The endothelium in sepsis
Rafat, Neysan
THE ENDOTHELIALM IN SEPSIS:
inflammatory response and progenitor cell involvement

Neysan Rafat
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This dissertation is dedicated to the Bahá’í youth in Iran.

Members of the Bahá’í-Faith in the Islamic Republic of Iran are denied access to higher education due to their religion, regardless of the fact that education is a fundamental human right according to Article 26, paragraph 1 of the Universal Declaration of Human Rights (UDHR), which also Iran adopted in 1948. As such, no Bahá’í youth in Iran would be permitted to conduct such a dissertation. Therefore, this dissertation should be considered on their behalf.
Paranimfen: Shamim Rafat
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CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THE THESIS
1.1 GENERAL INTRODUCTION

1.1.1 GENERAL ASPECTS OF SEPSIS

Sepsis describes a complex clinical syndrome, which is characterized by a systemic inflammatory response of the organism to the presence of an infection \(^1\). Due to their impaired immune system, the very young and very old are particularly prone to develop sepsis. Furthermore, persons of all ages are at risk, who are recovering from major surgery or trauma, who are receiving immuno-modulating drugs or total parenteral nutrition, or who have immuno-compromising diseases (e.g., cancer, diabetes, or AIDS). The severity of sepsis varies widely. Although in some patients the inflammatory response may be relatively mild and self-limited, in many patients sepsis may lead to organ dysfunction, organ hypoperfusion and hypotension. Septic shock occurs in a subset of patients suffering from severe sepsis and refers to a condition in which hypotension does not respond to fluid resuscitation.

Sepsis is a leading cause of death among the elderly, especially on the Intensive Care Unit. According to surveys of the German Sepsis-Network “SepNet”, about 300 per 100,000 people contract sepsis each year in Germany. This is comparable to the incidence of myocardial infarction (360/100,000), and clearly higher than the incidence of breast cancer (110/100,000) and of colon cancer (50/100,000). Findings in the United States of America, representative for industrialized countries, showed identical results (3 cases per 1,000 population) \(^2\). When looking at the general causes of death, sepsis has a death rate of 9% and therefore a similar importance as myocardial infarction \(^3\). In-hospital mortality among patients with severe sepsis was recently estimated to be 29%, although rates as high as 50% have been reported in the literature \(^2, 4\). For patients with septic shock, mortality is even higher \(^5, 6\). Not surprisingly, the economic costs of hospital care for patients with severe sepsis are substantial, >23,000 EUR per patient in Germany ($ 22,100 in the United States), which makes it one of the most cost-intensive diseases of our time \(^2, 3\). Despite considerable effort to understand the systemic inflammatory response and characteristics of severe sepsis and despite the progress in critical care management, no significant improvement in the mortality of septic patients could be noticed over the past decade \(^7, 8\).

1.1.2. PATHOGENESIS AND PATHOPHYSIOLOGY OF SEPSIS

The causes of sepsis are multifactorial and can include practically any infectious organism. For a long time, infections with Gram-negative bacteria have been considered to be the most frequent cause of this condition \(^9\). Recent studies however, have indicated
that Gram-positive infections are more frequently found in septic patients. An effective immune response to pathogens depends on the proper activation, regulation and effector function of immune cells. The primary function of innate immunity is to recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors, which trigger signals resulting in pro-inflammatory gene expression, leukocyte chemotaxis, phagocytosis, cytotoxicity and activation of adaptive immune responses. To restore the equilibrium, an anti-inflammatory response is induced subsequently. In a favorable case, homeostasis is re-established. However, an overreaction of the pro-inflammatory response can lead to a compensatory anti-inflammatory response in which the pro-inflammatory and anti-inflammatory responses counterbalance each other. The body is in a state of immune paralysis and unable to produce an adequate immune response.

To understand the pathophysiology of sepsis, we have to bear in mind that it comprises multiple derangements involving several different organs and systems. In general, sepsis develops when the initial, appropriate host response to an infection becomes amplified and then dysregulated, e.g., septic patients have substantial, life-threatening alterations in their coagulation system. In the past, it has been suggested that sepsis merely represents an excessive hyper-inflammatory response with patients dying from inflammation-induced organ injury. More recent data indicate that considerable heterogeneity exists in the inflammatory response of septic patients. Some patients appear to be immuno-stimulated, since increased levels of pro-inflammatory cytokines can be detected in the peripheral blood, whereas others appear suppressed and show decreased levels of pro-inflammatory cytokines. Heterogeneity is also found in the cellular changes. The function of some cells are enhanced such as neutrophils that remain activated for an extended time. Other cellular changes become accelerated in a destructive way including lymphocyte apoptosis. Furthermore, metabolic changes are observed in septic patients. To present knowledge no single mediator/system/pathway/pathogen drives the pathophysiology of sepsis, implying a highly complex pathology.

Inflammatory Response
The inflammatory response represents an important element of the immune response to pathogens, because an appropriate inflammatory response eliminates the invading microorganisms without causing too much damage to tissues or organs. During the onset of sepsis the inflammatory system becomes hyperactive and an array of pro-inflammatory mediators are released, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-8. The primary role of these cytokines is to enhance leukocyte infiltration into sub-endothelial compartments at the site of infection to control and clear the invading pathogen rapidly. Simultaneously, strong production of
acute-phase-proteins, such as C-reactive protein, occurs and the complement system is activated.

