Renal Microvascular Endothelial Heterogeneity In Sepsis

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CHAPTER 1
General Introduction, Aims & Thesis Outline
GENERAL INTRODUCTION

In science, ideas that are considered irrefutable one day can fade into irrelevancy the next, as new observations and thoughts reshape our understanding of the world. While theories may undergo transformations, certain elements persist as the foundation of continued scientific discourse. Such is the tale of the endothelium – a remarkable organ that takes center stage in this thesis. In 1661, Italian physician Marcello Malpighi made a landmark discovery by observing small blood vessels called capillaries, which connect the larger arteriolar and venous vessels that were known at the time. Almost 200 years later, German physiologist Theodor Schwann was the first to describe the endothelium, which encompasses the collective network of endothelial cells (EC) that form a thin lining along blood vessels permeating every organ in the body. Schwann’s original description of the endothelium as a passive barrier separating blood from underlying tissue endured for more than a century.

As we fast-forward to 2023, the endothelium has retained its name, yet its previous portrayal as a passive barrier is now rendered obsolete. From the 1980s to early 2000s, a series of scientific discoveries considerably expanded our understanding of the endothelium’s active and diverse biological roles. These include recruitment of white blood cells (leukocytes), modulation of blood clotting (coagulation), and control of microvascular barrier integrity to prevent blood components from leaking out of the blood vessel (Fig. 1). These discoveries underscore the physiological significance of the endothelium and emphasize growing awareness of its significance. In the following paragraph, I will delve deeper into these biological processes and elucidate the role of EC therein. Moreover, I will discuss endothelial heterogeneity, the involvement of EC in sepsis pathophysiology, and introduce omics approaches that allow unbiased analyses of specific classes of molecules.

The endothelium is a multifaceted organ

Endothelial cells (EC) exhibit responsiveness to bloodborne stimuli such as pro-inflammatory mediators tumor necrosis factor alpha (TNFα) and interleukins, which are released by circulating leukocytes and resident immune cells such as Kupffer cells, and lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. As a result, EC express cellular adhesion molecules (CAMs) on their surface, most notably E-selectin, vascular cellular adhesion molecule 1 (VCAM1), and intracellular adhesion molecule 1 (ICAM1). CAMs facilitate leukocyte tethering, rolling, activation, adhesion, and transmigration, thereby enabling leukocytes to move to a site of infection or tissue damage. This process is reviewed in more detail elsewhere. EC also secrete cytokines and chemokines to actively attract leukocytes and communicate with neighboring EC. Another unique feature of EC is their production of specialized granules known as Weibel-Palade bodies. The cargo of Weibel-Palade bodies is released into the bloodstream in response to extracellular stimuli, and includes both proteins involved in coagulation, such
as von Willebrand Factor (vWF) and tissue plasminogen activator (tPA), as well as those involved in inflammation, such as interleukin 8 (IL8) and angiopoietin 2 (Angpt2). While EC predominantly produce proteins that inhibit coagulation, such as thrombomodulin (THBD) and tPA, they can also be prompted to produce pro-coagulants such as plasminogen activator inhibitor 1 (PAI-1) upon exposure to inflammatory mediators. The process of coagulation and the roles of the endothelium therein are excellently reviewed elsewhere.

Additionally, under pro-inflammatory conditions the integrity of the endothelial barrier can become compromised through disintegration of junction molecules, leading to fluid leakage into the underlying tissue. This may give rise to edema formation, thereby impairing normal organ function.

