Therapeutic interventions in shock
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Chapter 6

Summary, Conclusions and Future Perspectives
Chapter 6

I. Summary

Shock initiated by hemorrhage and sepsis is frequently encountered in the intensive care unit. One of the main complications of shock, multiple organ dysfunction syndrome (MODS), remains a major determinant of morbidity and mortality in patients in the intensive care units. Due to the complex mechanisms underlying the pathogenesis of shock, the treatment of this condition is still a big challenge and new pharmacological strategies are urgently needed.

The vascular endothelium has a high sensing capacity and can swiftly respond to alterations in the local extracellular environment as occur under both physiological and pathophysiological conditions [1]. Microvascular endothelial cells play a crucial role in the pathogenesis of MODS in critical ill patients through sensing microcirculatory alterations and diminished blood pressure as well as regulating vascular permeability and leukocyte recruitment/transmigration into underlying tissue, leading to tissue injury and organ damage (as described in Chapter 1). Endothelial cells constantly face mechanical forces associated with the local blood flow, and they can sense alterations in these mechanical forces and transform them into biochemical signals, modulating their morphology and function [2]. Microvascular endothelium is highly activated to a pro-inflammatory state during shock-induced by either hemorrhage or sepsis - through the expression of cytokines, chemokines, and adhesion molecules [1, 3]. In this thesis, we investigated potential therapeutic strategies for the treatment of shock associated changes in microvascular endothelial behavior in hemorrhage and sepsis. Our aims were to understand the role of microvascular flow alterations in regulating endothelial behavior and test the effects of different therapeutic interventions on the inflammatory activation status of microvascular endothelial cells created by flow alterations and cytokines both in vitro and in vivo.

Shock is associated with a decrease and loss of intravascular blood flow. In Chapter 2, we investigated the role of flow cessation and the later recovery of flow as occur in shock and resuscitation in regulating endothelial behavior by dissecting the changes of flow from the other in vivo co-existing factors using an in vitro flow system [4]. We demonstrated that flow alterations per se contribute to endothelial pro-inflammatory
activation and that these alterations can interact with pro-inflammatory factors that co-exist in vivo during shock such as TNFα. The abrupt reflow related enhancement of cytokine-induced endothelial pro-inflammatory activation supports the concept that sudden regain of perfusion during resuscitation has an aggravating effect on endothelial pro-inflammatory activation, which may play a significant role in vascular dysfunction and consequent organ injury after recovery from hemorrhagic shock. This study implies that by improved resuscitation strategies and/or by pharmacological interference with pro-inflammatory signaling cascades during resuscitation following circulatory shock, the inflammatory responses of the microvasculature and associated organ dysfunction may be dampened.

The expression of genes is partly regulated by posttranscriptional modifications, including histone (de)acetylation. In acute injuries, the homeostasis of histone (de)acetylation is disturbed in favor of increased activity of HDACs. Furthermore, IkappaB kinase (IKK)/NF-κB signaling plays a crucial role in regulating endothelial inflammatory activation. Based on the effects of flow alterations on endothelial cells in Chapter 2, we next studied flow alterations-associated molecular changes, i.e., histone acetylation and NF-κB activation, as well as the effects of pharmacological interventions at the level of these changes, on endothelial responses in the absence and presence of cytokine stimulation. In Chapter 3, we demonstrated that histone acetylation in steady flow-preadapted endothelial cells was significantly disturbed during the period of flow cessation as well as during subsequent acute reflow, showing decreased level of histone acetylation, which suggested a potential beneficial effect of histone deacetylase inhibitor valproic acid (VPA) on these processes. VPA treatment before the installment of 8h flow cessation significantly attenuated loss of flow-induced endothelial pro-inflammatory responses, both in the absence and presence of pro-inflammatory cytokine TNFα. We furthermore showed that although the expression of IκB was not affected during loss of shear stress, pre-incubation with IKK inhibitor BAY11-7082 effectively inhibited the upregulation of pro-inflammatory molecules induced by flow cessation both in the absence and presence of TNFα. These results indicate that pharmacological intervention at the level of HDAC respectively NF-κB activity may represent a potential therapeutic approach for the
Chapter 6

treatment of shock induced endothelial pro-inflammatory activation.