Much attention has been given to the relevance of the pro-inflammatory response in the clinical outcome of septic patients. This was based on the observation that septic patients with increased serum-levels of specific mediators such as TNF-α are at increased risk for death. In animal studies, injection of TNF-α molecules resulted in widespread inflammatory alterations and tissue injury similar to that observed in septic patients. Furthermore, injection of lethal doses of the endotoxin lipopolysaccharide (LPS) resulted in elevated serum-levels of the same mediators and inhibition of these specific mediators improved survival in murine endotoxin shock models. These observations launched a series of clinical trials aimed at blocking TNF-α or IL-1β, but these trials failed to show improvements in sepsis survival. One confounding factor is that TNF-α levels were not always increased in patients, in part because of the rapid kinetics of the TNF-α response. Serum TNF-α and IL-1β reach toxic levels in mice and human volunteers within 1-2 h after LPS infusion and delayed treatment with anti-TNF-α or anti IL-1β failed to prevent late endotoxin deaths. Moreover, endotoxin-responsive mice treated with lethal doses of endotoxin often succumbed at latencies of up to 5 d, long after serum TNF-α and IL-1β have returned to basal levels. The insights gained from the failure of clinical trials of anti-TNF-α strategies, as well as the observations regarding the kinetics of TNF-α release compared with the onset of lethality, prompted a search for a late mediator of endotoxin-induced death. In this search high-mobility group box (HMGB)-1 was identified as a late mediator of endotoxin lethality in mice. Originally, HMGB-1 was identified as a nuclear DNA-binding protein that functions as a structural cofactor for proper DNA-transcriptional regulation and gene expression. Recent studies indicate that immune cells can liberate HMGB-1 into the extracellular milieu where it functions as a pro-inflammatory cytokine. Furthermore, HMGB-1 is recognized by the innate immune system as a necrotic marker to signal tissue damage.
Figure 1. Early versus late mediators of endotoxin lethality in mice. Mice treated with a lethal dose of LPS succumb at latencies of up to several days, long after serum TNF-α and IL-1β have returned to basal levels. HMGB-1 release occurs at a delayed stage of sepsis and parallels the onset of lethality. Reprinted with permission from Wang H, Yang H, Czura CJ, Sama AE, Tracey KJ. (2001) HMGB1 as a late mediator of lethal systemic inflammation. Am J Respir Crit Care Med;164:1768-73. Official Journal of the American Thoracic Society. © American Thoracic Society.

In a mouse model, serum HMGB-1 was minimally detectable at 8h after administration of a median lethal dose (LD₅₀) of LPS, and increased to a prolonged plateau level from 16 to 32 h after LPS treatment (fig.1) ³⁰. Delayed administration of antibodies to HMGB-1 attenuated endotoxin lethality in mice, and administration of HMGB-1 itself was lethal ³⁰. These characteristics distinguishes HMGB-1 from previously described early cytokine mediators of LPS lethality. In view of the late and prolonged kinetics of HMGB-1 in mice and considering that clinical signs of sepsis typically develop long after the early cytokine response to the acute infection, these results support the assumption that HMGB-1 might play a role in sepsis. In fact, in septic patients increased levels of HMGB-1 could be detected, whereas it was not detectable in healthy controls. In addition, serum HMGB-1 levels were significantly higher in septic patients who did not survive as compared with survivors ³⁰.

Another observation revealed that stimulation of the vagus nerve could attenuate production of inflammatory cytokines such as TNF-α ³⁶. The effect was mediated through the α7 subunit of the nicotinic acetyl choline receptor ³⁷, providing an innovative prospect for inhibition of the inflammatory cascade. Recently, transcutaneous vagus nerve stimulation was shown to not only reduce serum TNF-α levels, but also to inhibit serum HMGB-1 levels and improve survival in a murine sepsis model ³⁸. Whether this activity can be therapeutically utilized for septic patients has still to be proven.

The excessive pro-inflammatory response that occurs in sepsis is balanced by an array of counter-regulatory anti-inflammatory mediators that attempt to restore immunological equilibrium. Counter-inflammatory mediators include antagonists such as the soluble TNF receptors and IL-1 receptor antagonists, inactivators of the complement cascade and anti-inflammatory cytokines like IL-10, which lead to a decline in the production of many of the pro-inflammatory mediators. This is accompanied by a decrease in HLA-DR expression on monocytes ³⁹. Another aspect of down-regulation of immunity in the course of sepsis is the development of lymphocyte apoptosis. Extensive lymphocyte apoptosis is seen in animal models of sepsis and also occurs in septic patients, but much less in critically ill non-septic controls ⁴⁰, ⁴¹. Septic patients are usually lymphopenic, and analysis of autopsy tissue samples has demonstrated a selective depletion of B and CD4⁺ lymphocytes ⁴². The functional consequences of this comprise a more general immune
paralysis, characterized by T-cell hyporesponsiveness and anergy, a state of non-responsiveness to antigens. This counter-balancing response to the initial pro-inflammatory state can also be considered as an overresponse and an inadequate host defense against infection, therefore constituting a potential mediator of severe sepsis and progressive organ failure. This notion has prompted efforts to restore immune activation with agents such as interferon (IFN)-γ\textsuperscript{39}, granulocyte colony-stimulating factor (G-CSF)\textsuperscript{43} or granulocyte macrophage colony-stimulating factor (GM-CSF)\textsuperscript{44}. These clinical trials only showed little success in terms of improved survival\textsuperscript{45}. Taken together, these data indicate that the inflammatory response in septic patients is complex and multifactorial and not simply defined as enhanced or decreased. It rather displays a dynamic process and varies in time. While in the onset of sepsis the inflammatory system becomes hyperactive, in later stages the excessive pro-inflammatory response is counterbalanced by an anti-inflammatory response possibly resulting in immune paralysis (fig. 2). This was supported by the finding that PBMCs obtained from patients in earlier stages of sepsis, i.e., without organ dysfunction or shock, were hyperresponsive to LPS regarding production of inflammatory cytokines such as IL-6 and TNF-α\textsuperscript{46}. And in contrast to this, patients with severe sepsis and septic shock displayed a down-regulation of pro-inflammatory cytokines in whole blood\textsuperscript{47}.

Figure 2. Dynamic time-course of the inflammatory response during sepsis. Various stimuli can cause activation of different cell types and serum proteins, as well as of the coagulation and
complement system, leading to excessive production of pro-inflammatory cytokines and chemokines and up-regulation of adhesion molecules on endothelial cells and polymorphonuclear leukocytes (PMNs). Monocytes and PMNs release large amounts of granular enzymes and generate ROS in response to the original stimulus in the early (hyperreactive) phase of sepsis. As result of excessive pro-inflammatory mediator production, vascular permeability increases, tissue damage and organ failure occur and crucial innate immune functions become defective, resulting in increased susceptibility toward infection in the later (hyporeactive) phase of the immune response, often along with the occurrence of immune paralysis. DIC, disseminated intravascular coagulopathy. Reprinted with permission from Riedemann NC, Guo RF, Ward PA. (2003) Novel strategies for the treatment of sepsis. Nature Medicine;9:517-24. © 2003 Nature Publishing Group.

