Exploring cellular and molecular mechanisms underlying endothelial heterogeneity in sepsis
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Introduction and Aim of the thesis
Chapter 1

GENERAL INTRODUCTION

Sepsis is a complex disorder that is caused by a dysregulated host response to infection [1], which accounts for an estimated 2.8 million deaths per year [2]. Besides antibiotics, supportive therapies such as fluid and vasopressor administration aiming to restore the function of vital organs are the current mainstay of sepsis therapy [3]. Ideally, broad-spectrum antibiotics are administered within the first hour of sepsis recognition [4], although identity and source of infection remains unknown in 40% of patients with sepsis [5]. Even when the source is known, every hour of delay in antibiotic administration to resolve the underlying infection is associated with a 7% increase in risk of death in patients with sepsis [6]. The current management of sepsis does not effectively halt sepsis progression and eventually cause vital organs to fail [7]. Sepsis criteria scoring strategy such as Sequential Organ Failure Assessment (SOFA) is used to assess disruption in the function of vital organs, of which score of 2 points or more is associated with an in-hospital mortality greater than 10% [1]. If the patients eventually recover, 5-year mortality rate is as high as 81% [8], and most suffer long-term sequelae related to physical, psychological, and cognitive abilities [8].

Sepsis-related organ dysfunction

Sepsis-related organ dysfunction is the condition in which organs fail as a result of dysregulated host response to infection [1,9]. Any organ can fail because of sepsis, and the failure of one organ can lead to the dysfunction of another organ due to organ cross-talk [10], leading to multiple organ dysfunction syndrome (MODS). Sepsis-associated MODS is linked to higher mortality than sepsis without organ injury [11]. Many factors can contribute to the severity of sepsis and consequently MODS, such as type of pathogen, source of infection, route of administration, co-morbidities, co-medication, age, and genetic makeup [12]. Sepsis-related organ dysfunction is represented by clinical symptoms that can appear alone or collectively as a sign of failing of specific organs in patients. Therefore, it is difficult to establish whether organs will fail once sepsis symptoms become manifest, and when they do, which organ will fail first.
Microvascular disturbances are known to play an important role in organ dysfunction [13]. Tissue oedema [12], leukocyte infiltration [14], and microthrombi [15] are common findings in the microvasculature of failed organ in sepsis patients, though presence of all these abnormalities at the same time is not a pre-requisite for sepsis-related organ failure. The degree of vascular permeability, leukocyte infiltration, and coagulation impairment varies depending on organ, and the organ can fail even in the absence of one or more pathological manifestation(s) mentioned [12]. Failing lung shows fluid accumulation, while dysfunctional kidney is characterized by deteriorating glomerular sieving function leading to reduced glomerular filtration rate [16] and anuria [12]. Similarly, leukocyte infiltration is massive in failing lung, while kidney on the verge of failing can show minimal leukocyte infiltration [14]. Microthrombi are mostly found in the microvascular beds of sepsis-associated dysfunctional lung, but not as extensively in any of the other failing organs [17].

While studies in living sepsis patients are impossible, studies in mice are commonly used to investigate kinetics of cellular responses of different organs in sepsis [18]. The endotoxemia model is commonly used to study exaggerated inflammatory response related to early sepsis [19,20]. In this model, lipopolysaccharide (LPS) or endotoxin injection into mice results in organ-specific increase in vascular permeability [12,21], leukocyte recruitment [21], and pro-inflammatory responses, which are all processes that are also happening in human sepsis [22]. Some manifestations of endotoxemia such as systemic arterial hypotension, lactic acidosis, impaired myocardial contractility, and increased levels of circulating TNF-α and IL-6 resemble the clinical features in human sepsis [18]. Despite some resemblance with the systemic inflammatory response seen in clinic [23], LPS endotoxemia does not fully recapitulate the essence of the complex pathophysiology of human sepsis. Cecal ligation and puncture (CLP), a procedure of releasing faecal material into the peritoneal cavity by perforating the cecum [24], is considered to be the ‘gold standard’ experimental sepsis model that recapitulates human sepsis better [25]. CLP produces immune, hemodynamic, and biochemical responses [23] similar to that of intraperitoneal sepsis in human patients [26,27]. Studies done to assess efficiency of TNF-α antibody [28] and IL-1 receptor antagonist [29] showed comparable results with clinical trial findings [18], which implies some extent of face validity of CLP model in studying human sepsis.
Endothelial dysfunction in sepsis

The vasculature consists of a continuous network of blood vessels that supplies nutrients and oxygen to organs and are all covered by endothelial cells (EC). In organs, the blood vessels are comprised of arterioles, capillaries, and venules, which are collectively termed as the ‘microvasculature’ (Figure 1). By acting as ‘gatekeepers’ between blood and tissues [30], EC form a selective barrier along the microvasculature of organs and maintain homeostasis by regulating vascular permeability [31] and coagulation [30]. EC are also active responders to proinflammatory stimuli [32–35]. During sepsis, EC become activated, leading to impaired barrier permeability [12], leukocyte recruitment into organs [14], and dysregulated coagulation [17].

