In vivo endothelial cell and neutrophil responses to shock

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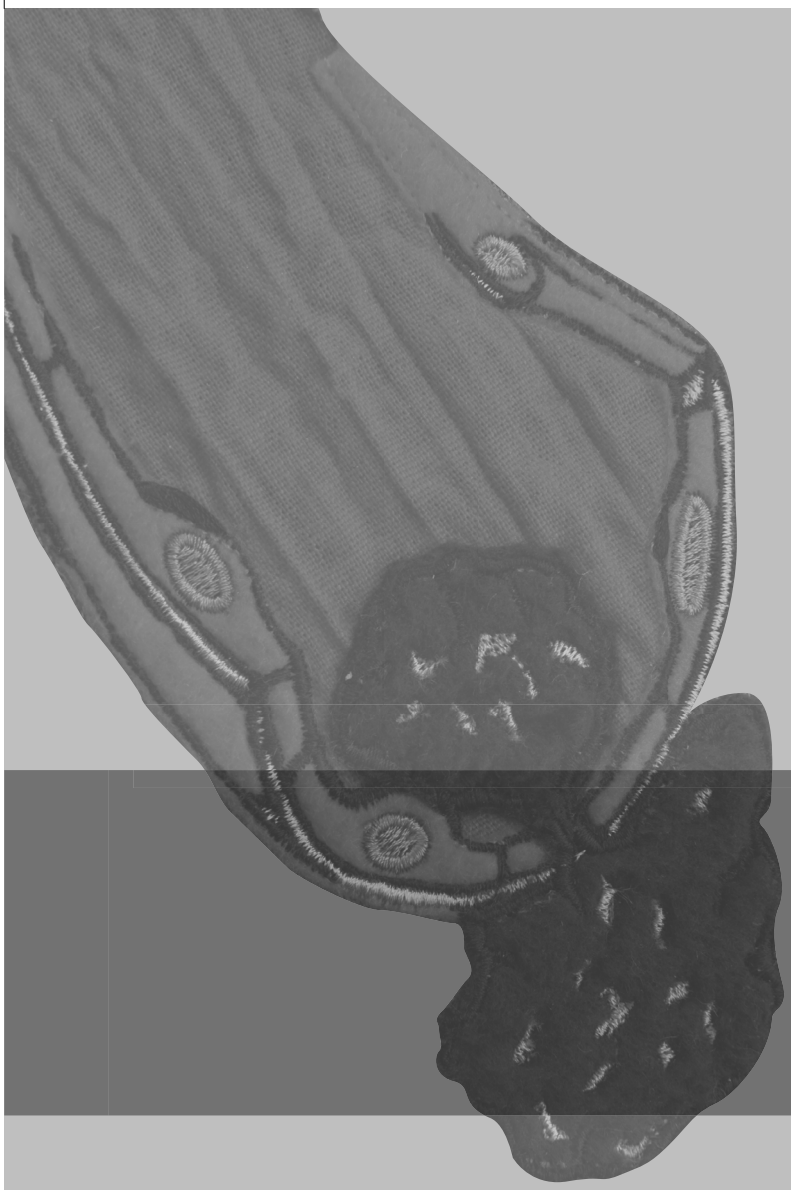

Chapter 1

Introduction

1.1 Introduction

1.1.1. Introduction

Multiple organ dysfunction syndrome (MODS), which is also called multi organ failure (MOF), is, despite advances in supportive care, still a major cause of late morbidity and mortality[1]. MODS is the result of a sustained systemic inflammatory response syndrome (SIRS). In sepsis this systemic activation of the inflammatory cascade is activated by an infectious pathogen. SIRS can also be the consequence of non-infectious provocateurs, such as hemorrhagic shock (HS), burns and trauma[2]. The endothelium is recognized as an important player in the pathogenesis of MOF via endothelial-polymorphonuclear cell (PMN) cross-talk. This cross-talk leads to migration of PMN, also called neutrophils into tissues, which is facilitated by adhesion molecules, chemokines and cytokines expressed by both cells types. Once in the tissue, PMN isolate and destroy infectious agents and trigger tissue repair. However, while fulfilling their function they can also cause innocent bystander injury leading to tissue injury and organ damage[3-5].

1.1.2. Inflammation

The inflammatory process is a response to an injurious stimulus. It can be evoked by a wide variety of insults including infections, hemorrhage, trauma, and burns. The first description of cardinal symptoms of inflammation with redness and swelling with heat and pain were reported by the Roman encyclopedist Celsus (ca 30 BC–50 AD) in *De Medicina as rubor et tumor cum calore et dolore.* Galen of Pergamon, the physician and surgeon of Roman emperor Marcus Aurelius, added a further sign of inflammation, the impaired function or *functio laesa*[6]. Although these signs of inflammation were used to describe local infection, they are also recognized in patients with systemic inflammation such as patients with hemorrhagic and septic shock. For example, nitric oxide (NO), an important gaseous cellular signalling molecule produced by the endothelium from L-arginine, oxygen and NADPH via nitric oxide synthase (NOS) enzymes, signals the surrounding smooth muscles to vasodilatation and thereby increases the calibers of arterioles and venules *(rubor)* [7]. As a result a decrease in systemic blood pressure occurs with compensatory tachycardia and an increased cardiac output. Furthermore, release of cytokines and prostaglandins cause a fever *(calor)* by elevating the set point of the thermoregulatory center in the hypothalamus [8]. Increase in permeability of the vascular endothelium causes loss of solutes and proteins to the extravascular space, known as an exudate, which leads to swelling and oedema formation *(tumor)*. Local increase in tissue turgor and the activation of the kinin cascade with the generation of particularly bradykinin and mediators released by leukocytes cause pain *(dolor)* via stimulation of nociceptors [9]. Finally, these processes could lead to organ failure *(functio laesa)* [10,11].

Inflammation is one of the first and essential responses of the immune system to pathogen or injury. To defend a body against pathogens or injury, our immune system consists of three lines of defense using several mechanisms. First we have mechanical, chemical and biological barriers that prevent the colonization of tissues by pathogens. Second, once infectious agents have penetrated tissues two further innate defense operations are activated, consisting of humoural actions such as the complement system, the coagulation system, lysozyme, and interferon and of cellular actions, such as neutrophils, macrophages, and natural killer cells [12].

The ability of the innate immune system to mount a quick inflammatory response is essential for the host to defend against environmental pathogens or injury. The third level of defense is the adaptive immune system, the delayed response to infection, which will here not be discussed further.

Components of the innate immune system are pre-existing defense mechanism, as they are able to mount rapid responses because of their activated or near-activated states. For this response it is of importance that the innate immune system recognizes pathogens and discriminates self versus non-self. Receptors recognizing molecules on pathogens are called pattern recognition receptors (PRRs), the molecules on pathogens are called pathogen associated molecular patterns (PAMPs). Not only pathogens but also endogenous ligands released from damaged or dead cells, the so-called damage associated molecular patterns (DAMPs) such as high mobility group box 1 (HMGB1), are recognized by PRR. It alerts the cell to danger and initiates the phagocytic process. Antigen presenting cells such as macrophages and dendritic cells play a critical role in this process as they express different classes of PRR. However, neutrophils, endothelial cells, lymphocytes, and epithelial cells also express PRR [13-15]. Not all PRR are located on the cell membrane, some PRR are located intracellular, and these PRR are able to detect pathogens in the cell cytoplasm such as *Shigella* [16]. Some PRR are secreted, and by this means they are able to enhance responses such as phagocytosis or activate the complement system. An example of a cell surface PRR is Toll like receptor (TLR)-4. Binding of lipopolysaccharide (LPS) to TLR-4, a PAMP, induces transcriptional activation, synthesis and secretion of pro-inflammatory cytokines and chemokines by macrophages and dendritic cells. Subsequent the adaptive immune system and endothelium are activated [17] (figure 1.1.).

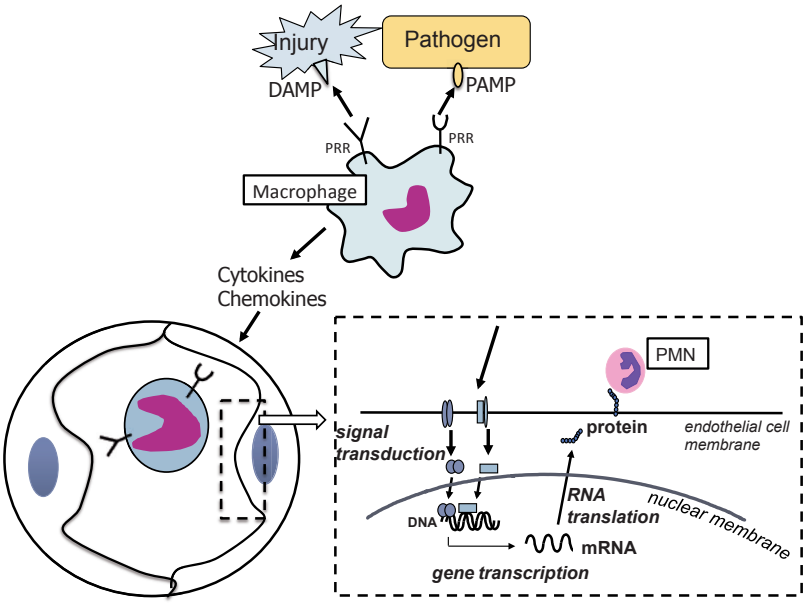

Figure 1.1: Activation of the endothelial cell via cytokines after recognition of patterns associated with pathogens or injury by the immune cell. Pattern recognition receptors (PRR) on the immune cell (for example macrophages, monocytes or dendritic cells) recognize the damage associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) that are associated with injury respectively pathogen. After signal transduction and gene transcription (not shown) cytokines are released from the immune cells. These cytokines lead to signal transduction in the endothelial cell (inset on the right). This is followed by transcription of pro-inflammatory genes in the endothelial nucleus. After transportation of mRNA to the endothelial cytoplasm the mRNA is translated into proteins including adhesion molecules, cytokines and chemokines which are expressed, respectively exocytosed by the luminal side of the endothelial membrane. This facilitates PMN influx into tissue.

Although the innate immune system is important to defend the host from potential invaders, this innate immune response may become exaggerated and sustained without apparent benefit and even with severe adverse consequences. While local responses to infection are beneficial to the host, the systemic responses could have more detrimental effects. Locally vasodilatation and altered vascular permeability increases the permeability of (mainly) postcapillary venules. This allows selective extravasation of plasma proteins and leukocytes (mainly neutrophils), which are normally restricted to the blood vessels, at the site of infection or injury. Locally activation of the blood coagulation cascade slows the hematogenous spread of infection. These processes help generate repair mechanism after localized injury. In contrast, in systemic activation these same processes lead to tissue oedema disrupting homeostasis, to systemic shock, disseminated intravascular coagulation and organ dysfunction [18].
The inflammatory cascade consists of pro-inflammatory and anti-inflammatory responses, the latter consisting of immunosuppressive mediators and loss of immune cells by apoptosis[19]. The condition characterized by systemic pro-inflammatory reactions is called the systemic inflammatory response syndrome (SIRS), while the presence of anti-inflammatory reactions is called *compensatory anti-inflammatory response syndrome* (CARS) [20]. Pro-inflammatory

cytokines, for example, are tumor necrosis factor (TNF-)α, Interleukin (IL-)1 β, IL-6, IL-8, IL-12, and platelet activating factor (PAF). Anti-inflammatory cytokines include IL-4, IL-10, and IL-13. A balance of both pro-inflammatory and anti-inflammatory cytokines is necessary to regulate inflammatory mechanisms and are thereby fundamental for homeostasis of tissue, organs and organisms [19,21]. There is evidence that immunosuppression as a consequence of the anti-inflammatory response also plays an important role in patients' outcome following sepsis or injury [22,23]. As mentioned above, release of cytokines following an insult affects the activation status of a variety of cells, including the endothelial cells, by receptor-mediated activation of intracellular signaling cascades resulting in changes in gene and protein expression.

1.1.3. The endothelium

Important cellular players in inflammation and resolution of inflammation are the endothelium and the neutrophil. After the explosion of endothelial cell research that began in the 1970s the endothelium is nowadays recognized as having a multifunctional nature and acts as an important player in the pathogenesis of MOF in critical ill patients.

In 1628, after the first description of blood circulation by William Harvey (1578-1657), a study by Marcello Malphigi (1628-1694) next described the physical separation between blood and tissues, which led to the concept of a network of vessels [24]. In the 1800s Friedrich Daniel von Recklinghausen (1833-1910) described that cells cover these vessels. The term endothelium was first introduced in 1865 by Wilhelm His (1831-1904), a Swiss anatomist, to distinguish it from the epithelium [25]. The endothelium is nowadays seen as a dynamic, multifunctional and heterogeneous structure [26,27]. It provides a cellular inner coating to all vessels in the blood circulatory system, and forms a structural barrier between the vascular space and the tissues. That endothelial cells are not inert but rather highly active was first shown by in electron microscopy studies of the vessel wall by George E. Palade (1912-2008) in 1953. He revealed the presence of characteristic organelles, including plasmalemmal vesicles (caveolae) and Weibel-Palade bodies [28] and by James L. Gowans in 1959 who showed interactions between lymphocytes and the endothelium of post-capillary venules [29].

Blood flows from the heart via the aorta and arteries into arterioles and next into capillaries. After tissue perfusion capillaries transform into venules where after they broaden and become veins. The endothelium has a wide array of physiological functions, which differ in time and place in the body. The endothelium acts as a semi-selective barrier between the vessel lumen and surrounding tissue, controlling the passage of nutrients and of white blood cells across the vascular wall. Other functions are its role in hemostasis, in the formation of new blood vessels via angiogenesis, and regulating the vasomotor tone via vasoconstriction and vasodilation of the surrounding layers of vascular smooth muscle cell and hence the control of blood pressure. An important role of the endothelium lies in inflammation, as endothelial cells in the smallest microvessels coordinate the recruitment of inflammatory cells to sites of tissue injury or infection. For this task, they produce cytokines, chemokines, adhesion molecules, and growth factors serving as communication signals.

The microcirculation consists of three main sections, i.c., arterioles, capillaries, and venules. The microenvironmental conditions and commands differ per organ and vascular organ bed and thus endothelial cells lining the blood circulatory system have different manifestations.

The arterioles are primarily responsible for delivery of blood to localized tissue areas and regulation of the rate of delivery via vasodilatation or vasoconstriction of the vessels. Arteriolar gap junctions appear to be important for conduction of vasomotor responses along the vessel. To achieve this they use electrical communication, via sympathetic nerve fibers, and chemical communication via gap junctions between the endothelial cell and the vascular smooth muscle cell [30,31]. The endothelium of capillaries and post capillary venules, which in contrast to arterioles have no muscle layer, forms a (semi-permeable) barrier and are important for selective transport of fluid and solutes, including plasma proteins such as albumin and immunoglobulins, between blood and tissue. The blood flow velocity is lowest in capillaries thus permitting more complete exchange of diffusible materials between interstitium and plasma [32]. The permeability of the endothelial capillaries differs between organs and organ beds because of heterogeneity in endothelial lining, depending on location[27]. A continuous endothelial lining can be fenestrated or non-fenestrated. A non-fenestrated lining for example, is present in skeletal muscle and skin, with tight junctions with numerous transport vesicles, and in the central nervous system, as part of the blood brain barrier, which is relatively impermeable with tight junctions with few vesicles. Only small molecules such as ions can diffuse through tight junctions. A fenestrated endothelial lining for example, is present in the renal glomerulus where small molecules and some proteins are able to diffuse through. Discontinuous lining for example are sinusoidal capillaries which are a special type of fenestrated capillaries. They are present in the liver and bone marrow. Permeability of these vessels is higher compared with those lined by fenestrated endothelial cells, due to the presence of large inter-cellular clefts and fewer tight junctions, which thus can accommodate passage of different serum proteins but also of red and white blood cells. Two cellular pathways have been identified in controlling endothelial barrier function, the transcellular pathway and the paracellular pathway. Under normal circumstances transvascular exchange is transcellular via transcytosis by vesicle-mediated transport of macromolecules through caveolae, vesiculo-vacuolar organelles and/or transcellular channels [33]. The paracellular pathway is induced during inflammation [34]. Second messengers such as thrombin, lipopolysaccharide (LPS), tumor necrosis factor (TNF) -α, vascular endothelial growth factor (VEGF) and platelet activating factor (PAF) increase the permeability. They initiate signaling pathways which lead to phosphorylation of junctional proteins and reorganisation of the actin-myosin apparatus eventually leading to disruption of the junctions and subsequent enabling transport of molecules and fluid into the interstitium [34]. The post-capillary venules are the side where paracellular but also transcellular transendothelial migration of inflammatory cells such as PMN predominantly takes place [11,35].

1.1.4. Endothelial-leukocyte cross-talk

The five main types of white blood cells are PMNs, eosinophils, basophils, lymphocytes, and monocytes. The PMNs are the most abundant type of white blood cells in humans, where they account for 40%–75% of all leukocytes, while in mice, this percentage is much lower (5%) [36]. Migration of PMNs to the site of injury is one of the fundamental components of the innate acute inflammatory response. In 1824 Henri Dutrochet (1776-1847) already reported that blood corpuscles escaped sideways out of the vessel wall and speculated about white blood

cell transmigration [37]. Elie Metchnikoff (1845-1916) suggested that 'phagocytes' might injure innocent bystander tissue as they fulfill their normal phagocytic function [38,39]. More recently it has become apparent that after migration into tissue, the release of cytokines, chemokines, growth factors, proteinases, and reactive oxygen species by PMNs plays an important role in organ dysfunction [3-5]. Furthermore, after migration into inflamed tissue, PMN release granule proteins, which direct monocytes and macrophages to the sites of inflammation and stimulate their ability to release cytokines and chemokines [40]. Endothelial cells play a pivotal role in facilitating the migration of PMNs into the peripheral tissues. In the majority of organs, PMN migration to a site of injury occurs in postcapillary venules and is mediated by the spatiotemporal expression and activation of adhesion molecules, chemokines, and cytokines by the vascular endothelium as well as by the neutrophils [27,41]. When transmigrated during inflammation, the lifespan of neutrophils, normally in blood 6 hours and in tissue 24-48 hours, can be extended for several days through inhibition of neutrophil apoptosis via endothelial derived factors [42].

Expression of pro-inflammatory adhesion molecules P-selectin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1) by the endothelial cells in the microvasculature are instrumental in the recruitment of PMNs into tissues. This recruitment cascade starts with tethering of the PMN, which is followed by rolling onto and then firm adhesion to the endothelium, where after migration into the tissue takes place. Each adhesion molecule has a specific role in this cascade. After activation of endothelial cells, P-selectin is secreted within minutes and mediates the initial tethering of free-flowing leukocytes and supports subsequent fast rolling interactions. In contrast, E-selectin supports rolling at slow velocities and thereby facilitates the transition from rolling to firm arrest. P-selectin is stored in Weibel–Palade bodies of endothelial cells and to a lesser extent in α-granules of platelets, while E-selectin, the counter ligand for sialyl LewisX on the leukocytes, is synthesized *de novo* and expressed on the endothelial cell surface following pro-inflammatory stimulation. In cooperation with selectin-mediated pathways there is chemokine-induced integrin-dependent neutrophil arrest[43]. Activation of leukocyte integrins, via for example endothelial chemokines[44], results in their increased binding to VCAM-1 and ICAM-1 on endothelial cells, which is firm enough to resist the continuous shear stress created by blood flowing through the vessels. Firm adhesion is followed by migration of the leukocytes out of the vasculature into tissue (figure 1.2.). ICAM-1 is constitutively expressed on endothelial cells but also on a variety of other cell types. Inflammatory mediators markedly augment ICAM-1 surface expression by the endothelium. Although VCAM-1 is expressed by some microvascular segments without concurrent proinflammatory activation, it is upregulated together with ICAM-1 in the course of acute inflammation induced by cytokines like IL-1 or TNFα that are e.g., released during different conditions of shock [45]. The cascade of events necessary for recruitment of PMN is not universally to all vascular beds. For example neutrophil recruitment in the lung and liver does not require E- or P-selectin [27].

After migration, PMN are able to phagocytose and kill invading pathogen or resolve injury. After the balance is shifted from proinflammatory to anti-inflammatory signals the neutrophil undergoes apoptosis, with the engulfment of apoptotic PMN by macrophages being a key event in the resolution of inflammation [46].

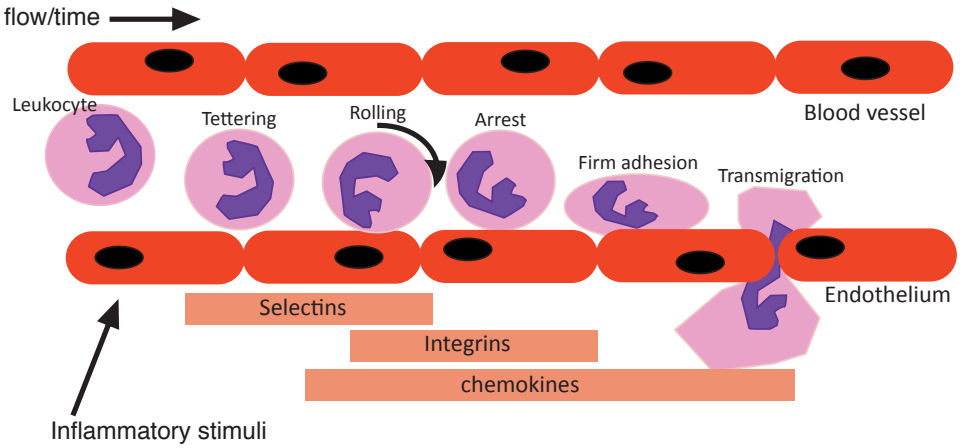

Figure 1. 2: Interactions between polymorphonuclear cell and endothelium.
Over time polymorphonuclear cells make contact with cytokine and chemokine induced activated endothelial cells. P-selectin mediates the initial tethering and E-selectin facilitates the transition from rolling to firm arrest. The integrins VCAM-1 and ICAM-1 are important for firm adhesion of the leukocyte to the endothelial cell whereafter the leukocyte transmigrates through the vessel wall and enters inflamed tissues. These processes mainly take place in the first part of the post capillary venules in organs and are subject to variation in molecular control.

1.1.5. Shock and inflammation

The survival of patients suffering from shock has increased as a result of advances in medical care, including improved resuscitation strategies, international guidelines and dedicated intensive care units. However, concomitant with this survival of shock, MODS became manifest [47,48]. Patients who develop multi-organ dysfunction syndrome following shock have a high morbidity and mortality [49]. The organs most affected in these patients are the lung and the kidney. Lung failure, the so called acute respiratory distress syndrome, is associated with lower short term mortality than AKI [50], although the long term mortality (5 years) is higher[1]. There is direct relationship between the number of organs failing and mortality [50]. Unfortunately, the pathophysiology of MODS following shock remains poorly understood and despite multiple clinical trials with adjunctive therapies to reverse MODS, there is still no effective therapeutic target or intervention. Treatment of MODS consists mainly of supportive measures, which are aimed at limiting and managing the symptoms, for example the use of vasopressors to support blood pressure, mechanical ventilation to address respiratory failure and renal replacement therapy to address kidney failure.

Shock is defined as a clinical state which occurs when an imbalance between oxygen supply and demand results in the development of tissue hypoxia. The original hypothesis that MODS is caused by tissue hypo-perfusion and 'oxygen debt', however, has been challenged over the last 30 years. It became evident that shock conditions have in common that patients can develop MODS based on an excessive and uncontrolled inflammatory response to the initial insult [51,52]. However, the pathophysiology of MODS following shock is complex and multifactorial and other aetiologies of MODS have been postulated to play a role in the

development of MODS, as summarized in table 1.1. in which these theories are summarized.

Table 1.1. Theories on the etiology of MODS.
Presumed pathologic process and manifestations, adapted from Abraham and Singer[53].

Pathologic Process	Manifestations
Tissue Hypoxia	Increased lactate
Uncontrolled infection	Nosocomial ICU-infection
Systemic inflammation	Cytokinemia (TNF, IL6, IL8), leukocytosis
Immune paralysis /immune suppression	Increased anti-inflammatory cytokines (IL10, IL-4, IL-13)
Endothelial activation	Leukocyte influx, capillary permeability
Dysregulated apoptosis	Decreased neutrophil apoptosis
Gut-liver axis / gut ischemia	Increased infection, Kupffer cell activation
Macrocirculation impairment	Changed tissue perfusion, vasoplegia

Trauma is a leading cause of morbidity and mortality, and there is an obvious relationship between trauma induced severe hemorrhage, the resulting MODS and clinical outcome[3-5,54]. Advances in hemorrhagic shock (HS) resuscitation have improved early survival, and as a result, the epidemiology of death from trauma has changed [6,47]. Survivors of the initial life-threatening insult are at risk to develop MODS [7,55]. Hemorrhage is a form of hypovolemic shock. The body responds to hypovolaemia through activation of the sympathic nervous system, which causes tachycardia and vasoconstriction in an attempt to maintain cardiac output and blood pressure. To preserve blood flow to the vital organs, there is marked vasoconstriction of cutaneous and other peripheral muscular blood vessels. Furthermore, the body actively retains fluid. Thus, HS is characterized by hypotension, tachycardia, oliguria, and by pale, cold, and clammy skin. The primary treatment of HS is control of the source of bleeding as soon as possible, and fluid replacement directed at optimizing perfusion and oxygen delivery to vital organs [8,56]. In general three animal HS models are being used in laboratories, the fixed pressure and fixed volume models and the uncontrolled hemorrhage model [9,57]. In the fixed-pressure hemorrhage model the mean arterial pressure (MAP) is lowered to a certain level for a defined period of time. One disadvantage of this model is that compensatory mechanisms are suppressed. In the fixed-volume hemorrhage model a pre-defined volume of blood is withdrawn. The disadvantage of this model is that the severity of hemorrhagic shock is uncertain since blood pressures are not measured [58]. The uncontrolled hemorrhage model

is considered more clinically relevant with physiologic compensation mechanisms, yet the results are highly variable and therefore this model is more difficult to employ [10,11,57]. In this thesis we employed the fixed-pressure hemorrhage model because of the reproducibility and reliability compared to the other models since the pressure-controlled HS induces a consistent shock depth with less variable systemic and organ inflammation [12,58].

The term sepsis is derived from the Greek word sepsin, which means 'to make putrid'. Van Leeuwenhoek made the first documented observations of living bacteria in 1674 although no relationship between bacteria and infectious disease was made at that time. Sepsis is a systemic inflammation in the setting of host microbial invasion. Severe sepsis is defined as organ dysfunction caused by sepsis. Persistent hypotension despite adequate fluid resuscitation characterizes septic shock [2,17]. To describe illnesses associated with inflammation diverse terms are used, table 2 is an overview of definitions of the diverse terms.

Table 1.2. Definitions of terms used to describe critical illnesses associated with inflammation, adapted from Levy et al[2,18] and Dellinger et al[19,59].

Infection	Pathologic process caused by the invasion of normally sterile tissue or fluid or body cavity by pathogenic or potentially pathogenic microorganism
SIRS	Systemic inflammation with or without infection Two or more of the following conditions: Temperature >38 °C or <36 °C Heart rate > 90 beats/min Respiratory rate > 20 breaths/min or $PaCO_2$ < 32 mm Hg WBC counts >12,000/mm^3, <4,000/mm^3, or >10% immature forms
Sepsis	Systemic inflammatory response to infection or suspected infection
Severe Sepsis	Sepsis-induced tissue hypoperfusion or organ dysfunction (thought to be due to the infection)
Septic shock	Sepsis with acute circulatory failure characterized by persistent arterial hypotension unexplained despite adequate fluid resuscitation
MODS	Altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention

WBC, white blood cell; SIRS, systemic inflammatory response syndrome; MODS, multi organ dysfunction syndrome

The mortality rates of patients admitted to the ICU with sepsis are high, approximately 27-29%. For patients with severe sepsis they are around 40%, while in patients with septic shock the rates are rising above 50% [20,50,60-62]. Common management strategies involve antibiotic therapy and a package of supportive measures, which are aimed at limiting and managing the systemic, multi-system consequences of sepsis. The guidelines published by the International Surviving Sepsis Campaign Guidelines Committee are being widely adopted, although the evidence level for many of the individual therapies is weak [19,21,59]. Thus it is not surprising that a recent study on therapy compliance with these guidelines found that for some recommendations, such as targeted steroid therapy, compliance was not associated with improved outcomes [24,60].

Several animal models are employed to mimic sepsis. First, injection of an exogenous toxin such as for example LPS, also known as endotoxin, is often employed. Second, alteration of the animal's endogenous protection barrier such as induction of intestinal leakage via for example cecal ligation and puncture is used to induce sepsis and lastly, administration of an exogenous bacteria is applied to induce sepsis [25,63]. In this thesis research, we employed a systemic LPS challenge model. This model, which was already developed by Arthus in 1903, is a frequently used animal model in which LPS is administered i.p to induce severe inflammation [32,64-67]. Lipopolysaccharide is derived from the cell wall of gram-negative bacteria and in many septic patients the presence of endotoxin in blood, called endotoxemia, is an important mediator of systemic inflammation and of the damage that Gram-negative bacteria cause[26,27,68,69]. In the LPS-induced inflammation mouse model, LPS administration induces systemic inflammation that mimics many of the initial clinical features of sepsis seen in patients, including increases in proinflammatory cytokines such as TNF-α and IL-1 [28,70], but without the occurrence of bacteraemia. Besides inducing TNF-a production, when administered *in vivo*, LPS can directly bind to receptors such as CD14 and toll-like receptor (TLR)-4 [29,71]. The binding of LPS to TLR-4 activates intracellular signal transduction pathways such as the NF-κB (nuclear factor κB) and mitogen-activated protein kinase (MAPK) pathways[72,73] and thereby affects the activation status, among others of endothelial cells. Activation of endothelial cells via systemically released cytokines and direct binding of LPS to receptors as described above results in changes in gene expression, endothelial adhesion molecules (E-selectin, VCAM-1, and ICAM-1) become upregulated, and PMN are recruited. Furthermore endothelial cell activation causes compromise of the barrier properties of the endothelium, resulting in vascular endothelial leakage. Administration of LPS to mice causes renal injury, including decreased GFR, and increased blood urea nitrogen (BUN) [30,31,74] which can also be seen in human septic patients.

1.1.6. Shock and Ageing

Ageing humans are more vulnerable to a wide variety of insults than younger humans[32,61,75,76]. In some cases this is the result of significant co-morbidities and reduced physiological reserve.

In the absence of significant co-morbidities, even 'healthy' ageing still seems to be associated with increased vulnerability to insults associated with systemic inflammation, resulting in significantly worse outcomes in terms of morbidity and mortality [27,61,77]. Advanced age is an important risk factor for the development of MODS [78]. Sepsis is the most frequent systemic insult in the elderly population, with the mean age of intensive care unit patients meeting the criteria for severe sepsis being 63.8 years [34,77]. Moreover, elderly patients account for 65% of sepsis cases, yielding a relative risk that is 13.1 times higher than that of younger patients[34,77]. In addition to being at higher risk of developing severe sepsis in response to an infection, standardised-mortality rates (adjusted for APACHE score) in elderly patients with severe sepsis are significantly greater than in younger patients, with case fatalities increasing from 10% in children to 40% in patients > 85 years of age [11,35,61,77]. As with sepsis, age is a significant factor in the outcome after trauma. Across a range of injury severity scores, age correlates positively

with the likelihood of death after trauma [36,79]. There is a threefold higher incidence of MODS in patients above 56 years of age [37,79]. The current understanding of the pathophysiology of MODS following shock, however, originates mainly from studies in young animals [33,80,81].
In humans, ageing without co-morbidity, or 'healthy aging', is associated with low-grade inflammatory activity, as measured by a 2– 4-fold increase in plasma TNF-α and IL-6 [38,39,82]. Moreover IL-6 levels start to increase in healthy people at about 50-60 years at age and high levels are referred to as predictors of morbidity and mortality in the elderly [3-5,83].

