator at Northeastern, Dr. Ebong, we established methods to assess the glycocalyx thickness and structure using both transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) (**Fig. 2**). Human brain microvascular endothelial cells (passage 7) were plated at a density of ~200,000 cells/cm^2 on gelatin-fibrin hydrogels. Once cells were confluent, gels were exposed to 0.5% BSA in media for 4 hours, fixed for TEM analysis and incubated with 0.15% Alcian blue. Optimized Alcian blue protocol was successful in staining the endothelial glycocalyx (**Fig. 2 A-B**) and thickness measurements ranged from 64-162 nm which is comparable to what is found in literature [8-10]. CSLM imaging of endothelial cells stained for Heparan sulfates, also confirmed presence of glycocalyx on cells cultured in 2D (**Fig. 2C**).

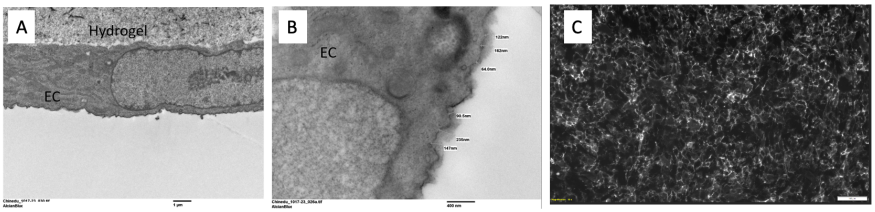

***Figure 2**. Optimized protocols for visualizing endothelial glycocalyx. (A-B) Ultrastructure images of Alcian Blue stained brain endothelial cultured on gelatin-fibrin hydrogel (C) Immunofluorescence staining of heparan sulfates, the mains component in the glycocalyx*

Characterize viral infection using SARS-CoV-2 pseudovirus treatment of endotheliallcells and vascular networks

To model infection in our vascular beds, we introduced a replication competent VSV pseudovirus. We created complex vascular networks by printing vascular beds using fugitive inks that are cleared leaving perfusable channels for endothelial cell lining. Engineered vessels were cultured under flow conditions until confluent. To characterize endothelium response, post-infection vessels were then treated with either SARS-CoV-2 spike pseudovirus and TNF alpha, or TNF alpha alone. Adapting our glycocalyx 2D staining protocols using labeled lectins, we treated our vascular beds and interestingly found higher expression of at incoming forks (**Fig. 3**). Pseudovirally treated vessels showed greater surface expression of VCAM-1 especially in atherogenically vulnerable vascular forks. We also noted broad spread abnormalities in nuclear structure in pseudovirally treated vessels, including syncytial formation and micronuclei. Through this work we demonstrate that engineered human vasculature can be used to model to study infectious disease under both diseased and healthy states.

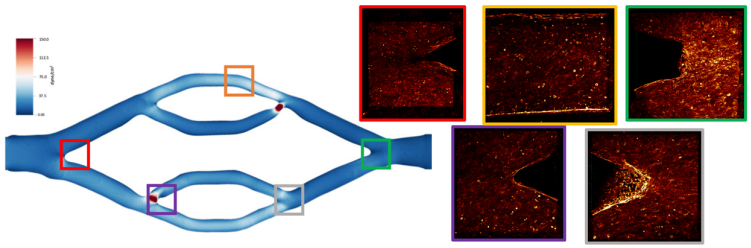

***Figure 3**. Staining patterns of fluorescently tagged wheat germ agglutinin, which binds to glycoproteins of the cell membrane that make up the glycocalyx, show more intense staining at incoming forks which correlate with areas of higher wall shear stress (Figure of wall shear stress distribution is from Pepona et al 2020*

Integrate innate immune components to the vasculature.

A key element of the vascular dysfunction in vasculotropic disease is activation of innate immune cells. These cells express toll-like and other pathogen associated molecular pattern receptors for detecting invading pathogens, and in response secrete inflammatory mediators such as TNF alpha, IL1B, or IL6, which contribute to vascular barrier dysfunction and coagulopathy. In our work we introduced circulating monocytes to our vascular model to compare how interactions between monocytes and the endothelium differ in infected or uninfected models. THP-1 cells were used as a model for primary human monocytes. These cells were cultured and labeled with lyophilic membrane dye. These labeled cells (300,000 cells/mL) were introduced into our recirculating media reservoir and allowed to run under flow. Results show minimal adhesion of immune cells to the healthy endothelium in the presence of un-activated immune cells. When compared to the inflammatory diseased state, cells exposed to TNF alpha, we saw an increased in THP-1 attached.