EC respond to LPS, a component of Gram negative bacteria found in the blood of sepsis patients [36], and various other sepsis-related proinflammatory stimuli, by expressing adhesion molecules such as P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1). The increased expression of these molecules promotes tethering, rolling, and adhesion of leukocytes to activated endothelial surface. Additionally, EC release various inflammatory cytokines and chemokines that enable recruitment of more leukocytes into the microvascular beds. Some of the recruited leukocytes will undergo trans-endothelial migration into the tissues either via the transcellular or paracellular route [37]. Adhesion molecules, such as cluster of differentiation 31 (CD31) [38], ICAM-1, and ICAM-2 [39,40] facilitate trans-endothelial migration (also called ‘diapedesis’) of leukocytes into the tissues. The endothelial expression of these proinflammatory molecules is among the earliest signals that promotes adhesion of leukocytes to the blood vessel walls, and thereafter influx of leukocytes into the tissue of organs, which further contributes to impairment of organ function in sepsis.

The endothelial barrier is maintained by tight regulation of endothelial adherens junctions and tight junctions. Gap junctions, which are made up of molecules mainly of the connexin family, are important in regulating cell-to-cell communication [41]. VE-cadherin, the main endothelial adherens junctional molecule, is important in regulating junctional integrity between EC of the microvasculature of all organs, particularly in heart [42], kidney [43], and lung [42,44]. Apart from adherens junction, tight junctional molecules such as occludins
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[45], claudin-5 [46], and junctional adhesion molecules (JAMs) [47] also function in maintaining vascular integrity in an organ-specific manner [12,46]. During sepsis, VE-cadherin [48] and claudin-5 [49]-regulated endothelial barriers are impaired, thus allowing the entry of fluid and leukocytes from the blood vessels into organs. VE-cadherin facilitates the exit of leukocytes from the microvasculature, evidenced by strong inhibition of leukocyte extravasation in cremaster, skin, and lung microvascular beds after the insertion of VE-cadherin-α-catenin fusion construct in mice [50]. Additionally, in sepsis, EC release coagulation factors including von Willebrand factor (vWF), which initiate the coagulation cascade, leading to thrombus formation in the organ microvasculature. Next to its platelet-activating properties, vWF promotes the adhesion of leukocytes to the EC [51]. vWF plasma levels were found to be higher in non-survivor septic patients with acute lung injury compared to sepsis survivors [52], while soluble VE-cadherin levels were increased in plasma of sepsis patient with acute kidney injury (AKI) [48].

A. Heterogenous endothelial response in sepsis

Endothelial cells are structurally and functionally diverse [53], which is partly attributed to microenvironmental factors [54] and epigenetic control [55,56]. Following inflammatory stimulation, the extent of endothelial activation varies depending on EC location [53] and the type of stimulus [53,57]. While it is established that EC between organs are phenotypically different in health and disease [53,58,59], the microvasculature within an organ also has EC with different identities that respond to inflammation to different extents [53,59]. The kidney microvascular segments are comprised of arterioles, capillaries (glomerulus and peritubular), and post-capillary venules [60]. In the kidney of mice injected with TNF-α [33] or LPS [21], E-selectin expression was shown to be more restricted to the glomerular segments, while VCAM-1 expression predominated in extra-glomerular microvascular segments [21,33]. Our research group showed that in the kidney of mice injected with TNF-α, restriction of VCAM-1 expression in the glomerulus was associated with high mIR-126 expression [33]. This observation implies the existence of microvascular bed-specific molecular control of endothelial responses to inflammation, which still largely remains elusive. Understanding molecular signatures of the microvascular segments of kidney and other organs
that are susceptible to sepsis is key in understanding the differential roles of microvascular EC in the pathogenesis of sepsis-related organ dysfunction.