Dysregulated Coagulation
In normal hemostasis there is a balance between the blood being fluid to allow free flow within the vessels and appropriate clotting to control bleeding (for more details on molecules involved in coagulation, see chapter 1.1.4). Inflammation can cause significant alterations at multiple levels within the coagulation system and the cells that regulate this system \(^{48}\). Septic patients frequently manifest disseminated intravascular coagulation (DIC) with consumption of platelets and prolongation of clotting times. In addition, the altered hemostasis leads to an inappropriate blood clotting, which results in clogged blood vessels and reduced blood flow. The interaction between the clotting system, circulating white blood cells and platelets, and the endothelium, covering all blood vessel walls in our body, adds another level to an already multifaceted picture. Due to the severe coagulation abnormalities, clinical trials with anticoagulant recombinant activated protein C (APC), which inactivates factors V and VIII of the clotting cascade, have been initiated following studies in the baboon model of Escherichia coli sepsis \(^{49,50}\). APC turned out to improve survival in patients with severe sepsis, but it is clearly not a panacea for all patients \(^{51}\). The most beneficial effects were observed in patients with the worst prognosis. On the other hand, patients at low risk for death had no improvement in survival and a significantly increased risk for bleeding when treated with APC \(^{51}\).

Cellular Dysfunction
Many cellular aspects become dysfunctional in sepsis. The induction of cellular apoptosis and necrosis is one of the areas of active investigation and has been reviewed elsewhere in detail \(^{52}\). Increased apoptosis may contribute to the pathogenesis of sepsis by delayed removal of those cells that should be removed, e.g., neutrophils, and early removal of those cells that should not be removed, e.g., lymphocytes.

Significant apoptosis of lymphocytes has been demonstrated in septic patients. These apoptotic lymphocytes were observed in all lymphoid organs including the spleen and thymus, but also in the gastric associated lymphatic tissue \(^{51}\). Blocking lymphocyte
apoptosis in peritonitis improved survival in murine sepsis models. Consequently, it has been speculated that prevention of apoptosis may be efficacious in sepsis by preventing immune suppression that occurs in the later phase of the immune response in sepsis. Polymorphonuclear neutrophils (PMN) with their potent oxidative and proteolytic potential are a critical component of the innate immune response to infectious challenges. Neutropenic patients, regardless of the cause of the neutropenia, and patients with PMN dysfunction are at increased risk for the development of infectious complications. An appropriate, strong PMN activation benefits the patient and helps to eradicate an infectious focus. It is difficult to define an appropriate response versus a hyperactive response. Patients who have suffered traumatic injury are at increased risk for the development of multisytem organ failure due to increased chemotactic responses of PMN to CXC chemokines. However, PMN isolated from septic patients demonstrate decreased chemotaxis toward IL-8 and depressed expression of CXCR2. In accordance with the above, it was confirmed in early trauma patients that high CXCR2 function correlated with the development of organ injury, e.g. acute respiratory distress syndrome, whereas low function predisposed to pneumonia and sepsis.

Another significant matter concerns PMN survival since inappropriate apoptosis of PMN occurs in septic patients. Neutrophils in the circulation typically have a short lifespan of only 5–6 hours following their maturation and release from bone marrow stores, and ≤24–36 hours when cultured in vitro. However, patients with sepsis have a delay in their neutrophil apoptosis, causing them to persist longer in the bloodstream. One of the reasons is a prolonged activation of nuclear factor κB (NFκB) and reduced caspase-3 activity. Consequently, the prolonged exposure of organs to the cytotoxic factors produced by PMN can lead to organ injury. So, on one side, the oxidant potential of neutrophils is increased, while at the same time functions such as chemotaxis and phagocytosis are often depressed.

1.1.3 SEPSIS AND MICROCIRCULATORY DYSFUNCTION

The microcirculation constitutes a functionally highly active system that dynamically interacts with circulating and tissue-associated cells (i.e., leukocytes, platelets, endothelial cells), and contributes to local, downstream and even upstream regulation of vascular tone. When this system is damaged, it can affect all participating cellular components, in particular endothelial cells, smooth muscle cells, as well as circulating blood cells. Activation, dysfunction and injury of microvascular endothelial cells may occur as a result of ischemia, inflammatory mediators, as well as adherent leukocytes, in particular neutrophils with their attribute to produce cytotoxic factors and proteolytic enzymes as mentioned in 1.1.2. Consequences of microcirculatory dysfunction include
the breakdown of endothelial and epithelial barrier function, leading to tissue edema and uncontrolled inflammatory cell infiltration. Furthermore, vasodysregulation occurs leading to the formation of arteriovenous shunts and/or the loss of peripheral resistance with severe macrohemodynamic consequences, disturbance of oxygen transport and utilization by tissue cells. In sepsis circulatory disturbances, including decreased peripheral vascular resistance and maldistribution of blood flow, as well as disturbance of oxygen transport occur, leading to focal tissue hypoxia and cell injury. This dysfunction of microcirculation is one feature of the sepsis-related multiorgan failure. The characteristic macrohemodynamic feature of sepsis in its advanced manifestation is a vasodilatory shock, characterized by hypotension as a result of loss of peripheral resistance due to the failure of vascular smooth muscle to constrict. The loss of peripheral resistance occurs despite markedly increased blood levels of catecholamines and is characterized by a poor response of the vascular smooth muscle cells to exogenous administration of catecholamines and other vasopressor agents. The loss of peripheral resistance can be linked to a maldistribution of blood flow at the microcirculatory level. In order to pass through the smallest segment of the microcirculation, i.e., the capillaries, blood cells are required to undergo changes in shape and circumference. The deformability of red blood cells (RBC) is clearly reduced in septic adult patients as well as in septic infants. The mechanisms involved in this RBC rigidity in sepsis have not been thoroughly delineated and may include peroxidation of RBC membrane lipids, increased cytosolic calcium concentrations as well as NO overproduction. Also, activated neutrophils, which can be found in increased numbers in the peripheral blood of septic patients show reduced deformability, thus contributing to microcirculatory dysfunction in sepsis. Likewise, leukocyte activation results in increased aggregability, which may contribute to vascular obstruction and impaired microvascular flow, and an increased expression of adhesion molecules on the surface of neutrophils of septic patients. Finally, septic patients often show DIC, which contributes to microcirculatory dysfunction through fibrin deposition and the occlusion of capillaries by microthrombi. Histological and ultrastructural studies on muscle and skin biopsies of septic patients demonstrated that myocyte and capillary damage is associated with the breakdown of endothelial barrier function and the local accumulation of neutrophils, macrophages, and mast cells. This loss of endothelial barrier function and increased microcirculatory permeability has also been confirmed in a study on septic patients using computer-assisted venous congestion plethysmography. Since microcirculatory obstruction develops at the level of capillaries, blood is thought to bypass the microcirculation through large emerging arteriovenous shunts, thereby contributing little to tissue oxygenation. In summary, the microcirculatory dysfunction in sepsis is characterized by loss of peripheral vascular resistance, maldistribution of blood flow, reduced deformability of
RBC and PMN, DIC, increased microcirculatory permeability and microthrombus formation.