1.1.7. Ageing and the endothelium
Until now there is limited knowledge about the effect of (healthy) aging on endothelial function. In ageing three important physiological functions of the endothelial cells have been shown to be affected, *i.c.*, hemostasis, angiogenesis and vasomotor tone. Hemostasis regulation is changed towards increased coagulability and decreased fibrinolysis [40,45]. The angiogenesis function is decreased. With reduced collateral vessel development in response to ischemia, and reduced expression of vascular endothelial growth factor (VEGF) in kidney, heart and lung, an essential growth factor which stimulates vasculo- and angiogenesis [27,41,84-86]. Dilatation function of the vasomotor tones is affected as the endothelium dependent vasodilatation in conduit arteries and resistance vessels progressively declines with age, independently of structural changes of the vascular wall as a result of reduced NO bioavailability[42,87-91]. Mechanisms proposed for this latter dysfunction are oxidative stress and inflammation since in vascular endothelial cells obtained from the brachial artery and/or antecubital veins of older humans, the expression of pro-inflammatory nuclear transcription factor NF-κB and pro-inflammatory cytokines IL-6, TNF-α and MCP-1 (monocyte chemoattractant protein-1) were increased [92,93].
Alterations upon aging are also seen in the renal vasculature, the kidney being one of the most prominent organs affected by aging [94]. In humans creatinine clearance is stable until age 30 to 40 years and then declines linearly at a rate of about 8ml/minute/1.73 m^2/decade in the majority of elderly persons without renal disease. Loss of functional kidney tissue in the elderly is closely related to loss of renal microvasculature [44,86]. Segmental glomerulosclerosis increases with increased age, while the number of intact glomeruli decreases [45,95]. In mice, ageing induces a decrease in the spatial density of both small and large cortical microvessels, associated with inhibition of VEGF production [27,86]. These ageing related changes in kidney function increase the susceptibility of elderly individuals for acute kidney injury and contribute to delayed tissue repair in older persons following a systemic insult.

1.2 Aim of this thesis

Multi organ failure following shock is still related to high morbidity and mortality, and is even more prominent in the elderly. The endothelial-polymorphonuclear cell cross-talk is considered a major molecular player in the pathophysiological processes leading to MODS, yet the exact nature of their actions is unknown. We hypothesised that shock induced stress activates microvascular endothelium towards a pro-inflammatory state. Since the endothelium is a dynamic, multifunctional and heterogeneous structure, we hypothesised that different

organs and different microvascular beds within organs would respond differently to the same systemic stress associated with shock (LPS respectively HS induced). Until now there is no detailed insight available in the kinetics of endothelial activation and leukocyte influx during the induction of HS. Understanding the kinetics and molecular control of these processes may assist to identify targets for therapeutic intervention.

Our first aim was to investigate the kinetics, profile, and organ specificity of microvascular endothelial cell activation during the earliest phase of HS (chapter 2). In chapter 3 our aim was to further investigate the role of microvascular endothelial activation by neutrophils in HS in the kidney, since PMNs might have an important role in organ damage via bystander injury[40,46]. Thus we hypothesized that depletion of neutrophils would prevent immunomodulation and attenuate endothelial cell activation, measured as endothelial adhesion molecules expression, and loss of kidney function. We examined the kidney since it is one of the organs most affected in MODS and because acute kidney injury (AKI) is strongly associated with patient morbidity and mortality [50,96].

Hemorrhagic shock induces pronounced and early endothelial activation (chapter 2 and 3), however from these studies it remained unclear whether endothelial activation was based on activation by pro-inflammatory cytokines induced by HS alone. Additionally, hypoxia too could play a (more) prominent role since tissue hypoxia is supposed to be a mediator of MODS (see table 1.1.). In our original mouse model of HS, the mice were spontaneously breathing. Since shock is defined as tissue hypoxia due to an imbalance between systemic oxygen delivery and oxygen demand [97], the clinical practice is to give patients additional oxygen often via intubation and mechanical ventilation. Mechanically ventilating the mice would be more clinically relevant and might prevent hypoxia induced stress. However, mechanical ventilation may induce an inflammatory reaction [98] and induce endothelial activation of the lung and in distant organs[99]. Thus the aim of this third study (chapter 4) was to use a more clinical relevant model and investigate the consequences of mechanical ventilation of mice subjected to HS on microvascular endothelial activation in the lungs and kidney. To separate the effects of shock, hypoxia and systemic inflammation on endothelial cell activation, we performed additional experiments in which mice were subjected to severe hypoxia without shock.

In critically ill patients advanced age is a major risk factor for the development of MODS. We hypothesised that the microvascular endothelium likely becomes 'primed' during (healthy) ageing, as a result of the low-grade inflammatory activity associated with healthy ageing. This potentially makes the elderly more susceptible to the harmful effects of a second inflammatory insult[53,100]. Thus increased age may 'set the stage', making the patient vulnerable to a 'second hit' such as a severe infection or HS, thereby explaining the increased incidence of MODS, and worse outcomes. Therefore, the aim of chapter 5 was to investigate whether in elderly the interaction between PMN and the endothelium is affected as a consequence of ageing, and whether ageing-related endothelial priming contributed to this process. For this purpose we studied the effect of LPS challenge of young (3 months) and aged mice (18 months) with focus on expression of endothelial adhesion molecules and members of the angiopoietin/TIE-2 system, and PMN influx in the kidney.

Finally, the results described in this thesis are summarized and future perspectives of the outcomes discussed in chapter 6.

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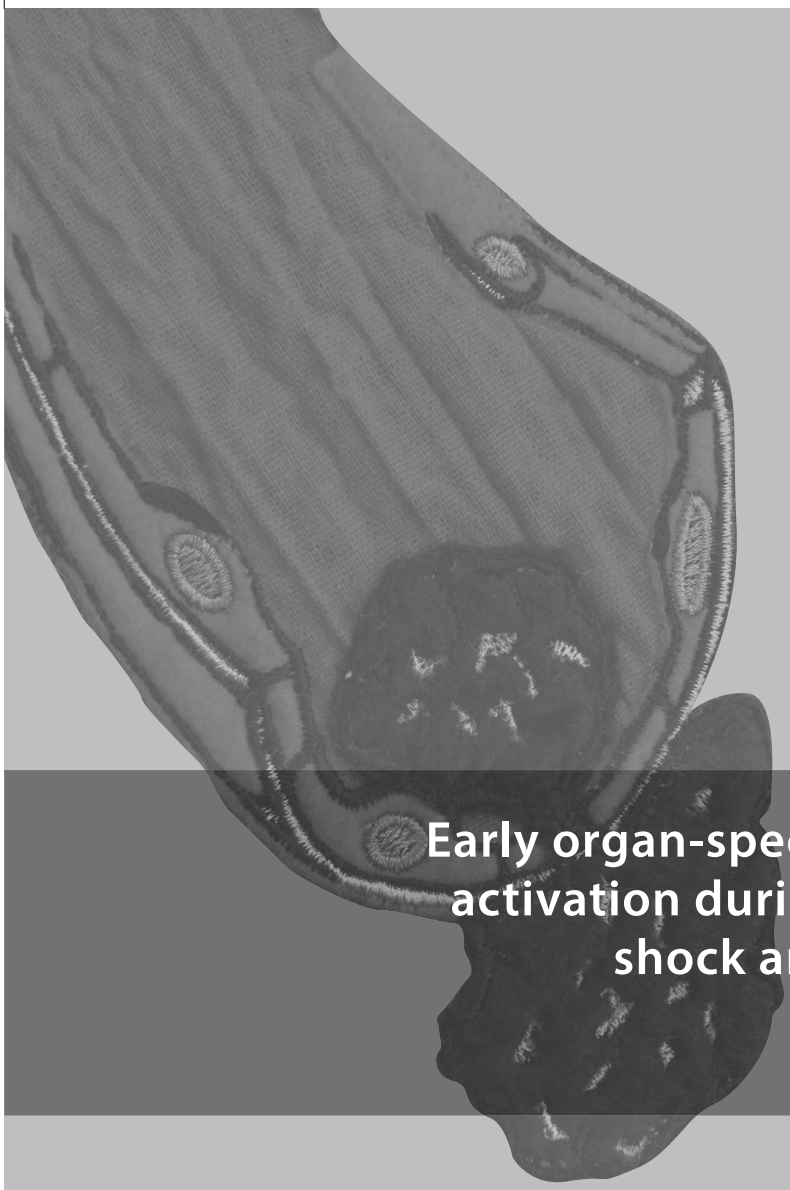

Chapter 2

Early organ-specific endothelial activation during hemorrhagic shock and resuscitation

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Abstract

Introduction

Multiple organ dysfunction syndrome (MODS) is a complication of HS (HS) and related to high morbidity and mortality. Interaction of activated neutrophils and endothelial cells is considered to play a prominent role in the pathophysiology of MODS. Insight in the nature and molecular basis of endothelial cell activation during HS can assist in identifying new rational targets for early therapeutic intervention. In this study we examined the kinetics and organ specificity of endothelial cell activation in a mouse model of HS.

Methods

Anesthetized male mice were subjected to controlled hemorrhage to a mean arterial pressure of 30 mmHg. Mice were killed after 15, 30, 60 or 90 min of HS. Following 90 min of HS, a group of mice was resuscitated with 6% hydroxyethyl starch 130/0.4 (Voluven®). Untreated mice, and sham shock mice that underwent instrumentation and 90 min of anesthesia without shock served as controls. Gene expression levels of inflammatory endothelial cell activation (P-selectin, E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1) and hypoxia-responsive genes (vascular endothelial growth factor and hypoxia-inducible factor 1α) were quantified in kidney, liver, lung, brain, and heart tissue by quantitative reverse-transcription-polymerase chain reaction. Furthermore, we examined a selection of these genes with regard to protein expression and localization using immunohistochemical analysis.

Results

Induction of inflammatory genes occurred early during HS and already before resuscitation. Expression of adhesion molecules was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial genes CD31 and VE-cadherin, which function in endothelial cell homeostasis and integrity, were not affected during the shock phase except for VE-cadherin in the liver, which showed increased mRNA levels. The rapid inflammatory activation was not paralleled by induction of hypoxia-responsive genes.

Conclusion

This study demonstrated the occurrence of early and organ-specific endothelial cell activation during HS, as presented by induced expression of inflammatory genes. This implies that early therapeutic intervention at the microvascular level may be a rational strategy to attenuate MODS.

Introduction

The development of multiple organ failure (MODS) following hemorrhagic shock (HS) is a problem in the care of patients who suffer major bleeding. MODS contributes significantly to morbidity and mortality1. Advances in medical care of HS patients, including the introduction of resuscitation fluids, trauma centers and intensive care units, have resulted in a significant decrease in early deaths caused by HS. Concomitant with recovering from a previously fatal condition, the systemic inflammatory response initiated by HS became manifest1. The inflammatory response is nowadays considered the leading cause for the development of MODS.

Although the precise mechanisms and pathways leading to organ injury after HS are still unknown, the neutrophil is thought to be a principal cellular mediator of tissue damage. Migration of neutrophils into tissue during HS leads to significant organ damage through release of proteases and oxygen derived radicals [2]. The interaction between neutrophils and endothelium is instrumental in the migration of neutrophils into different tissues [3]. This migration is regulated by adhesion molecules on both leukocytes and endothelium, the latter including P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1).

Most studies concerning MODS following HS have focused on the postresuscitation period, in which the shock is compensated by volume infusion. A few studies showed the occurrence of early cytokine production and activation of intracellular signaling pathways which are well-established triggers of endothelial cell activation [4-7]. Increased expression of P-selectin in liver and lung after 90 min of HS induction implicates early endothelial responses [8], which might be induced by hypoxia [6,9]. Based on these studies, we hypothesized that HS rapidly activates endothelial cells towards a proinflammatory status. Early endothelial activation could imply options for an early therapeutic window. Considering the organ-specific involvement in HS associated MODS, this activation likely presents differently in different organs. It is imperative to unravel the kinetics and pathophysiological mechanisms of endothelial activation to develop specific therapeutic strategies that can either prevent or attenuate the effects of HS on organ function and related morbidity and mortality.

The aim of this study was to investigate the kinetics, profile, and organ specificity of endothelial cell activation during the earliest phase of HS. We employed a mouse model of controlled arterial pressure of 30 mmHg during a time period of 90 min, after which designated groups of mice were resuscitated. We examined the expression levels of a series of adhesion molecules (P-selectin, E-selectin,VCAM-1 and ICAM-1) known to contribute to leukocyte recruitment, hypoxia-related genes hypoxia-inducible factor 1 α (HIF-1α) and vascular endothelial growth factor A (VEGF-A), and the proinflammatory cytokine TNF-α, by quantitative reverse-transcription-polymerase chain reaction (RT-PCR). Furthermore, immunohistochemistry was performed to detect endothelial adhesion molecule and HIF-1α proteins. Besides information on the extent of activation, this approach provided details regarding the microvascular location of endothelial activation within the organs.

Materials and methods

Animals

Eight to 12-week-old C57Bl/6 male mice (20–30 g) were obtained from Harlan Nederland (Horst, The Netherlands). Mice were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-hr light/dark cycle. All procedures performed were approved by the local committee for care and use of laboratory animals, and were performed according to strict governmental and international guidelines on animal experimentation.

HS model

Mice were anesthetized with isoflurane (inspiratory, 1.4 %), N20 (66%) and 02 (33%). Throughout the experiment mice were breathing spontaneously. The animals were kept on a temperature-controlled surgical pad (37-38 ˚C). The left femoral artery was cannulated with polyethylene tubing with an internal diameter of 0.28 mm and an external diameter of 0.61 mm for monitoring mean arterial pressure (MAP), blood withdrawal, and resuscitation. Hemorrhagic shock was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg within 15-30 min. Blood was collected in a heparinized 1-ml syringe to prevent clotting. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain MAP at 30 mmHg during this period. Mice were killed 15, 30, 60 or 90 min after MAP of 30 mmHg was achieved. In Europe, colloidal infusion fluids are frequently used in the resuscitation of shock victims. We therefore resuscitated, following 90 min of HS, additional groups of mice with 6% hydroxyethyl starch 130/0.4 (Voluven; Fresenius-Kabi, Bad Homburg, Germany), two times the volume of the blood withdrawn. After 1, 4 or 24 hours following volume resuscitation these mice where killed. Control mice were left untreated and received anesthesia only before they were killed. Sham shock mice underwent instrumentation and were kept under anesthesia for 90 min, however no blood was withdrawn. The HS 90-min shock and HS sham shock group consisted of five animals, the other groups consisted of three animals per group. After the experimental procedure, brain, heart, lungs, liver and kidney were harvested and snap-frozen in liquid nitrogen, and stored at -80 °C until analysis.

RNA isolation and quantitative RT-PCR

RNA was extracted from 20 (liver, brain and kidney) or 30 (heart and lung) 5-µm-thick cryosections and isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands), according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoreses. RNA yield (OD260) and purity (OD260/OD280) were measured by ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

One microgram of total RNA was subsequently used for the synthesis of first-strand cDNA with SuperScript III RNase minus reverse transcriptase (Invitrogen, Breda, The Netherlands) in a 20-µl final volume containing 250 ng of random hexamers (Promega Benelux) and 40 units of RNase OUT inhibitor (Invitrogen). After the RT reaction, 1 ml cDNA was used for each PCR reaction. Intron-overspanning primers and minor groove binder (MGB) probes used for quantitative RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Foster City, CA, USA)

primers included housekeeping gene beta 2 Microglobulin (B2M) (assay ID Mm00437762_m1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm99999915_g1), endothelial cell marker CD31 (platelet endothelial cell adhesion molecule 1, Mm00476702_m1), VE-cadherin (Mm00486938_m1), VEGF-a (Mm00437304_m1), HIF-1α (Mm00468869_m1),and P-selectin (Mm00441295_m1). TNF-α (Mm00443258_m1) primers and probes were purchased as pre-developed assay reagent for gene expression (Applied Biosystems). Primers and probes for E-selectin (forward primer: 5'-CAACGTCTAGGTTCAAAACAATCAG-3', probe: 5'-CACAAATGCAATCGTGGGA-3', reverse primer: 5'-TTAAGCAGGCAAGAGGAACCA-3'), ICAM-1 (forward primer:5'- ATGGGAATGTCACCAGGAATG-3', probe: 5'-CAGTACTGTACCACTCTC-3', reverse primer: 5'-GCACCAGAATGATTATAAGTCCAGTTATT-3') and VCAM-1 (forward primer: 5'-TGAAGTTGGCTCACAATTAAGAAGTT-3', probe: 5'- AACACTTGATGTAAAAGGA-3', reverse primer: 5'-TGCGCAGTAGTAGAGTGCAAGGA-3') were purchased as Assay-by-Design from Applied Biosystems, using the same quality criteria as for Assay-on-Demand.

The final concentration of primers and MGB probes in TaqMan PCR MasterMix (Applied Biosystems, Foster City, CA, USA) for each gene was 900 nM and 250 nM, respectively. Water was used as a negative control, to exclude unspecific signals arising from impurities, and consistently showed no amplification signals.

Taqman real-time RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Amplification was performed with the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 two step cycles of 15 s at 95 °C and 60 s at 60 °C. Triplicate real-time RT-PCR analyses were executed for each sample, and the obtained threshold cycle values (Ct) were averaged. According to the comparative Ct method described in the ABI manual *(http://www.appliedbiosystems.com)*, gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the ΔCt value. The average ΔCt value obtained from control, a non-treated, mouse was then subtracted from the average ΔCt value of each sample subjected to the experimental conditions described, yielding the ΔΔCt value. The gene expression level, normalized to the housekeeping gene and relative to the control, was calculated as 2-ΔΔCt.

Localization of adhesion molecule expression, leukocyte infiltration, and HIF-1α using immunohistochemistry

Localization of P-selectin, E-selectin, VCAM-1, and ICAM-1 expression was determined in kidney, liver, heart, lung, and brain by immmunohistochemistry. Leukocyte recruitment was analyzed by immunohistochemical staining with an anti-CD45 pan leukocyte marker antibody. Frozen organs were cryostat-cut at 5 µm, mounted onto glass slides, and fixed with acetone for 10 min. After drying, sections were incubated for 45 min at room temperature with primary rat anti-mouse antibodies: anti-E-selectin (MES-1, kindly provided by Dr. D. Brown, Celletech Group, Slough, UK), anti-CD31 (clone MEC13.3; Pharmingen BD Biosciences, Alphen aan den Rijn, The Netherlands), anti-CD45 leukocyte common antigen (Pharmingen BD Biosciences), anti-VCAM-1 (clone M/K-1.9; ATCC, Manassas VA, USA), and anti-ICAM-1 (clone YN1/1.7; ATCC) in the presence of 5% fetal calf serum. After washing, endogenous peroxidase was blocked by incubation with 0.1% H_20_2 in PBS for 20 min. This was followed

by incubation of 30 min at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies (Rabbit anti Rat-Ig, DAKO, Glostrup, Denmark). Conjugates were diluted 1:50 in PBS supplemented with 2% normal mouse serum. Sections with isotype matched controls, E-selectin, and VCAM-1 specific antibodies were further incubated for 30 min at room temperature with HRP-conjugated goat anti-rabbit antibody (Southern Biotech Association, Birmingham, Alabama, USA) diluted 1:100 in PBS. Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich Chemie, St.Louis, Missouri, USA) and sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands). No immunostaining was observed with isotype matched controls, demonstrating specificity of staining with the antigen specific antibodies.

For HIF-1α staining, 5 mm acetone fixed cryosections were incubated for 45 min at room temperature with primary HIF-1α goat polyclonal antibody (HIF-1α (Y-15): sc-12542, Santa Cruz Biotechnology, California, USA) in the presence of 5% fetal calf serum in PBS. Endogenous biotin was blocked by Biotin Blocking System (DAKO) according to manufacturer's protocol and peroxidase activity was blocked by incubation with 0.1% H_2O_2 in PBS for 10 min. Subsequently, sections were incubated at room temperature for 45 min with rabbit anti-goat antibody (DAKO; dilution 1:100 in PBS) in the presence of 2% normal mouse serum. Further staining procedure was performed as described above. Negative control samples were incubated with PBS instead of the primary antibody.

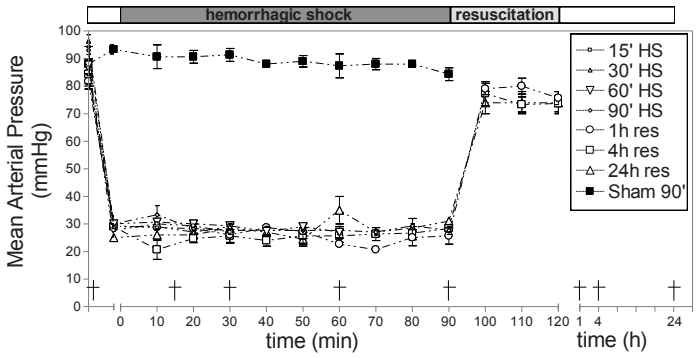

Figure 1: Study design and measured mean arterial pressure during experiment.

Hemorrhage was induced by blood withdrawal as described in "Materials and Methods" . Mice were sacrificed (†) at start of experiment, or after 15, 30, 60 or 90 minutes of HS. Following 90 minutes of hemorrhagic shock, a group of mice was resuscitated. Sham shock mice (■) underwent instrumentation and 90 minutes of anesthesia. Blood pressure is expressed as mean ± SEM, (n=5 in the 90' HS group and 90' sham shock, other groups n= 3).

Statistical analysis

Statistical significance of differences was studied by means of the analysis of variance (ANOVA) with post hoc Least Significant difference. Differences were considered to be significant when $p < 0.05$.

Table 1. **Expression levels of genes investigated in this study in healthy mouse organs**

	Kidney	Liver	Heart	Lung	Brain
CD31	5.9 (5.5-6.2)	7.1 (6.8-7.4)	5.5 (5.0-5.6)	0.9 (0.5-1.6)	7.2 (7.2-7.4)
VE-Cadherin	6.0 (5.7-6.1)	6.4 (6.3-7.7)	4.4 (4.2-4.6)	−0.3 (−0.6 to 0.5)	7.1 (7.1-7.2)
P-Selectin	14.9 (14.6-15.7)	12.4 (11.6-12.4)	14.4 (12.9-14.8)	8.3 (8.2-8.8)	18.0 (17.0-18.0)
E-Selectin	14.6 (14.4-14.7)	17.0 (16.8-17.3)	14.2 (13.6-14.4)	12.3 (11.5-12.6)	18.6 (18.1-18.9)
VCAM-1	9.4 (9.3-9.5)	8.9 (8.6-9.6)	10.2 (9.3-10.2)	6.4 (5.3-6.9)	8.2 (8.1-8.5)
ICAM-1	7.6 (7.4-8.1)	7.7 (7.6-8.3)	10.4 (9.9-10.7)	2.4 (2.1-3.1)	10.7 (10.1-11.1)
HIF-1α	6.1 (5.5-6.4)	7.0 (6.6-7.3)	7.5 (6.0-8.0)	4.4 (4.4-5.0)	6.3 (6.3-6.5)
VEGF-A	4.3 (4.1-4.3)	4.5 (4.1-4.8)	5.7 (4.8-5.7)	0.8 (0.8-1.6)	6.0 (5.9-6.2)

Table 1: Expression levels of genes investigated in this study in healthy mouse organs

Expression of CD31, VE-cadherin, P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in control mice not subjected to any experimental procedure was determined in lung, liver, kidney, heart, and brain using quantitative RT-PCR with GAPDH as housekeeping gene. Data represent values from three animals per group expressed as median ΔCT (minimum-maximum).

Table 2. **Kinetics of EC activation before resuscitation**

		0	30 min	60 min	90 min	Sham 90 min
CD31	Lung	1.0 (0.6-1.3)	1.4 (0.2-1.9)	0.6 (0.4-2.0)	0.6 (0.1-1.7)	0.4 (0.1-1.5)
	Liver	0.8 (0.7- 1.0)	0.5 (0.4-0.8)	0.6 (0.6-0.6)	0.7 (0.4-1.1)	0.8 (0.6-1.0)
	Kidney	1.0 (0.8-1.3)	0.7 (0.4-0.8)	0.8 (0.5-1.5)	0.5 (0.5-0.7)	0.7 (0.6-0.8)
	Heart	1.1 (1.0-1.5)	1.0 (1.0-1.0)	1.3 (1.1-1.3)	1.2 (0.9-1.3)	1.5 (0.2-2.7)
	Brain	1.0 (0.9-1.0)	0.6 (0.4-0.7)	0.7 (0.6-0.7)	0.7 (0.4-1.1)	0.6 (0.6-0.9)
VE-Cadherin	Lung	1.0 (0.6-1.2)	1.2 (1.2-1.6)	0.5 (0.4-1.8)	0.7 (0.1-1.7)	0.3 (0.1-1.7)
	Liver	2.4 (1.0-2.7)	3.3 (2.5-3.4)	3.1 (3.1-3.2)	3.3 (3.0-7.8)†	4.3 (2.7-5.0)
	Kidney	1.0 (1.0-1.3)	0.9 (0.2-1.0)	1.2 (1.2-1.5)	1.2 (0.9-1.3)	0.8 (0.7-1.0)
	Heart	1.0 (0.9-1.2)	1.1 (1.0-1.1)	1.2 (1.2-1.4)	0.9 (0.9-1.2)	1.3 (0.2-2.4)
	Brain	1.0 (0.9-1.0)	0.7 (0.5-1.0)	1.0 (0.8-1.0)	0.8 (0.5-1.4)	0.9 (0.7-1.2)
P-Selectin	Lung	1.0 (0.8-1.1)	2.3 (0.2-2.0)	1.4 (1.4-7.2)	8.4 (1.1-60)*	1.5 (0.5-2.1)
	Liver	0.6 (0.6-1.0)	0.7 (0.6-1.7)	1.4 (1.0-1.5)	10 (1.8-32)	3.1 (0.4-7.4)
	Kidney	1.0 (0.6-1.2)	2.1 (0.9-4.2)	5.2 (4.7-12)	25 (3.2-112)	4.7 (0.6-7.4)
	Heart	0.4 (0.3-1.0)	0.6 (0.6-3.0)	1.3 (0.5-1.6)	15 (6.4-70)	3.7 (0.5-11)
	Brain	0.5 (0.5-1.0)	1.7 (1.0-2.5)	7.4 (6.5-8.7)	66 (28-397)	26 (1.3-63)
E-Selectin	Lung	1.0 (0.8-1.6)	1.5 (0.4-127)	7.6 (5.6-26)	54.5 (9.0-301)*†	2.5 (2.2-11)
	Liver	1.2 (1.0-1.4)	9.3 (3.4-778)	135 (106-176)	666 (472-2,289)†	637 (1.2-1,261)
	Kidney	1.0 (1.0-1.2)	7.4 (2.6-63)	39 (18-90)	133 (18-313)*†	30 (1.6-64)
	Heart	1.1 (1.0-1.7)	2.9 (2.3-25)	4.7 (3.5-8.8)	52 (16-92)	3.5 (1.8-52)
	Brain	1.0 (0.8-1.4)	9.8 (1.4-25)	34 (21-36)	124 (47-477)*†	66 (3.9-156)
VCAM-1	Lung	1.0 (0.7-2.1)	2.8 (0.8-15)	3.6 (2.0-6.2)	6.4 (1.6-29)*†	2.0 (0.9-3.0)
	Liver	0.8 (0.5-1.0)	2.5 (1.2-3.4)	3.4 (2.7-3.4)	4.9 (4.3-9.4)†	10.8 (1.9-13)
	Kidney	1.0 (0.9-1.1)	1.3 (1.2-3.5)	3.1 (2.5-3.8)	6.0 (1.4-15)*†	3.8 (0.7-5.3)
	Heart	1.0 (1.0-1.9)	1.5 (1.3-8.7)	3.4 (2.0-4.9)	12.5 (5.4-17)	3.6 (0.6-15)
	Brain	1.0 (0.8-1.1)	0.6 (0.6-0.8)	0.8 (0.6-1.1)	1.8 (1.4-2.1)*†	1.0 (0.8-1.1)
ICAM-1	Lung	1.0 (0.6-1.2)	1.9 (0.5-5.1)	1.0 (0.7-3.0)	3.1 (0.5-19)*	0.6 (0.3-1.9)
	Liver	1.0 (0.6-1.0)	0.7 (0.7-6.9)	3.9 (3.8-5.9)	24 (17-41)†	37 (0.6-43)
	Kidney	1.0 (0.7-1.1)	1.4 (1.2-4.4)	5.5 (3.1-6.7)	12 (3.7-24)*†	3.3 (0.8-4.4)
	Heart	1.2 (1.0-1.8)	3.9 (2.7-30)	12 (6.2-14)	82 (25-195)	6.2 (3.5-125)
	Brain	1.0 (0.7-1.5)	1.8 (0.7-2.8)	2.6 (1.7-4.1)	12 (6.6-33)*†	5.1 (1.6-7.9)

Table 2: Kinetics of EC activation prior to resuscitation

Expression levels of P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in lung, liver, kidney, heart, and brain analysed by quantitative RT-PCR using GAPDH as housekeeping gene. RNA levels were normalized to their respective levels in RNA isolates of healthy organs obtained from untreated control mice. Data are expressed as median (minimum-maximum) (n=5 in the 90' HS group and 90' sham shock, other groups n= 3). $p < 0.05$ values marked with * represent 90 minutes of HS, compared with 90 minutes of sham shock (sham 90'). $p < 0.05$ values marked with ‡ represent 90 minutes of HS, compared with control, as described in Materials and Methods.

Results

Hemodynamic changes induced by HS

Figure 1 illustrates the time course of MAP during the experiment. All groups of mice exhibited initial MAP values in the range of 75-100 mmHg. In sham shock mice the MAP remained stable during 90 min between 80-100 mmHg. In the HS group the MAP was maintained between 20-40 mmHg. At the end of resuscitation, all groups experienced a comparable increase in MAP, which nevertheless did not reach the baseline values observed before HS induction. All mice survived the procedure until the end of the experiments.

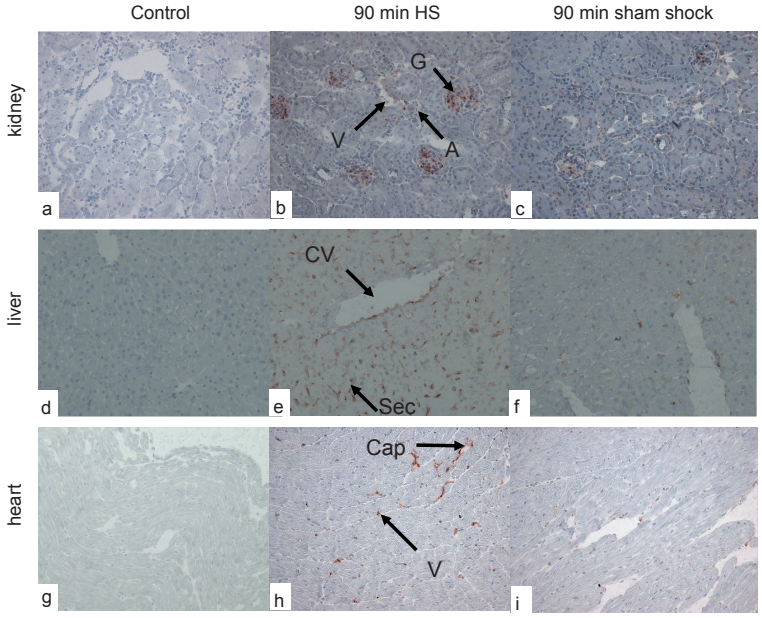

Figure 2: Localization of E-selectin expression in kidney, liver and heart during the early phase of HS

Immunohistochemical detection of E-selectin in healthy mouse tissue (a,d,g), after 90 minutes of HS (b, e, h), and after 90 minutes of sham shock (c, f, i). Staining was performed respectively on kidney (a-c), liver (d-f), and heart (g-i). Original magnification 200x. E-selectin is stained red, with increased staining in blood vessels after 90 minutes of shock. Arrows indicate: G = glomerulus, V = venule, A = arteriole, CV= Liver central vene, Sec = Liver sinusoidal endothelium, Cap = capillary.

Baseline gene expression levels in different organs

Quantitative RT-PCR was used to quantitatively measure the basal and HS-affected mRNA levels of adhesion molecules and the hypoxia-related genes HIF-1α and VEGF-A in the different organs. In control mice (Table 1) the levels of mRNA of the genes under study differed between genes within one organ, and between organs. CD31 and VE-cadherin expression levels within one organ were within the same magnitude. The low ΔCt values of endothelial marker genes CD31 and VE-cadherin in the lung (representing high mRNA levels) most likely reflects the high degree of vascularisation in this organ compared to the other organs. Although the ΔCt values do not directly represent protein levels, these data do imply that organ-specific vascular heterogeneity in microvascular endothelial cell status exists.