In Chapter 4, we next investigated the effects of VPA and BAY11-7082 administration to mice on microvascular endothelial responses and leukocyte adhesion in tissues during HS/resuscitation. In a model of pressure-controlled HS, the blood pressure was maintained at a mean arterial pressure (MAP) of 30 mmHg for 90 minutes, after which subgroups of mice were resuscitated with 4% human albumin as such, or supplemented with HDAC inhibitor VPA respectively IKK inhibitor BAY11-7082. Mice were sacrificed at 1 hour or 4 hours after resuscitation was completed. An acute induction of both systemic and local inflammation occurred both before and after resuscitation following HS. The administration of either HDAC inhibitor VPA or IKK inhibitor BAY11-7082 in the resuscitation fluid attenuated the pro-inflammatory activation of the microvasculature in kidney, lungs, and liver. The reduced protein expression of adhesion molecules was paralleled by diminished influx of polymorphonuclear leukocytes in kidney and liver after HS/resuscitation. The anti-inflammatory effects of VPA and BAY11-7082 administration in the resuscitation phase indicated that HDACs and IKK/NF-κB signaling are important targets for therapeutic intervention in the treatment of HS/resuscitation patients that warrant follow up studies.

As described in Chapter 1, the clinical hallmarks of shock, hypotension, vascular leakage, and leukocyte recruitment-mediated inflammation, are regulated at the level of endothelial cells. Endothelial cells are both active participants and mediators in the pro-inflammatory process. Thus endothelial-targeted delivery of drugs presents an interesting anti-inflammatory intervention that may also be able to assist in creating a better understanding of the role of endothelial cells in shock related inflammatory processes. Dexamethasone is a glucocorticosteroid which has pleiotropic anti-inflammatory and immunosuppressive effects. Local delivery of dexamethasone can be instrumental to counteract its adverse effects while maintaining its desired effects on the target cells of interest. In Chapter 5, we thus formulated dexamethasone into SAINT-O-Somes modified with antibodies against adhesion molecule VCAM-1, one of the molecular determinants expressed on the lumen surface of inflamed endothelial cells. To evaluate the targeting specificity and efficacy of dexamethasone loaded anti-VCAM-1 (Ab\textsubscript{VCAM-1}) SAINT-O-Somes,
Summary, Conclusions and Future Perspectives

An LPS “double hit” induced mouse model of sepsis was applied, in which animals were challenged with two consecutive injections of LPS, and $\text{Ab}_{\text{VCAM-1}}$ dexamethasone SAINT-O-Somes were administered in between. We showed that $\text{Ab}_{\text{VCAM-1}}$ dexamethasone SAINT-O-Somes were specifically delivered into LPS activated endothelial cells in the microvasculature in kidney, lungs, and liver. Moreover, compared to the administration of free dexamethasone and dexamethasone loaded conventional liposomes, $\text{Ab}_{\text{VCAM-1}}$ dexamethasone SAINT-O-Somes were capable of decreasing the expression of several pro-inflammatory genes in kidney and liver of mice challenged with two times of LPS. This work reveals that endothelial cell targeted delivery of dexamethasone by anti-VCAM-1 SAINT-O-Somes represents a potential therapeutic approach to interfere with sepsis associated endothelial activation. The real therapeutic impacts of this strategy in more clinical relevant model of sepsis need to be further investigated.

II. Conclusions

NF-κB signaling could be a potential target for therapeutic intervention of microcirculation alteration-associated endothelial inflammation. The subsequent in vivo study on pharmacological intervention during resuscitation after hemorrhagic shock demonstrated the anti-inflammatory effects on microvascular endothelial cells of HDAC activity inhibition and IKK/NF-κB signaling blockade, providing a lead for future therapeutic targeting of these two mechanisms in clinical trials. Furthermore, the administration of dexamethasone containing anti-VCAM-1 SAINT-O-Somes in a mouse model of LPS “double hit” induced endotoxemia achieved specific drug delivery to LPS activated endothelium and resulted in anti-inflammatory effects in kidney and liver. Future studies need to be performed with more clinical relevant animal models and with immunoliposomes loaded with more effective drugs to elucidate their real therapeutic beneficial in the treatment of sepsis.