1.1.4 SEPSIS AND ENDOTHELIAL DYSFUNCTION

The endothelium covers the surface of all blood vessels in our body and therefore resides at the critical interface between blood and the tissue \(^79\). Due to this location endothelial cells have an intense contact with blood cells and plasma proteins. Under physiologic conditions, endothelial cells exert various functions that are important for normal homeostasis. These functions include the maintenance of blood fluidity by prevention of coagulation, the orchestration of the migration of blood cells into the tissues by expression of adhesion molecules, the regulation of the microcirculation by controlling the tonus of the arterioles, and the regulation of vasopermeability \(^80\).

Under physiologic conditions, endothelial cells maintain blood fluidity by various mechanisms that inhibit coagulation throughout the vascular system (fig 3.). Endothelial cells express tissue factor pathway inhibitors (TFPIs) that block the initiation of coagulation (Fig. 3a). They have proteoglycans, such as heparan sulfate, on their surface, which bind anti-thrombin III and inactivate thrombin \(^81\) (Fig. 3a). They express thrombomodulin, a membrane protein, which binds thrombin and modifies its specificity as a procoagulant converter of fibrinogen to fibrin into an anticoagulant activator of protein C \(^82\) (fig. 3a). Protein C inactivates in the presence of its cofactor protein S activated factors V and VIII of the clotting cascade \(^83\). The endothelium also inhibits platelet adhesion and aggregation by producing nitric oxide (NO), generated by nitric oxide synthase 3 (NOS3)-mediated conversion of arginine, and prostacyclin \(^84\).

This process is also supported by the expression of a surface-bound adenosine diphosphatase, which hydrolyzes an important agonist of platelets, adenosine diphosphate \(^85\) (fig. 3a). NO also serves as a vasodilating agent as well as prostacyclin, which is also produced by endothelial cells. By these two vasodilating agents, the endothelium regulates the tonus of the arterioles via the smooth muscle cells in the medial layer of the vessel wall (fig. 3b), thereby regulating the microcirculation and decreasing blood pressure \(^86\). Furthermore, a capillary endothelial barrier, which is formed in most tissues by intercellular junctions containing tight and adherens junctions, prevents the passage of plasma proteins to tissues (fig 3c). Resting endothelial cells do not interact with leukocytes \(^87\), because they sequester leukocyte-interactive proteins, such as P-selectin and von Willebrand factor (vWF) within Weibel-Palade bodies (WPBs) (fig. 3d). The transcription of other adhesion molecules, such as E-selectin, vascular cell-adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 is also suppressed by resting endothelial cells.
Figure 3. Functions of resting endothelial cells. (a) All endothelial cells (EC) inhibit coagulation of the blood by expressing tissue factor pathway inhibitors (TFPIs), displaying heparan sulphate proteoglycans (HS) and Thrombomodulinon on their surface, producing nitric oxide-synthase 3 (NOS3)-mediated nitric oxide (NO) and sequestering von Willebrand factor (vWF) within their storage granules, known as Weibel–Palade bodies (WPB). (b) Arterial endothelial cells have a major role in regulating blood flow by controlling the tone of smooth muscle cells in the medial layer of the vessel wall by NOS3 produced NO. (c) Capillary EC are the principal regulators of transendothelial extravasation of plasma proteins by forming intercellular junctions. (d) Venular EC form the principal site of leukocyte trafficking from the blood into the tissues. Efficient recruitment of leukocytes requires that the EC are activated and express adhesion molecules that mediate leukocyte attachment. Resting EC sequester P-selectin into Weibel-Palade bodies (WPB) along with vWF. See text for details. Reprinted with permission from Pober JS and Sessa WC. (2007) Evolving functions of endothelial cells in inflammation. Nature Review Immunology;7:803-15. © 2007 Nature Publishing Group.

Sepsis and systemic inflammation induce rapid and profound changes in endothelial function. Upon interaction with inflammatory mediators, such as TNF-α or IL-1, the endothelium will become activated. By the loss of thrombomodulin and heparan sulfate it loses its anticoagulant properties and becomes a procoagulant surface. This is further intensified by the synthesis of tissue factor (TF) on the surface. Consequently, endothelial cells lose the clotting inhibitors TFPIs and antithrombin III, and the ability to activate protein C, and by the interaction of TF with clotting factor VII/VIIa they activate the extrinsic pathway of the coagulation system. Furthermore, the production of the vasoactive compounds that regulate the tonus of the arterioles,
including the vasodilators NO and prostacyclin, and the vasoconstrictor endothelin is strongly modified \(^93\). Under normal conditions, endothelial cells produce NO by a constitutive NO-synthase, eNOS, which is calcium-dependent \(^94\). Upon stimulation with pro-inflammatory cytokines endothelial cells may produce the inducible NO synthase (iNOS), which produces large amount of NO in a calcium-independent manner \(^95\) leading to rapid hypotension \(^96\). Also, increased levels of the vasoconstricting endothelins have been described in septic patients \(^97\), which may compromise the appropriate matching of flow to tissue needs \(^98\). Another very important alteration upon cytokine stimulation is the expression of adhesion molecules, such as P-Selectin, E-Selectin, VCAM-1 and ICAM-1 on the membrane of endothelial cells \(^99\). This leads to leukocyte binding, rolling over the endothelium, followed by strong adherence and finally the transmigration into the tissues \(^100\) enabling the leukocyte to enter the site of infected or damaged tissue (fig. 4). The activated endothelium further produces an array of inflammatory mediators, including cytokines, chemokines and complements factors, which all take part in the inflammatory response. As the level of inflammatory mediators produced by endothelial cells is highly heterogenous \(^101\), severity of inflammation in sepsis could be dependent on interindividual variations of endothelial cells to respond to inflammatory mediators.