Kinetics of expression of endothelial adhesion molecules and hypoxia-related genes during the shock phase

The kinetics of endothelial cell activation during onset and maintenance of HS were studied by quantitative analysis of P-selectin, E-selectin, VCAM-1 and ICAM-1 mRNA levels (Table 2). P-selectin, E-selectin, and ICAM-1 were strongly up-regulated at 90 min after initiation of HS compared to sham shock. Furthermore, especially E-selectin expression in the kidney and the lungs showed a trend towards early induction. Although the early increase was statistically not significant compared to the activation induced by 90 min of sham shock, it did show statistical significance when compared to control mice. Neither CD31 nor VE-cadherin expression changed under the influence of HS except for VE-cadherin expression in the liver, which showed increased mRNA levels. Resuscitation facilitated an additional up-regulation of the inflammatory endothelial genes compared with the activation status at 90 min of HS (Table 3). It is of note that especially in the liver and the kidney, VE-cadherin mRNA levels were strongly up-regulated at 1 h after resuscitation.

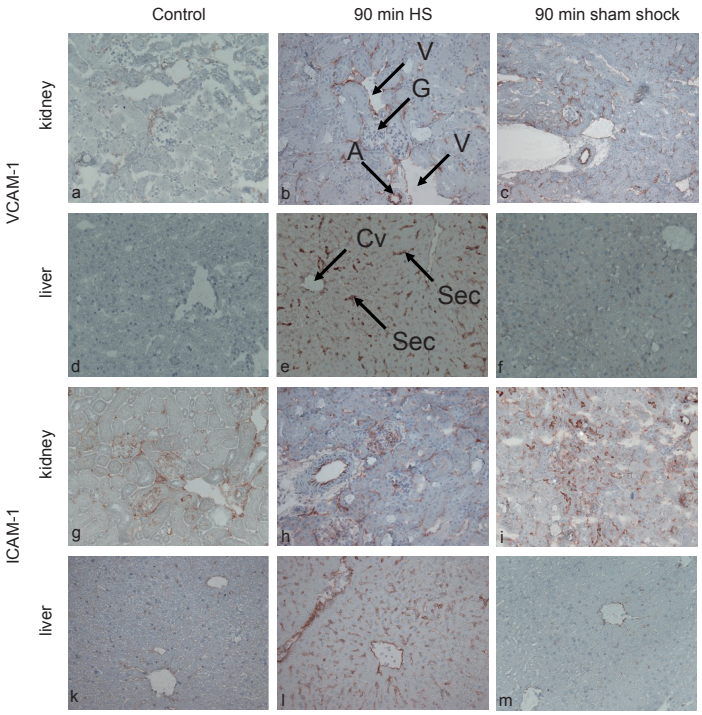

Figure 3: Localization of VCAM-1 and ICAM-1 expression in kidney and liver during the early phase of HS

Immunohistochemical detection of VCAM-1 and ICAM-1 in healthy mouse tissue (a, d, g, j), after 90 minutes of HS (b, e, h, k), and after 90 minutes of sham shock (c, f, i, l). Staining was performed respectively on kidney (a-c, g-i) and liver (d-f, j-k). Original magnification 200x. VCAM-1 and ICAM-1 and CD31 are stained red, with increased staining in blood vessels after 90 minutes of shock.

Table 3. **Kinetics of EC activation after resuscitation**

		90 min HS	1 h	4 h	24 h
CD31	Lung	0.6 (0.1-1.7)	0.1 (0.1-0.4)	0.5 (0.1-0.9)	0.8 (0.4-1.7)
	Liver	0.7 (0.4-1.1)	0.5 (0.5-0.8)	0.5 (0.4-0.5)	0.9 (0.6-1.0)
	Kidney	0.5 (0.5-0.7)	0.4 (0.4-0.4)	0.6 (0.3-1.0)	1.3 (0.8-2.7)
	Heart	1.2 (0.9-1.3)	1.0 (0.1-1.3)	0.7 (0.5-0.8)	2.0 (1.5-2.3)
	Brain	0.7 (0.4-1.1)	0.7 (0.6-0.7)	1.1 (0.5-1.2)	2.1 (1.1-2.9)
VE-Cadherin	Lung	0.7 (0.1-1.7)	0.2 (0.2-1.7)	0.7 (0.2-1.1)	0.7 (0.2-1.0)
	Liver	3.3 (3.0-7.8)	7.6 (4.9-8.1)*	6.6 (4.8-9.3)	3.0 (2.4-3.8)
	Kidney	1.2 (0.9-1.3)	1.6 (1.6-1.9)*	2.6 (2.4-2.9)	1.7 (0.8-2.1)
	Heart	0.9 (0.9-1.2)	0.8 (0.1-1.1)	0.9 (0.5-1.3)	1.5 (1.2-1.8)
	Brain	0.8 (0.5-1.4)	1.7 (1.1-1.7)	1.7 (0.9-3.2)	2.1 (1.1-2.4)
P-selectin	Lung	8.4 (1.1-60)	25 (18-65)	6.8 (5.3-28)	2.0 (1.9-6.3)
	Liver	10 (1.8-32)	118 (74-174)*	62 (6.0-75)	0.8 (0.5-1.2)
	Kidney	25 (3.2-113)	264 (255-311)*	94 (33-214)	9.3 (2.2-11)
	Heart	15 (6.4-70)	222 (33-287)*	119 (4.3-184)	2.1 (1.2-2.7)
	Brain	66 (28-398)	482 (253-9O1)*	26 (7.5-138)	13 (5.8-173)
E-selectin	Lung	55 (9.0-301)	103 (82-234)	3.9 (3.0-9.2)	6.2 (2.7-17)
	Liver	666 (473-2,290)	3,042 (2,196-3,059)*	230 (24-379)	6.3 (1.7-7.4)
	Kidney	133 (18-313)	252 (236-465)*	17 (9.5-46)	1.5 (0.6-1.7)
	Heart	52 (15.8-92)	492 (111-795)*	98 (1.5-301)	5.1 (1.6-7.5)
	Brain	124 (47-474)	287 (245-419)	30 (2.3-36)	40 (8.5-151)
VCAM-1	Lung	6.4 (1.6-29)	2.7 (2.6-7.3)	2.1 (2.0-6.4)	1.8 (1.6-2.7)
	Liver	4.9 (4.3-9.4)	12 (12-16)*	4.6 (2.5-7.8)	0.9 (0.9-1.0)
	Kidney	6.0 (1.4-15)	23 (21-27)*	6.4 (3.3-15)	1.5 (0.6-1.8)
	Heart	13 (5.4-17)	37 (2.5-46)*	11 (0.8-2)	1.9 (1.5-2.2)
	Brain	1.8 (1.4-2.1)	2.8 (2.2-3.3)*	0.8 (0.5-1.1)	1.4 (0.9-1.4)
ICAM-1	Lung	3.1 (0.5-19)	3.3 (2.9-7.8)	1.2 (0.9-5.8)	1.2 (0.9-2.8)
	Liver	24 (17-41)	33 (26-41)	9.7 (3.7-14)	1.3 (1.0-1.4)
	Kidney	12 (3.7-24)	25 (22-26)*	16 (4.2-20)	1.6 (0.7-2.6)
	Heart	82 (25-1 95)	356 (45-365)*	129 (4.4-236)	7.2 (2.2-9.1)
	Brain	12 (6.6-33)	27 (24-29)*	2.1 (1.4-11)	4.2 (1.4-6.6)

Expression levels of CD31, VE-cadherin, P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in the lung, liver, kidney, heart, and brain analyzed by quantitative RT-PCR using GAPDH as housekeeping gene. The RNA levels of adhesion molecules were normalized to their respective levels in RNA isolates of healthy organs obtained from untreated control mice. Data are expressed as median (minimum-maximum) (n = 5 in the 90-min HS group; other groups, n = 3). Hemorrhagic shock (90 min) was compared with 1 h postresuscitation group (1 h). P G 0.05 values are marked with * as described in "Materials and methods."

To corroborate the gene expression levels, cell adhesion molecules were immunohistochemicaly analyzed. In kidney, liver, and heart, E-selectin protein was strongly expressed at 90 min of shock, whereas in the sham shock group expression was minor or absent (Figure 2).

Immunohistochemical staining did not only show differences in expression between different organs, thereby supporting the gene expression data, but also demonstrated differences in activation between the diverse organ microvascular endothelial cells. In the kidney, for example, protein expression of E-selectin was mainly found in glomeruli (Figure 2), whereas VCAM-1 expression was seen primarily in the peritubular endothelial cells, arteries and venules,

but not in glomeruli (Figure 3). From this, it was concluded that HS under anesthesia strongly affected the activation status of microvascular endothelial cells in all organs. Unexpectedly, however, anesthesia and instrumentation without concurrent induction of HS also affected endothelial cell activation, as observed by up-regulation of adhesion molecule mRNA and protein levels (Figure 2, Figure 3, Table 2).
Hypoxia-related genes VEGF-A and HIF-1α were not transcriptionally affected during the 90 min of HS (Figure 4). Moreover, immunohistochemical staining did not reveal nuclear localization of HIF-1α in any of the organs (data not shown), implying absence of activation of the HIF-1α system during the HS phase.

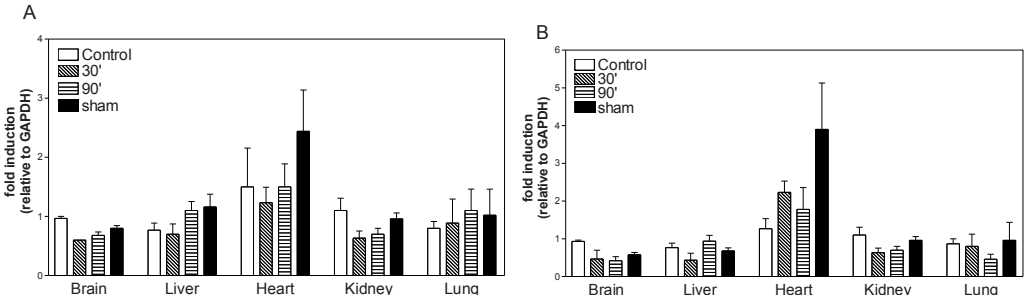

Figure 4: Effect of hemorrhagic shock on hypoxia related markers

Gene expression of HIF-1α (A) and VEGF-A (B) was determined in lung, liver, kidney, heart, and brain by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold induction of hypoxia related genes in comparison to the level of activity that was present in RNA isolates of normal healthy tissue. Data presented are the mean ± SEM of each group (n=5 in the 90' HS group and 90' sham shock, other groups n= 3). 90 minutes of HS was compared with 90 minutes of sham shock (sham 90'), no significant differences were found.

Early organ activation is paralleled by leukocyte influx

Adhesion molecule expression is instrumental in facilitation of leukocyte migration into the tissues. Influx of leukocytes into tissue, as determined with pan leukocyte marker CD45, was observed in kidney, lung, and liver at 90 min of HS (Figure 5). In contrast, in brain and heart no leukocyte influx was seen after 90 min of HS. No difference in leukocyte influx could be observed compared with control mice (data not shown) 24 h after volume resuscitation.

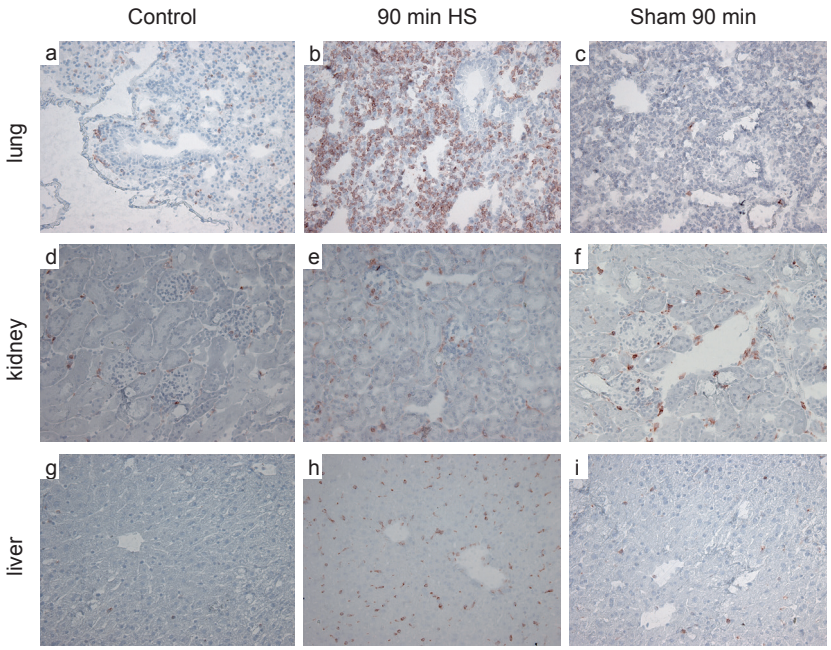

Figure 5: Influx of leukocytes in the different organs during HS

Immunohistochemical staining with an anti-CD45 pan leukocyte antibody showed influx of leukocytes in lungs (a-c), kidney (d-f), and heart (g-i). Staining was performed respectively on healthy mouse tissue (control) (a, d, g) and 90 minutes HS (b, f, h), and 90 minutes sham shock (c, e, i). Original magnification 200x. Leukocytes are stained red, with increased influx of CD45 positive cells is seen at 90 minutes of shock.

HS TNF-α is produced in kidney and heart tissue

Leukocyte-endothelial interactions are often initiated by pro-inflammatory cytokines. These cytokines are either produced locally in the organ where the inflammatory response is taking place or systemically released, for example, by the liver upon exposure to bacterial products. TNF-α gene expression analysis showed a significant TNF production in kidney and heart at 90 min after initiation of HS compared to sham shock mice (Figure 6). Interestingly, in the heart, this induction of TNF-α was not paralleled by leukocyte influx (data not shown).

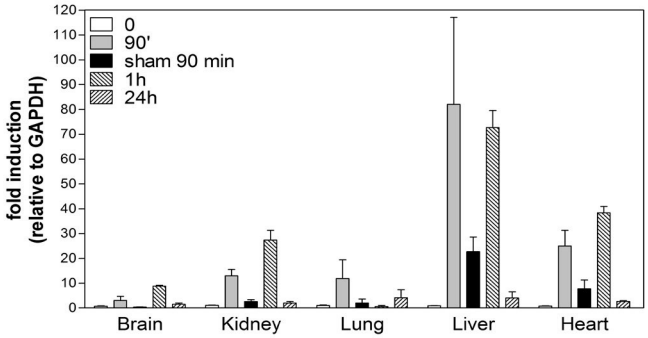

Figure 6: Effect of hemorrhagic shock on TNF-α in tissue

Gene expression of TNF-α was determined in lung, liver, kidney, heart, and brain tissue by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold induction of TNF-α in comparison to the level of activity that was present in extracts of normal healthy organs. Data presented are the mean ± SEM of each group (n=5 in the 90′ HS group and 90′ sham shock, other groups n= 3). 90 minutes of HS was compared with 90 minutes of sham shock (sham 90′). $p < 0.05$ values are marked with *.

Discussion

The cellular and organ response to HS is complex and results in profound changes in gene expression and organ function [10]. The earliest effects of HS on microvascular endothelial cells and its causes remain poorly understood. In models of HS, ample data exist regarding the detrimental influx of leukocytes into organ parenchyma. Because endothelial cell activation is instrumental in leukocyte recruitment, we aimed to investigate the kinetics and organ specificity of microvascular endothelial cell activation during the early phase of HS. We showed that induction of inflammatory gene expression is an early event that occurs before resuscitation is instituted. Expression of all adhesion molecules was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial integrity genes CD31 and VE-cadherin were only affected in the liver, while activation of the hypoxia HIF-1α pathway was absent during the shock period.

To our knowledge, we are the first to report the earliest effects of HS on the more complex pattern of microvascular endothelial cell activation and its organ specificity. Although no data on these early endothelial responses have been reported before, Xu et al [8] showed that in liver and lung, P-selectin, and ICAM-1 were up-regulated 3 hours after resuscitation in a 90 min HS model. The early endothelial activation during HS found in our study is in accordance with reports of early activation of inflammatory signal transduction routes in HS. In a volume-controlled HS model in which 25% of blood volume was withdrawn, the proinflammatory p38 mitogen-activated protein kinase (MAPK) activity increased in the kidney 60 min after initiation of HS5. McCloskey et al observed that Jun N-terminal Kinase (JNK) activation is an early event in the liver. After 30 min of HS with a MAP of 25 mmHg they observed an increase of JNK which persisted throughout the duration of the 120-min experiment [6]. Although in these studies these activated kinases were not assigned to specific cell types in the organ, these signaling pathways are known to control endothelial adhesion molecules expression[11]. During the progression of HS condition, the cytokine driven mitogen activated protein kinase (MAPK) and nuclear factor kappa B (NFkB) intracellular signaling pathways are likely to become activated in the microvascular endothelium [12,13]. Subsequent signaling via leukocyte integrin–endothelial immunoglobulin superfamily members is implied to take place in microvascular segments in which cell-cell interactions are most prominent. This signaling relays via small GTPase and p38 MAPK [14,15]. In an early stage of hemorrhage, changes in shear stress may furthermore affect endothelial activation in those microvascular beds where autoregulatory arteriolar control of blood flow cannot be sufficiently controlled [16]. Because most the studies on endothelial responses to inflammatory cytokine and shear stress changes have been performed in in vitro culture systems, and endothelial cells throughout the vascular tree are phenotypically heterogenic, the exact nature of signaling pathways leading to organ specific shock-related endothelial activation needs to be identified.

The expression of endothelial adhesion molecules is essential for endothelial-leukocyte interaction [3]. After activation of the endothelium, E-selectin is synthesized by de novo protein synthesis. Its expression on the endothelial cell membrane is induced a few hours after TNF-α stimulation of endothelial cells in culture [17]. We showed that E-selectin is up-regulated at mRNA and protein level within the first 90 min after initiation of HS in the kidney, lung, and the brain.

Of note are the organ-specific patterns of endothelial activation, which can theoretically form the basis for an organ-specific leukocyte recruitment process. Indeed, the early endothelial up-regulation of adhesion molecules was paralleled by early leukocyte recruitment in the liver, kidney, and lung. In contrast, the microvascular endothelium in the brain and the heart responded to HS with an increase in different adhesion molecule expression, but no leukocyte recruitment was seen in these organs. A similar organ heterogeneity in leukocyte recruitment was reported by Song et al [18], who showed an increase in lung neutrophil count after 1 h, being maximal at 4 h after HS, without any change in myocardial neutrophil counts. A more detailed study on chemokine and cytokine expression within the different organs combined with infiltrating leukocyte subset typing may shed light on this organ specificity of leukocyte recruitment.

Cellular hypoxia is considered to be an important mediator of MODS following HS [19]. During hypoxic conditions HIF-1α accumulates in the cell and forms a stable heterodimer with HIF-1β, where it translocates to the nucleus afterwards. HIF-1α can also be regulated at the transcriptional level as demonstrated in tumor models [20,21]. In a rat model of permanent focal ischemia of the brain, Bergeron et al [22] showed that mRNA of HIF-1α was significantly increased after 7.5 h of vascular occlusion. VEGF-A is a downstream target gene of HIF-1α that is primarily regulated at transcriptional level and a major controller of vascular permeability and angiogenesis. Under hypoxic conditions up-regulation of VEGF-A by HIF-1α occurs within minutes [23]. However, in our model, we did not see any significant induction of mRNA of HIF-1α and VEGF-A during the HS phase, nor did we see an increased nuclear localization of HIF-1α after 90 min of HS. This may imply that either the duration or the severity of hypoxia in the early HS phase is too short or too minor, respectively, to activate the HIF-1α system. On the other hand, Koury et al [24] showed that in a pressure-controlled HS model in the rat the HIF-1α level as measured by western blotting was increased in the ileac mucosa after 90 min of HS accompanied by a MAP of 30 mmHg. Although Hierholzer et al [25] observed an increase in HIF-1α DNA binding activity of 3.2 fold in the lung after a 40 mmHg MAP shock period of 2.5 h, no increased HIF-1α activation in livers of animals subjected to 40 mmHg MAP HS for 60 min was found [26]. Our data and those reported by others therefore suggest that although cellular hypoxia may play a role, it is not necessarily a key factor in the up-regulation of inflammatory genes in the early phase of HS.

Tumor necrosis factor is a pro-inflammatory cytokine, with the primary target including vascular endothelial cells. When TNF-α is administered to humans, it produces fever, inflammation, tissue destruction, and, in some cases, shock and death [27]. Effects on endothelial cells include protein independent changes in cell shape and motility as well as induction of proteins that regulate other parameters of the inflammatory response such as vasoregulation, leukocyte adhesion, leukocyte activation, and coagulation [13,28]. As such, TNF-α may be implicated in organ dysfunction in HS. Liu and Dubick [29] showed in rats with a HS to a MAP of 50 mmHg during 60 min that the mRNA encoding for TNF-α was up-regulated in ileum, kidney, liver and skeletal muscle. Serum TNF-α was increased in a mouse model already after 30 min after initiation of shock [12]. Combined with our increase in mRNA TNF-α levels in the various organs reported here, these studies indicate an early TNF-α response in HS. However, a direct

relation between local TNF-α production and early activation of endothelial cells with respect to leukocyte adhesion cannot be easily inferred. Additional experiments, with induction of HS in TNFR1/R2 knock out mice, need to be performed to get a more detailed view on the role of TNF on early endothelial cell activation in our HS model.

We showed that the combination of surgical instrumentation and anesthesia by itself strongly affected endothelial cell activation status. The administration of anesthetics may contribute to endothelial activation by exerting depressive effects on respiratory and cardiovascular functions, or by direct cellular actions. There also may be an effect associated with the use of heparin which is administered to maintain blood flow through the catheter [30]. Previous studies showing early increased pro-inflammatory IL-6 cytokine expression in mice that underwent a sham shock procedure [31,32], corroborate our findings of sham shock group proinflammatory responses [33].

In summary, our study revealed an early and organ-specific endothelial cell activation pattern during HS that occurred before resuscitation and was not per se hypoxia-driven. The early endothelial activation found in this study suggests that a temporal therapeutic window exists which can be used to attenuate endothelial cell activation at an early stage during resuscitation to prevent neutrophil sequestration in organs and subsequent multiple organ dysfunction syndrome. To identify potential targets for early therapeutic interference, we will, in future studies, asses complex kinase activity profiles [34] at different points after HS induction and resuscitation.

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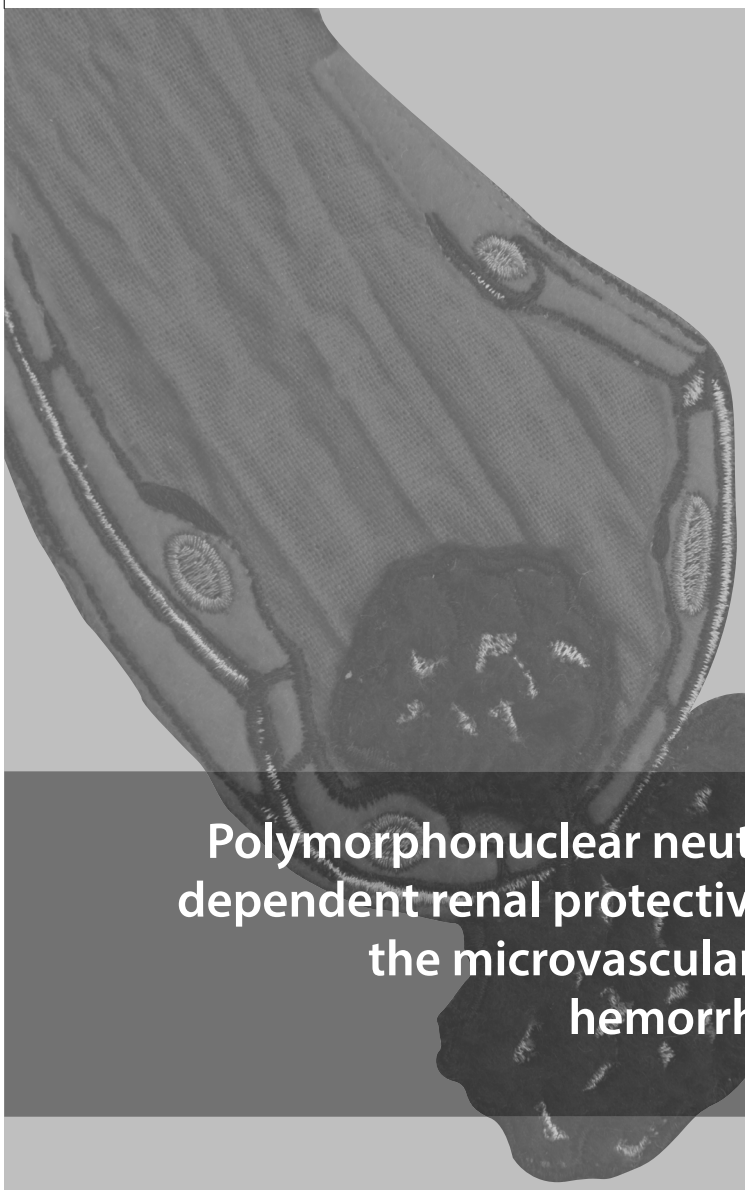

Chapter 3

Polymorphonuclear neutrophils play a time dependent renal protective role in activating the microvascular endothelial cell in hemorrhagic shock in mice

F.M. Wulfert, M. van Meurs, R.M. Jongman, J. Moser, M. Houwertjes, P. Heeringa, M.M.R.F. Struys, J.G. Zijlstra, G. Molema

Work in progress

Abstract

Introduction

Acute kidney injury in patients following hemorrhagic shock is a major determinant of morbidity and mortality. Endothelial-neutrophil interaction is supposed to be a principal cellular mediator of tissue damage. We hypothesized that depletion of neutrophils would prevent endothelial activation, influx of monocytes and macrophages and thereby attenuate acute kidney injury.

Methods

Male anesthetized mice were subjected to controlled HS with a MAP of 30 mmHg during 90 minutes followed by resuscitation with 6% hydroxyethyl starch. Mice were sacrificed before HS induction (control), after 90 minutes of HS, and 1, 8, and 24 hours after resuscitation. In selected animals, neutrophil depletion was established by i.p. injection of monoclonal rat anti-murine neutrophil antibody 24 hour prior to HS induction. mRNA of adhesion molecules (E-selectin, P-selectin, VCAM-1 and ICAM-1) measured in kidney by real time RT-PCR and protein expression of ICAM-1 ELISA were compared to expression in non-treated counterparts and controls.

Results

The role of PMNs in endothelial activation measured by adhesion molecules shows a biphasic pattern in hemorrhagic shock and resuscitation. In the early pre-resuscitation phase activation is inhibited by PMN depletion. After resuscitation PMN depletion has no significant effect on endothelial cell activation, moreover PMN depletion seems to compromise renal function.

Conclusion

In the early phage PMN are detrimental while in the later stage PMN are protective.

Introduction

Acute kidney injury in patients following hemorrhagic shock is a major determinant of morbidity and mortality [1,2]. Increased microvascular permeability is thought to be a main cause of organ damage. This is paralleled by the influx of neutrophils, which causes further detrimental effects [3]. Although the neutrophil is supposed to be a principal cellular mediator of tissue damage, the role of neutrophils in the exact nature of cellular and molecular processes and kinetics that cause MODS is largely unknown. The interaction between neutrophils and endothelium plays a pivotal role in facilitating and possibly directing the migration of neutrophils into tissues [4]. Migration to a site of injury mainly occurs in post-capillary venules and is mediated by the tightly controlled expression of chemokines and adhesion molecules by both the vascular endothelium and the neutrophils. The adhesion molecules P-selectin and E-selectin have a crucial role in the adherence of neutrophils to the endothelial cell layer while intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are important for the next step, firm adhesion. Firm adhesion is followed by migration of leukocytes from the vasculature into the site of inflammation. After migration into the tissue, neutrophils release proteolytic enzymes, and rapid production of reactive oxygen and nitrogen species augments the destructive effects [5,6]. Furthermore, during migration into inflamed tissue, PMN release granule proteins, which direct monocytes and macrophages to the sites of inflammation and stimulate their ability to release cytokines and chemokines [7].

Previously we have shown that increased expression of adhesion molecules is an early event already occurring 30 minutes after induction of hemorrhagic shock [8]. Furthermore we observed that endothelial cell activation was paralleled by leukocyte influx [8]. Based on these findings that hemorrhagic shock rapidly activates endothelial cells toward a pro-inflammatory status leading to neutrophil – endothelial cell interaction and considering the possible detrimental effects of this process, we hypothesized that depletion of neutrophils would prevent additional endothelial activation, influx of monocytes and macrophages and thereby attenuate acute kidney injury. To study this hypothesis, we depleted neutrophils prior to the induction of hemorrhagic shock in mice, and investigated its consequences for endothelial adhesion molecule expression and kidney function.

Materials and methods

Animals

Eight- to 12-week-old C57BL/6 male mice (20-30 g) were obtained from Harlan Nederland (Horst, the Netherlands). Mice were maintained on mouse chow and tap water ad libitum in a temperature-controlled chamber at 24°C with a 12-h light/dark cycle. All procedures performed were approved by the local committee for care and use of laboratory animals and were performed according to strict governmental and international guidelines on animal experimentation.

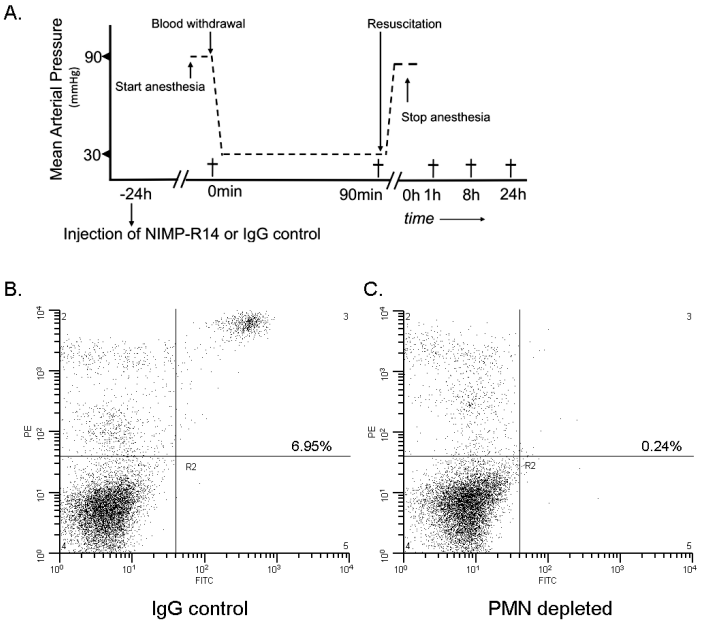

Figure 1: Study set up and effects of anti-NIMP Ab respectively control IgG antibody treatment on neutrophil counts in peripheral blood

A: Mice were treated with a neutrophil depleting antibody or control antibody 24 hours prior to indazn of monoclonal rat IgG (B.), or rat anti-murine neutrophil antibody (C.), whole blood samples were subjected to flow cytometric analysis to confirm neutrophil depletion as described in *Materials and Methods*.

Hemorrhagic shock model and neutrophil depletion

The hemorrhagic shock model was performed as described before (figure 1a) [8]. Briefly, mice were anesthetized with isoflurane (1.4%), N_2O (66%), and O_2 (33%). Throughout the experiment, mice were breathing spontaneously. The left femoral artery was cannulated with polyethylene tubing with an internal diameter of 0.28 mm and an external diameter of 0.61 mm for monitoring mean arterial blood pressure (MAP), blood withdrawal, and resuscitation. Hemorrhagic shock was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg within 15 to 30 min. Blood was collected in a heparinized 1-mL syringe. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain MAP at 30 mmHg during this period. Some mice were killed 90 min. after a MAP of 30 mmHg was achieved. We resuscitated additional groups of mice after 90 min of HS with 6% hydroxyethyl starch 130/0.4 (Voluven; Fresenius-Kabi, Bad Homburg, Germany), with two times the volume of the blood withdrawn. After resuscitation the canula was removed from the femoral artery and the artery and wound were sutured. Resuscitation and suturing time was approximately 30 minutes, after which anesthesia was discontinued. One, 8, or 24 h after recovering from anesthesia, these mice were sacrificed under anesthesia (figure 1). Control mice were left untreated and received anesthesia only before sacrifice. Sham shock mice underwent instrumentation and were kept under anesthesia for 90 minutes, however, no blood was withdrawn.