The active involvement of microvascular endothelial cells in the pathogenesis of shock and their responses to drug interventions suggest that these cells deserve more attention in the development of therapeutic strategies for the treatment of shock induced organ failure.
Chapter 6

III. Future Perspectives

Shock is associated with a high morbidity and mortality, and prompt recognition and medical management are essential to correct the cause of shock and stabilize the associated physiological disturbances [5]. Effective therapeutic strategies for the treatment of shock patients remain a challenging conundrum in clinical care. Microvascular endothelium, being highly sensitive to its extracellular dynamic environment, plays an important role in the pathophysiology of (micro) circulatory shock and its deranged function is associated with multiple organ dysfunction syndrome (MODS) following a systemic inflammatory response. Therefore, microvascular endothelial cells represent an attractive target for the development of novel therapies to reverse the inflammatory response in shock. A number of interesting issues are considered relevant for future research and will be discussed below.

Engineering a more physiological environment to study endothelial behavior in vitro

Current cell culture conditions for drug development fail to simulate the body’s cellular microenvironment and instead lead to loss of function in primary cell culture. To be specific, while endothelial cells in vivo are subjected and presumably adapted to natural levels of shear stress generated by blood flowing over their luminal surfaces, most studies have been performed with endothelial cells under static conditions. It is important to note that endothelial cells tend to adapt themselves to different microenvironments [6, 7]. The expression of certain genes may drift significantly in endothelial cells after being isolated from their organ microenvironment and cultured under static conditions for a few days [8]. This drift indicates that environmental factors such as hemodynamic forces play crucial roles in controlling endothelial functions in vivo. Even though over the past two decades in vitro studies on the influence of shear stress on vascular endothelial cell function have provided considerable insights [9], it is still urgently needed to engineer a more physiological environment for cell cultures to achieve in vitro results that better predict in vivo behavior.

Laminar shear stress exposure for a prolonged period of time will render endothelial cells in a more physiological state, facilitating the translation of in vitro study to in vivo conditions. Vascular endothelium
responds to alterations in the level of blood flow, which may contribute to the pathophysiology of diseases associated with an increase or decrease in tissue perfusion. A period of laminar shear stress adaptation is necessary to elicit a response of endothelial cells to changes in shear stress level [10], therefore flow adapted endothelial cells have been used in our studies to determine the consequences and molecular mechanisms of flow disturbances as occur in, e.g., sepsis and hemorrhagic shock. Our in vitro work provided evidence that removal of flow and subsequent application of acute reflow are capable of activating flow-preconditioned endothelial cells [4], indicating a contributing effect of blood flow alterations to shock associated pathogenesis. Abrupt flow cessation simulated ischemia in vitro was demonstrated to induce the generation of reactive oxygen species (ROS) in flow adapted pulmonary endothelial cells [11, 12], which recapitulated the events as occurred in an ex vivo lung model due to stopped perfusion [13, 14]. Collectively, these findings have helped us to understand loss of flow associated endothelial responses. What should be kept in mind is that vascular endothelial cells are highly heterogeneous in response to extracellular stimuli [15], thus it is possible that endothelial cells from different vascular segments adapt in a different way to local blood flow and will differently respond to mechanical stress depending on their position in the vascular system. Further studies are required to elucidate these differences to gain a full understanding of the role of this cellular milieu in determining endothelial function, and the potential of mimicking parts of it in an in vitro context.

To improve the in vitro context, co-culture of aortic endothelial cells with smooth muscle cells under shear stress has been used to be representative of arteries in the study of the pathogenesis of atherosclerosis [16]. Laminar shear stress induced increase of anti-inflammatory microRNA-146a expression in aortic endothelial cells co-cultured with smooth muscle cells declined due to flow stagnation, which was next validated in vivo in neointima formation in injured arteries [17]. In addition, it has been reported that the presence of pericytes surrounding perfused microvasculature has a dramatic effect on vessel functions such as vasoconstriction and permeability, and that human lung microvasculature can be mimicked by the co-culture of primary human lung microvascular endothelial cells and pericytes under perfusion [18]. This novel co-culture based vascular models
allows building tissue and disease specific microvasculature which are more suitable for tissue- or disease-specific drug testing *in vitro*.

