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Bioengineering and biophysics of viral hemorrhagic fever

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1

GENERAL INTRODUCTION

Viral hemorrhagic fever (VHF) is a group of acute diseases caused by highly infectious viruses. Its high mortality rate poses a high risk to public health, and there is urgent need to understand the effect of VHF viruses on host cells for further treatment strategies development. Thus, the aim of this thesis is to develop bioanalytical, lab-on-chip and single cell assays to investigate VHF viruses induced changes in vascular biology and macrophage immunometabolism. In this chapter, a general introduction to the involvement of endothelial cells and macrophages in VHF, the techniques (including Organ-Chip, acoustic force spectroscopy, live single-cell mass spectrometry) that applied here, and finally the outline of this thesis will be discussed in the followings.

Viral hemorrhagic fever

Viral hemorrhagic fever (VHF) refers to a group of severe systemic febrile illnesses caused by enveloped RNA viruses from several viral families [1]. VHF are associated with infections with viruses such as Ebola, Dengue, and Lassa viruses that cause relatively mild illnesses to severe, life-threatening disease with case fatality rates up to 80% [2]. These highly infectious viruses cause abnormal vascular regulation and vascular damage [3]. The vascular dysregulation manifests as mild hypotension, flushing of the skin, and vasodilation of the conjunctivae in the early course, as the disease progresses, vascular damage with capillary leakage may affect multiple organ systems and finally result in life-threatening shock [4]. Detailed understanding of the pathogenic mechanisms is still lacking. However, research has demonstrated that the mechanisms of hemorrhage in VHF include endothelial injury, activation of mononuclear phagocyte system, hepatic damage, and subsequent coagulopathy [4, 5]. The outbreaks of these diseases in African countries pose a great global health threat due to poor prognosis and lack of effective treatments. As such, it is necessary to understand the pathogenic roles of viral agents and the host response to viral infection.

Endothelial cell dysfunction in viral hemorrhagic fever

Key viral targets in VHF include vascular endothelial cells, monocytes, macrophages and dendritic cells, which enable dissemination through lymphatics to other organs after infection [6]. Although there are various distinct clinical features specific to each VHF, the involvement of vascular endothelium and the perturbation of vascular barrier integrity and coagulation abnormalities, has been recognized as common pathological signature [7, 8]. This malfunction of the endothelium can be caused either directly by the virus infection or indirectly by virus induced secreted factors that can cause endothelial activation [9]. For some of the VHF-causing viruses, such as Ebola, Lassa and Dengue, both direct infection of endothelial cells to produce a strong cytopathic effect and indirect effect on vascular

permeability via their shed glycoprotein (GP) from infected cells has been clearly demonstrated [10-14]. In contrast, direct infection of endothelial cells by dengue virus has been controversial, it is suggested that secreted viral proteins and induced inflammatory mediators (e.g., TNF-α) are likely to be contributing factors in increasing vascular permeability rather than direct infection of the endothelium [9, 15]. Dengue nonstructural protein-1 (NS1), a soluble viral protein secreted by infected host cells, has been shown to correlate positively with vascular permeability [16]. Further understanding the interaction of these viruses with the vascular endothelium and the dysregulation of endothelial cell barrier function are still needed in order to pave the way to define therapeutic targets for reducing disease severity.

Involvement of macrophages in viral hemorrhagic fever

Macrophages play a central role in VHF as initial viral targets and targets throughout the course of infection to facilitate the pathogenesis [17]. Infected macrophages result in aberrant production of proinflammatory cytokines, chemokines and tissue factor, recruiting additional susceptible cells to the site of infection and disrupting vascular permeability [18]. Noninfectious Ebola virus-like particles (VLPs) expressing the Ebola GP and matrix proteins (also studied in this thesis), were shown to be able to induce the activation of macrophages as well [19]. However, macrophages are not phenotypically homogenous upon the local microenvironment into either classically activated M1 or alternatively activated M2 macrophages. M1 macrophages are characterized by an upregulated expression of pro-inflammatory cytokines, the ability to mediate clearance of pathogens, and promote Th1 responses. In contrast, M2 macrophages are characterized by the involvement in production of immunomodulatory compounds, promote resolution of inflammation and wound healing [20, 21]. Given the critical role of macrophages in VHF, its polarization phenotypes may have significant influence on the progression of Ebola. There is evidence that M1 polarization can inhibit Ebola infection while M2 can promote infection by enhancing virus replication [21,

