Bioengineering and biophysics of viral hemorrhagic fever

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CONCLUSIONS AND OUTLOOK

Studies on viral hemorrhagic fever (VHF) have been hampered due to the non-availability of proper models and incomplete knowledge on the mechanism of VHF. In order to fill this gap, this thesis presented new bioanalytical, lab-on-chip and single cell assays to investigate changes in vascular biology and macrophage immunometabolism induced by VHF viruses.

Microvessels-on-chip recapitulates viral hemorrhagic shock syndrome

As Organ Chip-based assays provide accessibility to study virus-induced diseases in real-time and at high-resolution, they can open new avenues to uncover viral pathogenesis in a human-relevant environment and may eventually enable development of novel therapeutics and vaccines [1]. In **Chapter 3** and **4**, we presented a microvessels-on-a-chip platform to recapitulate Ebola and Lassa hemorrhagic shock syndrome [2, 3]. The hemorrhagic shock syndrome-on-a-chip described here is the first of model to fill the gap between human and nonhuman primate models. Despite its simplicity, this model is robust in mimicking the structure and functions of human microvessels and VHF disease-associated vasculopathy, and helps in understanding the effect and mechanism of experimental compounds, as demonstrated by our proof-of-concept analysis of two anti-Ebola VHF compounds. This platform can also make new discoveries about disease mechanisms as described in **Chapter 5**. Here, we investigated the modulation of endothelial barrier by Dengue NS1. The results not only support the notion that VE-cadherin, F-actin, and hyaluronan are the key molecules involved in vascular permeability of Dengue, but also the potential physiological relevance of this platform to study Dengue-induced microvascular syndromes at similar concentrations as shown in patients with Dengue virus infection [4].

Thus, this model can faithfully recapitulate the vasculopathy of VHF *in vitro* and aid as a high-throughput sensing platform. The model can also be of help in screening for therapeutic small molecules and monoclonal antibodies, aid in the efforts to develop effective pharmacokinetic model of drug treatments and assist in designing the proper dose regimens. It's also possible to monitor the release of molecules (i.e., bioactive lipids) into the liquid in the microvessels-on-a-chip after viral exposure and treatment, thus allowing for identifying biomarkers that report on disease progression and response to therapy. This platform can serve as a diagnostic tool to quantify the severity of VHF disease, to diagnose or predict susceptibility at an early stage and assess the risk for healthy subjects by perfusing blood plasma samples from patients to the chip. This opens new opportunities for developing efficient health management strategies against VHF.

An important future development would be in making individualized VHF model, beyond infusion of blood plasma samples. The application of human induced pluripotent stem cells (hiPSCs) to develop individualized model with the desired genotype and phenotypes to help overcome interindividual variabilities in VHF disease progression and facilitate personalized antiviral drug development. One can also integrate complex immune processes into the model to simulate VHF-related inflammatory responses, to improve the pathophysiological relevance of the models. This is however challenging but recent development of lymphoid tissues on a chip has made this a feasible possibility [5]. Ultimately, one may envision interconnecting multiple organs-on-chips to produce human body-on-chip models that provide new insights into VHF pathogenesis at a complex and systemic human level. The fabrication of multiple organs-on-chips may offer an appealing approach to fully recapitulate host pathophysiology and immune response, which would allow in-depth understanding of VHF pathogenesis at different disease stages characterized by different symptoms. Several multi-organ chips have been realized in recent years, by connecting simple units [6, 7]. Further advances are needed to be able to incorporate complexity of human organ systems and their response to VHF.

Development of organ chip models for the viral diseases mentioned in this thesis will make our society ready to address newly emerging viruses in the future. As we showed in this thesis, the platform can be easily adapted to study other VHFs, and other viruses that target junctional mechanics of human tissues.

Single cell lab-on-a-chip for VHF analysis: the Dengue VHF case study

In addition to organ chip models, we also decided to develop a lab-on-a-chip approach for VHF analysis. To demonstrate this, we focused on Dengue virus disease in **Chapter 5**. The damage to vascular endothelial cells by Dengue NS1 has been speculated to be involved in the pathogenesis of Dengue [8, 9], however, further insights are hampered due to the lack of sensitive detection tools. Thus, acoustic force spectroscopy (AFS) was applied for directly

probing mechanical dysregulation of human endothelial cells by NS1 at single-cell level.

NS1 had a measurable effect on the elasticity of endothelial cells, causing a reduction in overall stiffness measured by AFS. Here, we demonstrated the capability of AFS to measure inside a flow system, which presents as a powerful approach for capturing the heterogeneity of cultured adherent cells through rapid, real-time, high-throughput measurements of cellular viscoelasticity. This advanced single-cell technology will enable similar studies on other Dengue virus targeted host cells (e.g., immune cells, fibroblasts, etc.) [10, 11] to monitor the alterations of cellular mechanical stiffness so that evaluate the cytoskeletal structure and related cellular physiology under physiological conditions. More studies would be done to test the efficacy of drug molecules that may counteract the stiffness alterations of host cells led by Dengue virus. This technology can also be applied to investigate Ebola, Lassa, etc. Single cell information is important to learn whether the changes are cell intrinsic or an emergent collective response of a network of cells, for example as modeled by the organ chip models presented in this thesis.