Neutrophil depletion was established by i.p. injection of 0.5 mg monoclonal rat anti-murine neutrophil antibody NIMP-R14 (Hycult Biotech, Uden, the Netherlands) in 0.75 ml NaCl 0.9%, 24 hour prior to HS induction, as previously described [9]. The control groups received 0.5 mg of rat IgG (Sigma-Aldrich, St Louis, MO) in 0.75 ml PBS. Both compounds, rat IgG, and NIMP-R14 were tested for endotoxin contamination with the Limulus Amebocyte Lysate (LAL) test and found to contain low endotoxin levels of 0.319 EU/ml (IgG) respectively 0.213 EU/ml (NIMP-r14). Groups consisted of 5 or 6 animals. Kidneys were harvested at the indicated time points, snap-frozen on liquid nitrogen, and stored at -80°C until analysis.

Confirmation of neutrophil depletion

Neutrophil depletion was assessed in a selection of animals by flow cytometric analyses. Whole blood samples were collected 24 hours after i.p. injection of monoclonal rat anti-murine neutrophil antibody or rat IgG. For flow cytometry 500 µl buffer (0.15M NH_4Cl with 10mM $NaHCO_3$) was added to 70 µl of whole blood to cause lysis of the red blood cells, after which cells were incubated with primary Abs CD11b-PE (BD Biosciences, Breda, the Netherlands) and Ly6G-FITC (BD Biosciences) for 60 min. on ice. After washing three times with PBS supplemented with 2% fetal calf serum, cells were fixed in 2% paraformaldehyde. Cells were analyzed on a Calibur (BD Biosciences) flow cytometer using CellQuest software.

Gene Expression analysis by quantitative RT-PCR

Total RNA was extracted from 20 * 5 µm-thick kidney cryosections and isolated using the RNeasy Mini Kit (Qiagen, Westburg, Leusden, The Netherlands), according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoreses and consistently found to be intact. RNA yield (OD260) and purity (OD260/OD280) were measured by ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitative PCR amplifications were performed according to manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Applera Nederland, Nieuwerkerk a/d IJssel, The Netherlands). The Assay-on-Demand primers (ABI systems, Foster City, USA) used in the PCR reactions included housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm99999915 _g1), mP-selectin (assay ID Mm00441295_m1), mE-selectin (assay ID Mm00441278_m1), mVCAM-1 (assay ID Mm00449197_m1), mICAM-1 (assay ID Mm00516023_m1) and mTNF-α (assay ID Mm00443258_m1). Duplicate real time PCR analyses were executed for each sample, and the obtained threshold cycle values (CT) were averaged. According to the comparative CT method described in the ABI manual, gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the ΔCT value. The average, relative mRNA level per sample was calculated by 2- ΔCT.

Quantification of ICAM-1 protein levels in kidney by ELISA

ICAM-1 protein levels in kidneys from healthy and diseased mice were evaluated in duplicate using an ELISA kit (R&D Systems Inc. Minneapolis, MN) according to the manufacturer's instructions. For this 30 * 5 µm kidney slices were homogenized in 50mM Tris-HCl buffer (pH

7.5), containing 150mM NaCl and proteinase inhibitor cocktail, and centrifuged at 13,000g for 15 min. The amount of protein was determined by DC Protein Assay (Biorad, Hercules, CA) according to the manufacturer's instructions. ICAM-1 levels were normalized for total protein concentrations.

Cytokine analysis
TNF-α level in plasma was analyzed by ELISA according to the manufacturer's protocol (CytoSet, BioSource, Camarillo, CA).

Blood biochemistry
Plasma was collected by punction of the aorta at the time of sacrifice. Blood urea nitrogen (BUN) analyses were performed by enzymatic degradation assay (Roche, Woerden, The Netherlands). Plasma creatinine levels were measured by an enzymatic colorimetric method using Creatinine plus (Roche).

Statistical analysis
All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). We used two-tailed hypothesis testing with all tests. Several of the variables under study may violate the assumptions of parametric tests, so the results of the statistical tests were confirmed using nonparametric equivalents. We used two-way repeated measures ANOVA (timepoint × treatment) to compare the PMN depleted en PMN competent mice. Differences were considered to be significant when $P < 0.05$.

Results

Severe neutropenia is induced by administration of anti-NIMP antibody

FACS analyses of blood samples from a selection of mice 24 hours after intraperitoneal injection of anti-NIMP antibody respectively rat IgG confirmed that neutrophils were successfully depleted in the anti-NIMP treated mice (figure 1b).

*Minor upregulation of VCAM-1 and ICAM-1 24 hour after administration of anti-PMN antibody compared to contro*l

The effect anti-PMN antibody administration on endothelial activation was assessed by mRNA measurement of adhesion molecules 24 hours after the injection of anti PMN antibody and before installment of hemorrhagic shock. In the kidney, mRNA levels of VCAM-1, and ICAM-1 were statistically significantly up-regulated at 24 hour after injection anti-PMN antibody compared to non-treated controls (figure 2), though the upregulation was minor. Of note is that control rat IgG antibody also showed significant up-regulation at 24 hour compared to non-treated controls. As preparations were found to contain low endotoxin levels of 0.319 EU/ml (IgG) respectively 0.213 EU/ml (NIMP-r14), it is not likely that this regulation was caused by contamination of the protein preparations with endotoxin.

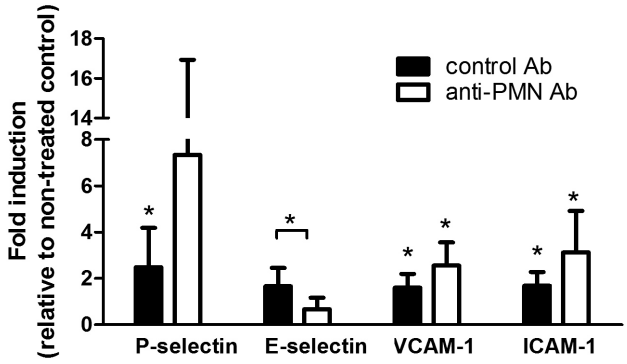

Figure 2: Effects of Ab treatment on mRNA levels of adhesion molecules in the kidney

In the kidney, antibody treatment exerted minor effects on mRNA levels of adhesion molecules. The kidney mRNA levels of adhesion molecules analyzed by quantitative RT-PCR using GAPDH as housekeeping, were normalized to their respective levels in RNA isolates of healthy organs obtained from untreated control mice. Mean values ± SD of 6 mice per group, * P <0.05.

Renal endothelial cells are 'primed' during the shock period yet fully develops after resuscitation

During the hemorrhagic shock period without any further manipulation of the animals, a slight, statistically not significant upregulation of all four major adhesion molecules was observed. The resuscitation procedure, however, in which hydroxy-ethyl starch was given intravenously to allow MAP to get back at 80 mmHg, strongly induced the expression of the cell adhesion molecules within 1 hour after resuscitation. Strongest induction was observed for P-selectin, showing a 131 fold increase compared to control mice. E-selectin showed an increase of 64,

while ICAM-1 and VCAM-1 showed a 44 respectively 30 fold increase compared to control. 24 hour after resuscitation their expression had returned to normal values (figure 3).

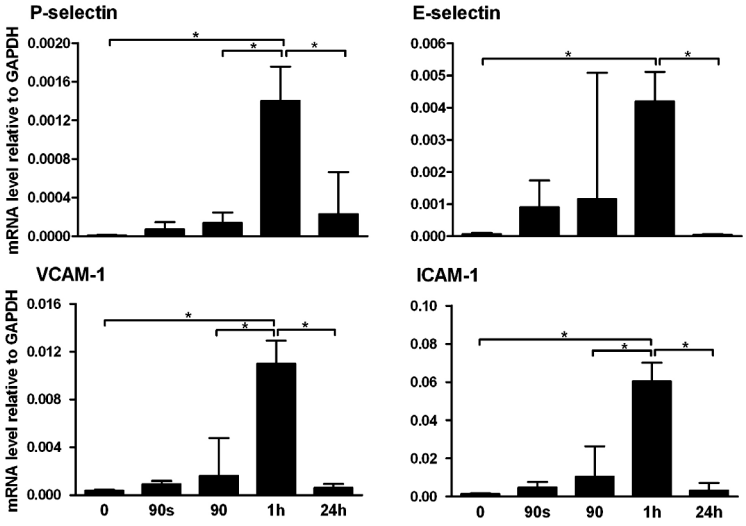

Figure 3: Gene expression of P-selectin, E-selectin, VCAM-1, and ICAM-1 is strongly upregulated in kidneys of mice subjected to hemorrhagic shock followed by resuscitation.

Mice were subjected to blood withdrawal to a mean arterial pressure of 30 mm Hg for 90 min, after which they were resuscitated with Voluven as described in *Materials and Methods*. mRNA levels shown are relative to GAPDH as housekeeping gene and were determined by quantitative RT-PCR as described in Materials and Methods. Mean values ± SD of 6 mice per group, * $P<0.05$.

Effects of neutrophil depletion on renal endothelial activation during shock and after resuscitation

During the hemorrhagic shock period, neutrophil depletion attenuated the activation status of the endothelial cells in the kidney, as witnessed by significant less induction of E-selectin, VCAM-1 and ICAM-1 at 90 minutes after induction of hemorrhagic shock (figure 4). Neutrophil depletion did not rescue resuscitation related endothelial activation (figure 4). Contrary, PMN competent and PMN depleted mice showed a significant induction of adhesion molecules after resuscitation, suggesting that activation of endothelial cells is only partly dependent of neutrophil activation.

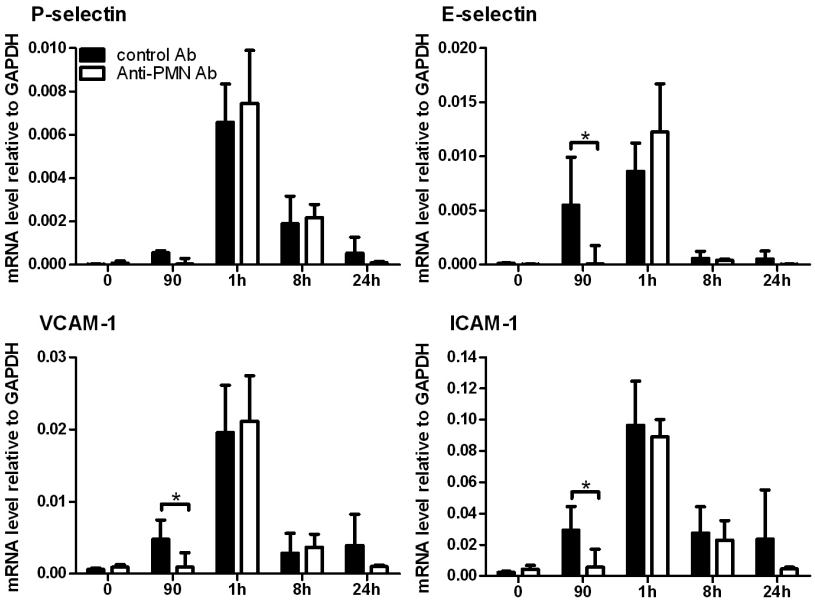

Figure 4: Neutrophil depletion affected endothelial priming during HS, after resuscitation with hydroxyethyl starch no differences between neutrophil competent and neutrophil depleted mice were seen.

Mice were treated with a neutrophil depleting antibody or control antibody 24 hours prior to induction of hemorrhagic shock by blood withdrawal to a mean arterial pressure of 30 mm Hg for 90 min. Some mice were resuscitated with Voluven as described in Materials and Methods. mRNA levels shown are relative to GAPDH as housekeeping gene and determined by quantitative RT-PCR as described in *Materials and Methods*. Mean values ± SD of 6 mice per group, * $P<0.05$.

No enhanced expression of ICAM-1 protein in neutrophil depleted mice

Previously it was demonstrated in an in vitro model that during migration into tissue PMN released granule proteins enhance ICAM-1 expression on endothelium, resulting in enhanced adhesion of monocytes [7,10]. We therefore analyzed the levels of this protein in our model. During hemorrhagic shock no difference in expression was observed between control and hemorrhagic mice and between IgG and anti-NIMP Ab treated mice. At one hour after resuscitation there was increased expression of ICAM-1 in both groups. At 24 hours after resuscitation a decrease to normal levels of ICAM-1 protein expression was seen in neutrophil depleted compared to PMN competent mice (figure 5).

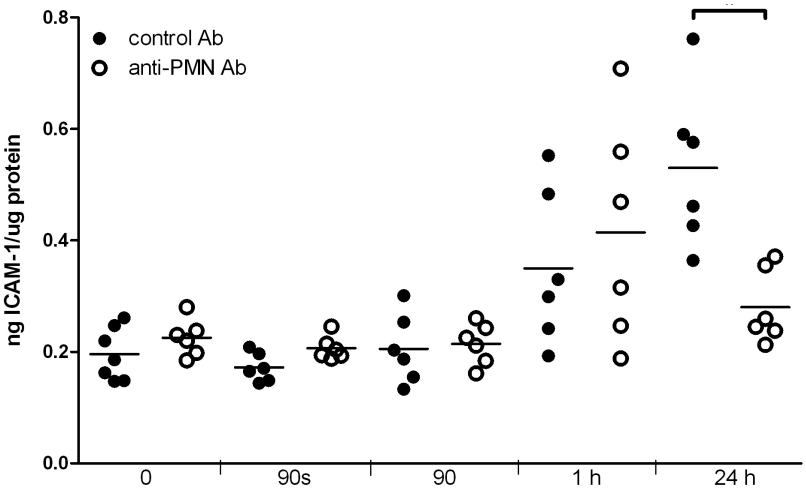

Figure 5: Effects of PMN depletion on ICAM-1 protein expression in kidney during hemorrhagic shock and resuscitation.

Mice were treated with a neutrophil depleting antibody or control antibody 24 hours prior to induction of hemorrhagic shock by blood withdrawal to a mean arterial pressure of 30 mm Hg for 90 min. Some mice were resuscitated with Voluven as described in Materials and Methods. ICAM-1 protein levels in kidney were measured by ELISA in neutrophils depleted (•) and in control antibody treated (o) mice. Values are means ±SD; n =6. *P < 0.05.

Tumor necrosis factor (TNF)-α production attenuated in neutrophil depleted mice during the hemorrhagic shock period but not after resuscitation

TNF-α is a pro-inflammatory cytokine, which affects endothelial cells in several ways, as it affects the expression of adhesion molecules, chemokines and cytokines, as well as contributes to endothelial permeability. We measured TNF-α mRNA in kidney and TNF-α in plasma (figure 6). PMN depletion was associated with lower local TNF-α production in kidney to lower systemic TNF-α release during hemorrhagic shock. This protective mechanism was lost during resuscitation, since1 hour after resuscitation TNF-α in both kidney tissue as well as in the systemic circulation was higher in neutrophil depleted mice than in control mice.
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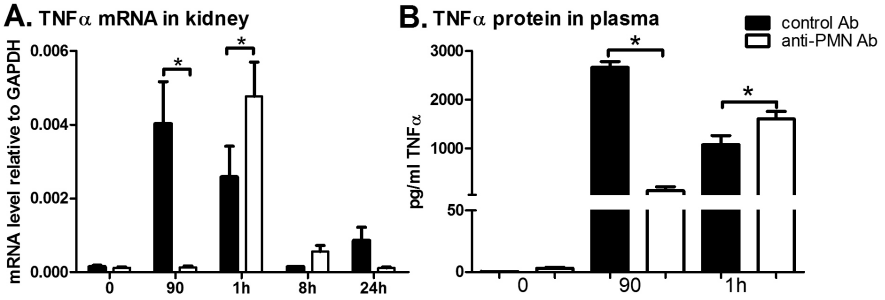

Figure 6: Effects of PMN depletion on pro-inflammatory cytokine TNF- α in kidney and plasma during hemorrhagic shock.

Mice treated with a neutrophil depleting antibody or control antibody 24 hours prior to induction of hemorrhagic shock by blood withdrawal to a mean arterial pressure of 30 mm Hg for 90 min, after which they were resuscitated with Voluven. BUN and Creatinine levels were measured in plasma described in *Materials and Methods*.

A. Gene expression level of TNF- α measured in kidney of control antibody (black bar) and neutrophil depleted antibody (white bar) treated analyzed by quantitative real-time polymerase chain reaction using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene.

B. Concentrations of TNF- α measured in plasma of control antibody (black bar) and neutrophil depleted antibody (white bar) treated mice. Mean values ± SD of 6 mice per group, * P<0.05.*

Temporally altered kidney function after neutrophil depletion and hemorrhagic shock

We observed no differences in BUN and creatinine levels at time point zero between neutrophil depleted, IgG treated and non treated control mice, suggesting that neutrophil depletion itself had no evident effect on kidney function (data not shown). Hemorrhagic shock caused a significant increase in serum creatinine irrespective whether PMN were present or absent in the mice, which normalized in both groups after resuscitation. A significant, but temporary increase in serum BUN concentration was observed at 8 hour after resuscitation in the neutrophil depleted mice, which was not observed in the IgG treated mice (figure 7).

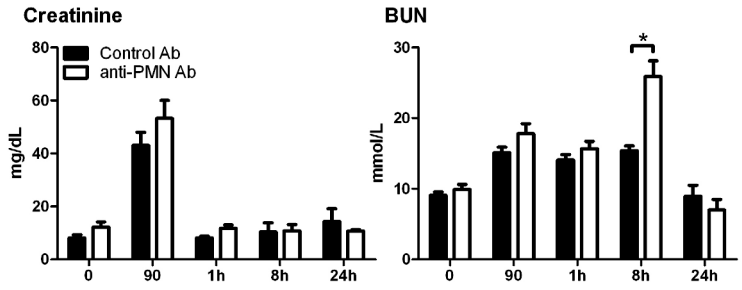

Figure 7: Creatinine and BUN levels represent kidney function in PMN depleted and competent mice during and after HS.

Mice treated with a neutrophil depleting antibody or control antibody 24 hours prior to induction of hemorrhagic shock by blood withdrawal to a mean arterial pressure of 30 mm Hg for 90 min, after which they were resuscitated with Voluven. BUN and Creatinine levels were measured in plasma described in *Materials and Methods*. Mean values ± SD, *P<0.05.*

Discussion

Multi organ failure and especially acute kidney injury following hemorrhagic shock is, despite advances in supportive care, still a major cause of late morbidity and mortality. The PMN is supposed to be a principal cellular mediator of tissue damage, although the exact nature of molecular and cellular processes and kinetics that guide neutrophil influx are largely unknown. The aim of this study was to determine whether depletion of PMN prevents the up-regulation of adhesion molecules, which are essential for leukocyte infiltration and the subsequent inflammatory response in HS. We showed that in neutrophil depleted mice during hemorrhagic shock early endothelial activation is attenuated. However, after resuscitation no difference between neutrophil depleted and PMN competent mice was seen. Moreover, late activation of endothelial cells, after resuscitation, was independent of the presence or absence of PMN. Depletion of PMN had no effect on kidney function. During hemorrhagic shock an increase in creatinine was observed in both PMN depleted and PMN competent mice. BUN a marker of kidney function was temporally increased in the PMN depleted mice only, indicating that PMN might be protective.

PMN play a central role in the early immune response. Via release of granule proteins monocytes and macrophages are activated and directed to inflamed tissue [7]. Fan et al showed in a hemorrhagic shock resuscitation model that TNF-α stimulation of the PMN results in the production of reactive oxygen species (ROS) via neutrophil NAD(P)H oxidase activation [11]. TNF-α and ROS are an important determinant of the NF-κB activation and ICAM-1 expression of the endothelial cell [12]. This suggest that neutrophils play an important role in the upregulation of ICAM-1 via NF-κB [12]. Our results corroborate these findings since 90 minutes after hemorrhagic shock, the mRNA expression of ICAM-1 in the PMN depleted mice was less compared to IgG treated mice. After resuscitation no differences in ICAM-1 expression protein in plasma and mRNA in kidney between neutrophil depleted and IgG treated mice were observed. Thus NF-κB induced ICAM-1 expression after resuscitation is irrespective of neutrophil NAD(P)H oxidase activation. Kin et al showed in an ischemia reperfusion model in rats that neutrophil depletion by means of anti-PMN serum led to a reduction in TNF-α in plasma 30, 60, and 180 min after reperfusion [13]. In our hemorrhagic shock model, we did observe a reduction in TNF- α during hemorrhagic shock, however, we did not observe a reduction in TNF- α after resuscitation. In contrast, TNF-α protein level in plasma was higher in the neutrophil depleted mice than in neutrophil competent mice 24 hours after resuscitation. This latter observation is similar to the one reported by Wu et al., who showed in an endotoxemia model that neutrophil depletion by GR-1 antibody led to higher expression of TNF- α, thereby concluding that there is a protective role for neutrophils in inhibiting LPS-induced TNF-α release [14]. Gresnigt et al observed that PMN are able to downregulate TNF- α production of human monocytes induced by endotoxemia, while IL-10 remains unaffected [15]. Our data corroborates these results. The above observations shows that role of the PMN in the development of MODS is complex. PMN possess a predominant role in mediating acute inflammation. In human they are the most abundant type of white blood cell, they are the first cells migrating to the site of injury and they modulate monocytes and macrophage function [7]. PMN depletion is, however, a "double-edged-sword", because polymorphonuclear leukocytes are an essential component of the innate immune system. Thus although PMNs may be a

significant contributing factor to organ dysfunction because of an exaggerated inflammatory response by release of reactive oxygen species after migration into tissue facilitated by adhesion molecules, they also have an important role in anti-inflammatory reactions [16,17]. Our data supports the double edge role of PMN since during HS PMN competent mice showed increased endothelial cell activation while after resuscitation PMN competent mice showed reduced endothelial activation compared to PMN incompetent mice. Thus during the shock period the PMN have detrimental effects on endothelial activation while after resuscitation the PMN has protective effects.

In this study we used anti-NIMP to deplete the PMNs. Other types of leukocytes such as monocytes are therefore still able to infiltrate the tissues. Macrophages release mediators such as pro-and anti-inflammatory cytokines and phagocytose microorganisms. This can explain why initially the expression of adhesion molecules is attenuated while later in time, after resuscitation, there are no differences in expression between PMN depleted and non-depleted mice. There is however, contradiction in the literature; while some authors demonstrate neutrophil independency of monocytes/macrophage migration [18] others demonstrate inhibition of monocytes/macrophage infiltration in neutrophil depleted animals[19]. Possibly the different methods used to prevent neutrophil migration may underlie the differences observed, while also the different disease models used can possibly contribute to the observation. While Henderson et al used an LFA-1 -/- knock out mice in their peritonitis model, Janardhan et al depleted neutrophils via an anti-neutrophil antibody prior to LPS injection. Both methods have their limitations, knock out mice might develop different phenotypic traits, while IgG antibodies engage the cellular immune system, which might lead to antibody dependent cell-mediated cytotoxicity [20]. Zhang et al observed that macrophages play an important role in recovery after AKI in an ischemia/reperfusion and diphtheria toxin mice model [21]. In our study the depletion of neutrophils might have inhibited the migration of macrophages and thereby preventing regeneration of kidney function. To investigate the role of macrophages in our model, flow cytometric analysis or immunohistochemical staining of macrophages are necessary followed by depleting macrophages, e.g., by treatment with antibodies or Clodronate, this will provide additional insight in the role of macrophages in endothelial responses in hemorrhagic shock.

Our study has several limitations. First, hydroxyethyl starch (HES) perse could affect the inflammatory response and organ function, in critically ill HES is associated with increased risk of AKI [22,23], however, use of HES to augment blood volume during surgery shows no indications of adverse effects [24]. The single effect of resuscitation cannot be measured because proper controls can not be constructed. Second, in humans the percentage of PMNs is 40%–75% of all leukocytes, in mice however, this percentage is much lower and thus effects of PMN on processes studied by us may be different from the PMN role in humans. Translating our data to the patient is therefore complex. Third, we acknowledge the fact that there is a controversy with regard to the validity of using creatinine and BUN as kidney dysfunction tests. Serum creatinine does not accurately reflect the glomerular filtration rate in critically ill patients with fluid shifts who are not in steady state. The increase in creatinine after 90 minutes of hemorrhagic shock seen in this study can be explained by dehydration through volume

depletion. BUN might also not be the best marker of kidney function. Levels of BUN increase during hypovolemia, as a result of less cardiac output and less renal blood flow, a so called prerenal cause of AKI. Fourth, hemorrhagic shock-induced organ failure is a multistep and dynamic process in time. In humans hypovolemia and hypotension leading to acute tubular necrosis is the most common cause of AKI. In our model late functional and histological impact was not awaited and thus we do not know if the model employed is severe enough to cause AKI. Mayeur 2011 et al observed acute renal failure and acute tubular necrosis (ATN) in mice subjected to 2-hrs HS with a MAP 35 mmHg [25] and thus in our model the duration of just 90 minutes of hemorrhagic shock might not be long enough to cause tubular necrosis. However, Mees et al observed in mice subjected to 90 min of HS with a MAP of 35 mmHg 48 hours after resuscitation, morphologic changes suggestive of ATN [26]. In our model we do see minor changes in tubules 8 and 24 hours after resuscitation following 90 min of HS suggesting that this model is severe enough to affect kidney function. Last, the use of an antibody to establish neutrophil depletion, although commonly used in preclinical research, is debatable because antibodies against neutrophils induce neutropenia but also cause blood flow cessation, and death via complement-dependent and independent mechanisms responses [27]. This may affect the outcome of our studies in a PMN depletion independent way. Furthermore, in our study we observed that control IgG pretreatment affected endothelial adhesion molecules expressed, which could be explained by a relatively the high protein load, the mice received via the rat IgG solution and IgG might interact in several ways with the immune system [20].

In summary, the role of PMNs in endothelial activation shows a biphasic pattern in hemorrhagic shock and resuscitation. In the early pre-resuscitation phase activation is inhibited by PMN depletion. This leads to the conclusion that in the early phage PMN are detrimental. After resuscitation PMN depletion has no significant effect on endothelial cell activation, moreover PMN depletion seems to compromise renal function. This leads us to the conclusion that in the later stage PMN are protective. Based on this pre-clinical study, techniques that remove PMN from the blood or inhibit their function, as has been performed in different patient categories [28] can not be recommended in patients suffering from hemorrhagic shock. Furthermore future studies are necessary to address the role of macrophages and other immune cells in the activation of endothelial cells in the development of AKI in post-hemorrhagic shock models to fully understand the kinetics and mechanism leading to AKI.

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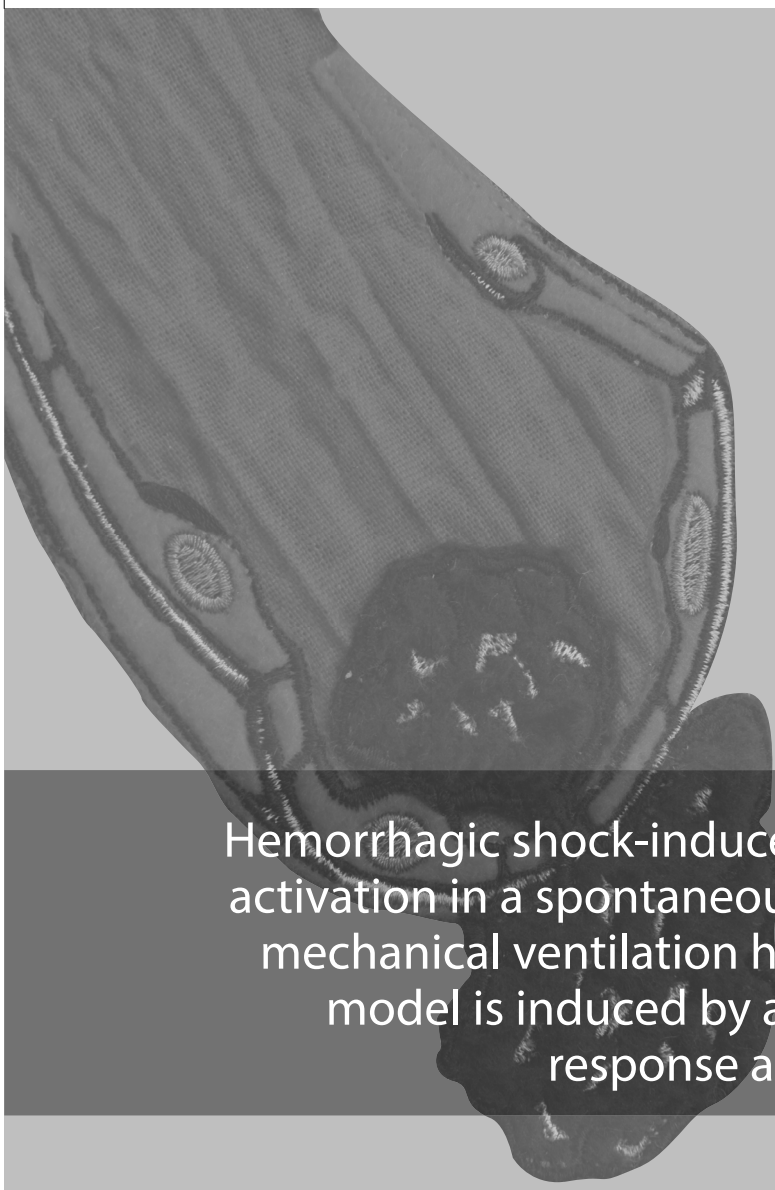

Chapter 4

Hemorrhagic shock-induced endothelial cell activation in a spontaneous breathing and a mechanical ventilation hemorrhagic shock model is induced by a proinflammatory response and not by hypoxia

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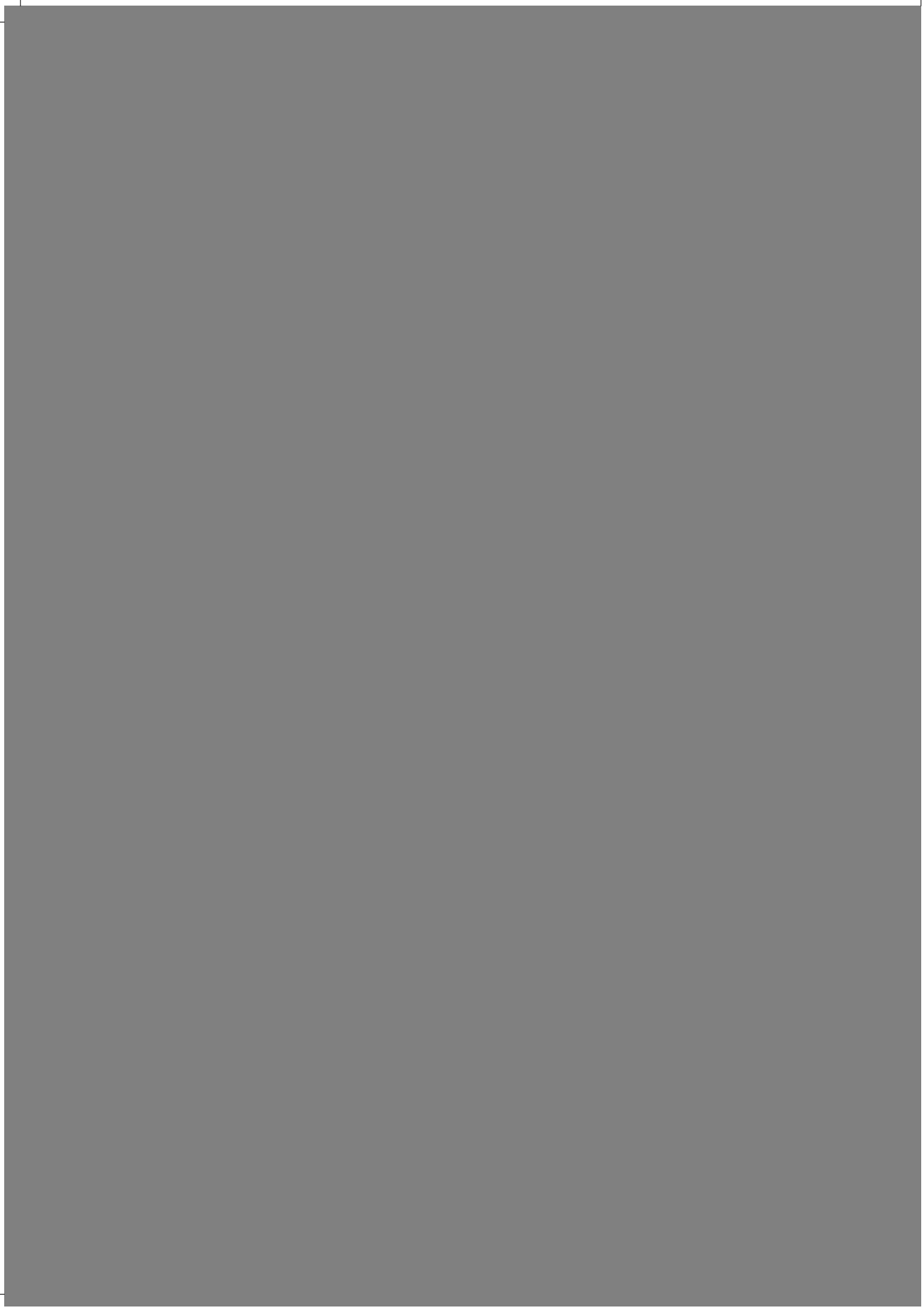

Abstract

Introduction

The interaction between neutrophils and activated endothelium is essential for the development of multiple organ dysfunction in patients with hemorrhagic shock (HS). Mechanical ventilation frequently is used in patients with HS. The authors sought to investigate the consequences of mechanical ventilation of mice subjected to HS on microvascular endothelial activation in the lung and kidney.