**Figure 1.** Endothelial cells (EC) cover the microvasculature lining of organs in the body. Figure above shows microvascular beds of lung and kidney, which are stained for CD31 (CD31; a pan-endothelial marker). Lung microvasculature is consisted of arterioles ('a'), pulmonary capillaries ('c'), and venules ('v'), while kidney microvasculature is consisted of arterioles ('a'), two form of specialized capillaries namely glomeruli ('g') and peritubular capillaries ('p'), and venules ('v').
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B. Endothelial-leukocyte interaction in sepsis

As described above, in sepsis, EC become activated leading to leukocyte recruitment to specific sites of inflammation. Neutrophils are the most common leukocyte subset found in the organs from sepsis patients [14,61] and in organs of animals in experimental models of sepsis [62]. Apart from neutrophils, macrophages are commonly found in sepsis kidney biopsies, particularly in the glomerular compartment [14]. Recruitment of leukocytes involves multiple steps, which includes transient rolling of leukocytes and subsequent adherence of leukocytes to the endothelial cell surface. The rolling of leukocytes is regulated by endothelially expressed selectins (P-selectin, E-selectin), and leukocyte-expressed selectin ligands (P-selectin ligand, E-selectin ligand, and CD44) [63]. In the glomerular compartment of the kidney, recruitment of leukocytes is mediated by platelet-derived P-selectin [64]. In vivo studies in cremaster, mesenteric, and skin tissues [65] showed that the ‘conventional’ recruitment of leukocytes occurs mainly in the post-capillary venules. This process was initially thought to be the same in all organs. However, intravital microscopy studies in liver, lung, and kidney [65], have shown that leukocyte recruitment is an organ- and microvascular bed-specific process [61,66,67]. Leukocyte recruitment in the sinusoidal capillaries of the liver is selectin-dependent and accounts for up to 70-80% of leukocyte recruitment compared to that in post-capillary venules of inflamed liver [66]. In contrast, blocking of E-selectin in a glomerulonephritis rat model did not affect glomerular leukocyte recruitment [68]. These studies imply that leukocyte recruitment in organs is stimulus- and microvascular compartment-specific, which could be attributed to remarkable heterogeneity of EC in different microvascular beds of organs. The mapping that connects the unique ‘configuration’ of endothelial molecular signatures in different microvascular segments of organs susceptible to sepsis such as kidney and lung, to recruitment of specific subset of leukocyte during sepsis, is yet to be established.

C. Lipopolysaccharide-driven endothelial signal transduction

Recognition of LPS by the innate immune system is among the earliest key event that leads to the inflammatory host response [69]. Endothelial cells recognize LPS via Toll-like receptor (TLR) 4 [70] and Retinoic inducible gene (RIG)-I [35]. LPS
interacts with LPS binding protein (LBP), and the internalized complex then binds to the TLR4-myeloid differentiation protein 2 (MD2) complex and CD14, further activating TLR4-mediated downstream signalling [71]. RIG-I recognizes LPS independent of TLR4 involvement and responds by activating its downstream signalling pathways via protein adaptor mitochondrial antiviral signalling proteins (MAVS) [35]. The activation of TLR4- and RIG-I- [35] mediated pathways result in downstream signalling cascades via p38 MAPK and NF-κB, leading to the expression of the proinflammatory molecules introduced above [72] (Figure 2).

Tyrosine kinases (TK) are important enzymes in signal transduction to maintain essential cellular activities, also in endothelial cells [73]. Additionally, TK are important components of Toll-like receptor-mediated signalling pathways, and are therefore critical in inflammation [74]. TK such as Pyk2 and Src were shown to control LPS-mediated inflammatory responses in endothelial cells via TLR4 [75–77]. Treatment with tyrosine kinase inhibitors was shown to increase survival and prevent lung oedema in mice exposed to LPS [78], and reduce the production of inflammatory mediators by LPS-activated human endothelial cells in vitro [76,79,80], and in murine experimental sepsis [81]. This underscores the importance of TK signalling in endothelial cellular response to sepsis and highlights the potential of TKs as druggable targets in alleviating sepsis-mediated inflammation.

**AIM AND OUTLINE OF THE THESIS**

As highlighted above, the pathophysiology of sepsis-related organ dysfunction is still not completely known, which hinders the development of effective therapies for sepsis. In health, EC keep vascular integrity, coagulation, and inflammation in check, but during sepsis, these processes are all compromised to different degrees. This is partly due to dysfunction of EC, leading to organ dysfunction. Organs fail differently in sepsis despite being exposed to the same insult, which suggests that impaired endothelial-regulated processes are distinctively controlled both at organ and at microvascular segment levels. The precise molecular control of inflammatory response of different microvascular segments of sepsis-affected organs remains
elusive. The questions that are fundamental to this thesis are, 1) how do endothelial cells respond to sepsis-related inflammatory insults?; 2) what are cellular and molecular differences between EC in different organs and microvascular segments?; and 3) what molecules regulate these cellular or molecular differences?

Figure 2. Schematic overview of LPS-mediated signalling transduction in endothelial cells. TLR4- and RIG-I-mediated signalling pathways are known to activate p38 MAPK and NF-κB pathways, leading to the expression of proinflammatory molecules (E-selectin, VCAM-1, ICAM-1, and cytokines).

