Modeling vascular inflammation with immune cell-vessel crosstalk in hiPSC-derived 3D vessels-on-chip

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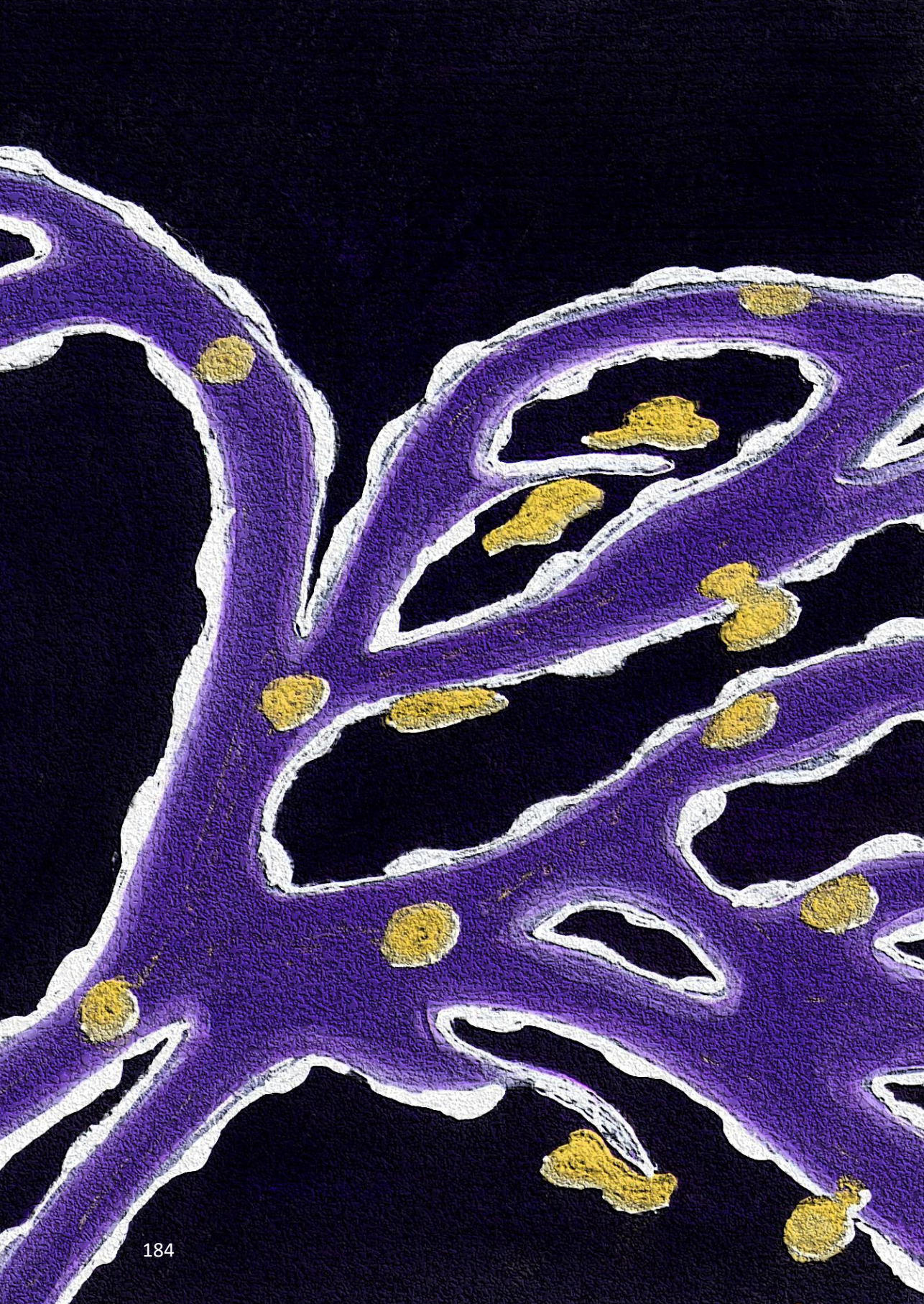