Ebola VLP induces cellular metabolic reprogramming to endothelial cells, M1 and M2 macrophages

Recent studies showcase that viral pathogenesis is significantly associated with hijacking metabolic mechanisms and resources of host cells [12, 13]. Yet, little is known about host metabolic reprogramming as a result to Ebola. In **Chapter 6**, we analyzed the impact of Ebola VLP on primary human endothelial cells and the different polarization status of macrophages (M1 and M2) by untargeted cellular metabolomics using direct infusion-mass spectrometry (DI-MS). Ebola VLP induced specific cellular metabolic reprogramming to endothelial cells, M1 and M2 macrophages. We determined that Ebola VLP exposure broke the metabolic homeostasis of the three cell types in a cell specific manner, caused metabolic reprogramming, and significantly affected metabolism mainly in fatty acid-, steroid-, and amino acid–related metabolism pathways. The alterations of these metabolites and

pathways reflected the cellular responses to Ebola VLP or the involvement in Ebola pathogenesis.

Ebola virus is a deadly zoonotic virus that requires extremely high biosafety containment measures for investigations using live virus; thus, non-infectious VLPs used here are a safe method to study aspects of Ebola infection and immune response to infection. Ebola GPs are critical in Ebola pathogenesis and can compare its effect with intact VLPs, as well as a control VLP that does not have GPs would provide a better comparison set by similar assay. Besides GPs, another composition of Ebola VLP–matrix protein VP40 is a major viral structural protein and plays a central role in virus assembly and budding, it can also, for example, activate endothelial cells and induce a decrease in barrier function [14]. Future complementary experiments (e.g., VLPs without GP, GP or matrix protein VP40 only) will contribute to better mechanistic understanding of the observed metabolic changes. Lastly, this assay can be adjusted for other hemorrhagic fever viruses that also show the ability to reprogram the host metabolism, such as Flaviviruses, Dengue virus, etc.

The results present here are the initial step towards understanding the potential metabolic alterations done to host cells upon Ebola VLP exposure. We intend to further confirm the possible identifications by MS/MS and more targeted analysis as future efforts. Other omics approaches (i.e., transcriptomics and proteomics) and assays will be applied in future to reach a more complete disclosure of the mechanisms. One can perform RT-PCR to investigate changes in the enzymes involved in the metabolic pathways involved as read out of changes in activities, or use of known inhibitors to interfere with the pathways cited as validation of the data presented in this study. Finally, one can investigate the potential of above-mentioned metabolic pathways as candidate targets for the development of prophylactic or therapeutic countermeasures.

The modulation of host metabolism by viruses is essential to complete virus life cycle, therefore, identifying metabolic biomarkers for therapeutic strategies maybe promising. However, conventional population-level metabolomic approaches do not consider the

inherent cellular heterogeneity of individual cells and instead average out the metabolic profile across a large number of cells [15]. Single-cell metabolomics provides the capability to overcome these limitations.

Live single-cell metabolomics coupled with machine learning: a new approach for studying immunometabolic changes in medicine and infectious diseases

While studying Ebola metabolism using direct infusion mass spectrometry, we recognized the need for resolving immunometabolic changes at the single-cell level. The metabolic differences between M1 and M2 macrophages that determine their distinct functional properties are not fully understood, especially on the single-cell level. The direct infusion approach requires minimal sample preparation and thus is well suited for small volume analysis, down to single cells. In **Chapter 7**, an untargeted live single-cell mass spectrometry based metabolomic profiling coupled with a machine learning data analysis approach was developed to address this knowledge gap for the first time. Results here show that M1 and M2 macrophages have distinct metabolic profiles, with differential levels of metabolites mainly belong to lipids. These differences can be linked to the pro- and anti-inflammatory nature of different macrophage subtypes. Putatively annotated metabolites, including fatty acyls, glycerophospholipids, and sterol lipids, known to play a role in inflammation and cell differentiation, were assigned by coupling with random forest algorithm.

The combination of random forest and live single-cell metabolomics provided an in-depth profile of the metabolome of M1 and M2 macrophages at single-cell level for the first time, which will pave the way for future studies targeting effect of pathogens on macrophages and other immune cells. Further studies involving targeted and quantitative analytical measurements at single-cell level will be performed to confirm the metabolomic alterations detected between different macrophage phenotypes. Besides, the findings present here showcase the potential of this approach to do phenotypical classification based on single-cell metabolomics data, and subsequently, gain a deeper understanding of the metabolome

of heterogenous cell populations. This will help fuel the future research that aims to explore modulatory mechanisms of other immune cell differentiation on the single-cell level. Despite the utility and flexibility of this approach, single-cell sampling process suffers from low throughput and requires highly skilled operators. Improvements will be made in future to address these challenges by applying automated methods for large scale single-cell studies.

Conclusions

The VHF-on-a-chip model represents a transformative system that can serve as a low-cost, easy-to-use and high-throughput platform for studying VHF viruses induced microvascular destabilization in a human-like setting but also for diagnostics and drug development. We developed and tested metabolomics approaches that can be applied to the organ chip models in the future to further expand the available possibilities. The untargeted metabolomics analysis demonstrates its strength to elucidate the effect of virus on host cells and screen potential biomarkers that can reveal fundamental VHF pathogenic mechanisms. Coupling single-cell metabolomics with random forest algorithm showcase the potential to do phenotypical classification based on single-cell metabolomics data, and subsequently, gain a deeper understanding of the metabolome of heterogenous cell populations.

Overall, the work in this thesis contributed to our understanding of the effect of VHF viruses on host cells. More importantly, the technologies developed in this thesis expectedly open up unprecedented opportunities for virologists that are at the front line of fight with the viruses that threaten our global society.

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