**Figure 4. The leukocyte adhesion cascade.** Upon stimulation with inflammatory mediators, adhesion molecules on endothelial cells will be up-regulated leading to interaction with leukocytes. This induces a cascade of different steps resulting in the transmigration of leukocytes into the tissue. The key molecules involved in each step are indicated in boxes. ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as αLβ2-integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular
addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as α4β1-integrin).


Endothelial activation is a physiological process in response to inflammatory mediators resulting in recruitment of leukocytes to the site of infection to eradicate the microbes. Several mechanisms may lead to endothelial dysfunction or damage, at which the permeability may become impaired leading to capillary leakage which complicates sepsis.

In vitro, endothelial cells will undergo apoptosis in response to several mediators, including some infectious agents. First of all, neutrophils, which adhere to endothelial cells via adhesion molecules are well able to cause endothelial damage by producing oxygen radicals and proteinases such as elastase, thereby inducing apoptosis. These degranulation products of neutrophils were increased in septic patients and correlated with clinical outcome. Also, pro-inflammatory cytokines such as TNF-α can induce apoptosis of endothelial cells in vitro. Furthermore, endothelial apoptosis can be induced by ischemia/reperfusion injury, since reperfusion of ischemic tissues can increase the local inflammatory reactions causing additional damage.

The morphological damage pattern includes the deformation and loosening of the endothelial cell texture, apoptosis and consequent destruction of the cell structure, and phagocytosis. In addition, destruction and rearrangement of intercellular contact molecules, and proteolysis of endothelial basal membrane can lead to detachment of endothelial cells. In fig. 5 mouse aortic endothelium is shown under septic conditions induced by cecal ligation and puncture (CLP) compared to healthy endothelium. The CLP technique is a common method to induce endotoxic shock and represents a model of sepsis which closely reproduces the clinical situation. By ligation and perforation of the cecum, feces will be allowed to enter the abdominal cavity and induce a pro-inflammatory response leading to endotoxic shock. The electron microscopic analysis shows significant morphologic abnormalities in the structure of the aortic endothelium after CLP including destruction of the endothelial cells and their detachment from the basal membrane.
Figure 5. Electron microscopic analysis of normal mouse aortic endothelium and aortic endothelium of mice subjected to septic shock. Mice were subjected to sham operation or cecal ligation and puncture (CLP). Aortic endothelium is shown of control mice (A), and mice 10 h (B) and 24 h (C) after subjected to CLP. The electron microscopic analysis shows partial detachment of some endothelial cells from the basal membrane at 10 h after CLP (B). The structure of the aortic endothelium 24 h after CLP exhibits a more significant morphologic abnormality (C), with most endothelial cells being swollen and appearing to be partially detached from the basal membrane. Reprinted with permission from Matsuda N, Hattori Y. (2007) Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction. J Smooth Muscle Res.;43:117-37 and by courtesy of Prof. Yuichi Hattori, Department of Molecular and Medical Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences University of Toyama, Japan.

In the process of destruction and detachment of endothelial cells, apoptosis and necrosis eventually lead to release of endothelial cells or endothelial microparticles into the circulation. This, in turn, can induce an inflammatory tissue reaction due to increased expression of adhesion molecules on the microparticles leading to enhanced interactions between leukocytes, endothelial cells and their microparticles. This apoptosis- and necrosis-induced endothelial cell damage could be confirmed clinically in septic patients, in which increased levels of vWF- and vascular endothelial growth factor receptor (VEGFR)-2-positive circulating endothelial cells could be identified during sepsis and septic shock. Also, the enhanced production of endothelial microparticles
with increased binding to leukocytes could be confirmed in patients with severe systemic inflammatory response syndrome (SIRS) \cite{111}.

Endothelial dysfunction and detachment of endothelial cells result in the development of interstitial edema. The decreased production of important anti-coagulative proteins and the dysfunction of fibrinolysis supports the coagulation-activating effects of cytokines. Eventually, these processes induce severe pathological alterations in the microcirculation with dysfunctions in oxygen-transport culminating in hypoxic organ-failure \cite{112}. Although these alterations seem to be similar in all septic patients, an improvement of the capillary perfusion was only found in sepsis-survivors, but not in sepsis-non-survivors \cite{113}. This finding supports the assumption, that endothelial dysfunction leading to impaired capillary perfusion plays an important role in the development of multi-organ-failure in the course of sepsis \cite{114}.

### 1.1.5 THE ROLE OF ENDOTHELIAL PROGENITOR CELLS IN SEPSIS

As mentioned in the chapter 1.1.4, altered endothelial function appears in the macro- and microcirculation in the course of sepsis and includes detachment of endothelial cells and capillary leakage. In these pathologic conditions, reconstitution of the endothelial layer is initiated. The recruitment of endothelial progenitor cells (EPCs) might play a role in this process, since many in vivo studies have demonstrated that vascular maintenance, repair, and neovascularization in ischemic tissue are partly mediated by recruitment of EPCs \cite{115,116,117}.