Besides flow, a variety of other important factors such as the diffusion of cell secretions as well as the concentration gradients of supplied nutrient and oxygen cannot be controlled under standard static conditions *in vitro*, which will lead to non-physiological cell environment and limit cellular development [19]. Recently developed options to mimic *in vivo* controlled microenvironment during *in vitro* culture include a multi-organ chip (MOC) microfluidic platform. It allows co-culture of multi-tissues, including primary cells, tissue equivalent, and tissue biopsies, under dynamic flow conditions over prolonged periods [20]. This MOC system enables prediction of interactions between co-cultured cells and tissues and better evaluation of drugs under development for their preclinical performance *in vitro*.

**Microcirculation as a therapeutic target in the treatment of shock**

Homeostasis of the microcirculation becomes severely altered during critical illness. Microcirculatory dysfunction has been considered to initiate a sequence of events that leads to MODS and represent a key factor for poor outcome [21]. We have investigated flow alterations-associated endothelial pro-inflammatory activation [4] and the involvement of histone deacetylation and NF-κB activation in these processes in our *in vitro* studies. Although not the scope of this thesis, the microcirculation as a therapeutic target in the treatment of shock needs further attention.

Observations of microcirculatory alterations in animals and patients have helped us to better understand the pathophysiology of shock-associated multiple organ failure [22]. Moreover, clinical research has identified various therapeutic approaches that have effectively modified the microcirculation. The administration of low-dose hydrocortisone has been reported to slightly improve sublingual microvascular perfusion in septic shock [23], although it did not improve survival or reverse shock at these doses [24]. Furthermore, the use of vasodilators in shock may increase the local blood flow in specific microvascular regions, thereby (re)perfusing hypoxic regions and improve organ functions [25]. In addition, besides effectively increasing arterial pressure, vasopressors such as norepinephrine could improve perfused capillary density in patients with baseline sublingual perfusion [26]. However, the effects
of vasodilators and vasopressors on different microvascular beds in organs are largely unknown. It is of note that the clinical effectiveness of these approaches may be affected by the considerable variation in the response of patients. It needs to be determined whether enhancing the microcirculation will contribute to improving the outcome of individual critically ill patients and include the amelioration of systemic inflammatory response, vascular permeability, and organ function.

In the clinic, a patient’s response to resuscitation following circulatory shock can be monitored by means of careful clinical evaluation and blood lactate measurements [5]. Bedside techniques are still required to allow the evaluation of the microcirculation, including determination of tissue perfusion and oxygenation [5]. Recently, the so called sidestream dark-field imaging technique has been applied to visualize and assess perioperative perfusion in the sublingual microcirculation during cardiopulmonary bypass [27], septic shock [28], and traumatic hemorrhagic shock [29]. The therapeutic endpoints of microcirculation for resuscitation have not been defined yet so that monitoring of the microcirculation is still not ready for routine clinical practice. Further preclinical studies with microcirculation improvement-controlled resuscitation strategies will provide more insight in the consequences of improved microcirculation on shock-associated responses such as the systemic and local production of pro-inflammatory cytokines, oxygen delivery, and tissue damage.

Improving preclinical animal models for sepsis study

Due to the limited availability of effective therapeutic strategies for sepsis, it is critically needed to improve our understanding of the pathophysiology of sepsis and to disclose novel targets for therapeutic interventions of critically ill patients. Despite substantial efforts, numerous therapeutic strategies that have had promising effects in animal models of sepsis have failed to demonstrate clinical efficacy in clinical trials [30], which may be attributed to the complexity of the causes and processes of sepsis in the patient and the limited clinical relevance of present animal models [30, 31].