Chapter 7

General Discussion and Future Perspectives

Introduction

This thesis has developed advanced Vessel-on-Chip (VoC) models to investigate vascular inflammatory responses and replicate key physiological processes during inflammation. By incorporating hiPSC-derived endothelial cells (ECs), mural cells, monocytes, and macrophages in these models, it has been possible to study immune-vascular interactions in both healthy and disease-specific states using hiPSCs derived from hereditary hemorrhagic telangiectasia (HHT1) patients. Two complementary VoC systems were developed: the single-lumen VoC model which allowed detailed studies of monocyte trans-endothelial migration (TEM) under physiological shear stress, revealing the role of hiPSC-mural cells in promoting TEM. The self-assembling VoC model investigated the behavior of HHT1 microvascular networks under inflammatory conditions and showed enhanced response of HHT1-hiPSC-ECs to pro-inflammatory stimuli. Additionally, the integration of hiPSC-derived macrophages (hiPSC-MΦs) into the self-assembling VoC model allowed for the investigation of perivascular macrophage (PVM)-like functions, including their anti-inflammatory capacity and interactions within the vascular microenvironment. Collectively, these findings demonstrated the versatility and potential of hiPSC-based VoC platforms for analyzing complex vascular and immune interactions, providing insights into disease mechanisms that could eventually contribute to drug discovery and precision medicine.

1. Modeling Inflammatory Responses using VoC models

Prolonged inflammation is a key driver in the pathogenesis of various vascular diseases, disrupting vascular homeostasis through sustained immune cell activation and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β[1].

In this thesis, TNF-α was used as an inflammatory trigger, given its established role in mediating vascular dysfunction by impairing endothelial barrier integrity, promoting leukocyte adhesion, and inducing cytokine secretion *in vitro*[2]. To study vascular inflammatory responses *in vitro*, we used two different VoC platforms, mentioned above: the tubular single-lumen VoC model and the self-assembling VoC model, each offering unique advantages for studying specific physiological functions.

The tubular single-lumen VoC model described in **Chapters 2 and 3**, has a predefined and uniform geometry, ensuring precise control over fluid flow and shear stress profiles. We demonstrated that this controlled microenvironment is particularly useful for standardized studies of endothelial barrier function and leukocyte TEM. However, since ECs and mural cells are arranged in a fixed spatial organization, this model may insufficiently support their dynamic interactions under certain experimental conditions. In contrast, the self-assembling VoC model used in **Chapter 4 and 5** closely mimics *in vivo* vasculogenesis and dynamic network remodeling, enabling the study of intrinsic vascular development, including lumen formation by ECs and mural cell recruitment. We showed that these features make it particularly valuable for investigating the development of the vascular abnormalities associated with disease pathologies like hemorrhage and vascular leakage. However, variability in network architecture and vessel diameter affects flow profiles, making it less suited for assays that require high standardization.

The choice between these two VoC platforms ultimately depends on the specific research questions. Together, these platforms were able to provide complementary insights into inflammatory responses and vascular pathologies, offering versatile tools for studying the complexities of vascular biology.

2. Studying Monocyte TEM using a Single-Lumen VoC model

Leukocyte TEM is essential for immune surveillance, but its dysregulation contributes to endothelial dysfunction and tissue damage in various diseases. Our premise is that by targeting proteins involved in leukocyte migration—such as selectins, integrins, and chemokines—would offer therapeutic strategies to selectively modulate leukocyte trafficking[3]. Studying these processes requires robust *in vitro* models that balance physiological relevance and practical applicability, one aim of this thesis.

We demonstrated that the tubular single-lumen vessel-on-chip (VoC) model described in **Chapter 2** offers an effective balance between robustness and physiological complexity, making it particularly well-suited for studying leukocyte adhesion and TEM studies *in vitro*. The VoC features a cylindrical collagen I hydrogel scaffold with a central hollow lumen, fabricated using a technique called viscous finger patterning (VFP)[4,5]. This collagen I-based lumen supports the formation of a confluent EC inner layer while allowing the embedding of mural cells within the ECM, facilitating direct interactions between mural cells and the endothelium. These cells further enhance the vascular barrier by depositing basement membrane components. The model can also be adapted to mimic tissue-specific vascular barriers, such as the blood-brain barrier (BBB), through the integration of astrocytes[6].

Although the single-lumen VoC model demonstrated significant advantages, it also has limitations. The use of manually fabricated polydimethylsiloxane (PDMS)-based chips presents challenges for standardizing chip production and scaling for high-throughput applications. Additionally, the chip design restricts access to the collagen I matrix to the luminal side, limiting assays that require stimulation from the abluminal side to mimic tissue injury or infection. Several similar VoC models have shown the potential of abluminal access, particularly by using chemokine gradients to promote leukocyte transmigration across hydrogels[7,8]. Modifications to chip design, such as incorporating side pockets, could address this limitation.

