Exploring cellular and molecular mechanisms underlying endothelial heterogeneity in sepsis
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SUMMARY

Patients suffering from sepsis are often confronted with organ dysfunction. Multiple organ dysfunction syndrome (MODS) is the most prevalent life-threatening sepsis complication, and kidney and lung are vulnerable to sepsis-associated injuries. The pathophysiology of kidney and lung organ dysfunction is not completely understood, hampering the development of effective therapy to stop the onset of sepsis to organs failure. What is known is that clinical manifestations of sepsis-associated organ dysfunction are linked to endothelial dysfunction. Endothelial cells (EC) are sentinel cells that regulate vascular permeability [1], coagulation [2], and leukocyte trafficking [3,4], which are the processes that are dysregulated in sepsis, and lead to organ dysfunction. Hence, preventing endothelial dysfunction in sepsis may be a useful strategy to prevent organ failure and thereafter sepsis-related mortality.

In chapter 1, I introduced the role of EC in health and in the pathophysiology of sepsis-associated organ dysfunction. Once thought of as inert bystanders, EC are now recognized as active responders in the onset of inflammation in sepsis. EC respond to infection by direct recognition of sepsis-related inflammatory stimuli, resulting in the expression of various inflammatory molecules, leading to leukocyte recruitment. As endothelial inflammatory activation, and thereafter endothelial-guided leukocyte recruitment, are organ- and microvascular-specific, the cellular and molecular attributes contributing to these processes in sepsis remain to be investigated. Therefore, in this thesis, I proposed to investigate the heterogeneous response of EC to sepsis-related inflammatory stimuli and the underlying molecular mechanism(s) that may explain these differences. The strategies were aimed to understand the endothelial response to sepsis at the organ, microvascular bed, and cellular level (Figure 1). The knowledge acquired will assist us to better understand the cellular and molecular basis of endothelial heterogeneous response in sepsis.

As kidney and lung are organs that are mostly affected by sepsis, in chapter 2, we compared the clinical manifestations of sepsis-associated acute kidney injury (sepsis-AKI) and sepsis-associated acute respiratory distress syndrome (sepsis-ARDS). We highlighted that differential responses of EC in the regulation of inflammation, leukocyte infiltration, and vascular barrier dysfunction are an important contributor to distinct clinical phenotypes seen in sepsis-AKI and sepsis-
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Figure 1: Schematic diagram of the relation of chapters in this thesis. (MODS=multiple organ dysfunction syndrome; *G=glomerulus; *PC=peritubular capillaries).
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ARDS. We furthermore concluded that understanding the cellular and molecular regulation of the microvascular beds of sepsis-afflicted kidney and lung is imperative in developing specific therapy to prevent organ deterioration in sepsis-AKI and sepsis-ARDS.

As introduced in chapter 2, endothelial-guided leukocyte recruitment occurs to different extents in sepsis-AKI and sepsis-ARDS. In chapter 3, we described the kinetics and the location of E-selectin and VCAM-1 expression in the kidney and lung of mice that underwent cecal ligation and puncture (CLP), a murine model to study polymicrobial sepsis. We explored the extent of endothelial activation in the arterioles, capillaries, and venules of the two organs that responded to CLP. E-selectin was transiently expressed in the renal glomerular capillaries, and in the pulmonary arterioles and venules of CLP mice. VCAM-1 expression in the kidney and lung was not visibly changed and mainly expressed in the arterioles and venules in both organs. In the capillaries, VCAM-1 was expressed in renal peritubular capillaries, and to lesser extents in pulmonary capillaries, yet was absent in renal glomerular capillaries until after 72 h of CLP. We furthermore investigated the kinetics of leukocyte infiltration in the two organs. In the kidney, neutrophil myeloperoxidase activity was persistently high throughout the 72 hours of CLP while it transiently increased in the first 7 hours of sepsis development in the lung. The neutrophil infiltration was much more extensive in the lung than in the kidney. Ly6G+ neutrophil infiltration was glomerular-compartment specific in the kidney while it was widespread in all microvascular beds of the lung in early CLP, and in pulmonary capillaries in late CLP. Summarizing, in CLP the expression of E-selectin and VCAM-1 in the kidney and lung was microvascular bed-dependent, and the presence of both molecules was not directly associated with Ly6G+ neutrophil infiltration in any of the microvascular beds.