Animal models are crucial in translational research. A clinical relevant animal model of sepsis should be capable of reproducing the complexity of human sepsis, including the pace and severity, and hyper-inflammatory to immunosuppressive stages [32]. An LPS “double hit”
mouse model was applied in our study to simulate the host response to more episodes of infection and to extend the severity and duration of the response as often occurs in sepsis patients. We found that the consecutive injection of LPS did not improve the model compared to traditional single injection of LPS. This may be explained by the doses of LPS used, time interval between the two injections, or LPS injection induced endotoxin tolerance of immune cells which is characterized by reduced sensitivity to endotoxin re-challenge [33]. Although endotoxin tolerance was initially thought to be a beneficial adaptive response of the host, it may be associated with immune dysregulation and the risk of secondary infections, which complicates the management of patients with severe sepsis and organ dysfunction [33]. For example, the initial endotoxin priming in the Shwartzman phenomenon is essential to induce lethality [34]. Low dose endotoxin priming (5 µg LPS, intradermal injection) induced endotoxin tolerance 24h prior to an LPS challenge (300 µg, intravenous injection) is accountable for the development of microvascular thrombosis in lung and liver via the Shwartzman reaction and for subsequent organ failure following sepsis [35]. The dose of the priming endotoxin and the timing interval until the secondary endotoxin challenge are critical determinants for the host responsiveness [33], and should be further studied to establish a more clinically relevant sepsis model.

Furthermore, sepsis is often encountered in the presence of other comorbidities such as other initial injuries including burn and traumatic hemorrhage [36], and other pre-existing diseases, e.g., chronic kidney disease [37], which will influence the host response to sepsis and should be taken into account in therapy. Therefore, complex animal models of human sepsis would be more suitable for testing therapeutics compared to simple animal models as they may more accurately predict drug responsiveness in humans. To this end, a two-stage mouse model consisting of pre-existing injuries or disease and subsequent sepsis may be more helpful to understand the pathophysiological mechanisms of sepsis patients and to better characterize the potential of therapeutic strategies. In the study by Gierer et al [38], pre-existing traumatic injury exaggerated the inflammatory response to subsequent LPS challenge. In addition, the incidence of sepsis increases with age, and elderly patients are more prone to sepsis [39, 40]. Moreover, from a clinical perspective, treatments comparable to the standard supportive therapy
Summary, Conclusions and Future Perspectives

for ICU patients should be included in animal studies to mimic the clinical conditions more optimally and to test the underlying benefit of new therapeutic interventions. By incorporating these variables in one animal model, we can distinguish the amplifying factors in sepsis and discover suitable therapeutic targets for different subpopulations of sepsis patients based on their distinct pathophysiological mechanisms. Analysis of (post-mortem) human biopsy samples is also an important tool to verify and extrapolate animal studies to human trials, complementing human plasma analysis to provide relevant human data of sepsis [41].

**Endothelial directed therapeutic strategies for the treatment of shock**

For the treatment of shock either induced by hemorrhage or sepsis, resuscitation is the first-line therapy, but it might not improve the outcome of shock patients although restoring systemic perfusion [42]. Therefore, to move forward, investigations have focused on potent pharmacological agents to add to resuscitation regimens to minimize cell and tissue injury and maintain or recreate physiological homeostasis [43]. The vascular endothelium is widely recognized as a critical component in maintaining physiological homeostasis and regulating pathological responses to injury. Thus, endothelial cells are important targets for therapeutic interventions in the treatment of shock.

In our study, the administration of histone deacetylase (HDAC) inhibitor valproic acid (VPA) respectively IKK inhibitor BAY11-7082 as an adjunct to resuscitation after hemorrhagic shock showed anti-inflammatory effects in the microvasculature, suggesting a possible role for these drugs in the treatment of shock associated multiple organ failure. However, systemic intervention of HDAC activity and IKK/NF-κB signaling likely will have side effects as both enzymes are of importance for maintaining cellular functions. First, the broad effects of VPA as a pan HDAC inhibitor on different cell types may be related to its reported side effects including coagulopathies, aplastic anemia, and hepatotoxicity [44]. The water-soluble property of the drug allows for formulation of VPA into immunoliposomes that can be modified to achieve endothelial specific delivery, thereby counteracting the underlying toxicity of systemic administration. However, we found that VPA leaked out rapidly after being formulated into liposomes (data not shown). This may be due to the amphiphilic structure of VPA with the
hydrophobic part buried in the phospholipid bilayer, which destabilizes the liposomal membrane. To address this problem, chemical strategies can be used to modify the hydrophobic part into a more hydrophilic part, thereby increasing the retention time of incorporated drug in the liposomal core and the stability of drug loaded liposomes. It is of interest in future studies to evaluate the efficacy of chemically modified VPA loaded endothelial targeted immunoliposomes in animal models of sepsis and hemorrhagic shock, to create a better understanding of the consequences of endothelial specific delivery of VPA for microvascular performance in these diseases.