2.1. The role of hiPSC-mural cells and shear stress in monocyte TEM

In **Chapter 3**, we used the single-lumen VoC model to investigate the role of hiPSC-mural cells and shear stress in the TEM of hiPSC-monocytes. Engineered 3D vessel lumens were connected to a microfluidic pump, which enabled the controlled perfusion of hiPSC-monocytes under inflammatory conditions induced by TNFα. By integrating continuous physiological shear stress, the model effectively replicated key cellular interactions under hemodynamic forces, providing insights into the dynamics of hiPSC-monocyte adhesion and TEM in both healthy and disease contexts.

Our findings revealed that hiPSC-mural cells play a critical role in shaping the inflammatory environment and promoting hiPSC-monocyte TEM. Bulk RNA sequencing (RNA-seq) and cytokine profiling showed that hiPSC-mural cells significantly contributed to the pro-inflammatory environment in TNFα-stimulated coculture VoCs. Both monoculture and coculture VoCs activated several similar inflammatory pathways. However, comparisons with monoculture VoCs provided limited insight into how coculture conditions influenced cell-specific responses to TNFα. To address this, single-cell RNA-seq would be helpful to elucidate cell-specific contributions of hiPSC-ECs and hiPSC-mural cells to the overall inflammatory phenotype in coculture VoC.

Multiplex bead assays further suggested crosstalk between hiPSC-mural cells and hiPSC-ECs in regulating pro-inflammatory cytokine release. However, these assays revealed combined cytokine release from cocultures in VoC, but the specific cell-source of cytokines remained unclear. Notably, while hiPSC-mural cells did not influence hiPSC-monocyte adhesion to the endothelium, they significantly enhanced TEM under physiological shear stress. This effect was likely mediated by chemokines secreted by mural cells, which may be present in both soluble and immobilized forms, prime monocytes for transmigration[9,10]. Future cytokine profiling and functional assays are needed to elucidate the precise mechanisms underlying mural cell-supported TEM.

Shear stress was critical in revealing the role of hiPSC-mural cells in promoting TEM. Shear stress likely facilitated the exposure of hiPSC-monocytes to EC-displayed chemokines, enhancing integrin activation and TEM[11–14]. The laminar shear stress applied in our VoC model was comparable to that in post-capillary venules, where it ranges from 1.5–4.5 dynes/cm^2 [15]. Bulk RNA-seq revealed upregulation of laminar flow-responsive- and barrier-protective genes, illustrating the physiological relevance of the flow profile[16]. However, extended application of shear stress may be necessary to determine long-term changes such as EC alignment and specification and their interaction with hiPSC-mural cells under flow.

Under flow conditions, we observed the upregulation of certain genes exclusively in coculture VoCs, which may contribute to maintaining endothelial barrier integrity during monocyte transmigration. Notably, *CXCL12* was exclusively upregulated in coculture VoCs. *CXCL12* encodes a chemokine that acts as an integrin activator displayed on inflamed ECs and interacts with leukocyte integrins to mediate firm adhesion and transmigration[14]. The coculture VoC condition also affected EC-specific gene expression under flow, particularly genes related to vessel barrier integrity and function, such as *PDGFB*, which regulates mural cell recruitment. These findings suggest that hiPSC-mural cells possibly modulate EC flow-responsive gene expression through mechanosignal transduction[17]. Endothelial function appears to be shaped by the combined effects of hemodynamic forces and interactions with surrounding hiPSC-mural cells.

Bulk RNA-seq provided valuable insights but could not distinguish cell-specific contributions from hiPSC-ECs, hiPSC-mural cells, and transmigrating hiPSC-monocytes in the VoC model. Future studies using single-cell RNA-seq could unravel distinct cell-type contributions to TEM, responses to shear stress, and inflammatory stimuli. Investigating how hiPSC-mural cells influence EC phenotypes and barrier integrity under flow, as well as how hiPSC-mural cell phenotypes evolve under shear stress, will deepen our understanding of their roles in vascular homeostasis and leukocyte TEM. Additionally, it would be interesting to investigate the changes in gene expression of hiPSC-monocytes during TEM in response to shear stress and upon interaction with hiPSC-mural cells[18,19].