22]. These findings show that modifying host responses maybe a potential therapeutic strategy, however, it is still necessary to uncover the details of immunological underpinnings of VHF.

Lab-on-a-chip technologies for viral hemorrhagic fever

Despite progress in disease model development, conventional *in vitro* culture and animal models sometimes fail to faithfully reflect human physiology and pathophysiology in response to pathogens [23]. This shortcoming is particularly seen in modeling VHF. As the epidemic and reemerge of VHF disease in West Africa poses a continued imminent threat to human health, the urgent need for developing innovative strategies to study VHF pathogenesis is evident. Organ Chip technology, however, offers a promising tool to recapitulate the complex 3D microenvironment reflecting the structure and function of the human organs so that better mimic the initiation and progression of host–microbe interactions [24]. Organ Chips are engineered microfluidic cell culture devices lined with human cells to replicate organ-level physiology by rebuilding tissue–tissue interfaces, mechanical cues, fluidic flow, and the biochemical cellular microenvironment *in vitro* with high fidelity [25]. Several Organ Chip platforms have been explored to study infection kinetics, virus–host interactions, viral pathogenesis and evolution, drug responses, and the development of resistance to drugs and vaccines [26]. As described in subsequent chapters of this thesis, our team has explored the application of Organ Chip technique for VHF for the first time by modeling Ebola, Lassa and Dengue induced vascular integrity loss and drug analysis in a high-throughput manner [27, 28]. These developments present a unique opportunity to bridge the gap between *in vitro* cell culture experimental models and *in vivo* human pathophysiology as well as an alternative for animal models [29]. In addition, the efficacy of candidate drugs, target the mechanical elements or signaling pathway, to counteract VLP-induced vascular leakage was demonstrated by this platform [26]. What’s more, experts stated that Organ Chip has the potential to reduce total R&D costs by 10–

26%, in particular in the lead optimization and preclinical phases of R&D, as well as improvements in success rates to drive savings [30]. These open new opportunities for developing effective therapeutic strategies by Organ Chip models.

VHF is a disease of mechanics and cellular mechanics is affected at various levels. The mechanics of endothelial cell junctions (as we demonstrate in this thesis), as well as migratory mononuclear immune cells are altered upon exposure to viral materials. The mechanical properties of cells, such as stiffness and viscosity, are closely related to their biological functions and are biophysical markers for determining cellular physiological state transitions or pathological changes [31]. Many techniques have been applied to study the mechanical properties of cells, such as acoustic force spectroscopy (AFS) [31]. AFS relies on acoustics and provides high-throughput tracking at single-cell level. Briefly, tens to hundreds of cells confined between the glass surface of a AFS microfluidic chip and silica beads, are instantaneously stretched by beads at a constant stress in the presence of acoustic forces. The movement of beads is tracked to record z-directional displacement in real time to quantify the stiffness of cells [31]. In 2021, AFS has been applied to measure the dynamic viscoelastic properties of endothelial cells upon variable conditions under controlled fluid flow [32]. As such, we used AFS as measurement tool to investigate the effect of Dengue NS1 on endothelial cells showing its capabilities to understand how the mechanical properties of key viral target cells vary as a function of VHF.