Lipopolysaccharide (LPS) is a Gram negative bacteria-derived mediator important in the pathogenesis of sepsis. LPS-mediated endothelial activation results in the expression of inflammatory adhesion molecules, such as E-selectin and VCAM-1. These molecules are known to be regulated by distinct receptors and intracellular signaling pathways, although it is unknown whether EC within a homogenous population respond similarly via identical receptors and intracellular signaling system. In chapter 4, we investigated LPS-induced E-selectin and VCAM-1 expression patterns in mouse renal microvascular compartments in vivo and in
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HUVEC in vitro. We found that E-selectin and VCAM-1 were expressed by EC to different extents between and within renal microvascular compartments of LPS-exposed mice. This observation was recapitulated in LPS-exposed HUVEC by the identification of four endothelial subpopulations discerned based on their E-selectin and VCAM-1 expression. These encompassed the following: E-selectin⁻/VCAM-1⁻ (-/-), E-selectin⁺/VCAM-1⁻ (E-sel+), E-selectin⁺/VCAM-1⁺ (+/+), and E-selectin⁻/VCAM-1⁺ (VCAM-1+). The occurrence of these subpopulations was regulated either via TLR4 only, or both TLR4 and RIG-I. Moreover, pharmacological inhibition showed important roles of NF-κB and p38 MAPK in the formation of E-sel+ and +/+ subpopulations. Interestingly, one EC subpopulation was devoid of the two adhesion molecules and did not express any cytokines or chemokines studied despite LPS exposure.

Based on the findings in chapter 4, we concluded that LPS activates different signaling pathways in EC that drive heterogeneous expression of endothelial inflammatory molecules. In chapter 5, we initiated an experimental strategy to study in more detail the differences in gene expression of the two distinct LPS-activated -/- and +/+ subpopulations. The -/- and +/+ subpopulations exhibited distinctive inflammatory responses despite similar exposure to LPS. Transcriptome analysis by RNA sequencing is currently being performed on these two subpopulations while this chapter is written. I expect that the outcome of this study will expand our understanding of gene expression profiles of the two subpopulations and possibly reveal molecular mechanisms controlling quiescence and exaggerated inflammatory responses of EC to LPS.

As described in chapter 4, despite the known involvement of specific receptors in regulating LPS-induced inflammatory activation, the intermediary molecules involved in LPS-mediated inflammatory signaling cascades in EC are understudied. Therefore, in chapter 6, we investigated the role of tyrosine kinases (TK) in regulating LPS-mediated endothelial inflammatory activation. TK profiling analysis revealed 58 TK that were involved in LPS-induced endothelial activation. Three of these TK were chosen for further studies. siRNA-based and pharmacological inhibition studies of two TK, namely focal adhesion kinase 1 (FAK1) and anaplastic lymphoma kinase (ALK), showed key roles in mediating LPS-induced endothelial inflammatory activation. Only pharmacological inhibition of FAK1, but not ALK, after the start of LPS exposure resulted in diminished expression of
inflammatory molecules. From this, we concluded that kinase profiling is a useful strategy to find TK as druggable targets to attenuate LPS-mediated endothelial inflammatory activation. Follow-up *in vivo* studies need be performed to assess the pharmacological consequences of inhibition of TK, such as FAK1, on endothelial activation in the microvasculature of organs affected by sepsis.

**CONCLUSIONS**

**EC respond in an organ- and microvascular bed specific manner in sepsis pathophysiology.**

In this thesis, I show that the extent of inflammatory activation of EC is different between the kidney and lung, and also between microvascular compartments of both organs. Moreover, we showed that regardless of the approach to systemically induce sepsis in mice, whether mice underwent CLP procedure (chapter 3) or LPS exposure (chapter 4), the endothelial inflammatory activation in the kidney is microvascular bed-specific.

As E-selectin and VCAM-1 are both involved in the recruitment cascade of leukocytes, one would expect that leukocytes are massively recruited in the CLP-activated microvascular compartments expressing E-selectin and/or VCAM-1. However, this is not the case, as the microvascular bed-specific localization of neutrophils did not necessarily coincide with the microvascular beds expressing E-selectin and/or VCAM-1. Additionally, despite the expression of E-selectin and VCAM-1 in the kidney microvascular compartments, neutrophil infiltration here was relatively limited compared to that of the lung, which suggests different mechanisms of neutrophil recruitment regulation in these two organs.

**EC heterogeneity goes beyond microvascular-specific attributes: EC inter- and intra-vascular heterogenous response to LPS is driven by the regulation of distinct receptor systems.**

In chapter 4, we found that the kidney of LPS-exposed mice expressed different extents of E-selectin and VCAM-1, between and within microvascular compartments. This finding was recapitulated by the identification of LPS-activated
EC subpopulations *in vitro* that either expressed E-selectin and/or VCAM-1, or none of these two molecules. The subpopulation that was devoid of E-selectin and VCAM-1 expression remained quiescent despite being exposed to LPS exposure. The LPS-activated subpopulations that either expressed E-selectin and/or VCAM-1 were shown to mediate inflammatory signal transduction via TLR4 and/or RIG-I and were activated via NF-κB and/or p38 MAPK. These findings suggest that *in vitro* LPS induces different extents of inflammatory molecules expression by activating different molecular regulation.