Methods

Anesthetized wild type C57BL/6 male mice were subjected to controlled hemorrhage; subgroups of mice were mechanically ventilated during the HS insult. To study the effect of acute hypoxia on the mice, the animals were housed in hypoxic cages. Gene expression levels was assessed by quantitative real-time polymerase chain reaction. Protein expression was assessed by immunohistochemistry and enzyme-linked immunosorbent assay.

Results

Ninety minutes after the shock induction, a vascular bed-specific, heterogeneous proinflammatory endothelial activation represented by E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1 expression was seen in kidney and lung. No differences in adhesion molecules between the spontaneously breathing and mechanically ventilated mice were found. Concentrations of the proinflammatory cytokines chemokine (C-X-C motif) ligand 1 (11.0-fold) and interleukin-6 (21.7-fold) were increased after 90 min of HS. Two hours of 6% oxygen did not induce the expression of E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1 in the kidneys and the lung.

Conclusions

Hemorrhagic shock leads to an early and reversible proinflammatory endothelial activation in kidney and lung. HS-induced endothelial activation is not changed by mechanical ventilation during the shock phase. Hypoxia alone does not lead to endothelial activation. The observed proinflammatory endothelial activation is mostly ischemiaor reperfusion-dependent and not related to hypoxia.

Introduction

MAJOR improvements in emergency medicine have led to decreased early deaths from hemorrhagic shock (HS) [1]. However, this renders patients vulnerable for secondary complications. A feared complication that can be caused by HS is multiorgan dysfunction syndrome. This syndrome causes severe morbidity and mortality and is a major challenge in critical care medicine. A systemic inflammatory response is considered the leading cause for the development of multiorgan dysfunction syndrome. Two failing organs are the lung and the kidney. Lung failure, the so called Acute Respiratory Distress Syndrome, and acute kidney injury are strongly associated with patient morbidity and mortality [2]. For symptoms of acute respiratory distress syndrome and acute kidney injury, patients are treated with mechanical ventilation and renal replacement therapy. The precise mechanisms leading to MODS after HS remain largely unknown. One of the proposed mechanisms is infiltration of neutrophils into the tissues, leading to significant organ damage through release of proteases and oxygen-derived radicals. The interaction between neutrophils and endothelium is essential for the migration of neutrophils into tissues [3]. This migration is regulated by adhesion molecules on both leukocytes and endothelium, including E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) [4]. We have recently shown that HS leads to early and organ specific pro-inflammatory microvascular endothelial activation [5]. This organ and microvascular bed specific endothelial activation is also seen in animal models subjected to septic shock, where it coincides with increased vascular leakage [6,7].

Hemorrhagic shock occurs frequently in the operating theatre as a result of difficult-to-control surgical bleeding. Patients with HS due to other causes including trauma or gastrointestinal bleeding require resuscitation and procedures to control the bleeding [8,9]. Therefore, patients with HS will be frequently intubated and mechanically ventilated to allow intervention procedures and warrant a patent airway and gas exchange [10]. Mechanical ventilation (MV) during conditions of hemorrhagic shock can act as a double-edged sword. Intubation and MV may protect organs from hypoxia and hypercapnia but also may initiate an inflammatory reaction [11] and induce pro-inflammatory activation in the lung and in distant organs [12,13].

To follow up on our previous observation of HS-induced microvascular endothelial priming during the shock phase5, our aim was to investigate the beneficial, neutral or harmful consequences of MV in mice subjected to HS on microvascular endothelial activation in the lung and kidney. Mechanical ventilation is started to prevent hypoxia and threatened oxygen delivery. HS by definition decreases oxygen delivery [14], yet the role of decreased oxygen availability in endothelial activation is not clear. To study the effect of a decreased oxygen delivery in vivo on endothelial cell (EC) activation we also evaluated endothelial proinflammatory adhesion molecule expression in hypoxic mice.

Materials and Methods

Animals

Eight- to 12-wk-old C57Bl/6 male mice (20–30 g) were obtained from Harlan Nederland (Horst, The Netherlands). Mice were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12:12-h light-dark cycle. All procedures were approved by the local committee for care and use of laboratory animals (DEC, University Medical Center Groningen, Groningen, The Netherlands) and were performed according to

national and international guidelines on animal experimentation.

Mouse shock model

The mouse HS model has been extensively documented elsewhere [1,15,16]. In short, mice were anesthetized with isoflurane 3.0% induction in N2 O (2 l/min) and O2 (1 l/min); after-anesthesia was continued with isoflurane (inspiratory, 1.4%), N_2O (66%), and O_2 (33%). The left femoral artery was canulated for monitoring mean arterial pressure (MAP), blood withdrawal, and resuscitation. HS was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain a pressure constant HS model with a MAP at 30 mmHg during this period. A subset of mice was resuscitated after 90 minutes of HS with 6% hydroxyethyl starch 130/0.4 (Voluven®; Fresenius-Kabi, Bad Homburg, Germany) at two times the volume of blood withdrawn and killed 24 h after HS induction. During sacrifice, blood was withdrawn via aortic puncture under isoflurane anaesthesia, and the kidneys and lung were excised, snap-frozen in metal cups on liquid nitrogen, and stored at –80°C until analysis.

Table 1. Sample Size of Different Experimental Groups

Time Point	Group	n
	Control	6
90 min HS	Spontaneous	6
	MV	7
24 h after HS	Spontaneous	5
	MV	5
2 h hypoxic cage	21% O_2	5
	10% O_2	5
	6% O_2	5

HS = hemorrhagic shock; MV = mechanical ventilation.

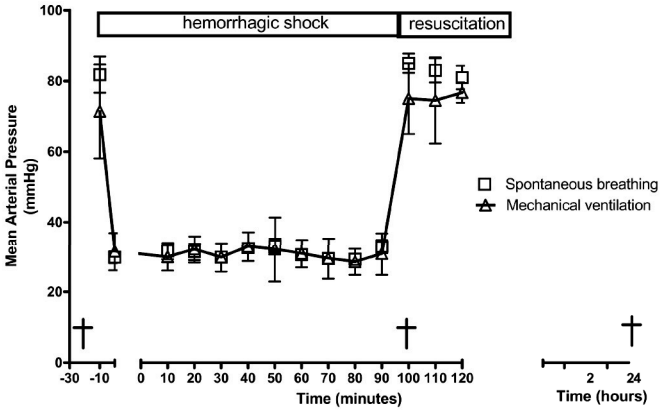

Figure 1: Study design and measured mean arterial pressure in mice during hemorrhagic shock in the presence or absence of mechanical ventilation.

Hemorrhage was induced by blood withdrawal as described in 'Materials and Methods'. Groups of mice were sacrificed (†) at the start of the experiment (control), or after 90 minutes of HS. Following 90 minutes of hemorrhagic shock, a subgroup of mice was resuscitated and sacrificed 24 hours after shock induction. Spontaneous breathing mice (■) were compared with mechanically ventilated mice (▲) . Data are expressed as mean ± SD, n>5 per group.

Mouse MV model
The mechanical ventilation model employed has been described previously [11,17]. In short mice were anesthetized with isoflurane inspiratory 3.0 % induction in N_2O (2 liter/min), and O_2 (1 liter/min). Animals were orally intubated under direct vision with an endotracheal tube (0.82 mm ID, 1.1 mm OD, length 25 mm). Endotracheal tube position was confirmed by bilateral chest excursions. Subsequently, animals were connected to the ventilator (MiniVent®; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Tidal volume was set at 180 µl; frequency was set at 150/min. All animals received 4 cm H_2O positive end-expiratory pressure. After intubation, anesthesia was continued with isoflurane (inspiratory, 1.4%), N2O (66%), and O_2 (33%). The anesthetic regimen between the spontaneously breathing and mechanically ventilated mice was the same. The investigators were not blinded to the treatment.

Arterial Blood Gas Analysis
Blood for blood gas analysis was withdrawn at three time points directly after shock induction via the arterial line, 90 min after shock induction just before sacrifice, and 24 h after shock induction via aortic puncture during anesthesia. Samples were analyzed immediately after collection on an ABL800 blood gas analyzer (Radiometer, Zoetermeer, The Netherlands).

Mouse hypoxia model
To examine the role of acute hypoxia on endothelial pro-inflammatory and hypoxia driven genes, a subset of mice was housed for 2 hours in respiratory cages to manipulate oxygen concentration. Oxygen concentration was set at 21%, 10%, and 6%, respectively. Hypoxia-exposed mice and unexposed control mice were killed during isoflurane anaesthesia, after which blood was withdrawn and kidneys and lung were harvested and handled as described in the mouse shock model section of Materials and Methods.

Gene Expression Analysis by Quantitative Real-time Polymerase Chain Reaction
RNA was extracted from 20 x 5-µm cryosections from kidney and mouse lung, and isolated using the RNeasy Mini Plus kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield and purity were measured by an ND-1000 Ultravioloet-Visual spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The Assay-on-Demand primers (ABI Systems, Foster City, CA) used in the polymerase chain reaction included the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (assay ID Mm99999915 _g1), E-selectin (assay ID Mm00441278_m1), ICAM-1 (assay ID Mm00516023_m1), VCAM-1 (assay ID Mm00449197_m1), tumor necrosis factor-α (TNF-α) (assay ID Mm00443258_m1), interleukin-6 (IL-6) (assay ID Mm00446190_m1), monocyte chemotactic protein-1 (MCP-1)(assay ID Mm00441242_m1), hypoxia-inducible factor-1α (HIF-1α) (assay ID Mm00468869_m1),

(vascular endothelial growth factor-A) VEGF-A (assay ID Mm00437304_m). Duplicate real-time PCR analyses were executed for each sample, and the obtained threshold cycle values (CT) were averaged. According to the comparative CT method described in the ABI manual, gene expression was normalized to the expression of the housekeeping gene, yielding the Δ CT value. The relative messenger RNA (mRNA) level was calculated by 2-Δ CT and per group averaged.

Cytokine analysis

TNF-α, interleukin (IL)-6, and chemokine (C-X-C motif) ligand 1 (CXCL-1) concentrations in plasma were analyzed by enzyme-linked-immunosorbent assay (ELISA) (TNF-α, IL-6, CytoSet, BioSource, CA; CXCL-1: ELISA-Kit, R&D Systems, Minneapolis, MN). Lower detection limits: 32 pg/ml for TNF-α; 160 pg/ml for IL-6; 160 pg/ml for for CXCL-1.

Localization of adhesion molecule expression using immunohistochemistry

Localization of CD31, E-selectin, VCAM-1, and ICAM-1 expression was determined in kidney by immunohistochemistry. Snap-frozen organs were cryostat cut at 5 µm, mounted onto glass slides, and fixed with acetone for 10 mins. After sections were dried, they were incubated for 45 mins at room temperature with primary rat anti-mouse antibodies recognizing CD31 (clone MEC13.3; Pharmingen BD Biosciences, Alphen aan de Rijn, The Netherlands), E-selectin (MES-1, kindly provided by Dr. D. Brown, UCB Celltech, Brussels
Belgium), and ICAM-1 (clone YN1/1.7; ATCC) in the presence of 5% fetal calf serum. After the sections were washed, endogenous peroxidase was blocked by incubation with H_20_2, O.1% in phosphate buffered saline for 20 mins. This was followed by incubation for 30 mins at room temperature with horseradish peroxidase conjugated secondary antibodies (rabbit anti rat-Ig, DAKO, Glostrup, Denmark). Conjugates were diluted 1:50 in PBS supplemented with normal mouse serum, 2%. Sections with isotype-matched controls and E-selectin antibodies were further incubated for 30 min at room temperature with horseradish peroxidase-conjugated goat antirabbit antibody (Southern Biotech Association, Birmingham, Alabama, USA) diluted 1:100 in phosphate buffered saline. Between incubation with antibodies, sections were washed extensively with phosphate buffered saline. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich Chemie, St.Louis, Missouri, USA) and sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands).

Statistical analysis

Statistical significance of differences was studied by means of unpaired independent Student's t-test or one-way ANOVA with post hoc comparison using Dunnett correction. First 90-min time points and 24-h time points HS were compared with HS combined with MV. When there were no differences, HS and MV at the same time points were pooled, for increased statistical power. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). We used two-tailed hypothesis testing with all tests. Several of the variables under study may violate the assumptions of parametric tests, so the results of the statistical tests were confirmed using nonparametric equivalents. We used a two-way repeated measures

ANOVA (group x time) to compare MAP during the shock phase between the spontaneously breathing and mechanically ventilated mice. Differences were considered to be significant when $p < 0.05$.

Results

During anesthesia, mice were intubated and mechanically ventilated or allowed to breathe spontaneously while blood was withdrawn to reach a MAP of 30 mmHg (table 1). No statistically significant differences in blood pressure were observed between the groups, neither during the shock phase nor during the post shock phase (figure 1). The initial blood pressure after induction in the spontaneous breathing group (mean 82 mmHg, SD 16.2) was not significantly different statistically from the blood pressure in the MV group (mean 71 mmHg, SD 13.8). Mice in all the groups remained normoxic and and did not became hypercapnic (table 2). Mechanically ventilated mice had increased metabolic acidosis at 90 min of HS compared with the spontaneously breathing mice with a statistically significant lower pH and lower base excess at 90 min after shock induction. This metabolic acidosis was resolved 24 h after the HS insult (table 2).

Table 2. Arterial Blood Gas Analysis of Mice that Were Only Instrumented (Instrumentation), and Mice 90 min and 24 h after Initiation of Hemorrhagic Shock Induction, in the Absence or Presence of Mechanical Ventilation

Time Point	Group	pH	$Paco_2$ (kPa)	Pao_2 (kPa)	BE (mM)	Lactate (mM)
Instrumentation	Spontaneous	7.31 ± 0.05	4.49 ± 0.58	28.8 ± 3.13	−8.7 ± 2.30	4.44 ± 1.19
	MV	7.35 ± 0.10	3.45 ± 0.83*	27.8 ± 2.49	−10.3 ± 3.9	5.79 ± 2.68
90 min	Spontaneous	7.40 ± 0.05	2.87 ± 0.61	27.8 ± 1.39	−10.6 ± 3.09	5.72 ± 0.93
	MV	7.24 ± 0.10*	3.11 ± 1.59	20.8 ± 7.47	−16.8 ± 4.67*	7.40 ± 1.2
24 h	Spontaneous	7.40 ± 0.05	2.85 ± 0.471	27.8 ± 4.23	−10.8 ± 3.03	5.40 ± 1.70
	MV	7.37 ± 0.04	3.23 ± 0.76	45.53 ± 15.46*	−10.3 ± 2.22	4.32 ± 0.84

Instrumentation: blood gas analysis directly after shock induction; 90 min: blood gas analysis directly before sacrifice at time point 90 min; 24 h: blood gas analysis directly before sacrifice at time point 24 h. Data are presented as mean ± SD

* $P < 0.05$ MV *vs.* spontaneous breathing.

BE = base excess; MV = mechanical ventilation; $Paco_2$ = partial pressure of arterial carbon dioxide; Pao_2 = partial pressure of arterial oxygen.

Ninety minutes after shock induction, an endothelial pro-inflammatory activation was observed, as reflected by increased E-selectin, VCAM-1, ICAM-1 mRNA expression levels (figure 2). After 24 h, the proinflammatory endothelial activation genes in both the MV and the spontaneously breathing group were back to baseline (figure 2). No differences between the spontaneous breathing and mechanically ventilated mice were found for E-selectin, VCAM-1, and ICAM-1 at 90 min or 24 h after shock induction. The lung and the kidney have different endothelial response patterns during HS. VCAM-1 mRNA levels were up-regulated in the lung after shock (figure 2d) but unchanged in the kidney (figure 2c). In the kidney and the lung, both mRNA of E-selectin and ICAM-1 were up-regulated after 90 min of HS (figure 2a, 2b, 2e and 2f).

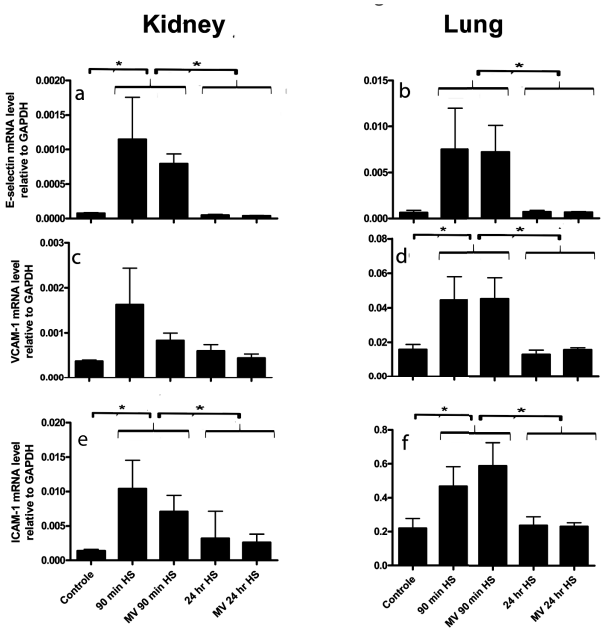

Figure 2: Kinetics of mRNA changes of endothelial cell adhesion molecules during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation.

Gene expression levels of E-selectin (a, b), VCAM-1 (c, d), and ICAM-1 (e, f) in kidney (a, c, e) and lung (b, d, f) were analysed by quantitative RT-PCR using GAPDH as housekeeping gene. Data are expressed as mean ± SEM, n>5, * p< 0.05.

To further extend our knowledge on microvascular bed specific differences of endothelial activation in HS, we examined these endothelial pro-inflammatory activation differences within the kidney by immunohistochemistry of different renal microvascular beds. The pan endothelial marker protein CD31 was expressed in all microvascular beds including arterioles, glomeruli, the peritubular vascular capillaries and venules in control mice (figure 3a) and did not change after shock induction with or without MV (figure 3b and 3c). In the kidney, E-selectin was absent in the control kidney, but was strongly up-regulated 90 min after shock induction in glomerular capillaries both with and without MV (figure 3d, 3e, 3f). In contrast in peritubular and arteriolar vascular beds, E-selectin could not be detected after shock induction (figure 3e and 3f). In control kidneys, constitutive ICAM-1 expression was observed in all vascular beds. In HS kidney, increased ICAM-1 expression was observed most prominently in the peritubular capillaries (figure 3h and 3i). Overall, no visible differences were found in the expression of adhesion molecules in the kidney related to MV.

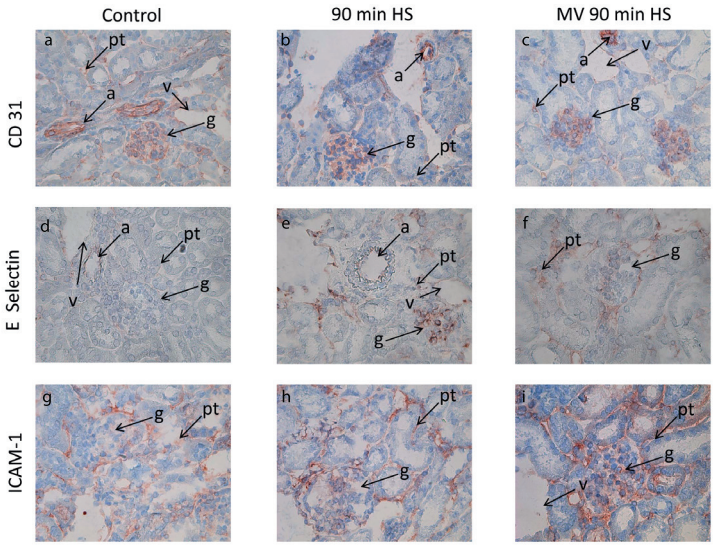

Figure 3: Endothelial marker gene CD31 and endothelial cell adhesion molecules E-selectin and ICAM-1 expression during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation. Immunohistochemical detection of CD31, E-selectin, and ICAM-1 in mouse kidneys. Staining was performed in healthy mouse tissue (a, d, g), after 90 minutes of HS (b, e, h), and after 90 minutes of Mechanical Ventilation in HS (c, f, i). Original magnification 200x. CD31 (a, b, c), E- selectin (d, e, f), and ICAM-1 (g, h, i) are stained red, while cells are stained blue. Specific renal microvascular beds are indicated by arrows: a = arteriole, g = glomerulus, pt = peritubular vasculature, and v = venule.

Endothelial pro-inflammatory activation can be caused by proinflammatory cytokines [18] and hypoxia [19]. First, we investigated the mRNA expression levels in lungs and kidneys of the pro-inflammatory cytokines TNF-α, IL-6 and MCP-1 in time. No differences between the spontaneous breathing and mechanically ventilated HS mice were found for TNF-α, IL-6, and MCP-1 90 minutes after shock induction (figure 4). No differences between the spontaneously breathing and mechanically ventilated HS mice were found for TNF-, IL-6, and MCP-1 90 min after shock induction (fig. 4, A, B, C, D, E, and F). In the kidney and lung, 90 minutes HS induced a small increase in mRNA for TNF-α (figure 4a, 4b) and IL-6 (figure 4c and 4d), whereas MCP-1 mRNA levels were unchanged. (figure 4e, 4f). To investigate whether the pro-inflammatory cytokines TNF-α, CXCL1 and IL-6 were produced in remote organs we measured soluble cytokine proteins in plasma.

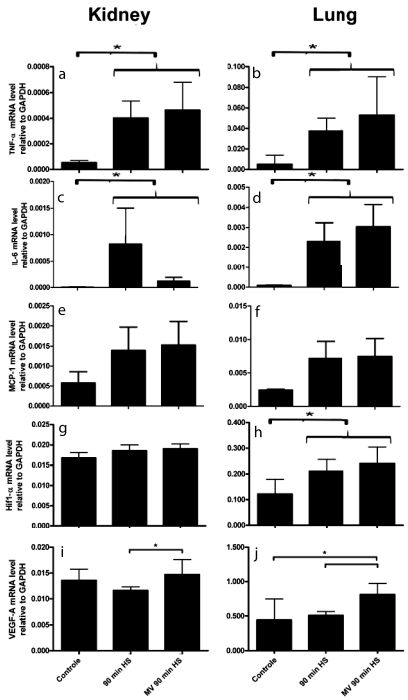

Figure 4: Kinetics of expression of pro-inflammatory and hypoxia related genes in kidney and lung during hemorrhagic shock in the presence or absence of mechanical ventilation.

Gene expression levels of the pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 and the hypoxia related molecules HIF-1α and VEGF-A in kidney and lung analysed by quantitative RT-PCR using GAPDH as housekeeping gene. Data are expressed as mean ± SEM, n>5. $p < 0.05$ values are marked with *

Because of insufficient blood sample, we were not able to perform cytokine enzyme linked immunosorbent assay in one mouse in the 90-min HS group. Although TNF-α protein in plasma was not changed at 90 min (figure 5a), concentrations of the proinflammatory cytokines CXCL-1 (11.0-fold) and IL-6 (21.7-fold) were significantly increased after 90 min (figure 5b, 5c). MV during 90 minutes of hemorrhagic shock did not affect these HS induced changes in IL-6 and CXCL-1 in the systemic circulation (figure 5). At 24 h after the shock period, all proinflammatory cytokines in the plasma were back to baseline (figure 5).

Hypoxia can induce HIF-1α both via transcriptional control and via posttranslational processes affecting the protein level, with an increase in VEGF-A as one of the downstream consequences. Thus, we therefore investigated whether shock induced changes in cellular oxygen levels influenced HIF-1α and VEGF-A, in our model. During the shock period, HIF-1α mRNA was not induced in the kidney, whereas in the lung it led to HIF-1α mRNA up-regulation, with no differences between HS alone and HS combined with MV (figure 4). VEGF-A mRNA showed a small but significant up-regulation in the 90 minutes with MV compared to HS alone, both in lung and kidney compared to the spontaneous breathing mice (figure 4i and 4j).

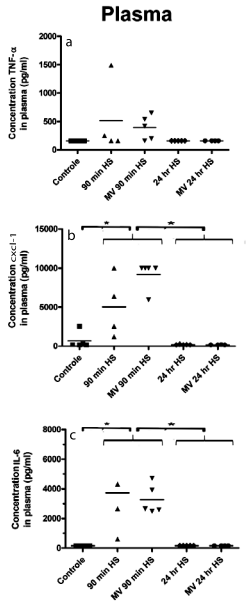

Figure 5: Pro-inflammatory cytokines in plasma during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation.

Levels of TNF-α, CXCL-1, and IL-6 in control (C), HS during 90 minutes, with and without mechanical ventilation and 24 after the HS insult with or without mechanical ventilation. Data are expressed as mean ± SD, n>4 for all groups. * p< 0.05 compared with control mice.

The up-regulation of HIF-1α in the lung and the up-regulation of VEGF-A in the MV group implies that a hypoxic condition may have occurred during the shock period in the absence and presence of mechanical ventilation. To determine the potential contribution of tissue hypoxia to the pro-inflammatory endothelial activation observed, we next studied endothelial pro-inflammatory activation in response to short term exposure to severe hypoxia only (table 1). To expose the mice to hypoxia during a similar period of time as the HS mice, including the time needed for instrumentation and anesthesia, mice were housed for 120 minutes in hypoxic cages with three different oxygen levels (figure 6). Neither 120 minutes of oxygen, 6%, nor oxygen, 10% (data not shown), induced the expression of the proinflammatory genes E-selectin, VCAM-1 and ICAM-1 in the kidneys and the lung of mice (figure 7). Surprisingly, this acute and severe hypoxia also did not increase the mRNA expression levels of HIF-1α and VEGF-A in lung and kidney (figure 7m-p). However, the severe hypoxia, did induce a proinflammatory response in the lung; 2 h of 6% hypoxia led to the up-regulation of proinflammatory cytokines IL-6, and MCP-1 mRNA, whereas TNF-α mRNA was unchanged (figure 7). In the kidney no differences in mRNA for IL-6, MCP-1 and TNF-α were observed between normal oxygen levels and severe hypoxia (figure 7g-k).

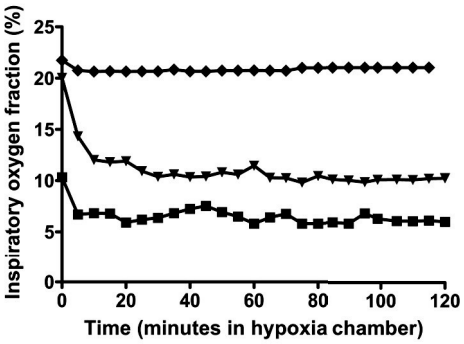

Figure 6: Inspiratory oxygen concentration of mice housed in hypoxic cages for 120 minutes

Levels of inspiratory oxygen were controlled during a 120 minutes stay in hypoxic cages. Inspiratory oxygen levels were maintained at 21% (diamonds), 10% (triangles) and 6% (squares). Mice were sacrificed after 120 minutes

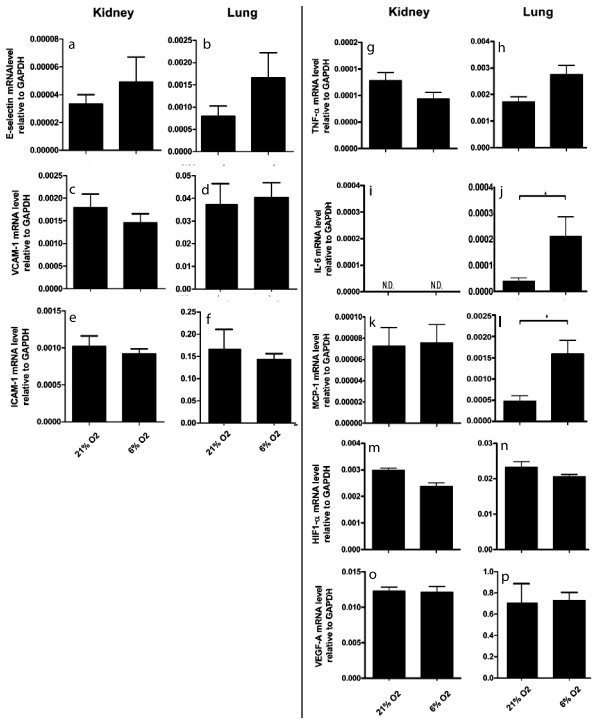

Figure 7: Kinetics of mRNA of endothelial cell adhesion molecules, pro-inflammatory cytokines and hypoxia related genes during acute hypoxia.

Gene expression levels of E-selectin (a, b), VCAM-1 (c, d), and ICAM-1 (e, f), and the pro-inflammatory cytokines TNF-α (g, h) , IL-6 (i, j), and MCP-1 (k, l) and the hypoxia related molecules HIF1-α (k, l) and VEGF-A (m, n) in kidney (a, c, e, g, i, k, m, o) and lung (b, d, f, h, j, l, n, p). Gene expression was analysed by quantitative RT-PCR using GAPDH as housekeeping gene after 120 minutes of acute hypoxia, during normoxia (21%) and hypoxia (6%). Data are expressed as mean ± SEM , n>5. N.D. = not detectable.

Discussion

The interaction between neutrophils and activated endothelium is crucial for the development of multiple organ dysfunction in patients suffering from hemorrhagic shock, but the effects of MV on this endothelial activation are not known. In this study of MV during HS in mice, we demonstrated that the proinflammatory EC activation in the lung and the kidney was not prevented nor augmented by MV. In addition, we found that the organ-specific endothelial activation in HS is not induced by an impaired oxygen delivery but merely by a systemic proinflammatory response induced by the HS.

To our knowledge, we are the first to report the effect of MV during HS and its effects on EC activation in mice. MV alone is able to induce an inflammatory response in mice and man[11,20]. In mice, MV induced TNF-α, IL-6 and IL-1β in mice lung 60 minutes after MV initiation, whereas plasma protein levels followed some time thereafter [11]. In addition, MV in mice led to endothelial activation in lung and distant organs within 120 min after MV initiation [12]. The data in our studies show that, compared to the HS insult, no additional detrimental effects on endothelial inflammation are induced when MV is performed in animals with HS. This implies that the insult given is too mild or too short to add additional detrimental effects.

Our results on the early proinflammatory cytokine release during HS corroborate several other animal studies. First, previous work from our own group shows that mRNA for

TNF-α is up-regulated in kidney and heart 90 min after the initiation of the HS5. Liu and Dubick showed in rats with an HS to a MAP of 50 mmHg during 60 minutes that the mRNA encoding for TNF-α was up-regulated in kidney and liver, and [21]. In a mouse model, TNF-α measured in serum was increased 30 min after shock onset [22].