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**Figure 1 | Endothelial cells are involved in leukocyte extravasation, coagulation, and microvascular barrier function loss**

In response to damage or infection, endothelial cells (EC) secrete chemokines and express cell adhesion molecules (CAMs) on their cell surface to recruit circulating leukocytes. Leukocytes then associate with EC through interactions with endothelial CAMs, resulting in transmigration of leukocytes through the endothelial cell layer into the underlying tissue. Blood clotting is determined by a balance between pro- and anti-coagulation factors. Under resting conditions, EC predominantly contribute to anti-coagulation through expression of thrombomodulin (THBD), endothelial protein C receptor (EPCR), and tissue plasminogen activator (tPA). When exposed to inflammatory stimuli, EC also produce and release pro-coagulants such as von Willebrand Factor (vWF) and plasminogen activator inhibitor 1 (PAI-1). EC maintain tight barriers by using junction molecules to connect with neighboring cells. If these junctions are impaired, microvascular leakage occurs, in which blood constituents can freely move out of the blood stream. Figure was created with BioRender.com.
Microvascular endothelial heterogeneity

The elaborate network of blood vessels, encompassing arteries, arterioles, capillaries, venules, and veins, orchestrate the delivery of oxygen and nutrients to every part of the body. Large arteries supply tissue with oxygenated and nutrient-rich blood, the exchange of which occurs in capillaries, which at less than 10 µm in diameter represent the smallest blood vessels in the body (Fig. 2A). Subsequently, venules collect the blood which is now depleted of oxygen and nutrients and filled with waste products, and transport it back to the heart in veins. Collectively, arterioles, capillaries, and venules constitute the microvasculature, and each represents a unique microvascular compartment that contains resident microvascular EC. Moreover, EC from different microvascular compartment have adopted unique properties to facilitate the highly specialized functions of organs. For instance, in the kidney, the main subject of my research in this thesis, afferent arterioles transport blood to a unique microvascular compartment called glomeruli. Blood is filtered in glomeruli to clear waste products from the body, and after reabsorption of water and important solutes by tubular epithelial cells results in the production of urine (Fig. 2B). The remaining blood next enters the efferent arterioles, which transition into peritubular capillaries, which supply the kidney with oxygen and nutrients and assist tubular epithelial cells with the reabsorption of water and solutes. From the peritubular capillaries blood drains into postcapillary venules. Postcapillary venules are exposed to lower blood pressure conditions compared with arterioles, which may assist more effective recruitment and transmigration of leukocytes. In most organs, leukocyte transmigration occurs primarily in venules, with occasional participation of capillaries, but seldom involve arterioles.

Figure 2 | Schematic illustration of blood vessel branching and the renal microvasculature

(A) Oxygen and nutrient-rich blood is supplied by arteries, which branch off into smaller arterioles. Gas and solute exchange in tissues occurs in the capillaries, after which venules and larger veins carry off oxygen- and nutrient-poor blood containing waste products and gases. (B) Illustration of a nephron, the functional unit of the kidney, indicating the four microvascular compartments of the renal cortex. The horizontal dotted line represents the approximate boundary between kidney cortex and medulla. Panel B was adjusted from chapter 5, where it was included as Fig. 1A. Both figures were created with BioRender.com.
EC associate with different vascular support cells depending on the microvascular compartment. Arterioles are encircled by smooth muscle cells that regulate blood flow by modulating the vessel diameter through contraction and relaxation. Capillaries and venules largely lack smooth muscle cells yet are in close contact with vascular support cells called pericytes, although many capillaries lack support cells to ensure efficient diffusion of oxygen and nutrients into the tissue²⁵.

Microvascular compartments not only differ in terms of their function and the identity of their associated vascular support cells, but also exhibit differences regarding gene and protein expression patterns of resident EC. These differences are collectively described as microvascular endothelial heterogeneity³⁰. In the kidney, endomucin (EMCN) is an example of a heterogeneously expressed protein, as EC in arterioles, which are positive for pan endothelial platelet/EC adhesion molecule 1 (PECAM1), do not express EMCN. In contrast, EC in glomeruli, peritubular capillaries, and postcapillary venules express both EMCN and PECAM1 (Fig. 3). While other genes and proteins with differential microvascular endothelial expression patterns have been described³¹–³³, the functional implications of this heterogeneity are inadequately understood.

**Figure 3** | Heterogeneous microvascular expression of endothelial markers PECAM1 and EMCN

Protein detection of pan endothelial platelet/endothelial cell adhesion molecule 1 (PECAM1) and endomucin (EMCN) demonstrates microvascular endothelial heterogeneity with regard to protein expression patterns in mouse kidney. All microvascular endothelial cells express PECAM1, while endothelial cells in arterioles lack EMCN expression. A, arterioles; G, glomeruli; PTC, peritubular capillaries; PCV, postcapillary venules.