Metabolomics for viral hemorrhagic fever and immunometabolic profiling

Metabolomics refers to the investigation of large set of small-molecule metabolites and has been applied to study a variety of infectious diseases and understand host-pathogen interactions. Metabolites represent the end products of cellular regulatory processes therefore provide the most downstream knowledge between molecular change and phenotype [33]. Thus, metabolomics is an informative approach to understand host-virus interaction, as it generates immediate information regarding the exact downstream effects

and identify the ultimate metabolites/lipids and pathways that are differentially affected during VHF [34]. Recently, metabolomics proved its capability in aiding to understand virus-host interactions by providing a direct snapshot of dynamic and vulnerable serum metabolic profile in the blood of patients with infection of Ebola, Lassa, Dengue, etc. [35, 36]. However, exploitation of the VHF virus-cell interaction by metabolomics is limited. In Chapter 6, we performed an untargeted cellular metabolomic to investigate the metabolic alterations of primary human endothelial cells, M1 and M2 macrophages upon exposure to Ebola VLPs. The most common application of metabolomics is to screen disease-specific biomarkers for either prognosis or diagnosis, as well as discover metabolic enzymes as druggable targets and metabolic regulators [33]. Its usage to reveal fundamental VHF pathogenic mechanisms of the virus-host interaction as well as the virus-cell interaction may provide valuable information to combat emerging VHF and decide novel potential targets for therapeutic intervention.

Immunometabolism describes the connection between metabolic programs and functions of immune cells during both health and disease [37]. Its dysregulation is linked to various diseases including but not limited to cancer and chronic inflammatory diseases, however, there is limited knowledge regarding the immunometabolic remodeling in response to pathogens due to the complex cell phenotypes involved in the progress [38]. Single-cell metabolomics is a recent development in immunometabolomics to introspect the cellular metabolic heterogeneity of individual cells that is often masked in pooled analyses. To overcome the major hurdle in single-cell metabolomics, multiple methods have been developed for the isolation of single target cell. In Chapter 7, a direct sampling by nano-electrospray ionization tips followed by mass spectrometry measurements of the tip contents is selected to study macrophages upon different polarization, due to its minimum sample requirement and processing along with high throughput data generation [34]. Resolving immunometabolism at single-cell resolution with these state-of-the-art technologies can provide an additional layer of insight into the heterogeneous distribution of metabolites with different functions of macrophages upon different polarization.

Outline of this thesis

The aim of this thesis is to develop bioanalytical, lab-on-chip and single cell assays to investigate VHF viruses induced changes in vascular biology and macrophage immunometabolism. Firstly, a microvessel-on-a-chip was developed to mimic the hemorrhagic shock syndrome caused by VHF viruses *in vitro* and tested new experimental drug candidates. In addition, AFS approach was applied to investigate the effect of Dengue on the cellular viscoelastic properties of endothelial cell at single-cell level. Then, metabolic profiling of the endothelial cells and macrophages, the two critical viral cell targets, upon Ebola VLPs exposure was performed on bulk-level. Finally, the immunometabolism of primary human macrophages upon polarization were investigated on single-cell level by the live single-cell metabolomics, setting the stage for future host-pathogen studies at the single cell level.

Chapter 2 summarizes the recent application of Organ Chips to recreate complex pathophysiological features of human viral infections *in vitro*. Human Organ Chip microfluidic culture devices enable to recapitulate tissue–tissue interfaces, fluid flows, mechanical cues, and organ-level physiology so that narrow the gap between *in vitro* experimental models and human pathophysiology. To mimic host–microbe interactions specifically, several Organ Chip platforms (e.g., liver, gut, nervous system, kidney, lung and microvessel) have been developed to study both pathogenic and nonpathogenic bacteria, and most recently, viral infections. Among the possibilities being explored are the use of Organ Chip to study infection kinetics, virus–host interactions, viral pathogenesis and evolution, drug responses, and the development of resistance to drugs and vaccines. Here, we provide an overview of Organ Chip technologies described for virology application to date and we discuss opportunities and challenges that lie ahead.