2.2. Insights into hiPSC-monocyte function

Our protocols for differentiating monocytes from hiPSCs through hemogenic endothelium and hematopoietic progenitor cell stages successfully generated monocyte-like cells expressing key surface markers, including CD45, CD14, and CD16. Despite developmental differences from peripheral blood monocytes, hiPSC-monocytes exhibited functional similarities, such as firm adhesion to TNFα-stimulated ECs under flow. However, they displayed increased surface expression of α4β1 integrins, which enhanced their adhesion[20].

Interestingly, our unpublished data showed that hiPSC-monocytes had limited expression of certain chemokine receptors, such as CCR2, which is critical for CCL2-mediated monocyte extravasation[21]. Our findings in **Chapter 3** support the hypothesis that hiPSC-monocytes may be in a naïve state post-differentiation, requiring activation by pro-inflammatory chemokines and shear stress provided in the coculture VoC model to initiate TEM[11,14,22,23]. This highlights that the phenotype and function of hiPSC-monocytes can be shaped by the microenvironment.

Patient-specific hiPSC-derived monocytes offer unique opportunities to study hereditary diseases characterized by monocyte dysfunction. We next explored the utility of this model in studying monocyte function in hereditary hemorrhagic telangiectasia (HHT1).

3. Modeling Inflammatory Responses in HHT1 using hiPSC-derived VoC Models

As described in **Chapter 4**, HHT1 is a complex vascular disease characterized by impaired blood vessel integrity and dysregulated immune responses, both of which contribute to its clinical symptoms. In addition to causing deficient TGF-β signaling that impacts vascular function, *ENG* haploinsufficiency also impairs the recruitment of mononuclear cells, such as monocytes, to sites of inflammation or injury, thereby reducing their capacity to restore tissue homeostasis[24,25]. This compromised immune response leads to persistent inflammation and defective vascular repair, as demonstrated in studies using *ENG* heterozygous mouse models of chronic colitis and ischemia[25,26]. Persistent inflammation and insufficient vascular repair are thought to act as a “second hit” trigger, leading to EC dysfunction, predisposing the vascular beds with *ENG* haploinsufficiency to localized vascular malformations, further exacerbating the progression of HHT1 pathology[27,28].

A key advantage of using hiPSC-derived models to study HHT1 is the ability to generate isogenic mutant and genetically corrected wild-type control pairs, ensuring that observed disease phenotypes are directly attributable to *ENG* haploinsufficiency rather than variations in genetic background. To investigate the inflammatory responses characteristic of HHT1 *in vitro*, we used both of our VoC platforms: (1) the single-lumen-based VoC model to assess the recruitment of HHT1 hiPSC-monocytes under shear flow in **Chapter 3**, and (2)

the self-assembling VoC model to investigate how an inflammatory trigger influences the development and remodeling of 3D microvascular networks formed by HHT1-hiPSC-ECs in **Chapter 4**. Together, these studies provide complementary insights into the role of inflammation as trigger in HHT1 pathology, from hiPSC-monocyte recruitment defects to vascular dysfunction under inflammatory conditions, as described in the next sections.

3.1. Investigating monocyte function in HHT1 using the single-lumen VoC model

Recent studies suggest that ENG, expressed on activated monocytes, acts as a counter-receptor for leukocyte integrins via its RGD motif[29]. In **Chapter 3**, we showed that HHT1-hiPSC-monocytes with *ENG* haploinsufficiency exhibit impaired adhesion to TNFα-stimulated hiPSC-ECs in 2D microfluidic channels under shear flow. This result was independent of whether the hiPSC-ECs carried the *ENG* mutation. Their impaired adhesion was also observed on immobilized VCAM-1 in 2D microfluidic assays, implicating the β1-integrin complex (α4β1), a receptor for VCAM-1, in this process. β1-integrins are crucial in the leukocyte recruitment cascade during inflammatory responses, and we demonstrated that activating β1-integrin rescued adhesion of HHT1-hiPSC-monocytes, whereas inhibiting it reduced adhesion in both mutant and isogenic wild-type hiPSC-monocytes. These findings suggest that interaction of monocyte-specific ENG with β1-integrin on monocytes potentially mediates outside-in signaling leading to integrin activation. Consequently, *ENG* haploinsufficiency impaired their adhesion to VCAM-1 due to the reduced β1-integrin activation[30].