Ever since the description of postnatal vasculogenesis, a process in which EPCs are recruited and differentiate into mature endothelial cells (EC) to form new blood vessels \cite{115,116,118}, growing evidence suggests the involvement of EPCs also in other pathological conditions. EPCs may also be recruited and incorporated into sites of active neovascularization during e.g., vascular trauma, tumor growth and inflammation. Moreover, expansion and mobilization of EPCs might represent an effective method to stimulate angiogenic activity of resting mature endothelial cells \cite{119,120}.

The majority of EPCs reside in the bone marrow in close association with hematopoietic stem cells (HSC) and the bone marrow stroma. EPCs have the capacity to proliferate, migrate and differentiate into endothelial lineage cells, but have not yet acquired characteristics of mature endothelial cells (EC), such as VE-cadherin or vWF. In animal ischemia models, mobilization of bone marrow-derived EPCs resulted in EPC homing to sites of active neovascularization and differentiation into EC \cite{121}. Mobilization of EPCs from the bone marrow critically depends on the activation of metalloproteinases and up-regulation of adhesion molecules. This is most likely mediated by soluble factors such as VEGF, granulocyte-macrophage colony stimulating factor (GM-CSF) and
erythropoietin (EPO). Serum concentrations of these factors dramatically increase under pathologic conditions, concomitantly with an increase in the number of cEPCs \(^{122-124}\). Many other factors are described to have important roles for the mobilization of EPCs. Among them are placental growth factor \(^{125}\), angiopoietin-1 \(^{126}\), pro-inflammatory cytokines such as G-CSF \(^{124}\), chemokines such as stromal cell-derived factor (SDF)-1 \(^{127}\), and hormones such as estrogens \(^{128}\). Interestingly, lipid-lowering \(^{129}\) and anti-diabetic drugs \(^{130}\), as well as physical activity \(^{131}\) also stimulate EPC mobilization.

**Definition and characterization of endothelial progenitor cells**

EPCs represent with 0.001-0.05\% a minor subpopulation of blood mononuclear cells (MNCs) \(^{132}\). In vitro culture methods have been developed to select and expand this population. Three culture methods have been described for isolating EPCs (fig. 6) \(^{133}\). The original method by Asahara et al. \(^{115}\) has been modified subsequently by others \(^{134, 135}\) and the resulting colonies, comprised of round cells centrally with spindle-shaped cells sprouting at the periphery, are nowadays referred to as colony-forming unit-ECs (CFU-ECs) or colony-forming unit-Hill cells \(^{134}\). These CFU-EC colonies could be cultured from a heterogeneous MNC population enriched for either CD34 or VEGFR-2. Another widely employed and methodologically similar approach, has been used in several studies \(^{117, 136, 137}\). Unfractionated MNCs are cultured in supplemented endothelial growth media for 4 days, whereupon the non-adherent cell fraction is removed. The resulting culture of adherent cells display features of an endothelial phenotype through binding of the endothelial-specific lectin Ulex Europeus Agglutini-1, and uptake of acetylated low-density lipoprotein (acLDL) \(^{117, 137}\). These cells appear similar to CFU-ECs in surface marker expression and in in vitro function, and as a result, both have often been grouped together in the literature under the name EPCs. These adherent cultured cells have also been referred to as circulating angiogenic cells (CAC) in recognition that these cells appear to promote neovascularization in animal models of critical limb ischemia or myocardial infarction (MI) \(^{117}\). A third type of EPCs is termed ‘endothelial colony-forming cells’ (ECFCs). To obtain these, MNCs are plated onto collagen I-coated plates in endothelial-specific growth media. Non-adherent cells are discarded during washing steps. ECFC colonies emerge from the adherent cell population 10-21 days after plating and display cobblestone appearance typical for ECs \(^{138, 139}\). Thus far, ECFCs have proven to be phenotypically indistinguishable from mature ECs, and exhibit de novo vessel-forming ability \(^{140, 141}\). Because ECFCs emerge much later in culture as compared to both CFU-ECs and CACs, ECFCs have been called ‘late outgrowth’ EPCs, while CFU-ECs and CACs have been called ‘early outgrowth’ EPCs \(^{142-144}\).
Figure 6. *In vitro* methods of EPC culture. (Method A) Culture of colony-forming unit – endothelial cells (CFU-EC) includes a 5-day process wherein non-adherent MNCs give rise to the EPC colony. (Method B) Circulating angiogenic cells (CAC) are the adherent mononuclear cells of a 4- to 7-day culture procedure. CAC cultures typically do not display colony formation. (Method C) Endothelial colony-forming cells (ECFCs) are derived from adherent MNCs cultured for 7–21 days in endothelial conditions and colonies display a cobblestone morphology. Reprinted with permission from Prater DN, Case J, Ingram DA and Yoder MC. (2007) Working hypothesis to redefine endothelial progenitor. Leukemia;21:1141-1149. © 2007 Nature Publishing Group.
GENERAL INTRODUCTION

When using cell culture to define and characterize EPCs, the starting cell population is typically heterogeneous, making it difficult to determine the precursor cell that gives rise to the final EPCs. Alternatively, EPCs can be prospectively identified without the need for culture, by selecting sub-populations of MNCs based on specific cell surface antigen expression. The definition and characterization of EPCs by utilizing cell surface antigens as markers allows for the selection of a more homogenous population. However, the definition of EPCs via this method is complex due to the absence of any single and restricted surface marker for EPCs versus detached circulating ECs (CECs). Furthermore, it is complicated due to the overlap observed between cell surface antigens expressed on the surface of putative EPCs and those expressed on cells of the hematopoietic lineage. While the cell surface antigens CD34, CD133, and VEGFR-2 are utilized to identify EPCs, it is important to note that they are also expressed on human hematopoietic stem cells (HSCs), as well as on various sub-populations of hematopoietic progenitor cells (HPCs), including low proliferative potential- and high proliferative potential-colony forming cells.