**Endothelial involvement in sepsis**

Sepsis, a complex disorder often referred to as blood poisoning, arises from a dysregulated host response to infection³⁴. Infectious agents such as bacteria that cross the body’s barriers
through internal or external injuries are typically cleared effectively, but in some cases can elicit a disproportionate response of the host. These responses can inflict damage to organs, leading to organ dysfunction and failure. Sepsis accounts for 19.7% of global mortality, and despite extensive clinical trials, no effective therapeutic interventions to counteract sepsis-associated pathophysiological processes exist. While all organs can start to fail following sepsis, the kidney is particularly susceptible, resulting in sepsis-associated acute kidney injury (sepsis-AKI) in one out of every three sepsis patients. The onset of sepsis-AKI is linked to heightened mortality rates and prolonged stays in the intensive care unit. During sepsis-AKI, the filtration function of the kidney becomes compromised, resulting in diminished urine output and accumulation of waste material in the blood. Additionally, sepsis-AKI can result in chronic kidney damage, which lowers the quality of life of sepsis survivors.

Sepsis-AKI research initially centered around renal immune and tubular epithelial cells. However, in the last decades, microvascular EC have emerged as important players in sepsis-AKI pathophysiology. EC are implicated in many of the dysregulated processes during sepsis, including leukocyte recruitment, disrupted coagulation, and microvascular leakage. Modulating endothelial responses in sepsis-AKI can improve disease outcome. For instance, induction of sepsis in mice lacking adhesion molecules E- and P-selectin resulted in improved kidney function and reduced lethality, and administration of an agonist of endothelial-enriched receptor Tie2 in mice exposed to LPS reduced microvascular leakage and improved kidney function based on glomerular filtration rate. It is of note that not all microvascular compartments respond similarly to sepsis-AKI and sepsis-like stimuli such as LPS and TNFα, as in the kidney, EC in glomeruli and peritubular capillaries express E-selectin in response to sepsis in mice, whereas arteriolar and venous EC do not. Furthermore, TNFα induces the expression of VCAM1 in all renal microvascular compartments, except for glomerular EC. These examples illustrate the heterogeneity in responses to sepsis and sepsis-like stimuli in renal microvascular EC, although the functional implications of differential microvascular responses are still poorly understood. Taken together, the endothelium represents a promising target for therapeutic interventions in sepsis-AKI.

Omics analyses
In the past decades, tremendous advances have been made in the field of omics approaches. Omics is a collective term for high throughput analyses that exhaustively characterize and quantify a specific type of biological molecule. In this manner, samples can be analyzed in an unbiased manner, which promotes in-depth descriptions of the molecular profiles and processes.

In response to extracellular stimuli, specialized proteins called kinases phosphorylate their protein substrates, and as such they are key components in relaying signals via intracellular signal transduction pathways to change the behavior of the cell. This can lead
to activation of transcription factors and subsequent gene transcription, resulting in the production of specific messenger RNAs (mRNAs). These mRNAs can be translated into corresponding proteins, which are the main functional constituents of the cell. MicroRNAs (miRNAs) are small non-coding RNAs that bind to target mRNAs and can thereby repress the translation of mRNA to protein. Omics can be employed to study mRNAs, miRNAs, proteins, or kinases through transcriptome, miRNome, proteome, or kinome analyses, respectively. By combining multiple omics analyses, additional information can be obtained about potential interactions between molecules, which subsequently may contribute to revealing molecular differences underlying, for example, endothelial heterogeneity.

**AIMS**

As discussed above, microvascular endothelial cells are important players in sepsis-AKI pathophysiology and represent promising therapeutic targets. Microvascular compartments respond differently in sepsis, yet detailed information on the molecular basis underlying heterogeneous microvascular responses in sepsis is lacking.