**Tyrosine kinases are important components in LPS-mediated inflammatory signal transduction that can be pharmacologically targeted to attenuate endothelial inflammatory activation.**

In chapter 6, we showed *in vitro* that TK are crucial components of the LPS signaling machinery in mediating endothelial inflammatory activation. Among these TK, focal adhesion kinase 1 (FAK1) and anaplastic lymphoma kinase (ALK) can be pharmacologically targeted to attenuate LPS-induced endothelial inflammatory activation. Only inhibition of FAK1 after LPS activation has started was capable of attenuating the inflammatory activation of HUVEC.

**FUTURE PERSPECTIVES**

**Non-replaceable role of *in vivo* studies in sepsis research**

In investigating the role of EC in a condition as complex as sepsis, *in vitro* approaches oversimplify the real situation of sepsis [5]. In sepsis, multiple cell types are affected, and these activated cells cross-talk to augment host inflammatory response. EC are capable of responding directly to sepsis-associated pathogens via TLR, NOD, and RIG receptors [6,7], but EC inflammatory responses are to some extent also dependent on leukocyte-derived humoral components [8] and require physical interaction with the leukocytes, platelets [9,10], and various other organ-specific supporting cells [11,12] to contribute to sepsis pathophysiology. These complex interactions are influenced by the unique landscape of organs [13,14], and therefore cannot be fully mimicked *in vitro* and can only be studied in animal models or in human-derived tissues.
To understand endothelial responses in specific organs, the responses of EC native to these organs should be exclusively studied. The organ-derived microvascular EC in vitro retain certain characteristics of in vivo EC, but the majority of the characteristics of these cells drift once removed from their native environments and cultivated for cell studies [15]. EC are known to have a distinct organ- [13,16] and vascular bed-specific [15,17] molecular identities in health and diseased states. Transcriptomic studies of lung EC for example have shown the presence of genes that are typically expressed by the parenchymal tissues of the lung, such as Surfactant Protein C (Spc) and Mucin1 (Muc1), which suggest that unique characteristics of EC are dependent on their microenvironment [13]. Next to this study, a recent single-cell transcriptomic analysis revealed a distinct profile of tissue-specific markers of the capillaries, which suggested plasticity of these capillaries in adapting to their tissue microenvironment [14]. Taken together, these studies imply that the dynamic interaction between EC and the cellular landscapes of organs in which these EC reside should be understood, to develop therapies targeting specific individual microvascular beds affected by sepsis. This can only be achieved by implementation of in vivo studies using appropriate models into sepsis research.

**Optimization of translational studies**

The use of animals in modelling sepsis is controversial [18], as responses seen in animals do not fully recapitulate the real sepsis scenario in human [18,19] and treatments given to ICU patients cannot be fully mimicked in rodents. As much as it is important to use animal models in understanding endothelial heterogenous response in sepsis, no animal model is perfect for this purpose. Therefore, the findings obtained from animal studies should also be complemented by supporting studies, preferably using tissues of human origin. An ex vivo method using precision-cut organ slices has been developed to study the development of diseases such as fibrosis [20], and to assess drug effects [21]. The advantage of using this approach is that any changes induced experimentally will affect the cells which are surrounded by local supportive structures [22], which mimic, in part, the native topography of human tissue. In the context of sepsis research, future studies can be dedicated to optimizing ex vivo methods using precision-cut organ slices from human organ biopsies. This translational model can be used to study the
consequence of pharmacological inhibition of tyrosine kinases [21] such as FAK1 on endothelial inflammatory response in various microvascular beds in organ slices exposed to sepsis inflammatory stimuli.

**Characterizing endothelial heterogeneity in sepsis at organ and microvascular bed levels**

In this thesis, I showed that the neutrophil infiltration in CLP occurred more extensively in the lung compared to the kidney. The microvascular EC of the kidney and lung responded differently to CLP sepsis conditions, which suggests that regulation of leukocyte infiltration occurs at organ and microvascular bed level. These functional differences could be attributed to molecular diversities of the microvascular beds activated by CLP-induced sepsis. It is therefore interesting to investigate differences in the expression levels of endothelial genes from different organs and microvascular compartments in the kidney [15] and lung [13] and assess the changes in the expression of these molecules in a defined time course of CLP sepsis. The information obtained will provide comprehensive insights into molecular underpinnings and regulatory pathways underlying endothelial heterogeneity between microvascular compartments of organs in sepsis.