Our results complement recent studies on the role of endothelial ENG in mediating leukocyte adhesion and transmigration through interactions with leukocyte integrins[31]. Together, these findings suggest a complex process where *ENG* mutations in both ECs and leukocytes may collectively impair leukocyte recruitment in HHT1. While our 2D assays assessing β1-integrin activation in HHT1-hiPSC-monocytes were performed under static conditions due to experimental limitations of the assay, future studies should examine how shear flow affects β1-integrin activity in HHT1-hiPSC-monocytes to provide a more comprehensive understanding of mechanotransduction in this context[12].

Using the single-lumen VoC model, we confirmed the impaired adhesion of HHT1-hiPSC-monocytes under shear flow. This model, incorporating hiPSC-mural cells and physiologically relevant shear stress, provided a platform to evaluate TEM of HHT1 hiPSC-monocyte through inflamed 3D vessels and assess the role of β1-integrin activity in this process. Notably, our VoC model incorporated hiPSC-ECs and hiPSC-mural cells derived from healthy donors, focusing primarily on HHT1 hiPSC-monocyte function. VoC models incorporating HHT1 hiPSC-ECs and hiPSC-mural cells from the same patient would enable a more comprehensive investigation of the cell-cell interactions underlying inflammatory responses. This modular VoC model could also facilitate the study of cell type-specific effects

of *ENG* mutations by integrating mutant and wild-type isogenic pairs in various combinations.

3.2. Investigating inflammatory responses in HHT1 using the self-assembling VoC model

In **Chapter 4**, we explored how TNFα affects EC-mural cell interactions and vascular remodeling in the self-assembling VoC model. HHT1-hiPSC-ECs with *ENG* haploinsufficiency, co-cultured with hiPSC-mural cells from healthy donors, self-assembled into 3D microvascular networks within a fibrin hydrogel. These HHT1 microvascular networks were more sensitive to TNFα stimulation compared to isogenic controls. Remarkably, TNFα stimulation exacerbated disease-related phenotypes, such as impaired EC-mural cell interactions and elevated inflammatory cytokine secretion. However, recapitulating the full complexity of vascular malformations, such as arteriovenous malformations (AVMs), remains as a challenge in our current model. Introducing shear stress into the self-assembling VoC model may be an essential step towards inducing AVM formation *in vitro*, as HHT1 ECs exhibit impaired mechanotransduction and migration responses to fluid shear stress. Shear flow regulates critical vascular processes, including EC proliferation, mural cell recruitment, and vessel stabilization, which are often compromised in HHT1[32–35]. Advanced microfluidic systems that replicate physiologically relevant flow conditions are essential to enhance the relevance of self-assembling VoC model for studying HHT1 pathology[36].

Vascular malformations in HHT1 vary significantly between vascular beds in different organs, despite the common *ENG* haploinsufficiency. Interestingly, a study using mouse and human ECs obtained from various organs showed that baseline ENG expression levels differ between vascular beds. For instance, vascular beds in the skin and lungs have inherently lower baseline ENG expression and are therefore more prone to EC dysfunction when these levels are reduced in mutant ECs. In contrast, those with higher baseline ENG expression (e.g. liver) are less affected due to sufficient ENG production from the remaining wild-type allele[37]. This may explain why some tissues have vessels with telangiectasias and some do not. In this context, secondary hits such as inflammation can be critical to drive the *ENG* loss-of-function. These findings emphasize the need to establish critical ENG expression levels in hiPSC-ECs to effectively study HHT1 pathology in VoC models. To address this, developing genetically engineered complete *ENG* knock-out hiPSC-ECs with an inducible *ENG* locus that could be incorporated into the VoC model, allowing temporal control over ENG expression during microvascular network development would be of benefit. While it might be challenging with current methods, generating hiPSC-ECs with tissue-specific baseline ENG levels representative of vascular beds in HHT1 patients would be a compelling strategy. Single-cell transcriptomic profiles of patient-specific or engineered hiPSC-ECs extracted from the VoC model could be benchmarked against publicly available human

tissue datasets to determine the alignment of their ENG expression with ECs in various vascular beds[38].

An important factor to consider when developing *in vitro* models of HHT1 is the substantial inter-patient variability in disease phenotypes. Variations in genetic backgrounds among patients can significantly influence the manifestation in timing and severity of HHT1 symptoms. Incorporating hiPSC lines from multiple donors with diverse genetic profiles could enhance the relevance of these models, enabling a more comprehensive understanding of disease heterogeneity and patient-specific therapeutic responses[39].