Decreased tissue oxygen delivery, leading to cellular hypoxia is considered to be an important mediator of MODS after HS23. During hypoxic conditions, HIF-1α accumulates in the cell, translocates to the nucleus, and forms a stable heterodimer, after which it induces gene transcription. HIF-1α can also be regulated at the transcriptional level as demonstrated in tumour models [24,25]. Vascular endothelial growth factor A is a downstream target gene of HIF-1α that is primarily regulated at the transcriptional level and a major controller of vascular permeability in shock states [6]. Under hypoxic conditions, up-regulation of VEGF-A by HIF-1α occurs within minutes [26]. In cellular experiments using the human umbilical vein cell line, EA.hy9 [26], incubation for 16 hours in oxygen, 1%, induced HIF-1 nuclear translocation. This was accompanied by ICAM-1 and E-selectin mRNA up-regulation compared to cells that were incubated in normoxia19. However, in human aortic endothelial Cells incubated for 8 h in oxygen (4%0 hypoxia, no up-regulation of E-selectin, VCAM-1 or ICAM-1 could be observed by Illumina gene microarray analysis [27]. We did not see any statistically significant induction of mRNA of HIF-1α or VEGF-A during HS alone, but MV combined with HS led to a small increase in HIF-1α and VEGF-A mRNA. In addition, HIF-1α and VEGF-A were not affected during our acute hypoxia experiments in mice without shock. Our data are contrary to Koury et al. [28], who showed that in a HS model in the rat, HIF-1α levels as measured by Western blotting were increased in the ileac mucosa after 90 minutes of HS. Hierholzer et al. [29] observed an increase in HIF-1α nuclear activity in the lung after a 40 mmHg MAP shock period of 2.5 h. No increased HIF-1α activation in livers of animals subjected to 40 mmHg MAP HS for 60 minutes

was found[30]. From these studies it becomes clear that there are large organ-, insult- and time-frame-dependent differences that regulate HIF-1α responsiveness in HS models. Thus, our data and those reported by others suggest that severe whole-body hypoxia is not the driving factor for EC activation in HS in kidney and lung but that large interorgan differences might exist.
Our animal model of HS with or without MV has several limitations. First, in our pressure-constant HS model, increased metabolic acidosis was observed in the mechanically ventilated mice at 90 min of HS compared with the spontaneously breathing mice. This suggests the occurrence of decreased organ perfusion in vital organs in the MV group. This increased organ hypoperfusion could lead to deleterious outcomes later. In addition, we used a short shockperiod to mimic the short and severe nonresuscitated HS seen in the clinic, but the described proinflammatory effects of MV might be more pronounced after longer periods of MV [11,12].

Possibly that, after the shock insult has resolved, ongoing MV may lead to additional organ damage. However, this clinical important question cannot be tested in this small rodent model because of instabilities of this model after prolonged MV. The absence of evidence of differences in endothelial activation in a pressure-constant mouse HS model induced by MV at 90 min of HS is not evidence of the absence of the effects of MV during longer periods or after the resolution of HS on endothelial activation in vital organs. A larger animal model with more physiological monitoring might in the future provide more insight in the consequences of longer MV exposure after the shock insult on EC activation. In addition, in our hypoxic cage experiments we did not measure blood gases nor oxygen delivery capacity. However, in man, it has been shown that 12% normobaric hypoxia is sufficient to cause hyperlactatemia as an indicator of insufficient oxygen transport [31]. Thus, we hypothesised that oxygen, 6%, is sufficient to induce an impaired oxygen delivery. This oxygen concentration leads to an estimated pO2 of 4-5 kPa and an oxygen saturation of 50-60%. In our model the severe hypoxia induced a proinflammatory response in the lung; 2 hours of 6% hypoxia lead to the up-regulation of IL-6, and MCP-1 mRNA. Finally, the translation of our rodent results to patients with HS is difficult. In our experiments, we used young male, otherwise healthy mice, which do not model older patients with multiple comorbidities. The effects of aging and comorbidity on the systemic and microvascular responsiveness to HS and MV are now being investigated.

The early endothelial activation found in this study suggests that the therapeutic window to attenuate endothelial cell activation takes place early during the shock insult. This EC activation is induced at least in part by a proinflammatory response induced by proinflammatory cytokines, whereas in the mouse, whole-body cellular hypoxia does not induce endothelial adhesion molecules in the same time frame. In our model of HS, MV per se does not add to the proinflammatory endothelial activation seen in lung and kidneys. The observed proinflammatory endothelial activation is mostly ischemia- or reperfusion-dependent and not related to hypoxia. Additional studies will investigate potential therapeutic strategies to

diminish proinflammatory endothelial activation in HS on endothelial dysfunction.

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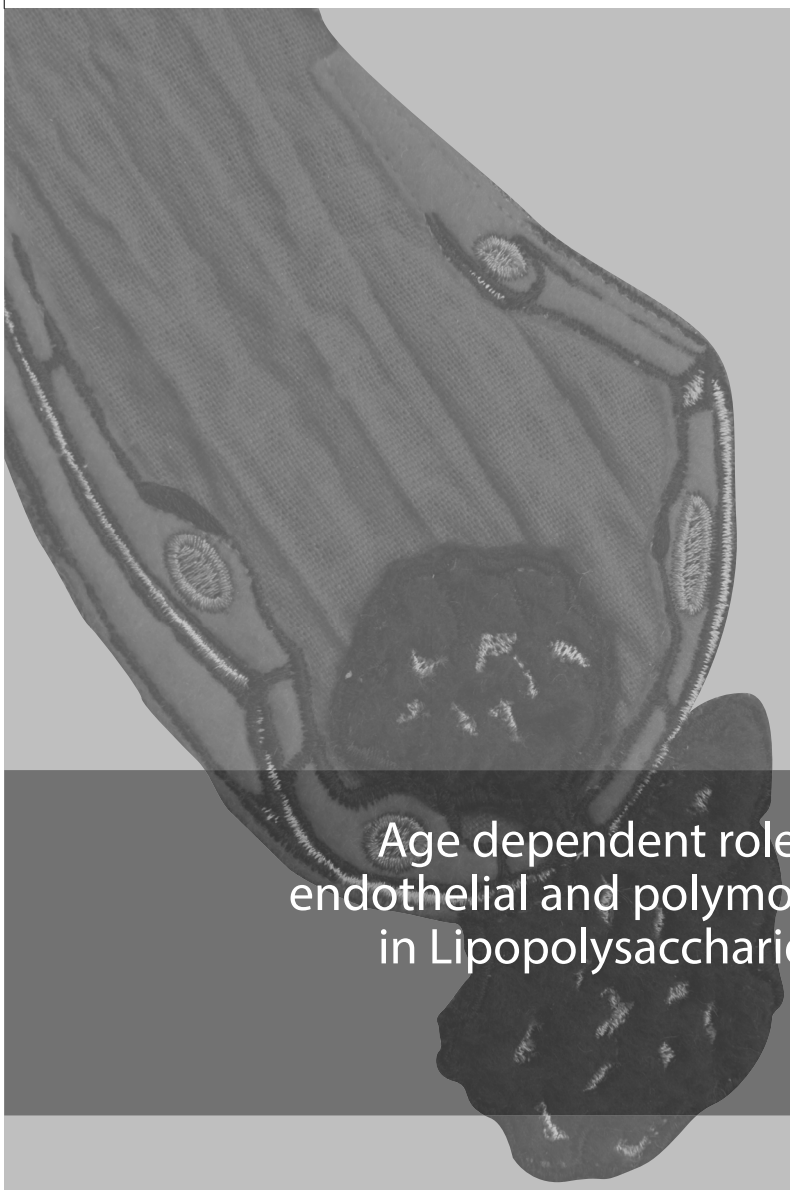

Chapter 5

Age dependent role of microvascular endothelial and polymorphonuclear cells in Lipopolysaccharide-induced acute kidney injury

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Abstract

Introduction

The incidence of acute kidney injury following severe sepsis is higher in elderly. We hypothesized that microvascular endothelium is 'primed' by ageing and that sepsis represents a 'second hit' resulting in more severe microvascular complications.

Methods

Three and 18 months old mice were intraperitoneally injected with 1,500-endotoxin units/gram body weight lipopolysaccharide and sacrificed after 8 hours. Flow cytometry and Myeloperoxidase ELISA determined neutrophils in plasma. Quantitative reverse transcription polymerase chain reaction was used to analyze messenger ribonucleic acidlevels of P-selectin, E-selectin, Vascular cell adhesion protein 1, Intercellular Adhesion Molecule 1, Tie-2, and Ang-1 and Ang-2. In kidney tissue we assessed neutrophil influx and E-selectin protein expression. Neutrophils were depleted with the monoclonal Ab NIMP.

Results

At basal conditions, microvascular endothelial cell activation status was similar in both groups, except for a higher Ang-2 expression ($p<0.05$) in the kidney of aged mice. Lipopolysaccharide induced increase in neutrophil count was higher in old (fold change 3.3) compared with young mice (fold change 2.2). Messenger Ribonucleic acid analysis showed higher upregulation of P- and E-selectin ($p=0.0004$, $p=0.0007$) after lipopolysaccharide administration in kidneys of elderly mice, which was confirmed at the protein level for E-selectin. Renal neutrophil influx in lipopolysaccharide treated aged mice was increased (fold induction 2.5 in aged and 2.1 young, $p<0.0001$). Polymorphonuclear cell depletion exaggerated the lipopolysaccharide induced kidney injury.

Conclusion

Ang-2 is increased in older mice, which might cause priming of the endothelial cells. Endothelium responded by a more extensive increase in expression of P- and E-selectin in older mice and increased polymorphonuclear cell influx.

Introduction

Ageing humans are more vulnerable to a wide variety of insults than younger subjects. In some cases this is the result of co-morbidities and reduced physiological reserve. However, in the absence of significant co-morbidities, 'healthy' ageing still seems to be associated with increased vulnerability to insults associated with systemic inflammation, resulting in significantly worse outcomes in terms of morbidity and mortality [1,2]. In humans, ageing without co-morbidity is associated with low-grade inflammatory activity, as measured by a 2-4-fold increase in plasma tumor necrosis factor (TNF-α) and interleukin (IL-6) [3]. As a consequence, the microvascular endothelium is likely 'primed' by (healthy) ageing potentially making the elderly more susceptible to the harmful effects of a second inflammatory insult.

Such an increased sensitivity to a second hit could explain the increased incidence of sepsis, and worse outcomes of sepsis in aged patients [1,2]. Sepsis is a well-described, but poorly understood, condition involving a systemic inflammatory response to infection and leading to multi organ dysfunction syndrome. Apparently, the kidney is vulnerable since acute renal failure is a frequent complication of sepsis. The current understanding of the pathophysiology of sepsis originates mainly from studies in young animals. From these studies, it has become clear that polymorphonuclear neutrophils (PMNs) are directly engaged in the pathophysiology. The interaction between PMNs and the endothelium plays a pivotal role in facilitating the migration of PMNs into the peripheral tissues. In the majority of organs, PMN migration to a site of injury occurs in post-capillary venules and is mediated by the spatiotemporal expression and activation of adhesion molecules, chemokines and cytokines by the vascular endothelium as well as by the PMNs[4]. After migration, activated PMNs release proteolytic enzymes and produce reactive oxygen species augmenting tissue injury [5]. It is thought that PMNs play a major role in the induction of organ damage in sepsis [6-8].

We hypothesized that in elderly the PMN – endothelium interaction is increased as a consequence of ageing related endothelial priming, which in turn is responsible for the increased susceptibility of elderly to a 'second hit' like sepsis resulting in increased organ damage. To investigate this we used lipopolysaccharide induced acute endotoxemia in mice as a model and compared renal inflammation in young (3 months) and aged (18 months) mice focusing on expression of a number of endothelial adhesion molecules and members of the Angiopoietin/Tie2 system, and PMN influx. To delineate the contribution of PMNs, similar experiments were performed in neutrophil depleted mice.

Materials and Methods

Animals

Mice were obtained from Harlan Nederland (Horst, the Netherlands) and maintained on chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-h light/dark cycle. All procedures were approved by the local committee for care and use of laboratory animals (DEC, University Medical Center Groningen, Groningen, The Netherlands) and were performed according to strict governmental and international guidelines on animal experimentation.

Lipopolysaccharide endotoxemia model

96 mice, 8 mice per group (see table I), initially entered the study, prior to the experiment one aged mouse died of unknown cause and was not replaced. Mice were first subdivided in groups of young and old mice, after which we randomly picked 8 mice per treatment group. All further experiments were blinded to murine age and treatment.

For the induction of endotoxemia, wild-type female C57BL/6 mice, 3 months and 18 months old, were intraperitoneally injected with 1,500 EU/g body weight lipopolysaccharide (Lipopolysaccharide; Escherichia coli, serotype O26:B6; Sigma-Aldrich, St Louis, MO; for the specific batch of lipopolysaccharide used the dose was 0.5 µg/g body weight). Animals were supplied with water and food during the whole experiment. After 8 hours mice were anesthetized with isoflurane, blood was drawn via aortic puncture, and kidneys were harvested. Control mice were, where appropriate, subjected to similar conditions as the lipopolysaccharide treated mice prior to and during sacrifice. In a random selection of animals, PMN depletion was established by intraperitoneally injection of monoclonal rat anti-murine PMN antibody NIMP-R14 (Hycult Biotech, Uden, the Netherlands) at 0.5 mg in 0.75 ml of PBS 24 hour prior to endotoxaemia induction, as previously described.9 The control groups received rat Immunoglobulin G (IgG) (Sigma-Aldrich) at 0.5 mg of IgG in 0.75 ml of PBS. Rat immunoglobulin G and NIMP-R14 were both tested for Endotoxin contamination by the Limulus Amebocyte Lysate (LAL) test and contained 0.319 EU/ml (IgG) and 0.213 EU/ml (NIMP-r14), respectively.

After harvesting the kidneys they were partly snap-frozen on liquid nitrogen and stored at -80 °C for gene and protein analyses, and partly fixed in 4% formaldehyde and embedded in paraffin for histopathological evaluation by periodic acid Schiff staining according to standard procedures.

PMN and leukocyte count

PMN count was assessed in blood of a random selection of animals by flow cytometric analysis. Whole blood samples were collected before and after lipopolysaccharide injection. 500 µl buffer (0.15M NH_4Cl with 10mM $NaHCO_3$) was added to 70 µl of whole blood to cause lysis of the red blood cells, after which cells were incubated with primary Abs CD11b-PE (BD Biosciences) and Ly6G-FITC (BD Biosciences, Breda, the Netherlands) for 60 min on ice. After washing three times with phosphate buffered saline supplemented with 2% fetal calf serum, cells were fixed in 2% paraformaldehyde. Cells were analyzed on a Calibur (BD Biosciences, Breda, the Netherlands) flow cytometer using CellQuest software (BD Biosciences) Total leukocyte counts was also performed see Supplemental Digital Content 1 for materials and method and, see Supplemental Digital Content 2, figure 1 which represents total leukocyte counts in control and lipopolysaccharide treated mice, and lipopolysaccharide treated mice pretreated with IgG respectively NIMP-R14.

Blood biochemistry

Plasma was collected by aortic puncture at the time of sacrifice. Neutrophil gelatinase associated lipocalin (NGAL)10 in plasma was determined by ELISA (BioPorto Diagnostics A/S,

Gentofte, Denmark) according to the manufacturer's instructions. Blood urea nitrogen (BUN) analyses were performed by enzymatic degradation assay (Roche, Woerden, The Netherlands). The investigator performing the analyses was blinded to the treatment.

Gene Expression analysis by quantitative RT-PCR

Total RNA was extracted from 20 * 5 µm-thick kidney cryosections and isolated using the RNeasy Mini plus kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield (OD260) and purity (OD260/OD280) were measured by ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, the Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitative PCR amplifications were performed according to manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Applera Nederland, Nieuwerkerk a/d IJssel, Netherlands), using the following Assay On Demand primers: for housekeeping gene mouse- Glyceraldehyde 3-phosphate dehydrogenase (assay ID Mm99999915_g1), mP-selectin (assay ID Mm00441295_m1), mE-selectin (assay ID Mm00441278_m1), mouse Vascular cell adhesion protein 1 (assay ID Mm00449197_m1), mouse Intercellular Adhesion Molecule 1 (assay ID Mm00516023_m1), mAng-1 (assay ID Mm00456503_m1), mAng-2 (assay ID Mm00545822_m1), mTie-2 (assay ID Mm00443242_m1), TNF-α (assay ID Mm00443258_m1) and IL-10 (assay ID Mm00439614_m1).

The investigator performing the analyses was blinded to the treatment. Calculations were performed as previously described.11;12 Glyceraldehyde 3-phosphate dehydrogenase was shown not to change with age13, and was in our model not affected by lipopolysaccharide administration, as was concluded from the threshold cycle values for this gene that did not differ in the samples generated via our standardized protocol. These threshold cycle values were 20.2 +/- 0.7 (young) and 20.5 +/- 1.3 for the control groups, and 20.5 +/- 0.3 (young) and 20.8 +/- 0.6 (old) for the lipopolysaccharide treated groups.

Immunohistochemistry

For the detection of E-selectin and PMNs in tissue slides, an anti-rabbit peroxidase-based Envision®+ system (DakoCytomation, Carpinteria, CA) was used, according to the manufacturer's instructions. In short, 5 µm cryosections were incubated for 60 min with MES-1 (a kind gift from Dr. Derek Brown, Ph.D., scientist, UCB Celltech, Brussels, Belgium), rat anti mouse Ly6G (clone 1A8; BD Biosciences) or isotype control (IgG2a; Antigenix America, Huntington Station, NY, USA). Peroxidase activity was blocked for 5 min by incubation with blocking solution (DakoCytomation), subsequently sections were incubated for 45 min with rabbit-anti-rat secondary antibody (Vector Laboratories, Burlingame, CA), diluted 1:300 in PBS supplemented with 5% fetal calf serum and 2% normal mouse serum, and 30 min with anti rabbit-polymer-HRP.

After detection of peroxidase activity with 3-amino-9-ethylcarbazole, sections were counterstained with Mayer's haematoxylin. No immunostaining was observed with isotype-

matched controls, demonstrating specificity of staining with the antigen specific antibodies.

Quantification of myeloperoxidase levels in kidney and plasma by ELISA

Myeloperoxidase protein levels in kidney tissue and plasma from healthy and lipopolysaccharide treated mice were measured in duplicate using an ELISA kit specific for mouse myeloperoxidase (HK210, Hycult Biotech) according to the manufacturer's instructions and as described previously [14]. Myeloperoxidase levels in the kidney were normalized to total protein concentrations in the tissue homogenate and expressed as pg myeloperoxidase per µg of total protein. The investigators were not blinded to the treatment.

Statistical analysis

Statistical significance was determined by means of unpaired independent Student t test (two tailed) (figure 2A) and using one-way analysis of variance with Bonferroni's multiple comparison tests with selected pairs of groups. All statistical analyses were performed with GraphPad Prism software (GraphPad Prism Software Inc. San Diego, CA). We used two-tailed hypothesis testing with all tests. Several of the variables under study may violate the assumptions of parametric tests, so the results of the statistical tests were confirmed using nonparametric equivalents.Differences were considered to be significant when $p < 0.05$. The sample size was calculated from previously published results from our group [11].

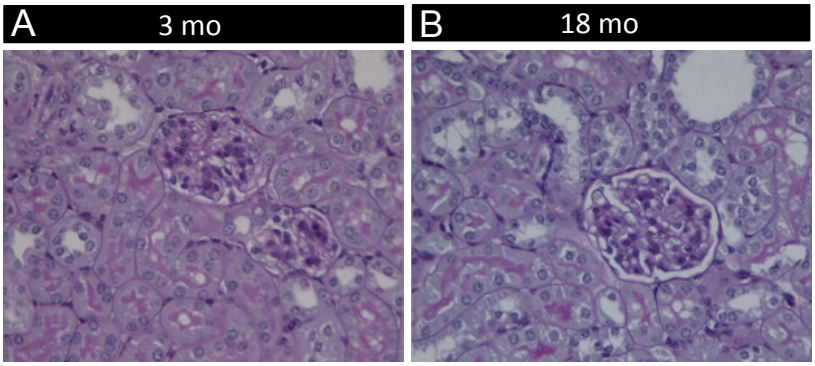

Figure 1: No morphological differences were found between the kidneys of young and aged mice. Kidney tissue samples of untreated young (A) and aged (B) mice were fixed in 4% formaldehyde and embedded in paraffin for histopathological evaluation using periodic acid-Schiff staining. Original magnification of x400.

Results

Morphology

No morphologic differences were found between kidneys of young and of aged mice.

Histomorphological assessment of PAS-stained renal sections revealed no differences in glomerular morphology between young and aged mice. Neither the glomerular vascular segments nor any other microvascular segments showed aberrant sclerosis or vascular injury (Figure 1).

The number of circulating PMNs is increased in aged mouse after lipopolysaccharide challenge
We assessed numbers of circulating PMN in aged and young animals since an overall increase in neutrophil numbers may affect general neutrophil migration in tissue. The increase in PMN count measured in whole blood samples after lipopolysaccharide exposure was higher in old compared to younger mice (Figure 2A).
Myeloperoxidase is abundantly present in azurophilic granules of PMN and is rapidly released when polymorphonuclear leukocytes are activated [15]. Higher levels of serum myeloperoxidase were observed in aged mice after lipopolysaccharide challenge (3.3 fold induction in aged versus 2.2 fold induction in young mice, Figure 2B), indicative of a higher PMN activation status in the older mice.

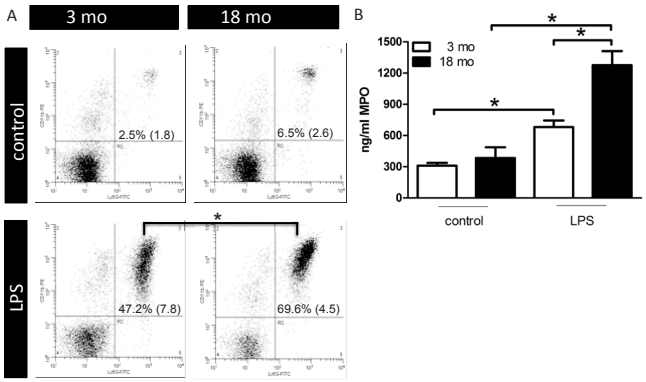

Figure 2: In aged mice, the increase in number of circulating polymorphonuclear cells after lipopolysaccharide challenge is higher than in young mice.

A. Flow cytometrical analysis of representative blood samples of a 3-months and 18-months-old mouse with and without lipopolysaccharide injection. In each scatter plot, the right upper quadrant represents polymorphonuclear cells (Ly6G and CD11b positive). The relative increase in polymorphonuclear cells count after lipopolysaccharide administration is lower (* $p<0.05$) in 3 months compared with aged mice. Value as % of polymorphonuclear cells of total WBC count (± SD) of n=3.

B. Quantification of Myeloperoxidase levels in plasma assessed by ELISA (mean [SD], N=8 per group; * $P<0.05$, by ANOVA with post hoc comparison of all groups using Bonferroni correction). LPS = lipopolysaccharide.

Increased expression of endothelial adhesion molecules P-selectin and E-selectin in kidney in aged mice after lipopolysaccharide challenge
No differences in basal gene expression were seen between old and young mice before lipopolysaccharide challenge. Endothelial cell activation before and 8 hours after lipopolysaccharide challenge were evaluated by analysis of P-selectin, E-selectin, Vascular cell adhesion protein 1, and Intercellular Adhesion Molecule 1 messenger (m)RNA levels in kidney tissue (Figure 3A) in both young and aged mice. All four adhesion molecules were strongly upregulated ($P<0.0001$) in old and young mice 8 hours after lipopolysaccharide challenge compared with controls. Eight hours after lipopolysaccharide administration, the fold induction in P-selectin was 87 in old mice compared to 51 in young mice and was significantly higher ($p<0.0004$). Fold induction of E-selectin in young mice after treatment

with lipopolysaccharide was 4.5 compared to 8.5 in old mice which was significantly higher ($p<0.0007$). Lipopolysaccharide challenge also increased the levels of soluble endothelial adhesion molecules in plasma of both young and old mice (Supplementary Digital Content 2, figure 2), but the differences between young and old mice were not statistically significant. In neutrophil depleted mice, lipopolysaccharide challenge did not result in changes in soluble Intercellular Adhesion Molecule 1 and soluble Vascular cell adhesion protein 1 whereas sE-sel increased equally in both age groups. Immunohistochemical staining of E-selectin (Figure 3B) confirmed the mRNA data. An increase in expression of E-selectin protein was seen 8 hours after administration of lipopolysaccharide in both groups. This increase was higher in the aged mice compared to the young mice. Immunohistochemical staining of E-selectin furthermore demonstrated striking microvascular heterogeneity in reaction to the stimulus. In young mice expression of E-selectin was mainly found in glomeruli, while only minor expression occurred in the peritubular endothelial cells, and in arterioles and venules. In aged mice, increased expression of E-selectin was found in both the glomeruli and in the peritubular microvasculature. Lipopolysaccharide injection resulted in an increase in both TNF-α and IL-10 levels in plasma and of mRNA in kidney. Kidney mRNA levels of TNF-α and IL-10 were significantly different ($p<0.0001$) between young and old mice, yet no differences in plasma TNF-α and IL-10 levels were found between young and old mice (Supplementary digital content 2, figure 3).

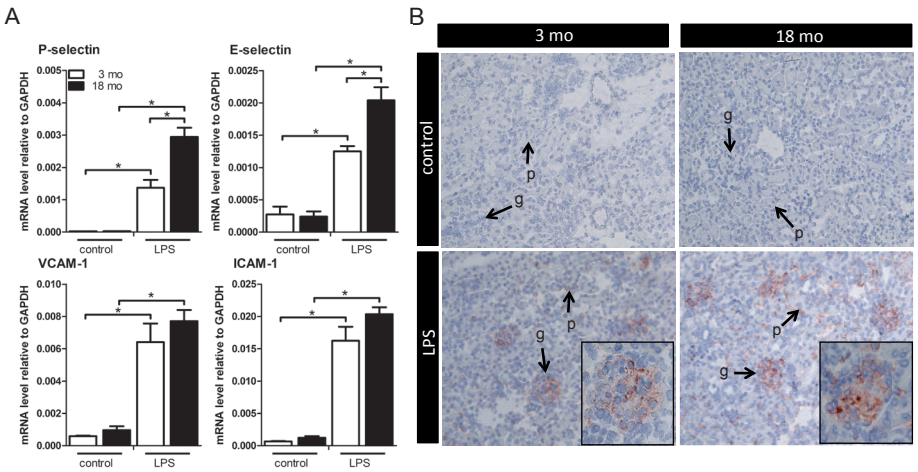

Figure 3: In the kidney, the expression of P- and E-selectin was more extensively induced by lipopolysaccharide in aged mice compared with young mice.

A. Expression of renal messenger RNA of P-selectin, E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1 in young, 3-months-old (white bars) and elderly, 18-months-old (black bars) mice subjected to LPS. Young and elderly mice were treated with lipopolysaccharide and sacrificed 8 hours later. Messenger RNA levels shown are relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene and were determined by quantitative Reverse transcription polymerase chain reaction. (Mean [SD], N=6 per group; * $p<0.0001$, by ANOVA with post hoc comparison of all groups using Bonferroni correction).

B. Young and elderly mice were treated with lipopolysaccharide and sacrificed 8 hours later. Frozen kidney tissue section were immunohistochemically stained for E-selectin (red), expression is visible in glomeruli (g) and peritubular (p) vasculature. Original magnification x200, Inserts: x400.

In aged mice Ang-2 mRNA levels in kidney were higher before and after lipopolysaccharide challenge

Since competition with Ang-1 for binding to the receptor Tie-2 by Ang-2 induces inhibition of Tie-2 signal transduction and is associated with inflammation and vascular leakage, we quantified Ang-1, Ang-2, and Tie-2 mRNA levels in kidney tissue [12,16]. No differences in basal gene expression of Ang-1 was seen between the age groups (Figure 4A). Basal expression of Ang-2, however, was significantly higher ($p<0.05$) in aged mice compared to young mice (Figure 4B). Basal expression of Tie-2 (Figure 4C) showed no differences between the age groups however, after lipopolysaccharide challenge Tie-2 expression was downregulated significantly ($p<0.0006$) in both groups, as previously reported in young mice.12 In both groups Ang-1 was not significantly different. Ang-2 on the other hand was significantly higher expressed after lipopolysaccharide challenge in the aged mice compared to the young mice, although in both groups the absolute increase induced by lipopolysaccharide was approximately 10 fold.

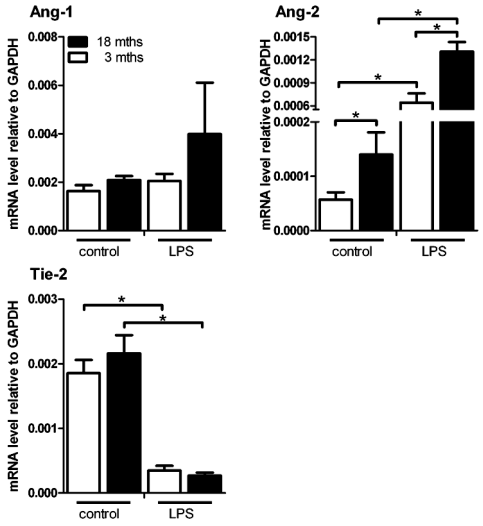

Figure 4: In aged mice, angiopoietin-2 messenger RNA (mRNA) levels in kidney were higher before and after lipopolysaccharide challenge. Angiopoietin-1 (A), angiopoietin-2 (B), and TIE-2 (C) in young, 3-month-old (red bars) and elderly, 18-

month-old (blue bars) mice subjected to lipopolysaccharide. Young and elderly mice were intraperitoneally injected with lipopolysaccharide and sacrificed 8 h later. mRNA levels shown are relative to glyceraldehyde 3-phosphate dehydrogenase

as housekeeping gene and were determined by quantitative reverse transcription polymerase chain reaction.bvMean (SD), n = 6 per group; *P < 0.0001, by ANOVA with post hoc comparison of all groups using Bonferroni correction. Ang-1 =angiopoietin-1; Ang-2 = angiopoietin-2; Tie-2 = endothelium-specific receptor tyrosine kinase.

Higher PMN count and higher expression of E-selectin and P-selectin in the aged mice after lipopolysaccharide challenge is paralleled by a higher PMN influx in kidney

Immunohistochemical staining of leukocytes using Ly6G antibody showed that, in contrast to young mice in old mice a small number of PMNs is present in glomeruli already before lipopolysaccharide exposure. The influx of PMNs in kidney tissue 8 hours after lipopolysaccharide administration (Figure 5A) is moreover markedly higher in aged mice. The increased influx of PMNs in aged mice was associated with increased myeloperoxidase levels (Figure 5B) with a fold induction of 2.5 in aged mice after lipopolysaccharide and a fold induction of 2.1 in young mice after lipopolysaccharide.

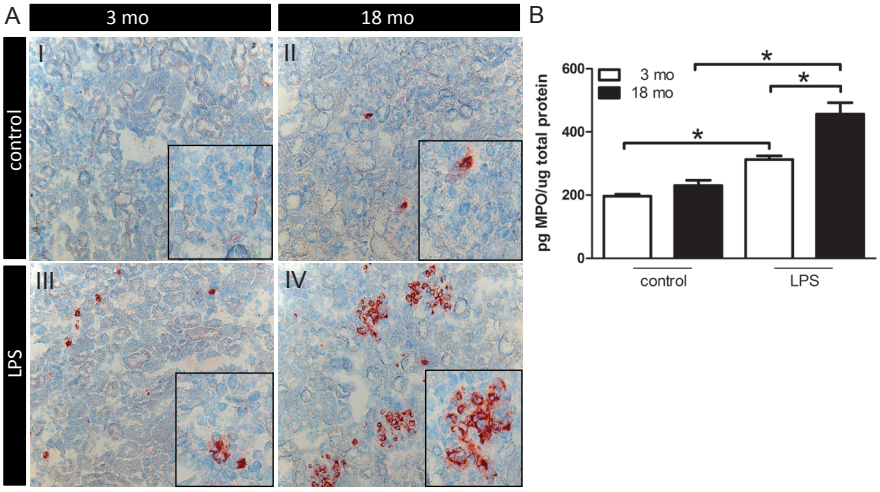

Figure 5: Polymorphonuclear cell infiltration in the kidney 8 hours after LPS injection is higher in aged mice.
Young and elderly mice were treated with lipopolysaccharide and sacrificed 8 hours later. A. Frozen kidney tissue sections were immunohistochemically stained for PMN (red) using Ly6G antibody. Original magnification x200, Inserts: x400. B. Quantification of polymorphonuclear cell infiltration in kidney tissue was performed using MPO ELISA. (Mean [SD], N=8 per group; * P<0.05, by ANOVA with post hoc comparison of all groups using Bonferroni correction). LPS = lipopolysaccharide.

Loss of kidney function in aged mice after lipopolysaccharide challenge cannot be prevented by PMN depletion

To study whether increased PMN influx in kidney of aged mice was accompanied by an increase in functional loss we measured NGAL and BUN levels in plasma. In quiescent conditions, no differences in NGAL and BUN levels between young and aged mice were observed. After lipopolysaccharide challenge there was a significantly higher ($p<0.0001$) increase of NGAL and BUN concentration in plasma of older mice (Figure 6A and B). It is of note that not for al mice enough blood or urine was available for kidney function measurements.