The comparison of the profile of molecule expression between microvascular beds of the organ can be done by laser microdissection (LMD) technique. In our research group, we have recently applied LMD to dissect microvascular compartments of kidney [23] and lung EC (M. Luxen, ongoing project) from their native environments. These microdissected EC can be subjected to transcriptomic and proteomic studies to obtain in a high-throughput manner profiles of genes and proteins expressed in different microvascular beds of the organs under study. Such studies will hopefully allow us to better understand the molecular basis that is unique to specific microvascular compartments in responding to sepsis.

Sepsis pathophysiology is not limited to leukocyte recruitment, but also includes impaired vascular permeability and dysregulated coagulation, both regulated by EC. In this thesis I did not look into molecules that control endothelial barrier and coagulation, which are processes that were also shown to be differentially regulated by EC per organ during inflammatory conditions [5,24]. In sepsis, endothelial barrier-regulating molecules were controlled to different extents
in response to LPS in the kidney and lung, corroborating the observation of diverging oedema patterns in the kidney and lung in sepsis patients [5]. Additionally, van Willebrand factor, a pro-coagulant molecule produced, stored, and released by EC when activated, in the lung of mice induced by hypoxia was regulated via Sp1 and YY1 protein, while in the heart, it was GATA6-regulated [4]. Heterogeneity in transcription factors in the capillaries in healthy and diseased mice was described in the kidney [25] and liver [26]. As CLP is a ‘gold standard’ animal model in sepsis research, it is interesting to investigate whether the transcription factors controlling endothelial barrier and coagulation are also differentially regulated at the microvascular compartment level in kidney and lung of mice affected by sepsis-induced CLP.

Characterizing endothelial heterogeneous response to LPS at cellular level

In chapter 4 of this thesis, we investigated the receptor and intracellular signaling system that regulate differential expression of E-selectin and VCAM-1 in EC in vitro. We found that this differential expression is attributed to distinct activation of TLR4/RIG-I receptors and NF-κB/p38 MAPK signaling pathways, which underscores the role of pattern recognition receptors and transcription factors in the regulation of endothelial heterogeneous response. Transcription factors were previously described to be differentially expressed in vitro in activated EC from different origin [16]. Follow up studies would be to identify pattern recognition receptors, transcription factors, and/or other regulatory molecules that may be distinctly regulated in LPS-activated EC subpopulations studied, as discussed in chapter 5. As single-cell transcription factor network analysis has been proven useful to predict different transcriptional networks of individual EC [14], it is interesting to pursue single-cell transcriptomic analysis of the kidney and lung of control and LPS-exposed mice to investigate the genes that drives heterogeneous phenotype in individual EC within organs.

Tyrosine kinases as druggable targets in sepsis

In chapter 6 of this thesis, we showed that pharmacologically inhibiting TK can be a strategy to reduce inflammatory activation in EC. In our study, we showed that
inhibition of FAK1 with drug FAK14 is able to attenuate LPS-mediated inflammatory activation of EC \textit{in vitro}. However, FAK1 is known to be important in integrin-mediated signal transduction, which is crucial in cellular signal transduction of both healthy and diseased cells [27]. It is interesting to investigate the consequence of FAK1 inhibition to microvascular beds of organs affected by CLP. The next thing to do is to investigate whether FAK1 inhibition can attenuate \textit{in vivo} activation of EC without negatively affecting any of the microvascular compartments of organs affected by sepsis.

Summarizing, I showed that kidney and lung were susceptible to CLP-induced sepsis inflammation, and that the EC from both organs were activated by sepsis in a microvascular bed-specific manner. Moreover, the extent of leukocyte infiltration differed per organ, and per microvascular-bed compartments of both organs. I furthermore showed that differential expression of E-selectin and VCAM-1 in LPS-exposed EC \textit{in vitro} was regulated by distinct TLR4/RIG-I receptor and NF-κB/p38 MAPK regulatory mechanisms. Additionally, in this thesis, FAK1 and ALK were shown to be important mediators of LPS-regulated EC inflammatory activation, and pharmacological inhibition targeting these two kinases attenuated this activation \textit{in vitro}. I here describe future studies that can be pursued to further understand endothelial heterogeneity in sepsis. Unravelling the molecular regulation of endothelial heterogeneity at organ, microvascular bed, and cellular levels will assist in development of therapies targeting specific microvascular beds affected by sepsis.
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