Human circulating EPCs have typically been identified as cells expressing CD34, CD133, and VEGFR-2 (fig. 7). Peichev et al. reported that mature ECs do not express CD133, and that nearly all the CD34+/VEGFR-2+ circulating EPCs in peripheral and human umbilical cord blood express CD133. Furthermore, culture of CD34+ cells from human fetal liver with VEGF and fibroblast growth factor-2 (FGF-2) resulted in differentiation of non-adherent CD133+/CD34+ cells into adherent CD133-/VEGFR-2+/acetylated LDL-uptake+ cells with an EC morphology. In an in vivo human model, the neo-intima formed on the surface of left ventricular assist devices was found to be colonized with CD133+/VEGFR-2+ cells. These data suggested that the circulating CD34+ cells co-expressing CD133 and VEGFR-2 are a phenotypically and functionally distinct population of EPCs that may play a role in neovascularization.

Many clinical studies designed to determine the role of EPCs in a variety of vascular disorders have used flow cytometry to investigate and enumerate the EPC concentration in the blood of patients with vascular disease. In these studies, the widely accepted definition of EPCs co-expressing CD34, CD133, and VEGFR-2 has been applied. But since these antigens are also expressed on primitive hematopoietic progenitor cells (HPCs), Case et al. isolated CD34+/CD133+/VEGFR-2+ cells from human umbilical cord blood and granulocyte colony-stimulating factor-mobilized peripheral blood, and assayed them for either EPCs or HPCs. They found that CD34+/CD133+/VEGFR-2+ cells did not give rise to early outgrowth EPCs and were devoid of vessel forming activity. In contrast, CD34+/CD133+/VEGFR-2+ cells gave rise to HPCs that expressed the hematopoietic lineage-specific antigen CD45. Therefore, they tested whether EPCs could be separated from HPCs by immunoselection for CD34 and CD45. Their results showed that CD34+/CD45+ cells gave rise to HPCs but not EPCs, while CD34+/CD45- cells gave rise to EPCs but not HPCs. These data, in contrast to the observations of Peichev et al.,
lead to the conclusion that CD34+/CD133+/VEGFR-2+ cells represent HPCs that do not yield EC progeny.
In summary, flow cytometry and colony forming assays are the two main methods used for identification and functional assessment of endothelial progenitor cells. However, both of these techniques have serious limitations. Although endothelial cells-colony forming units (EC-CFU) are widely accepted as a surrogate estimate of EPC number and function in cell culture, some important limitations may restrict the assumption that EC-CFUs reflect EPC numbers accurately. Shantsila et al \cite{156}, comparing CFU units to flow cytometry, described that endothelial CFU counts represent the cumulative characteristics of EPC quantity and their functional characteristics, and cannot be reliably used for the estimation of EPC numbers in peripheral blood or the bone marrow. They suggest that flow cytometry may be the better technique for EPC quantification.

![Diagram of cell lineages](image)

GENERAL INTRODUCTION

Homing of endothelial progenitor cells to activated endothelium
Homing of endothelial progenitor cells to the target tissue is a multi-step-process involving various chemokines, cytokines, adhesion molecules and proteases (fig. 8). While the homing of leukocytes to sites of inflammation is well studied (see chapter 1.1.4, fig. 4), the molecular mechanisms of progenitor cell homing to sites of ischemia or injury are poorly understood. From a process point of view the homing of endothelial progenitor cells to sites of ischemia and to sites of injury share at least some common features with the homing of leukocytes to sites of inflammation. In a mouse model, embryonic EPCs (eEPC) arrested within tumor microvessels extravasated into the interstitium and incorporated into neovessels suggesting that adhesion and transendothelial migration are also involved in the recruitment of endothelial progenitor cells to sites of tumor angiogenesis. The fact that these cells can contribute to tumor angiogenesis indicates that eEPCs, although they are primarily programmed to form blood vessels during embryonic vascular development, retain this ability within an angiogenic environment in the adult and may be comparable to adult EPCs.

Also, adhesion molecules, which play a critical role in rolling a firm adhesion and transmigration of leukocytes were identified as key regulators of EPC homing. Recently, evidence was provided that the initial steps of this process are mediated by P-selectin and E-selectin. Activation of the ephrin family member EphB4 in EPCs leads to a higher expression of PGSL-1, a selectin ligand, leading to an increased adhesion to P-selectin and E-selectin. siRNA for P-selectin suppresses this response indicating that PGSL-1 expression facilitates the recruitment of EPCs and thus enhances their proangiogenic capacity. Other studies have shown that also E-selectin potentiates angiogenesis in ischemic hindlimbs, partly by mediating EPC-endothelial cell interactions.

β2-integrins expressed on the cell surface of EPCs mediate the firm adhesion and transmigration of EPCs to the damaged endothelial monolayer. Activation of the β2 integrins was shown to improve the homing and the neovascularization capacity of EPCs in a mouse model of hindlimb ischemia. Interestingly, HMGB-1, which is released extracellularly upon activation of cells by inflammatory cytokines and during cell necrosis, and a late marker of sepsis, was recently reported to activate β1 and β2 integrins on the surface of endothelial progenitors, thereby guiding EPC adhesion and homing to ischemic areas. The importance of β2 integrins in homing of EPCs is also highlighted by studies focusing on ICAM-1. Up-regulation of ICAM-1 during ischemia was shown to associate with enhanced EPC recruitment to ischemic limbs.

α4 integrin, expressed on EPCs, also seems to play a crucial role in progenitor cell homing. It promotes homing of circulating endothelial progenitors to the sites of active tissue remodelling and improves blood flow recovery and tissue preservation. This supports the assumption, that interaction between EPC surface molecules with their
counter ligands on the dying endothelial cells or sub-endothelial matrix proteins plays a major role in EPC homing.
The receptor c-Kit and its membrane-bound form of Kit ligand (KitL) are involved in the mobilization of EPCs from the bone marrow\textsuperscript{165}. Observations that the soluble KitL also promotes endothelial cell migration and survival\textsuperscript{166} raised the possibility that c-Kit and KitL might be also involved in EPC homing to activated endothelium. Recently, Dentelli et al.\textsuperscript{167} demonstrated that inflammatory activation induced the expression of the KitL on microvascular endothelial cells in vitro and in vivo. The inflammatory activation mediated also the recruitment of EPCs. Moreover, they showed that depletion of endogenous c-Kit or inhibition of c-Kit enzymatic activity prevented adhesion of EPCs to activated ECs both in vitro and in vivo, indicating that functional c-Kit on EPCs is essential. Since the chemokine SDF-1 mediates homing of stem cells to bone marrow by binding to CXCR4 on circulating cells\textsuperscript{168}, Ceradini et al.\textsuperscript{127} investigated the regulation of SDF-1 expression and its physiological role in peripheral tissue repair. They showed that the recruitment of CXCR4-positive EPCs to regenerating tissue is mediated by hypoxic gradients via HIF-1-induced expression of SDF-1.