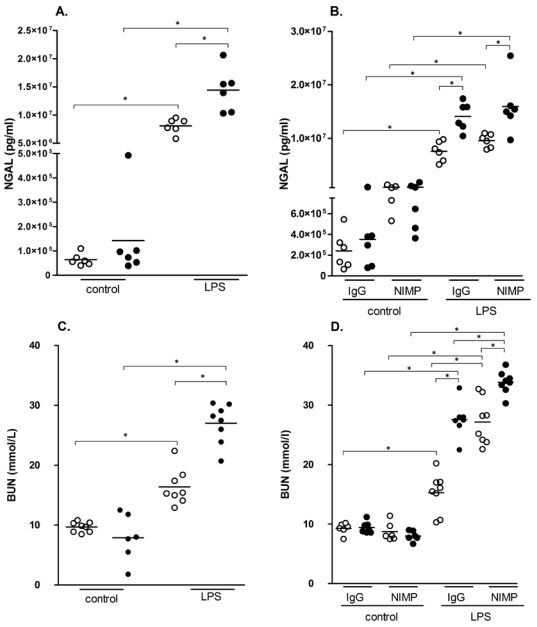

Fig 6: Plasma neutrophil gelatinase associated lipocalin (NGAL) and Blood Urea Nitrogen (BUN) levels in young and old mice. NGAL (A) and BUN (B) were measured as described in Materials and Methods in plasma of 3 months old (white circles) and of 18 months old (black circles) mice. Subset of mice were treated with anti-NIMP antibody 24 h before lipopolysaccharide challenge to deplete polymorphonuclear cells (neutrophils) or control Immunoglobulin G antibody that did not affect polymorphonuclear cell count wereafter NGAL (C) and BUN were measured (D). At time 0, mice were ip challenged with LPS at 0.5 µg/g and killed 8 h later. Untreated mice served as controls. Values are means +/- SD; n=8; *P ≤ 0.05 by ANOVA with post hoc comparison of all groups using Bonferroni correction. IgG = Immunoglobin G; LPS = lipopolysaccharide.

Since PMNs are associated with increased loss of vascular integrity and kidney damage we next depleted the PMNs before lipopolysaccharide administration to examine the role of neutrophil-endothelial interaction in increased kidney function loss in aged mice [5]. Fluorescent-assisted cell sorting analysis of whole blood of mice 24 h after injection of an NIMP antibody demonstrated that the mice had become severely neutropenic. In PMN depleted mice, NGAL and BUN levels (Figure 6C and D) were higher than in untreated controls. After PMN depletion, there was a significantly higher increase in NGAL in aged mice compared to younger mice after lipopolysaccharide administration, with a 12 fold increase in younger mice and a 19 fold increase in older mice (Figure 6B). It is of note that both IgG and NIMP injection raised NGAL levels in young and old mice. This observation was however not seen in the BUN measurements.

Discussion

Since sepsis is a disease striking hardest in the elderly, the aim of this study was to investigate the differences in renal microvascular endothelial and PMN responsiveness to lipopolysaccharide challenge between young and aged mice, in order to study differences in susceptibility of the kidney to lipopolysaccharide. This model shows that lipopolysaccharide in mice increased NGAL and BUN, which is suggestive of acute kidney injury. In older mice this phenomenon is more pronounced. We demonstrated that at basal conditions, microvascular endothelial cell activation status was similar in both groups of mice, except for a higher Ang-2 expression in the kidney of aged mice.

The PMN increase in plasma is higher and P-selectin and E-selectin levels in the kidney are higher in aged mice after lipopolysaccharide challenge compared to that in young mice. In older mice there was also involvement of the peritubular endothelial cells with upregulation of E-selectin not only in the glomeruli. The higher PMN count in plasma and higher expression of P-selectin and E-selectin after lipopolysaccharide challenge was paralleled by a higher renal PMN influx. We also demonstrated that older mice are more prone to kidney failure as measured by NGAL. Loss of kidney function in aged mice after lipopolysaccharide challenge was not counteracted in PMN depleted mice, implying that the PMNs are not major participants in kidney dysfunction.

Acute renal failure in sepsis patients is an independent predictor of mortality [17]. Therefore we focused on renal injury. Although lipopolysaccharide injection is a model with limited resemblance with sepsis we were able to induce renal failure in this model that might share pathophysiological mechanisms with sepsis. We hypothesized that a 'set point change' of endothelial cells by 'healthy ageing' might play a role in the increased vulnerability of the aged. To determine whether this 'priming' exists in the microvascular compartment, we studied the expression of proinflammatory adhesion molecules that are used by the endothelial cells to recruit PMN into tissue. A higher increase of P-selectin and E-selectin in aged mice after lipopolysaccharide challenge was observed. P-selectin and E-selectin mediate leukocyte rolling and thereby represent the first step in neutrophil migration across the wall of microvessels into tissue[4]. Intercellular Adhesion Molecule 1 and Vascular cell adhesion protein 1 were not significantly different in aged mice. At first sight this observation is in contrast to data published by Saito et al, who observed a higher increase in Intercellular Adhesion Molecule 1 in the heart of old mice compared to young mice [18]. This possibly can be explained by endothelial heterogeneity between organs as we previously showed in a hemorrhagic shock model[11].

A dual role of PMNs and macrophages has been described before, e.g., humans with signs of downregulation of the cellular components of the innate immune system have a higher mortality in sepsis [19]. This was explained by a deficient immune system resulting in a lower clearing capacity for infection. However, our current study and others suggest that leukocytes activation can also have a protective role other than in elimination of an infection. The classical innate immune response does not seem to be the explanation for vulnerability of

the kidney. The finding that PMN depletion does not save kidney function also supports this. On the contrary, in PMN depleted mice kidney function after lipopolysaccharide challenge was significantly worse. The increase in myeloperoxidase and PMN in kidney tissue after lipopolysaccharide in aged mice compared to young mice has an analogue. Gomez et al did a similar observation in lung tissue in aged mice after lipopolysaccharide challenge [20]. We observed that the kidney function, measured as plasma BUN levels, in PMN depleted mice after lipopolysaccharide challenge was significantly worse both at young and older age, while NGAL was statistically not different. Polymorphonuclear leukocytes are an essential component of the innate immune system. Pathogens are ingested and killed by means of phagocytosis and the generation of reactive oxygen species and the release of toxic granular enzymes [21]. Kasten et al have shown that during sepsis neutrophils are producers of IL-10, an anti-inflammatory cytokine[22]. In an lipopolysaccharide model employing young mice, Wu et al, furthermore showed an increase in TNF-α and, in accordance with our data, in BUN in PMN depleted mice after lipopolysaccharide challenge[23]. Depletion of PMNs might therefore lead to decreased IL-10 and increased TNF-α production, both having been suggested to be contributing to organ failure. PMN depletion possibly prevented the occurrence of the anti-inflammatory reactions and as a consequence an increase in kidney injury occurred in both young and aged mice after lipopolysaccharide challenge.
On the other hand, PMN may be a significant contributing factor to organ dysfunction due to an exaggerated inflammatory response by release of reactive oxygen species after migration into tissue facilitated by adhesion molecules [21]. Hoesel et al showed that in a caecal legation and puncture model in young mice delayed depletion of PMN after caecal legation and puncture resulted in improved liver and kidney function. This implies that, by depleting the PMNs before lipopolysaccharide challenge, we may have blocked both functions of the PMN, accelerating and resolving inflammation. Important to note is that, the antibody used in the study of Hoesel et al (RB6-8C5) is not PMN specific but also depletes the monocyte/macrophage populations [24]. As macrophage function involves release of mediators such as cytokines and phagocytosis of microorganisms, they also play a major role in organ function, and hence it is not surprising that their depletion played a role in improved organ outcome[25]. We saw an increase in macrophages in the kidney in aged mice prior to lipopolysaccharide treatment (data not shown), rendering it possible that macrophages play a role in loss of kidney function.

We investigated the Angiopoietin/Tie system, because an imbalance of this system is associated with inflammation and vascular leakage. In quiescent conditions, Ang-2 mRNA levels were higher expressed in aged mice compared to young mice, suggesting a change in basal endothelial activation status in aged mice. Competition with Ang-1 binding for the receptor Tie-2 by Ang-2 induces inhibition of Tie-2 signal transduction and is associated with inflammation and vascular leakage [16]. In humans an increased Ang-2 level in blood is associated with more severe disease and worse outcome [26]. The higher expression of Ang-2 could imply that the vasculature is more prone to inflammation as reflected by the higher number of PMN present in the glomeruli of aged mice. Fiedler et al showed that Ang-2 sensitized endothelial cells to TNF-α [27]. Since we demonstrated in our study that aged mice have higher levels of

Ang-2, it can be hypothesized that aged mice may be more sensitive to TNF-α exposure [27]. To investigate a possible role of Ang2 in sensitizing microvascular endothelial cells in older mice, local and systemic Ang2 levels would be informative. Unfortunately, methods to measure these protein levels in mice are at present not available. Future studies in aging mice in which Ang-2 is neutralized, e.g., by treatment with antibodies [28] will provide additional insight in the role of Ang-2 in endothelial responses in endotoxemia and sepsis during aging.
Others have shown that aging is associated with higher levels of TNF-α in healthy elderly, in mice after lipopolysaccharide challenge, and in mice after caecal legation and puncture [3,29-32]. TNF-α induces endothelial cell activation with an increased expression of adhesion molecules [33], which could explain the increased P-selectin and E-selectin expression in aged mice in our study. Cunningham furthermore showed that TNF-α is a key mediator of acute renal failure in mice [34]. Taken together, higher levels of TNF-α in the aged mice might cause more endothelial activation and kidney injury through Ang-2 sensitization of endothelial cells for TNF-α. Although we did not find any differences in TNF-α and IL-10 levels between young and old mice 8 hours after lipopolysaccharide injection (Supplementary digital content S3), this does not rule out organ specific differences in cytokine production nor differences in pro-inflammatory cytokine levels in plasma at earlier time points, as suggested by Chorinchath et al [30].
Our mouse model, to represent patients with sepsis, has some shortcomings. Although lipopolysaccharide induced shock is a widely used laboratory model for sepsis, there are more clinically relevant animal models. We chose to use our model based on the fact that these highly standardized and frequently used single hit animal models are reproducible and make comparison with the published research possible. However, in mouse lipopolysaccharide models, lipopolysaccharide dosing is highly variable
Previously, others have shown that lipopolysaccharide induced shock causes renal injury, including decreased glomerular filtration rate, increased blood urea nitrogen (BUN), and increased renal PMN infiltration [35,36] We had several reasons for choosing the mouse model employed. First, most of the antibodies antibodies to knock out PMNs, including NIMP-r14, cannot be used in rats. Secondly, the availability of knock out mice is of importance for future follow-up studies. Third, in our previously experiments with an lipopolysaccharide model in young mice with a relatively low dose of lipopolysaccharide (0.5 μg/g) we measured an increase in renal PMN infiltration and blood urea nitrogen [12].

The here reported new observations in this model now calls for further studies in more complex models such as those employing multiple insults which is seen in sepsis patients[37]. In addition, several morphologic changes that are reported in literature to occur with aging, are in fact caused by associated comorbidities such as diabetes, arteriosclerosis and hypertension. Moreover, evidence exists that structural as well as functional changes occur in the human aging kidney, independent of related illnesses [38]. In contrast to findings in the human situation, where as a result of aging an increasing number of glomeruli is affected by glomerulosclerosis [39], in our animal model no difference in morphology between young and old mice was observed. This suggests that while in the clinical situation a secondary hit, for example sepsis, might have more extensive effects, this is not expected to occur in the mouse

model we have employed. An important role in the development of glomerulosclerosis in humans is the high caloric western diet. Mice subjected to a high caloric western diet were recently shown to develop vascular and renal activation and inflammation[40], which provides an interesting perspective for the design of future experiments to combine western style diet in ageing mice with an lipopolysaccharide challenge. Limitations of our depletion study is the possible confounding effect of the IgG and NIMP-r14 antibody as these antibody on itself might have effect on NGAL levels.

Although animals were supplied with water during the experiment we cannot rule out that elderly mice were more ill and therefore drank less and had more fluid extravasation leading to increased volume depletion. As dehydration is considered a factor for acute kidney injury this could have influenced the higher NGAL and BUN levels in elderly mice. It would be interesting for follow up studies to explore the role of fluid suppletion.

A number of limitations may be taken into account for the use of NGAL as a marker of kidney injury. First of all, large-scale population-based 'normal ranges' of NGAL are lacking at this moment. Furthermore, the preciseness of NGAL as a marker need to be further substantiated since published studies of NGAL show wide variation in levels and wide confidence intervals [41]. Another potential limitation of our study is the possible confounding effect of the IgG and NIMP-r14 antibody as this antibody on itself might have effect on NGAL levels.

In summary, in aged mice no obvious proinflammatory endothelial cell activation was found in the microvascular segments of the kidney as measured by classical markers of endothelial activation. In contrast, Ang-2, an autocrine activator of endothelial cells, was significantly increased ($p<0.05$) in older mice before lipopolysaccharide exposure, possibly priming the endothelial cells for an exaggerated response to an inflammatory stimulus. Upon endotoxaemia, representing a "second hit", the endothelium responded by a more extensive increase in expression of P-selectin and E-selectin in comparison with young mice. Moreover, in aged mice circulating PMN count increased significantly upon lipopolysaccharide exposure (fold 3.3), which was paralleled by an increase in PMN in glomeruli. Surprisingly, these PMNs might have a protective role, in young as well as older mice, as demonstrated in PMN depletion experiments. The observed striking differences between young and aged mice in our sepsis model and other studies [30,32,42] have important impact for future drug development in sepsis research because the underlying pathophysiological mechanisms between young and old subjects might show important differences. Detailed clinical observations and analyses of (biochemical) parameters in aged patients are needed to understand age dependent mechanisms of organ dysfunction.

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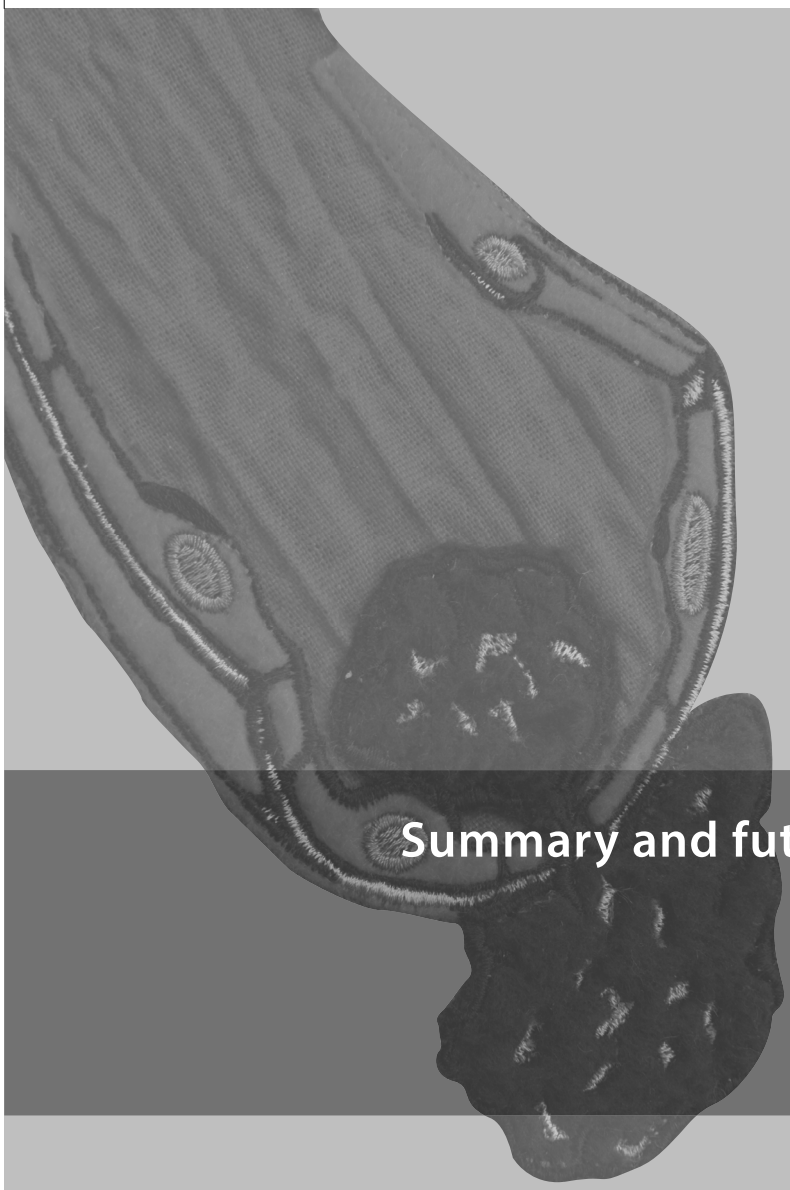

Chapter 6

Summary and future perspective

6.1 Summary and Conclusions

Multi-organ dysfunction syndrome (MODS) following shock states is still a major determinant of morbidity and mortality [1-3]. The endothelium is an important player in the pathogenesis of MODS in critically ill patients, as it facilitates polymorphonuclear leukocytes (PMN) migration in tissue, which is thought to play a pivotal role in organ dysfunction. Besides a package of supportive measures, which are aimed at limiting and managing the systemic, multi-system consequences of sepsis and trauma there is, until now no clinical treatment directed at the cause of MODS available which improves outcome. We hypothesize that understanding the spatial-temporal pattern of endothelium activation in shock states can assist in identifying new rational targets for early therapeutic interventions to prevent the development of MODS. In this thesis, we employed two mouse shock models, hemorrhagic shock (HS) and endotoxaemic shock, to investigate the microvascular responses to shock induced stress. We focussed on inflammatory endothelial cell activation and leukocyte influx patterns as well microvascular bed differences in response to shock.

In **Chapter 2** we examined the kinetics and organ specificity of endothelial cell activation in a fixed pressure hemorrhagic shock mouse model in which the blood pressure was maintained at a mean arterial pressure of 30 mmHg for different time periods before sacrifice. After 90 minutes of HS additional groups of mice were resuscitated with 6% hydroxyethyl starch. Outcomes were compared with untreated mice and sham shock mice that underwent instrumentation and 90 minutes of anesthesia without shock. Gene expression levels of adhesion molecules (P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM- 1)) and hypoxia responsive genes (vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1alfa (HIF1α)) were quantified in the different organs by quantitative, or real time, reverse-transcription polymerase chain reaction (RT-PCR). Furthermore, we examined a selection of these genes with regard to protein expression and localization using immunohistochemical analysis. Expression of adhesion molecules was significantly induced in all organs, albeit to a different extent depending on the organ. Resuscitation and thus reperfusion was an additional insult, which induced an additional increase in adhesion molecule expression. Early organ activation measured as an increase in adhesion molecule expression was paralleled by leukocyte influx, in the kidney, lung, and liver at 90 min of HS. The rapid inflammatory activation was not paralleled by induction of hypoxia-responsive genes.
We thus demonstrated that during hemorrhagic shock there is early and organ-specific endothelial cell activation pattern, which occurred before resuscitation and was not per se hypoxia-driven.

The interaction between the neutrophil and the activated endothelium is supposed to be a principal cellular mediator of tissue damage following hemorrhagic shock. As already evident from chapter 2, leukocyte influx in kidney is an early event in hemorrhagic shock.
In **Chapter 3** we hypothesized that depletion of PMN would attenuate endothelial activation and attenuate leukocyte influx in kidney and thereby organ function loss. In this study we

focused on the kidney as the kidney is one of the organs most extensively affected in MODS and because acute kidney injury is related to high morbidity and mortality[2,4]. We employed the same HS mice model as in the previous study, in which mice were subjected to controlled HS with a MAP of 30 mmHg for 90 minutes followed by resuscitation with 6% hydroxyethyl starch. Mice were sacrificed before HS induction (control), after 90 minutes of HS, and 1, 8, and 24 hours after resuscitation. In addition to the previous study, selected animals were PMN depleted via i.p. injection of monoclonal rat anti-murine neutrophil antibody 24 hour prior to HS induction. Flow cytometric analysis of whole blood samples confirmed neutrophil depletion. mRNA levels of adhesion molecules (E-selectin, P-selectin, VCAM-1 and ICAM-1) measured in kidney by real time RT-PCR and protein expression of ICAM-1 analyzed by ELISA were compared between PMN competent and PMN depleted mice. The role of PMNs in endothelial activation as represented by adhesion molecule induction showed a biphasic pattern in hemorrhagic shock and resuscitation. Ninety minutes after induction of HS, in the pre-resuscitation phase, activation was inhibited by PMN depletion. In contrast, after resuscitation the absence of PMN had no significant effect on endothelial cell activation, moreover PMN depletion seemed to compromise renal function with increased levels of BUN 8 hours after resuscitation in the PMN depleted mice. The observed detrimental effects of PMN depletion may be related to the protective function of PMNs in innate immune defences, which is part of the body's reactions to HS.
In summary, in a hemorrhagic shock mouse model that activation of adhesion molecules after resuscitation is independent of "activated" PMN and thus depletion of neutrophils in this model did not attenuate organ function loss.

Mechanical ventilation (MV) is frequently used in patients with HS not only to allow intervention procedures but also to warrant a patent airway and gas exchange thereby preventing hypoxia. Preventing hypoxia by supplemental oxygen is one of the cornerstones in the treatment of patients in shock as shock is defined as global tissue hypoxia secondary to an imbalance between systemic oxygen delivery and oxygen demand. Cellular hypoxia is supposed to be an important mediator of MODS after HS[5]. However, MV by itself may initiate an inflammatory reaction of the lung and distant organs[6] and thus act as a double edge sword. In **Chapter 4** the aim was to use a more clinically relevant model and investigate the consequences of mechanical ventilation of mice subjected to HS on microvascular endothelial activation in the lungs and kidney. As previously observed in chapter 2 there was a vascular bed specific, heterogeneous pro-inflammatory endothelial activation in kidney and lung, measured by increased expression of E-selectin, VCAM-1 and ICAM-1 mRNA 90 minutes after induction of HS. No differences in endothelial activation were seen between spontaneously breathing and mechanically ventilated mice. Also mRNA levels in the lung and kidney of (endothelial) pro-inflammatory cytokines (TNF-α, IL-6 and MCP-1) were not different between the spontaneously breathing and the mechanically ventilated mice. Similarly, no differences between spontaneous breathing and mechanical ventilation mice at 90 minutes after HS induction were seen in CXCL-1 and IL-6 levels in plasma, which were increased in both groups. mRNA level of the hypoxia responsive gene HIF1α was upregulated in the lung but not in

the kidney 90 minutes after HS, however, no differences were seen between spontaneous breathing and mechanical ventilating mice. The role of hypoxia as mediator of endothelial activation was lastly investigated by studying endothelial activation in response to severe hypoxia by exposing mice to two hours of 6% hypoxia. This did not upregulate the expression of adhesion molecules, in either kidneys or in lungs of the mice.
We concluded that in our model of HS, MV per se does not add to the proinflammatory endothelial activation seen in lung and kidneys. Moreover the observed proinflammatory endothelial activation is mostly ischemia- or reperfusion dependent and not related to hypoxia.

The incidence of acute kidney injury (AKI) following severe sepsis is higher in the elderly than in younger patients. The endothelium of aged but healthy patients is suggested to be more susceptible to inflammatory insults when compared with younger patients. As a result, the kidneys and other organs are more vulnerable in situations such as sepsis. In **Chapter 5** we demonstrated that in healthy aged mice no obvious proinflammatory endothelial cell activation was found in the microvascular segments of the kidney, as measured by classic markers of endothelial activation. In contrast, Ang2, an autocrine sensitizer of endothelial cell activation, was significantly increased ($P < 0.05$) in older mice before lipopolysaccharide exposure, possibly priming the endothelial cells for an exaggerated response to an inflammatory stimulus. Upon LPS administration induced endotoxaemia, the endothelium of aged mice responded with a more extensive increase in expression of P-selectin (87-fold induction in aged vs. 51-fold induction in young mice) and E-selectin (8.5-fold induction in aged vs. 4.5-fold induction in young mice) in comparison with young mice. Moreover, in aged mice, circulating PMN count increased significantly upon lipopolysaccharide exposure (3.3-fold induction in aged *vs.* 2.2-fold induction in young mice), which was paralleled by an increase in PMN influx in glomeruli. Increased PMN influx in kidney of aged mice was accompanied by an increase in functional loss following LPS administration in aged mice compared to young. There was a significantly higher ($P < 0.0001$) increase in NGAL and BUN concentrations in plasma of aged mice. PMN depletion did not attenuate loss of kidney function either in young mice, or in aged mice.
In summary, there are no obvious differences in basal gene expression of pro-inflammatory endothelial adhesion molecules in aged compared to young mice. Upon endotoxaemia induction there was increased expression of endothelial adhesion molecules P-selectin and E-selectin in kidney was observed in aged mice which was paralleled by a higher influx of PMN in glomeruli of aged mice compared to young. This increased PMN influx in aged mice was accompanied with increased kidney function loss, which was not associated with PMN behaviour.

Conclusion
During shock states the endothelial cell function was altered in a spatial temporal pattern. During hemorrhagic shock there is organ and vascular bed specific early activation of the endothelium. Although neutrophil endothelial interaction is thought to play an important

role in MODS, PMN depletion prior the hemorrhagic shock or endotoxaemia induction did not attenuate endothelial activation. In contrast, kidney function was less affected in the PMN competent mice. Another important aetiology of MODS is hypoxia. However, in our study the pro-inflammatory reaction following hemorrhagic shock in kidney and lung does not seem to be related to hypoxia. Moreover in our model mechanically ventilating by itself did not add to the pro-inflammatory reaction of lung and kidney. Thus mechanical ventilation of mice subjected to HS did not exert positive or negative consequences on microvascular endothelial activation in the lungs and kidney. Lastly, from our investigations on the effects of aging on microvascular endothelial activation in endotoxaemic shock, demonstrated a significant increase basal expression of angiopoietin-2 expression in aged mice. Whether this is related to the higher inflammatory response of endothelial cells observed in aged mice needs to be further established.

6.2 Future perspectives

Anaesthesiologists and intensivists are increasingly confronted with the challenging care of patients with multi-organ dysfunction syndrome (MODS) following severe inflammatory stress such as trauma, burn, endotoxaemia, and sepsis. Due to improved primary care, the aging population, and the prevalent use of treatments and interventions for example in cancer, transplantation or autoimmune diseases that compromise host immunity, patients more often develop MODS. MODS is still related to high mortality and morbidity[7] despite decades of clinical trials with a variety of drug candidates, which have largely failed to reverse MODS. To date management of patients with MODS consist of supportive therapies, treating the symptoms through maintaining normal values of physical parameters such as blood pressure by application of, for example, vasopressors, mechanical ventilation, and dialysis.

Multi organ failure, a complex syndrome

Different inhibitors and biological antagonists have been developed to modulate one or more of the numerous steps in the sequence of systemic inflammatory response syndrome (SIRS) and MODS but are not associated with improved outcome. A good example of a promising therapy was inhibiting TNF-α. TNF-α is a key mediator in inflammatory responses, when administered to humans, it produces fever, inflammation, tissue destruction and, in some cases, shock and death[8]. Several randomized controlled trials in sepsis where conducted with an monoclonal anti-tumor necrosis factor antibody or $F(ab')^2$ fragment of a murine antibody[9]. Although the first results looked promising [10,11] a large randomized controlled trial (NORASEPT II) found no beneficial effect on mortality[12]. Two following randomized trials (RAMSES and MONARCS study) stratified patients according their IL-6 levels and only found modestly reduced mortality rates in the subset of septic patients with high IL-6 levels[13,14].

Why did these promising therapies fail in clinical trails? Several considerations can be made. First, multi organ dysfunction syndrome following SIRS is a complex syndrome with high variability between patients: the causality of disease, timepoint of diagnosis and thus starting point of treatment, and the patient characteristics such as co-morbidities, age, sex, and

weight varies. When designing a clinical trial it is important to define which patients should be treated and when, thus careful selection of patients is essential. Results from these studies should provide clinicians instruments to identify subpopulations, which would benefit from a certain treatment. An example of a subpopulation could be aged patients. In chapter 5 of this thesis we observed that the inflammatory response to LPS in aged mice is higher than in young mice. It is likely that inflammatory responses in elderly patients are not the typical response of young, healthy individuals. Clinical research with detailed clinical observations and analyses of biochemical parameters in aged patients are needed to understand age-dependent mechanisms of organ dysfunction. Standards of care for elderly patients in the acute-care setting might need to be different than for young patients.

Second, the pathogenesis of MODS is complex and several systemic pathways are contributing to organ dysfunction including immune dysregulation, hormonal alterations, metabolic changes, mitochondrial dysfunction, microvascular dysfunction, coagulation dysfunction, and cell apoptosis[15]. Despite extensive research we still do not understand the full nature of this complex orchestration of reactions. The current knowledge is mainly based on necessary reductionistic approaches in which we simplify the complex syndrome and complex patients to gene level or pathway levels. An important change in this approach requires multidisciplinary collaborations in which extensive data are combined to understand the behavior of an entire system. Since the pathogenesis of sepsis is so complex, the question further arises whether single therapies to modulate the inflammatory would be effective or are combinations of therapies the necessary approach.

Third, in inflammation the pro-inflammatory cascade (SIRS) is opposed by the anti-inflammation cascade (CARS)[16]. In the development of MODS this system seems to be off balance and is excessive, sustained, or spatially and/or temporally misplaced[17], causing a hyperactive immune response or immunosuppression. Thus strategies should be directed at tempering, fine-tuning and recalibrating the systemic inflammatory responses but at the same time preventing immunosuppression and in some patient boosting the immunity. Monitoring the Immune functional status of individual shock patients is necessary to identify patients which will benefit from tempering or boosting the immune system. Thus patients with a predominantly pro-inflammatory response might benefit from anti-inflammatory therapy such as anti-TNF-α. Another example of the dual role of our immune system is demonstrated in this thesis. PMN influx in tissue is thought to play a role in tissue damage following injury. However in our model we observed, in PMN depleted mice, a biphasic pattern in endothelial activation in hemorrhagic shock and resuscitation. Thus PMNs might, besides detrimental effects, also have a protective role, in young as well as older mice, since kidney function was worse in PMN depleted mice. It is possible that in the pro-inflammatory phase depleting or supressing PMN is necessary while in the immunosuppressive phase a boost of the immune system is more important. In future studies reintroducing PMNs at varies time points in PMN depleted mice will provide more insight regarding the dual role of PMN in shock states.

Another important consideration in developing successful therapies is acknowledging the heterogeneous nature of the endothelium. The endothelium plays a part in inflammation and in homeostasis. This is for example evidenced by increased levels of the endothelial biomarkers (sFlt-1, PAI-1, sICAM-1, and sVCAM-1) in sepsis indicating that the endothelium is activated during the pathophysiological process[18,19]. Given its pivotal role, further studies on endothelial-directed therapies are warranted. In directing therapies to the endothelium it is important to realize not all vascular beds are affected to the same extent and via the same mechanism. Thus in order to develop pharmacologically effective drugs it is essential to design therapeutics that specifically target vascular beds to prevent unwanted effects on endothelium at non diseased locations. To accomplish this it is important to unravel the molecular and functional meaning of microvascular endothelial heterogeneity. An important step is already made in our group by laser microdissecting microvascular beds from tissue sections, thereby making it possible to investigate mRNA and possibly in the near future protein expression and kinase activity in specific vascular beds instead of in whole tissue homogenates[20]. By this means, the local niche of the endothelium remains known, as is its association with disease activity.

Models in shock research

Giving the complex nature of MODS, the multifactorial causes and diverse patient characteristics, the translation of laboratory mechanisms of disease into treatment is difficult. An increased understanding of the pathophysiology of MODS is necessary to therapeutically attenuate the development of MODS. Translational research is indispensable to accomplish this. Research in patients faces certain limitations. For example tissue biopsies are limited, for ethical and medical reasons. This is often resolved by measuring soluble molecules, which are shed by diseased cells, or in other ways released from the cells in the blood stream, and by examining easily available tissue such as skin. However, as previously mentioned the microvascular endothelium is heterogeneous, and for example skin tissue is not a good representative for kidney and the aorta is not a good representative for the glomeruli[21].