4. Towards Advanced VoC Inflammation Models: Investigating Perivascular Macrophages in Vascular Microenvironments

Perivascular macrophages (PVMs), a specialized subset of tissue-resident macrophages (TRMs), play crucial roles in maintaining vascular homeostasis. Their high phagocytic and secretory activity enables them to clear apoptotic cells, eliminate pathogens, mediate inflammatory responses, and regulate tissue remodeling. However, many *in vitro* vascular inflammation models lack tissue-resident immune components, limiting their ability to capture the intricate cascade of cell-cell interactions involved in the inflammatory responses.

In **Chapter 5,** we successfully integrated hiPSC-derived macrophages (hiPSC-MΦs) into the self-assembling VoC model to investigate PVM functions within a microvascular environment. These hiPSC-MΦs were derived through hemogenic endothelium induction and developmentally resemble TRMs[20,40], making them well-suited for exploring how the microvascular niche in the VoC model shapes their phenotype and function[41,42]. In this model, hiPSC-MΦs closely associated with the microvascular network and acquired a PVM-like phenotype, as evidenced by enhanced LYVE-1 expression, particularly in hiPSC-MΦs localized near the vessel surface. This highlights the VoC platform's utility for studying PVM functions in vascular homeostasis and inflammation. Mouse studies have shown that PVMs regulate the recruitment of circulating leukocytes, such as monocytes and neutrophils, into various inflamed tissues[43–45]. Besides the self-assembling VoC model, the single-lumen VoC described in **Chapters 2 and 3** could serve as a complementary platform, offering controlled perfusion to assess the influence of hiPSC-MΦs on the TEM of hiPSC-monocytes under physiological shear stress[46].

A key finding in **Chapter 5** was the anti-inflammatory capacity of hiPSC-MΦs, which attenuated pro-inflammatory cytokine release induced by the fibrin hydrogel during microvascular network formation. This effect may be attributed to their efferocytotic activity[47]. Further studies are essential to uncover the molecular mechanisms driving this

anti-inflammatory effect. Detailed characterization of their efferocytotic activity could be achieved through advanced imaging techniques and transcriptomic analyses. Additionally, assessing efferocytosis in 3D by inducing localized inflammation[48] and apoptosis, or by experimentally blocking efferocytosis, would help clarify its contribution to the observed anti-inflammatory effects in the VoC model.

The activation states of TRMs exist along a spectrum, influenced by their developmental origin and tissue-specific niche. Conventionally, MΦ activation has been classified into two extreme sides of the spectrum: M1 (pro-inflammatory) and M2 (anti-inflammatory) subtypes, typically based on their stimulation with defined sets of factors *in vitro*. However, this framework has been criticized for oversimplifying the heterogeneity and complexity of TRM activation states observed *in vivo*[49,50]. These observations highlight the need for more nuanced approaches to assess TRM function *in vitro*. Advanced culture systems like our self-assembling VoC model offer a controlled microenvironment to study MΦ functions in a more physiologically relevant context. Transcriptional profiling of hiPSC-MΦs cultured in the VoC could provide deeper insights into their phenotypes and functional states under defined conditions. Benchmarking these data against reference datasets, such as the Stemformatics human myeloid transcriptome atlas[51], could help evaluate their alignment with native human MΦs[52].

Our self-assembling VoC model integrating hiPSC-MΦs holds potential for studying disease-specific TRM functions. For example, in the HHT1 mouse model, *ENG*-deficient TRMs exhibit impaired phagocytosis and reduced leukocyte recruitment during infection[53]. Incorporating HHT1 patient-derived hiPSC-MΦs into the HHT1 VoC model described in **Chapter 4** could provide valuable insights into the mechanisms underlying the persistent inflammation associated with HHT1 pathology. Overall, this fully hiPSC-based VoC model represents a powerful platform for investigating TRM-driven mechanisms in vascular inflammation and disease, with the goal of exploring therapeutic strategies targeting pathological conditions linked to TRM dysfunction.

5. Future Perspectives

Vessel-on-Chip (VoC) models are emerging as transformative tools for studying vascular biology and disease modeling *in vitro*. By combining VoC platforms with tissue-specific cells, these models allow the investigation of specialized vascular functions tailored to distinct tissue environments. However, achieving effective vascularization in human tissue models, such as organoids, remains a significant challenge.