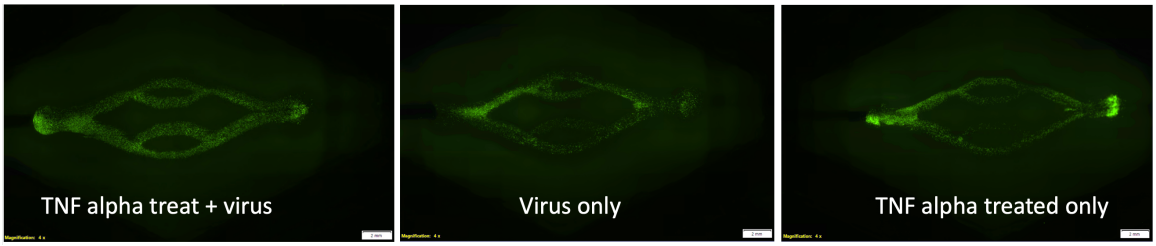

***Figure 4**. Immune attachment in diseased (TNF alpha treated) and viral exposed vessels Labeled THP-1 (green) cells showed greatest attachment in the inflammatory diseased state that was also exposed to virus.*

(varied concertation and labeled them)

Mission Impact

The work generated in this LW builds on two of the lab's core competencies: Advanced Materials and Manufacturing as well as Bioscience and Bioengineering. In this work, with the resources available at LLNL (functional vascular beds and infectious diseases expertise), we developed a platform to interrogate the interactions of pathogens, the innate immune system, and endothelium leading to vasculopathy. Establishing an infectable human *in vitro* model holds paramount importance in unraveling the complexities of severe diseases and devising robust countermeasures. This model marks a significant stride in comprehending host-pathogen interactions, aligning with LLNL's global security objectives. Ultimately, this research strengthens the scientific, technological, and engineering capabilities essential to the NNSA mission, reinforcing the Laboratory's expertise in bioscience and bioengineering. Partnership with Northeastern is anticipated to aid in developing a pipeline of talent in biomedical and materials engineering.

Conclusion

This work delves into the impact of comorbidities on vascular health during pathogen infection, specifically focusing on endothelial dysfunction. Through this work we have demonstrated the effectiveness of our *in vitro* human vasculature platform in analyzing viral pathogenesis in both diseased and healthy states. These engineered vessels exhibited increased inflammatory marker expression and structural abnormalities following pseudovirus treatment. Additionally, the integration of innate immune components into the vascular model revealed differences in immune

cell adhesion between healthy and diseased endothelium, highlighting the increased attachment of immune cells in the inflammatory state induced by TNF alpha. A manuscript highlighting the utility of this device and findings is under preparation and submission is anticipated by Spring 2024. The work from this study will serve as the preliminary data for future grant submissions.

Publications, Presentations, and Patents

E Zarate-Sanchez, SC George, ML Moya*, and C Robertson*. "Vascular dysfunction in hemorrhagic viral fevers: opportunities for organotypic modeling". Biofabrication (submitted)

"Bioprinted functional human vasculature and mechanisms of SARS-CoV-2 vasculitis". Poster presentation at 2022 Chemical and Biological Defense Science & Technology Conference, San Franciso, CA 6th-9th December 2022

"Engineering Human Vascular Beds To Study Endothelial Susceptibility To SARS-CoV-2." Oral Presentation at Tissue Engineering and Regenerative Medicine Annual Conference, Boston, MA 11th-14th April 2023

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References Cited

1 Konradt, C. & Hunter, C. A. Pathogen interactions with endothelial cells and the induction of innate and adaptive immunity. *Eur J Immunol* **48**, 1607-1620 (2018). https://doi.org:10.1002/eji.201646789

2 Mai, J., Virtue, A., Shen, J., Wang, H. & Yang, X.-F. An evolving new paradigm: endothelial cells--conditional innate immune cells. *J Hematol Oncol* **6**, 61-61 (2013). https://doi.org:10.1186/1756-8722-6-61

3 Perico, L. *et al.* Immunity, endothelial injury and complement-induced coagulopathy in COVID-19. *Nature Reviews Nephrology* **17**, 46-64 (2021). https://doi.org:10.1038/s41581-020-00357-4

4 Hynes, W. F. *et al.* Examining metastatic behavior within 3D bioprinted vasculature for the validation of a 3D computational flow model. *Science Advances* **6**, eabb3308 (2020). https://doi.org:10.1126/sciadv.abb3308

5 Jang, L. K. *et al.* Three-dimensional bioprinting of aneurysm-bearing tissue structure for endovascular deployment of embolization coils. *Biofabrication* **13**, 015006 (2020). https://doi.org:10.1088/1758-5090/abbb9b

6 Pepona, M. *et al.* Investigating the Interaction Between Circulating Tumor Cells and Local Hydrodynamics via Experiment and Simulations. *Cellular and Molecular Bioengineering* **13**, 527-540 (2020). https://doi.org:10.1007/s12195-020-00656-7

7 Trevelin, S. C., Carlos, D., Beretta, M., da Silva, J. S. & Cunha, F. Q. Diabetes Mellitus and Sepsis: A Challenging Association. *Shock* **47**, 276-287 (2017). https://doi.org:10.1097/SHK.0000000000000778

8 Ando, Y. *et al.* Brain-Specific Ultrastructure of Capillary Endothelial Glycocalyx and Its Possible Contribution for Blood Brain Barrier. *Sci Rep* **8**, 17523 (2018). https://doi.org:10.1038/s41598-018-35976-2

9 Walter, F. R. *et al.* Blood-brain barrier dysfunction in L-ornithine induced acute pancreatitis in rats and the direct effect of L-ornithine on cultured brain endothelial cells. *Fluids Barriers CNS* **19**, 16 (2022). https://doi.org:10.1186/s12987-022-00308-0

10 Hempel, C. *et al.* The need to freeze—Dehydration during specimen preparation for electron microscopy collapses the endothelial glycocalyx regardless of fixation method. *Microcirculation* **27**, e12643 (2020). https://doi.org:https://doi.org/10.1111/micc.12643