To address the first two questions, the first aim of this thesis was to investigate heterogenous response of EC to sepsis-related inflammatory stimuli and the underlying molecular mechanism that may contribute to these differences. Additionally, as tyrosine kinases (TK) are essential components in inflammatory signalling including such as observed in EC in sepsis, the next aim of this thesis was to identify which TK are involved in endothelial inflammatory signalling and investigate whether these kinases can be pharmacologically targeted to attenuate the inflammatory response.
Chapter 1

In **chapter 1**, I introduced the concept of microvascular dysfunction which leads to impaired vascular permeability, coagulation, and inflammation. In failing organs, these processes are affected, albeit to different extents. Kidney and lung are the two organs that are most commonly affected in sepsis. In **chapter 2**, we reviewed the clinical manifestations of sepsis-associated acute kidney injury (sepsis-AKI) and sepsis-associated acute respiratory distress syndrome (sepsis-ARDS). The focus was on the phenotypic differences in microvascular endothelial responses between lung and kidney which may contribute to the distinct clinical manifestations seen in both organs. We summarized the relevant *in vivo* and *in vitro* studies showing cell type- and stimulus-specific heterogenous endothelial responses associated with organ-specific failure in patients with sepsis.

EC respond differently to external stimuli depending on their location, and the type of stimuli [53]. Microvascular bed-specific endothelial responses have been shown *in vivo* [21,33], but the ramification of this heterogenous response for the recruitment of specific leukocyte subsets is not known. Using cecal ligation and puncture (CLP), the 'gold standard' model of experimental sepsis, in **chapter 3**, we therefore investigated the nature, kinetics, and location of activation of microvascular EC in lung and kidney in mice during CLP-induced sepsis development. We investigated to what extent activation was associated with leukocyte recruitment within the lung and kidney of CLP mice.

Since LPS exposure resulted in organ- and microvascular segment-specific heterogenous expression of endothelial inflammatory molecules in mouse endotoxemia [21], we hypothesized that LPS activated different intracellular mechanisms leading to differential expression of inflammatory molecules. In **chapter 4**, we investigated LPS-activated EC subpopulations based on their E-selectin and VCAM-1 expression. Using siRNA interference and pharmacological approaches, we investigated the role of TLR4 and RIG-I, as well as NF-κB and p38 MAPK, in the formation of LPS-activated EC subpopulations and propose a molecular mechanism that regulates LPS-induced phenotypic heterogeneity in endothelial inflammatory activation.

Based on the knowledge generated in chapter 4, we hypothesized that LPS-activated EC subpopulations are regulated by specific regulatory mechanisms leading to their distinct heterogenous profile of LPS-induced expression of inflammatory molecules. In **chapter 5**, we designed an experimental approach to
compare gene expression profiles of two LPS-activated subpopulations sorted based on their E-selectin and VCAM-1 expression. The two subpopulations were the ‘Quiescent’ E-sel⁻/VCAM-1⁻ cells (a subset of cells exposed to LPS that did not express E-selectin and VCAM-1) and the E-sel⁺/VCAM-1⁺ (LPS-exposed cells with the expression of both E-selectin and VCAM-1). I discuss the experimental strategies used to characterize these different LPS-activated EC subpopulations, preliminary data obtained so far, the expected outcomes, and thereafter potential challenges.

We hypothesized that TK are crucial in endothelial LPS-mediated signalling transduction, and that inhibition of TK activity can reduce LPS-induced endothelial inflammatory activation. Therefore, in chapter 6, we characterized the TK network that regulates LPS-mediated signalling in endothelial cells and identified 58 TK that can potentially serve as targets. Using siRNA and tyrosine kinase inhibitors (TKIs), we further investigated three TK for their roles in EC inflammatory signal transduction and then investigated whether pharmacologically targeting them could diminish LPS-induced inflammatory activation in HUVEC.

Finally, in chapter 7, I summarize the findings of the research presented in my thesis and discuss them within the context of our current knowledge and recent literature, and I discuss the implication of my thesis for future studies.

REFERENCES


Chapter 1


Introduction and Aims of the thesis


[33] S.A. Ásgeirsdóttir, C. van Solingen, N.F.
Chapter 1


[52] L.B. Ware, M.D. Eisner, B.T. Thompson, P.E. Parsons, M.A. Matthay, Significance of Von Willebrand Factor in Septic and Nonseptic Patients with Acute Lung
Introduction and Aims of the thesis


[69] R.J. Ulevitch, P.S. Tobias, Recognition of Gram-negative bacteria and