In **Chapter 6**, we reviewed emerging strategies for generating functional and perfusable vasculature within human organoid models. These strategies address critical limitations to

creating physiologically relevant organoid models, including oxygen and nutrient delivery, tissue maturation, and vascular stability. While direct differentiation or co-culturing of ECs within organoids has yielded vascular segments, these structures often lack perfusion and eventually regress, resulting in limited tissue maturation and functionality. Integrating organoids into VoC platforms has proven effective in overcoming these issues by promoting fully perfusable and stabilized vascular networks, thereby enhancing tissue maturity. Moreover, incorporating immune components such as macrophages can further enhance the functionality and integration of vascularized tissues, providing vital support for tissue viability and immune-vascular interactions[54–56].

Despite the recent advances, significant challenges remain in scalability, reproducibility and long-term stability, all of which constrain the translational potential of OoC systems. The lack of standardized methodologies and assay readouts could limit the integration of these models into regulatory and commercial pipelines. Ensuring that these models are fit-for-purpose requires defining their context-of-use and establishing robust, reproducible readouts[57]. Future efforts should focus on standardization and qualification processes, which are critical for ensuring consistent results across different laboratories and users. Additionally, integrating biosensors for real-time monitoring of biomarkers and advanced imaging could enable continuous tracking of dynamic processes such as inflammation and drug response. Benchmarking quantitative readouts against *in vivo* physiology would validate their predictive value and support their application in drug development, aligning with the principles of the 3Rs (replacement, reduction, and refinement of animal use). These efforts will be crucial for bridging the gap between academic innovation and regulatory acceptance, thereby expanding the applicability of these technologies[58].

Multi-lineage hiPSC-derived platforms offer transformative opportunities for disease modeling and drug discovery by enabling the differentiation of diverse cell types from a single, patient-specific source. This is particularly important when generating immune cells, such as T- and B-cells, where maintaining an isogenic background ensures compatibility with other differentiated cell types from the same hiPSC line. In such cases, using autologous immune cells minimizes the risk of immune rejection, a key consideration for disease modeling and personalized therapeutic applications. This approach is especially relevant in studying immune cell-vessel interactions, autoimmune disorders, and cell-based immunotherapies, where non-isogenic sources could trigger unintended immune responses. Integrating hiPSCs into OoC systems enhances their relevance for personalized medicine by allowing patient-specific drug testing and the identification of therapeutic targets under controlled conditions. To achieve widespread adoption, it will be critical to develop well-characterized panels of hiPSC lines with standardized differentiation protocols that are reproducible across laboratories. However, due to the inherent line-to-line and clone-to-clone variability in hiPSCs, standardization alone may not resolve issues related to heterogeneity. Variability between hiPSC lines should be systematically reported to improve

the reliability of hiPSC-based models in research. Implementing advanced screening methods for hiPSC differentiation, such as image-based machine learning strategies[59], alongside standardized protocols, could improve consistency and help address variability, ultimately strengthening the translational potential of hiPSC-based OoC systems in pharmaceutical research and precision medicine[60,61].

Recent developments highlight growing interest from regulatory agencies in OoC technology and hiPSC-based models. The Food and Drug Administration (FDA) Modernization Act 2.0 and Innovative Science and Technology Approaches for New Drugs (ISTAND) pilot program reflects increasing recognition of these models as potential alternatives to animal testing[62]. These initiatives represent a significant step forward in integrating advanced *in vitro* systems into regulatory frameworks, helping to speed up drug development and improve approaches in personalized medicine[63].

Advanced OoC platforms are best positioned as complementary tools to animal model-based biomedical research, enabling the study of physiological processes using human-derived cells. These models hold promise for predicting human drug responses before clinical testing. However, they cannot fully replicate whole-organ and systemic interactions, including multi-organ crosstalk, systemic immune responses, hormonal regulation, and drug metabolism. While multi-organ-on-chip (i.e. Body-on-a-Chip)[64] systems aim to bridge these gaps, the absence of a fully functional circulatory, endocrine, and nervous system remains a constraint. The success of next-generation OoC platforms depends on addressing current limitations, fostering collaborations across disciplines, and aligning technological innovation with regulatory standards to ensure broader implementation and impact. Ultimately, these platforms have the potential to revolutionize disease modeling, drug discovery, and precision medicine by providing comprehensive, multicellular *in vitro* models that closely mimic human physiology.

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