Furthermore, general blockade of NF-κB signaling is capable of ameliorating the pathological profiles of several inflammatory diseases such as rheumatoid arthritis [45], hemorrhagic shock [46], and sepsis [47]. However, NF-κB activation is also involved in the resolution of inflammation and thus may differently affect inflammatory processes depending on the cell type in which it is active and the development stage of the disease [48]. Moreover, NF-κB is of importance in maintaining cell survival and various organ functions, and general blockade of NF-κB signaling by small molecule inhibitors is associated with serious side effects [49]. Therapeutic strategies targeting NF-κB signaling specifically in endothelial cells help to determine the effects of endothelial specific inhibition of NF-κB signaling on tissue damage in these inflammatory diseases. Using a recombinant “sneaking ligand construct” (SLC)-1 which contains an E-selectin targeting domain, a Pseudomonas exotoxin A translocation domain, and a NF-κB essential modifier-binding effector domain, specific inhibition of NF-κB activation in activated endothelial cells in arthritis was achieved [50]. This strategy revealed that the inflammatory processes were critically dependent on endothelial NF-κB activation, thereby providing a proof of concept for the therapeutic application of endothelial cell specific NF-κB blockade in this immune disease [50].

In addition, new molecular drugs such as small interfering RNAs (siRNAs) offer high molecular specificity and potent silencing of disease associated genes [51]. Due to the physicochemical properties, siRNAs need to be formulated into a delivery system in order to modify their pharmacokinetic profiles, reduce off-target toxicity, and to improve their therapeutic efficacy [52]. The cellular and molecular concepts of targeted delivery of siRNAs to activated endothelial cells have been reviewed by
Summary, Conclusions and Future Perspectives

Kowalski et al [53]. NF-κB subunit RelA siRNA loaded SAINT-O-Somes conjugated with antibodies against VCAM-1 expressed on activated endothelial cells have been demonstrated to be capable of attenuating endothelial inflammatory response in the renal microvasculature towards LPS induced endotoxemia through silencing the expression of RelA in endothelial cells [54].

Although potential benefits of endothelial targeted therapeutics have been demonstrated, it cannot be ignored that multiple cell types are participants and/or regulators in the complex processes of systemic inflammation and tissue injury [55]. In our in vivo study of hemorrhagic shock, we observed that hemorrhagic shock and subsequent resuscitation resulted in the activation of neutrophils and monocytes in the circulation (Figure 1), which corroborated other previous studies [56, 57]. Furthermore, the recruitment and transmigration of leukocytes

**Figure 1. Activation status of neutrophils and monocytes in systemic circulation of mice after hemorrhagic shock and resuscitation.**

Neutrophils (A) and monocytes (B) from fresh whole blood were stained for Ly6G, CD11b, CD62L, and CD80. The percentage of CD11b positive and CD62L negative neutrophils relative to the total number of Ly6G stained neutrophils as well as the percentage of CD80 positive monocytes relative to total number of monocytes was quantified. Data represent mean ± SD of 4 mice per group. *, P<0.05, 1h respectively 4h after resuscitation vs. control. R, resuscitation.
into underlying tissues plays a pivotal role in tissue injury [58]. Therefore, effects of pharmacological interventions on leukocytes may significantly influence the outcomes of shock associated inflammation. It thus needs to be established what the real extent of effects on disease outcome is of endothelial cell targeted therapeutic interventions.

In summary, shock is often a fatal disease in the intensive care unit that involves complex pathophysiological processes. Early recognition and monitoring of risk factors as well as effective therapeutic modulation of the inflammatory immune response should be the aim of future therapy of shock. Preclinical in vitro and in vivo translational models are required to better understand the vascular consequences of MODS in critically ill patients based on which novel therapeutic options can be developed in future investigations.

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Summary, Conclusions and Future Perspectives


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Summary, Conclusions and Future Perspectives


Chapter 6