Chapter 3 reports the first high-throughput chip-based model for Ebola induced vascular integrity loss and drug analysis. Luminal infusion of Ebola VLPs leads to features of the endothelial dysfunction associated with the hemorrhagic shock syndrome via Rho/ROCK

pathway activation and subsequent changes in actin stress fibers. This process is independent of the infectious property of the virus and can be induced by noninfectious particles and Ebola GP as shown in the results. The critical Ebola-induced vasculopathy phenomenon can be blocked by targeting the VE-cadherin or Rho/ROCK pathways with FX06 or melatonin, respectively. Thus, we also demonstrated the utility of the approach by studying these two molecular drug candidates, which have never been experimentally tested for their potential effects on Ebola before.

Chapter 4 reports the first chip-based model for Lassa hemorrhagic syndrome. The model includes a perfusable and endothelialized microvasculature-on-a-chip featuring a collagen hydrogel that minimally mimics the extracellular matrix of host tissues. Luminal infusion of Lassa VLPs leads to dramatic increase in vascular permeability in a viral load-dependent manner, the resultant impairment of vascular integrity can be readily quantified. In addition, we demonstrated that Fibrin-derived peptide FX06 is able to suppress the Lassa-induced vascular integrity loss. Our proof-of-concept study provides a low-cost, easy-to-use and high-throughput platform that will not only be useful for studying the pathogenesis of Lassa in a human-like setting but also be critical for diagnostics and drug development.

Chapter 5 presents the application of microvessel-on-a-chip and AFS for investigating the direct contribution of Dengue NS1 to endothelial cells. The impact of Dengue NS1 on vascular integrity was assessed by the high-throughput microvessel-on-a-chip platform featuring the human endothelium that is partly surrounded by the extracellular matrix and allows parallel analysis of organ-level microvessel pathophysiology. Perfusing the microvessels with NS1 results in markedly increased leakage of the microvessels at physiologically relevant concentrations, besides the alterations of VE-cadherin and F-actin, higher level of hyaluronan, predictive marker for dengue severity, was also observed. AFS monitored the significant reduction of endothelial cellular mechanical stiffness after NS1 exposure at single-cell level in a high-throughput manner. Further understanding of underlying cellular mechanisms with the aid of these two advanced techniques may help

the development of treatments to prevent Dengue induced plasma leakage.

Chapter 6 presents the first metabolic profiling of the endothelial cells and macrophages upon Ebola VLPs exposure. They are the main cellular targets, which can be affected through direct interactions with pathogenic viral proteins even without being infected. These interactions affect cellular mechanics and immune processes, which are tightly linked to other key cellular functions such as metabolism. Therefore, a direct infusion mass spectrometry-based untargeted cellular metabolomic approach was performed to investigate the metabolic alterations of primary human endothelial cells, M1 and M2 macrophages upon exposure to Ebola VLPs. The results show that Ebola VLPs led to significant metabolic changes among them. Differential metabolites abundance and perturbed signaling pathway analysis further identified specific metabolic features, mainly in fatty acids and sterol lipids related metabolism pathways. The study presented here highlights the important roles of those host cells in Ebola virus pathogenesis.

Chapter 7 presents an untargeted live single-cell mass spectrometry based metabolomic profiling coupled with a machine learning data analysis approach to investigate the metabolic profile of M1 and M2 macrophages on the single-cell level. This methodology succeeded in capturing the metabolic signature unique to M1 and M2 macrophages, and then leveraging it to classify each phenotype with a high degree of selectivity and sensitivity. Putatively annotated metabolites show that M1 and M2 macrophages have different levels of fatty acyls, glycerophospholipids, and sterol lipids, which are important components of plasma membrane and involved in multiple biological processes. These findings showcase the combination of single-cell metabolomics with random forest models to do phenotypical classification based on single-cell metabolomics data, and subsequently, gain a deeper understanding of the metabolome of heterogenous cell populations, which will pave the way for future studies targeting differentiation of other immune cells.

Finally, in **Chapter 8**, a general discussion of this thesis is presented. A critical evaluation of the research is revealed together with an outlook on how these findings and approaches

will facilitate the understanding of VHF viruses as well as accelerate the development of treatment strategies.

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