Animal models are thus crucial in translational research. The animal model most widely used for the study of the pathogenesis of diseases is the mouse (Mus Musculus). Mouse models have various advantages. First, the mouse is evolutionarily very close to humans (99% of human genes are conserved in the mouse [22]. Second, mouse models are cost-efficient being a small size animal with a short generation time[23]. Third, molecular and genetic tools such as knock out mice are readily available and hence helpful in clarifying how a gene, which has been sequenced, may cause or contribute to disease.

It is important that these models are clinically relevant. Much of the current understanding of the pathophysiology of MODS originates from studies in young animals while in most clinical trials related to MODS the mean age of patients is 60 years. Aging is an important independent factor for increased morbidity and mortality, and in chapter 4 we confirmed that in aged mice the inflammatory response is more severe, measured as a higher increase in adhesion

molecule expression and increased influx of neutrophils in tissue compared to the young mice. Other considerations that need to be taken into account, relate to co-morbidities. Patients with MODS often have comorbidities such as obesity, diabetes mellitus and cardiovascular diseases. Wielinga et al showed that mice subjected to a high-caloric Western diet develop vascular and renal activation and inflammation[24]. There is evidence that gene expression and biological function are considerably altered in the aged and obese patient[25-27]; it may be valuable to apply the models to aged or obese or aged and obese mice, which may reflect the elderly and obese population more accurately. Future studies in animal models should thus incorporate aged mice, and even better, mice which resemble obese patients. There are several limitations when evaluating the data from studies with aged animals. At what age do we consider a subject 'old' and which age should mice have to represent an elderly person? With normal mouse and human survival studies Turnbull et al showed parallel survival curves between mice and human[28]. The strain and supplier of the animal used in the experiment is important, as there is significant genetic variation between strains and mice from different suppliers, which may affect the processes that are important in shock. Also survival curves may differ between animals from different strains and animal suppliers[23,29].

Recent studies into the temporal gene expression patterns in white blood cells of humans and mice in conditions producing severe inflammatory stress such as trauma, burn, endotoxaemia, and sepsis, suggest that the current murine models poorly reflects the human disease[30]. In patients the changes in gene expression following trauma, endotoxaemia and burns were similar for these conditions, thus regardless of aetiology the mechanism underlying SIRS in man seems to be similar[30-32]. Several interventions such as fluids, drugs, surgery, and life support as part of the initial clinical treatment of patients, likely alter gene expression of white blood cells that are not captured in murine models[30]. Furthermore mice are highly resilient to inflammatory challenge and maybe higher levels of injury are necessary to mimic the same response seen in patients[30]. A promising technology to make the mouse model more clinically relevant is 'genomically' humanized mouse models in which human genomic loci are transferred into the mouse genome[33].

The role of the PMN in shock states

PMN-endothelial interactions may play a role in endothelial adaptation to shock. In this thesis we employed a mouse model in which we *in vivo* depleted neutrophils before shock induction. The results of this PMN depletion study are at least difficult to interpret. In our HS model we observed a biphasic pattern in endothelial activation in hemorrhagic shock and resuscitation, moreover we observed higher levels of BUN in PMN depleted mice. Also in our LPS model we observed in PMN-depleted mice higher levels of NGAL and BUN levels compared to untreated controls. Possibly, this outcome is a result from the dual role of PMN since PMN contribute to tissue injury but is also involved in the termination of inflammatory processes[35]. The crucial role of PMN in our innate immunity could make them an important target for therapeutic strategies in inflammatory conditions such as MODS following sepsis or hemorrhagic shock. Understanding the mechanism and kinetics by which PMN endothelial

interactions play a beneficial or harmful role in the development of MODS is important for future research. However, research to unravelling the mechanisms of PMN-endothelial interactions faces several difficulties. *In vitro* studies to unravel the role of the neutrophil-endothelial interactions are for several reasons difficult to employ. First, neutrophils exhibit a short life span and are terminally differentiated, preventing growth in tissue culture. Immortalized cell lines do not reflect the functional diversification of neutrophils and neutrophils studied *in vitro* do not mimic the complex biology seen in tissues or circulation. *In vivo* studies of neutrophil function also raise concerns. Mouse neutrophils differ in important aspects from their human equivalents. An example of this is the different numbers of PMN in circulation (30% versus 70% in mice and humans, respectively)[36]. In man, studies are often performed on easily available circulating PMN isolated from blood. Exudated PMN exhibit other functions than their circulating counterparts and thus may circulating PMN not be a good representative of their local counterparts in the tissues[37]. Despite these difficulties it is of importance to get more insight in the many functions and kinetics of the PMN *in vivo* in order to develop therapeutic strategies to find the therapeutic window in which the beneficial and harmful effects of the PMN can be fine-tuned.

Conclusion

MODS following shock is a complex syndrome. During shock states there is early organ and vascular bed specific activation of the endothelium. For future research it is necessary to broaden our knowledge via multidisciplinary collaboration to get a better view on the complexity of the underlying processes. Therapies should be investigated that are directed at balancing the endothelium, preferably via a vascular bed specific manner. For clinical trials to be successful it is important that patients stratified for treatment are much more specifically and narrowly defined, using the above mentioned multidisciplinary approach that maps the integrated complexity of the syndrome.

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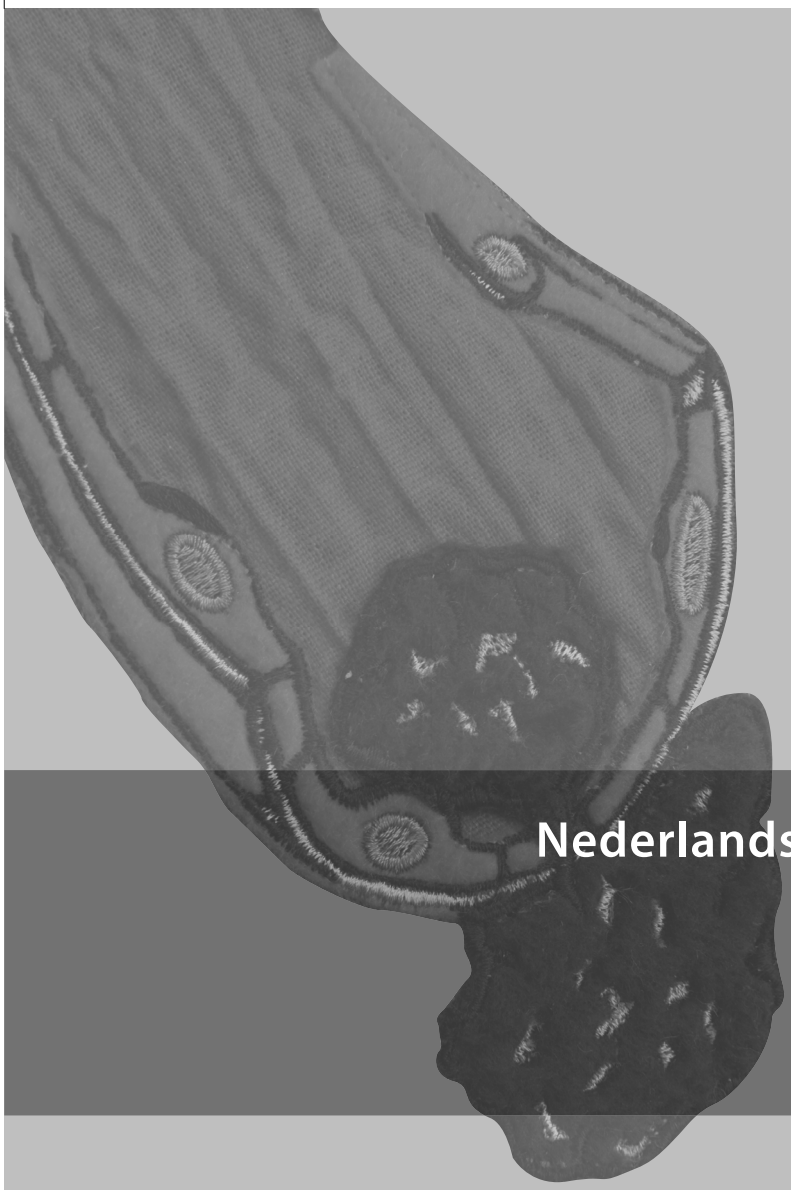


Chapter 7

Nederlandse samenvatting

Nederlandse samenvatting

Wanneer een traumapatiënt met verbloedingsshock wordt opgevangen op de eerste hulp staat er een heel team klaar om deze patiënt te behandelen. Deze behandeling bestaat uit het aanvullen van het verloren bloedvolume en het opsporen van de bloedingsfocus om deze zo snel mogelijk te stelpen. Door deze zogenaamde "damage control" - opvang is het aantal patiënten dat de directe gevolgen van verbloedingsshock overleeft in de laatste decennia verbeterd. Na een succesvolle initiële eerste opvang heeft de traumapatiënt echter kans op het ontwikkelen van een ernstige complicatie. Het functioneren van organen, die niet noodzakelijkerwijs aangedaan zijn door het primaire letsel, kan namelijk gestoord raken. Dit syndroom noemt men Multi-orgaanfalen (MOF). Een ander veelvoorkomende aandoening die aanleiding kan geven tot de ontwikkeling van MOF is een bloedvergiftiging, ook wel sepsis genoemd. Er zijn diverse theorieën betreffende de ontstaanswijze van dit syndroom, echter de oorzaak van MOF is nog niet opgehelderd. De therapie bestaat momenteel uit de behandeling van de symptomen en het onderliggend ziektebeeld, zoals het stelpen van de bloeding en, in geval van sepsis, het behandelen van de onderliggende infectie door onder ander het toedienen van gerichte antibiotica. Daarnaast wordt geprobeerd om de orgaanfunctie waar mogelijk tijdelijk medicamenteus of machinaal te ondersteunen, bijvoorbeeld door beademing of nierfunctievervangende therapie.

Het endotheel speelt een belangrijke rol in het ontstaan van MOF. Endotheelcellen vormen de binnenste bekleding van onze bloedvaten en zijn de grens tussen bloed en weefsel. Het endotheel speelt een rol bij diverse belangrijke functies zoals bijvoorbeeld de regulatie van influx van ontstekingscellen en de balans tussen stolling en antistolling. Verder reguleert het endotheel de bloeddruk doormiddel van regulering van de tonus van de vaten en de doorlaatbaarheid (permeabiliteit) van de haarvaatjes (ook wel capillairen genoemd) voor zuurstof en nutriënten. Indien het endotheel niet meer optimaal kan functioneren spreekt met van endotheeldisfunctie.

In dit proefschrift gaan we uit van het principe dat MOF ten gevolge van shock door verbloeding of sepsis mede veroorzaakt wordt door activatie en disfunctioneren van het endotheel. Wij richten ons in dit proefschrift op het endotheel in de capillairen, ook wel microcirculatie genoemd. Alhier treden de ontstekingscellen uit en vindt lekkage door de vaatwand plaats ten tijde van MOF. Het endotheel heeft zeer uiteenlopende eigenschappen naargelang hun locatie in het vasculair systeem. Het is belangrijk om het gedrag van endotheelcellen gedurende shock in hun natuurlijke omgeving te begrijpen. Wanneer, waar, en hoe raakt het endotheel geactiveerd gedurende shock? Hiermee zouden we namelijk aanwijzingen kunnen vinden om in de toekomst therapieën te ontwikkelen waarmee we vroegtijdig een gerichte behandeling kunnen geven voor MOF.

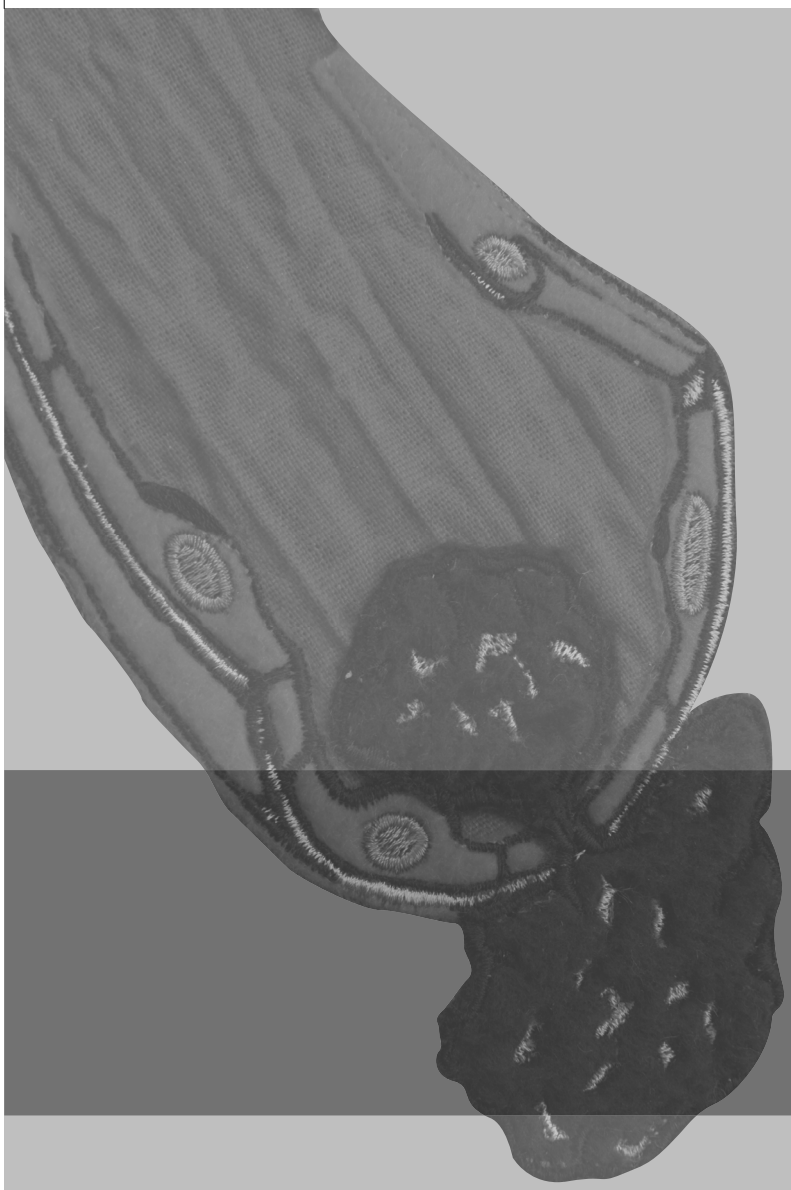
In **hoofdstuk 2** gebruiken we een muismodel waarin we verbloedingsshock nabootsten door het verwijderen van een gedeelte van de bloedvolume van de muis gevolgd door een behandeling met vocht om de bloeddruk weer op niveau te krijgen zoals dat ook gebeurt

bij bijvoorbeeld traumapatiënten. We laten in dit model zien dat er al heel vroeg tijdens verbloedingsshock activatie van endotheelcellen plaats vind in de microcirculatie in organen. Tegelijkertijd zagen we dat gedurende de shock perioden en na de behandeling met vocht de hoeveelheid ontstekingscellen in weefsels toenam. Hoewel elk orgaan endotheelactivatie liet zien was het patroon wel verschillend per orgaan en daarnaast ook specifiek per vaatbed binnen een orgaan. Er was dus sprake van een heterogeen beeld.
Ontstekingscellen zoals neutrofielen zijn een belangrijk onderdeel van ons immuunsysteem en spelen een rol in de bestrijding van infecties. De neutrofiel is een witte bloedcel. Andere witte bloedcellen zijn bijvoorbeeld lymfocyten en monocyten. Bij mensen vormt de neutrofiel de grootste groep witte bloedcellen. De belangrijkste rol van neutrofielen bestaat uit het opnemen en verteren van met name bacteriën. Echter neutrofielen hebben echter ook een schaduwzijde. Wanneer ze eenmaal uit de bloedbaan zijn getreden kunnen ze ook schadelijk zijn voor gezond weefsel. In **hoofdstuk 3** beschrijven we een studie betreffende de rol van neutrofielen in het verbloedingsshock-model. In deze studie werden de muizen eerst behandeld met een antilichaam zodat de neutrofielen niet meer functioneren. We richtten ons specifiek op het functioneren van de nier omdat dit een van de organen is die frequent faalt bij verbloedingsshock en omdat we na inductie van shock in hoofdstuk 2 veel neutrofielen in nierweefsel aantroffen. We konden echter laten zien dat het verwijderen van de mogelijk schadelijke neutrofielen geen afname van endotheelactivatie en geen vermindering van nierschade veroorzaakte.

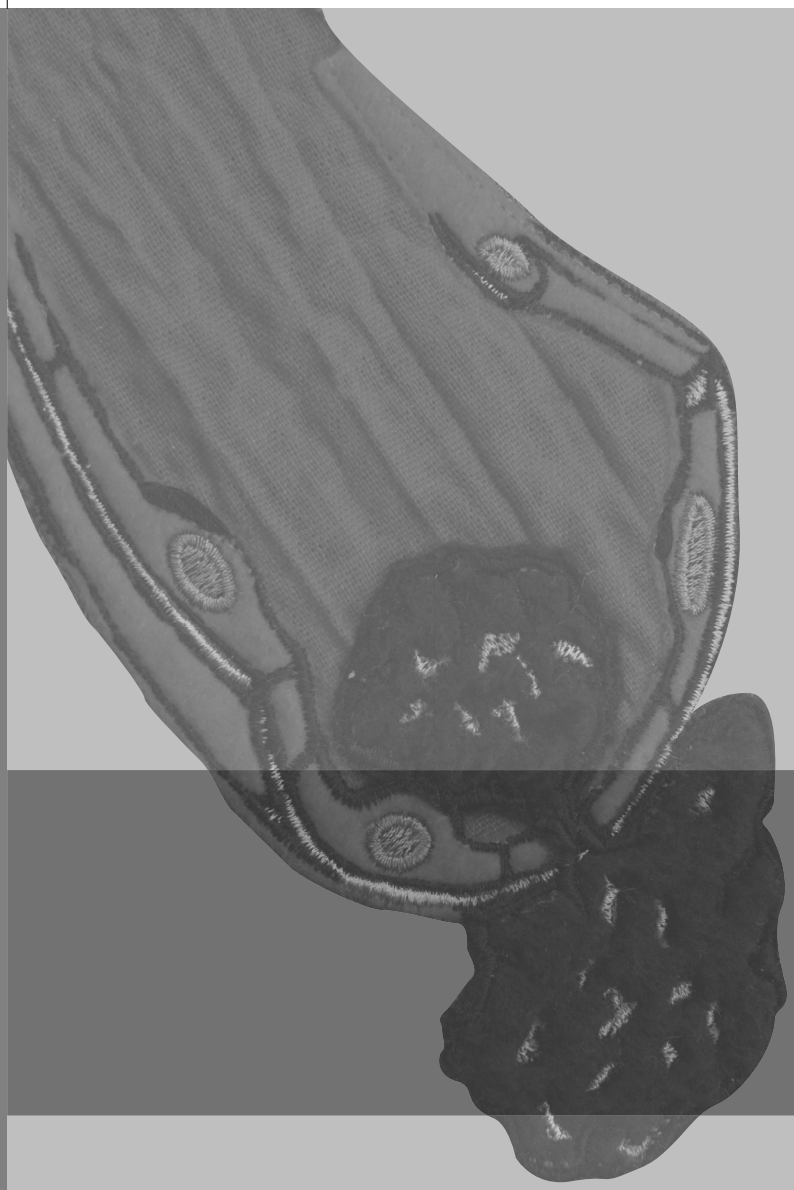
Gedurende de behandeling van patiënten met verbloedingsshock is het belangrijk dat patiënten voldoende zuurstof krijgen en dat koolzuurgas op normaal niveau blijft. Daarnaast is het zo dat deze patiënten vaak operatieve ingrepen ondergaan om de bloeding te stelpen waarbij deze patiënten veelal worden beademd. Kunstmatige beademing kan echter ook ongewenste bijeffecten hebben. Niet alleen kan reeds aanwezige longschade worden verergerd, het kan ook longschade induceren en leiden tot endotheelactivatie in andere organen. In **hoofdstuk 4** observeerden we dat kunstmatige beademing in het muismodel niet resulteerde in toegenomen endotheelactivatie. Experimenten waarin de rol van zuurstoftekort op endotheelactivatie nader werd onderzocht lieten zien dat zuurstoftekort *per se* in weefsels niet leidt tot endotheelactivatie.

Oudere patiënten hebben een groter risico dan jonge patiënten op het ontwikkelen van orgaanfalen na shock. Veelal is dit omdat ouderen meer onderliggende ziekten hebben dan jonge patiënten, echter ook gezonde ouderen zijn gevoeliger voor het ontwikkelen van orgaanfalen met een verhoogd risico op overlijden. Wij veronderstelden dat het endotheel van ouderen mogelijk reeds geactiveerd is als gevolg van (kleine) beschadigingen gedurende het ouder worden en dat ouderen daarom kwetsbaarder zijn voor de gevolgen van shock. In **hoofdstuk 5** gebruikten we een muismodel voor bloedvergiftiging (sepsis) waarin jonge en oude muizen een bestanddeel van bacteriën toegediend kregen om een ontstekingsreactie te induceren. We observeerden dat oude muizen meer activatie van het endotheel hebben en dat het aantal neutrofielen dat uittreedt in de weefsels groter is bij oudere muizen in vergelijking met de jonge muizen.

Samenvattend tonen de experimenten beschreven in dit proefschrift aan dat endotheelactivatie vroegtijdig tijdens shock plaatsvindt en dat dit gebeurt op een orgaan- en vaatbed-specifieke wijze. Hoewel neutrofielen een belangrijke oorzaak kunnen zijn in het ontstaan van orgaanfunctie stoornissen, laat het verwijderen van neutrofielen in ons muismodel van shock geen verbetering van de nierfunctie zien. Een andere oorzaak van MOF is zuurstof-tekort, echter ook zuurstof-tekort in weefsels lijkt in ons model geen belangrijke rol te spelen in endotheelactivatie. De leeftijd van de muis daarentegen speelt wel een rol. MOF is een complex ziektebeeld, en toekomstige therapieën moeten gericht zijn op het in balans brengen van het endotheel bij voorkeur op een orgaan- en vaatbed- specifieke wijze.



Appendices



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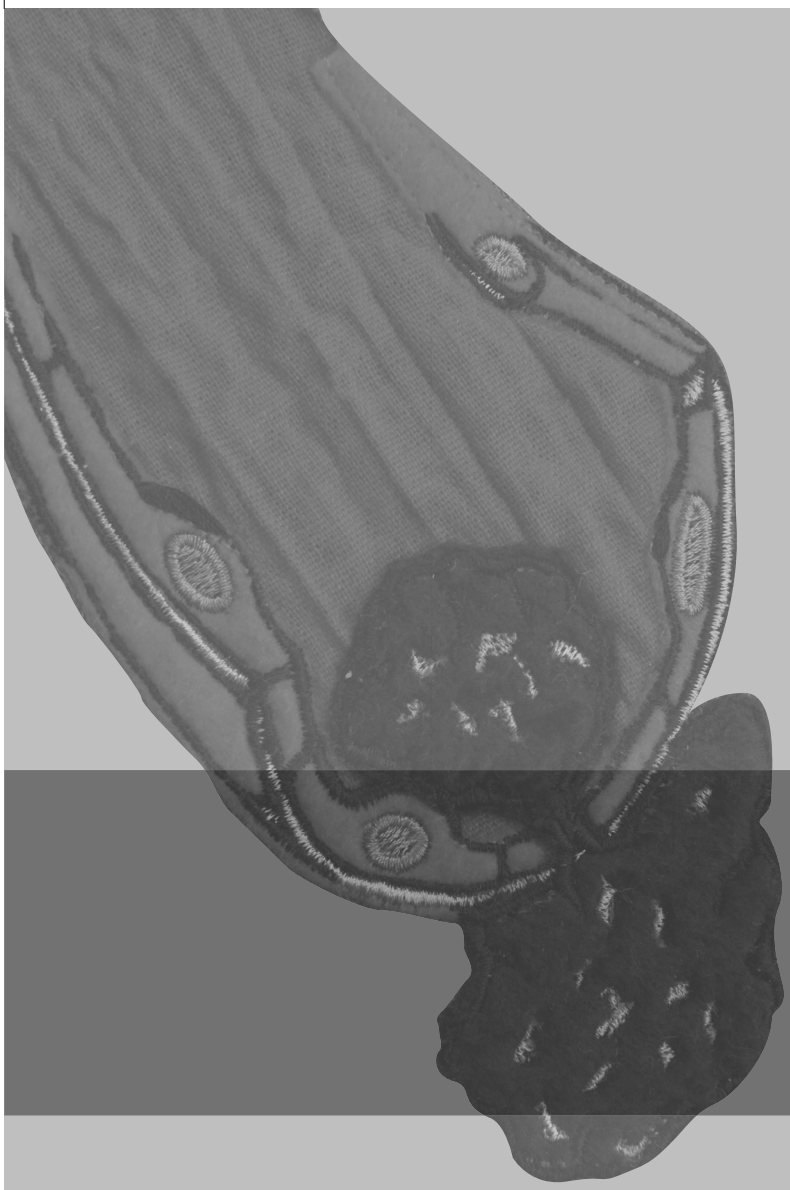

Biografie/Biography

Biografie

Francis Wulfert is geboren op 19 september 1974 in Heerhugowaard (Noord-Holland). Na het behalen van haar VWO-diploma is zij in 1995 gestart met haar geneeskunde studie aan de Universiteit van Amsterdam. In 2002 rondde zij deze studie af. Hierna heeft zij als arts-docent, als AGNIO chirurgie in het Westfriesgasthuis te Hoorn en als eerste hulp arts in het Waterlandziekenhuis te Purmerend gewerkt. In september 2005 verhuisde Francis naar Groningen om daar bij de afdeling anesthesiologie en de afdeling medische biologie van het Universitair Medisch Centrum Groningen (UMCG) onderzoek te doen naar endotheel activatie als gevolg van shock onder begeleiding van Prof. dr. Ingrid Molema en Prof. dr. Leon Aarts. In 2006 is zij gestart met de opleiding tot anesthesioloog, waarbij zij In 2007 naar Almelo is gegaan om daar in het Ziekenhuis groep Twente ziekenhuis haar perifere stage jaar voor de opleiding anesthesiologie te volgen. In 2007 nam Prof. dr. ing. Jan Zijlstra de begeleiding van Prof. dr. Leon Aarts over en kreeg ook Prof. Michel Struys een rol in de begeleiding van het onderzoek. In 2014 leidde de resultaten van het onderzoek tot dit proefschrift waarvan een groot deel tot stand kwam tijdens de opleidingsperiode. Sinds mei 2012 na het succesvol afronden van de opleiding tot anesthesioloog werkt Francis als staflid anesthesiologie in het UMCG.

Biography

Francis Wulfert was born on September 19th 1974 in Heerhugowaard, the Netherlands. After graduation from high school in 1995 she started her medicine study at the University of Amsterdam. She completed this study in 2002 and worked subsequently as a medical teacher, as a resident in Westfriesgasthuis in Hoorn and as a physician at the emergency department in Waterlandziekenhuis in Purmerend. In September 2005 she moved to Groningen to start as a PhD student at the department of Anesthesiology and the department of Pathology and Medical Biology at the University Medical Center Groningen (UMCG). Under supervision of Prof. dr. Ingrid Molema and Prof. dr. Leon Aarts she studied endothelial responses in shock conditions. In 2006 she started as a resident in training in Anesthesiology. One year of her residency was followed in Twente Ziekenhuis Almelo. In 2007 Prof. dr. ing. Jan Zijlstra followed up Prof. dr. Leon Aarts as supervisor and in addition Prof. dr. Michel Struys took part in supervision of the research project. In 2014 the results led to this thesis. Since May 2012, after successful completion of her residency in anesthesiology, Francis started working as a faculty member of the department of Anesthesiology at the UMCG.

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1. van Meurs M*, Wulfert FM*, Knol AJ, De Haes A, Houwertjes M, Aarts LPHJ, Molema G: Early organ-specific endothelial activation during hemorrhagic shock and resuscitation. Shock 2008; 29:291-9 (*authors contributed equally)
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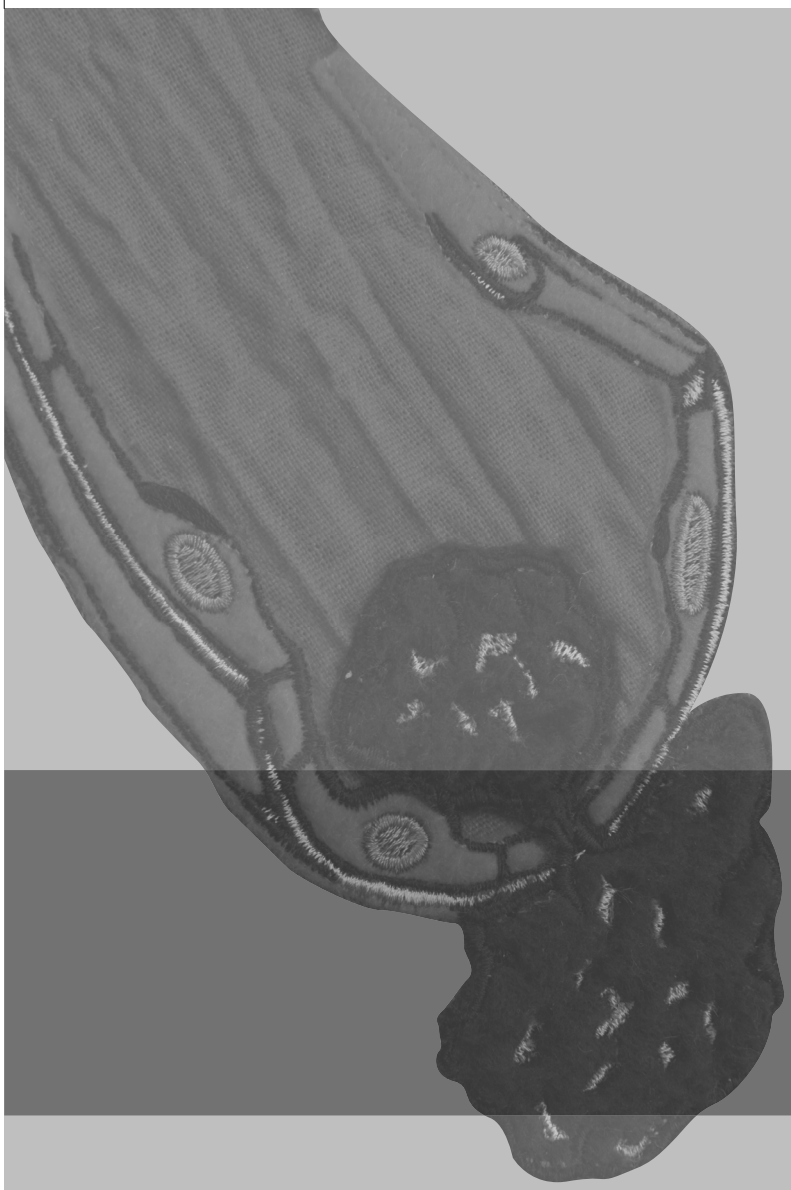

Dankwoord

Dankwoord
Dit avontuur is geëindigd! Het ging over heuvels en dalen maar de top was de moeite zeker waard. Onderweg heb ik veel geleerd over mezelf en anderen. Ik heb dit avontuur zeker niet alleen beleefd.

Prof. dr. Ingrid Molema, jij opende voor mij een nieuwe wereld, de basale wetenschap. Ik heb me iedere keer weer verbaasd over jou onuitputtelijk enthousiasme. Op inhoudelijk vlak kon ik me geen betere begeleider wensen. Je leidde me op tot onderzoekster, je gaf me de tools om zelfstandig het pad van onderzoek te bewandelen maar ving me op als ik dreigde te struikelen.
Prof. dr. ing Jan Zijlstra, jij vertelde me dat promoveren vooral ook leuk moet zijn. Bedankt voor jou bijdrage en inzet, je ondersteunde me juist op het moment dat ik dat nodig had.
Prof. dr. Michel Struys, je bent het team pas in een later stadium komen versterken. Ik kijk ernaar uit in de toekomst nog mooie wetenschappelijke paden met jou te bewandelen.

De leden van de beoordelingscommissie prof. dr. Henning, prof. dr. Tony Absalom en prof. dr. Leon Aarts dank ik voor het kritisch lezen en beoordelen van het manuscript. Prof. dr. Rob Henning wanneer ik gedurende mijn promotie met jou sprak werd ik altijd geïnspireerd door de energie waarmee jij met onderzoek bezig bent. Prof. dr. Tony Absalom, ons avontuur gaat nu beginnen, er zal nog mooi onderzoek volgen.
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Francis
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