The overarching research aim of this thesis was to investigate the molecular identity of (microvascular) endothelial responses to sepsis-like insults *in vitro* and to sepsis in the kidney *in vivo*, while taking into account the existence of endothelial and microvascular heterogeneity. To this end, I employed kinomics, miRNomics, and transcriptomics as unbiased, explorative analytical tools.

The questions that I sought to answer in this thesis are as follows:

- Which kinases are involved in endothelial responses to sepsis-like stimuli?
- Is basic renal microvascular endothelial heterogeneity associated with unique miRNA and mRNA signatures, and if yes, what is the extent and identity of these differences?
- Do renal microvascular compartments exhibit heterogeneity in their induction of miRNAs and mRNAs in response to sepsis-AKI, and if yes, what is the molecular signature of this heterogeneity?

By pursuing these questions, I expected to identify potential therapeutic targets in sepsis-AKI that can be further investigated in follow-up studies.
The endothelium is introduced as a multifaceted organ with a crucial role in biological processes in health and disease in chapter 1. Microvascular EC exhibit heterogeneity with regard to function, yet the underlying molecular mechanisms of this heterogeneity are poorly characterized. In sepsis, a severe and frequently fatal disorder, EC are involved through their engagement in leukocyte recruitment, coagulation, and microvascular leakage. Chapter 2 zooms in on intracellular signaling transduction pathways involved in endothelial responses to sepsis, and discusses the involvement of kinases and phosphatases in these intracellular
signaling pathways as potential therapeutic targets. Kinases are crucial molecules in relaying signals from extracellular stimuli further downstream toward changes in cellular behavior. Endothelial responses to pro-inflammatory stimuli are dependent on kinases, yet the number of kinases with demonstrated involvement in these processes is limited, as summarized in chapter 2. Therefore, in chapters 3 and 4 we employed kinome analyses to identify the kinases involved in endothelial responses \textit{in vitro} to LPS respectively TNF\alpha. Moreover, we investigated whether pharmacological inhibition of the identified kinases was able to ameliorate endothelial inflammatory activation.

Microvascular EC have unique characteristics depending on their microvascular compartment, and I hypothesized that differential miRNA and mRNA expression profiles underlie these differences. To test this hypothesis, in chapter 5 I used laser microdissection (LMD) to collect microvascular compartments from the renal cortex of healthy mice, and subjected them to small RNA sequencing and RNA sequencing to obtain microvascular miRNome and transcriptome profiles, respectively. The results were validated using RT-qPCR, miRNA in situ hybridization, and immunohistochemistry. To incorporate miRNA and mRNA data, we performed miRNA-mRNA pair analysis and assessed whether putative functional consequences of miRNA-based mRNA repression could be identified.

We were interested to investigate the changes in microvascular EC miRNA transcription following sepsis. To that end, in chapter 6 I performed small RNA sequencing of renal microvascular compartments from cecal ligation and puncture (CLP)-sepsis mice, followed by assessing microvascular miRNA levels in patients with sepsis-AKI and functional \textit{in vitro} studies.

Microvascular EC are important players in coagulation and inflammation during sepsis, although the contribution of different microvascular compartments to these processes is incompletely understood. Therefore, in chapter 7 I studied the microvascular transcriptome in sepsis by combining LMD and RNA sequencing. Heterogeneous microvascular responses associated with coagulation and inflammation were investigated at mRNA and protein level, and I determined the functional involvement of identified genes in endothelial coagulation and inflammatory activation \textit{in vitro} using siRNA-based gene knockdown.

In chapter 8, I summarize the main findings of the research presented in this thesis, and return to the main research aim. Furthermore, I discuss the implications and future perspectives of the research presented in this thesis.
Interleukin 6-Mediated Endothelial Barrier Disturbances Can Be Attenuated by Reduced Glomerular Endothelial Thrombomodulin Is Associated with Glomerular Permeability characteristics of cultured endothelial cell monolayers. (1839).

PAI-1 is a vascular cell–specific HIF-2–dependent angiogenic factor that promotes retinal neovascularization in diabetic patients. (1661).

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