\textbf{Figure 8. EPC homing to activated endothelium.} After vascular injury causing local inflammation, the endothelial monolayer is activated as a consequence of which rapid platelet aggregation occurs. Platelets and activated endothelial cells secrete high levels of SDF-1, while elevated levels of VEGF are also observed. PGSL-1, a ligand for selectins, is up-regulated in these cells through activation of the EphB4 pathway. Extremely high levels of β2 integrins can also be found in EPCs. All these molecules will interact with their ligands P-selectin, E-selectin, and ICAM-1 that are expressed on the activated endothelial cells. Additionally, necrotic ECs express high mobility group box protein (HMGB)-1 that further enhances the interaction of β2

EPCs need to transmigrate to the injured tissue to be able to influence neovascularization. Their invasive capacity is crucial for tissue repair and restoration of organ function. Cathepsin L was reported to play an important role in this process. This protease is highly expressed in EPCs and essential for matrix degradation and invasion. Cathepsin L-deficient mice displayed impaired recovery following hindlimb ischemia, and infused cathepsin L-deficient EPCs neither homed to sites of ischemia nor augmented neovascularization \(^{169}\). 

MMP-2 was also found to affect the invasive properties of endothelial progenitors \(^{170}\). EPCs from MMP2/- mice exhibit reduced extracellular matrix degradation and as a result, MMP2/- mice respond poorly to hindlimb ischemia because of reduced neoangiogenesis \(^{170}\).

In conclusion, homing of EPCs is a complex process and involves various mediators that recruit them to activated endothelium in response to a damage-induced inflammation. EPCs have been extensively studied in cardiovascular diseases and accumulating evidence highlights their importance in vascular repair and tissue remodeling.

The role of EPCs in sepsis has not been studied so far. In patients with acute lung injury (ALI) EPC colony numbers were significantly higher compared with healthy control subjects, but did not differ between patients with ALI and intubated control subjects. Increased cEPC numbers were associated with improved survival in the ALI group. Septic shock was present in 44% of the patients with ALI and its incidence was statistically not different in survivors and nonsurvivors \(^{171}\). Whether the recruitment of circulating EPCs might have a beneficial effect on the clinical course in sepsis has still to be elucidated.

1.1.6 CYTOKINE-INDUCED SIGNALING IN ENDOTHELIAL CELLS

TNF-α induced signaling pathways

Several aspects of the interaction between host and pathogen must be considered in drawing a picture of the immune events that underlie the cause of sepsis. The initial activation of innate immunity by, e.g., LPS, may lead to release of e.g., TNF-α, IL-1, IL-6 and HMGB-1. The molecular changes underlying cellular dysfunction are hence a likely result of cytokine activation.
The signaling pathways in endothelial cells induced by pro-inflammatory cytokines comprehend a complex system. As in most other cells in the body, TNF-α-induced effects are mediated by TNFR1. Upon binding of TNF-α to TNFR1 on the endothelial cells, multiple pathways are activated leading to an inflammatory status of the endothelial cell (fig. 9). TNFR1 activation provides a docking site for accessory proteins that form the branching point for the pro-inflammatory and pro-apoptotic signaling pathways, eventually leading to the activation of the transcription factor nuclear factor κ B (NFκB), a dimer usually consisting of p65 (RelA) and p50 (NFκB1). In this cascade NFκB-inducing kinase NIK activates inhibitor of κB (IκB) kinases IKKα and IKKβ. IKKs form a catalytic subunit responsible for phosphorylation, site-specific ubiquitylation and subsequent proteasomal degradation of IκB. Upon degradation of IκB, the nuclear localization sequence of NFκB targets the protein into the nucleus and binds to DNA. By interaction with co-activators and other components of the gene transcription machinery, NFκB activates transcription of a range of inflammatory genes. Besides being controlled by IKK activity, reactive oxygen species prominently affect NFκB activity. Following TNF-α induced NFκB activation, endothelial cells express functionally related genes, including the pro-inflammatory adhesion molecules E-selectin, ICAM-1, and VCAM-1, cytokines IL-6 and IL-8, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). In parallel, genes involved in controlling cell signaling and apoptosis/cell proliferation are down-regulated. NFκB dependent transcription is only transiently activated upon stimulation due to feedback mechanisms including the induction of IκB gene transcription and translation by NFκB, and IKKβ autophosphorylation in its inhibitory loop.

Besides inducing a pro-inflammatory status, NFκB signaling also leads to the expression of protective genes, such as zinc finger protein A20, bcl-related gene A1 and inducible hemeoxygenase-1 (HO-1). It is assumed that these protective genes prevent the endothelium from going into apoptosis upon activation. At the same time, they down-regulate the endothelial pro-inflammatory response.

Upon TNF-α activation also mitogen-activated protein kinase (MAPK) become activated in endothelium. The 3 major MAPK signaling pathways comprise the extracellular-regulated protein kinase (ERK) pathway, the c-Jun NH2–terminal kinase (JNK) pathway, and the p38 MAPK pathway. They are involved in a network of signaling routes leading to cell proliferation, transformation and differentiation (ERK regulated), and apoptosis, stress responses and inflammation (JNK and p38 MAPK regulated). Of the MAPK-family, p38 MAPK is involved in TNF-α -driven IKK activation. Furthermore, p38 MAPK can control mRNA stability of inducible cytokines TNF-α, IL-1 and IL-8. JNK activation in HUVEC resulted in ICAM-1 and VCAM-1 expression.
Figure 9. Simplified representation of the signaling pathways that become active in endothelial cells upon interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α activation. The activation leads to the expression of pro-inflammatory genes, including cell adhesion molecules, cytokines and chemokines, and cell survival genes. Abbreviations: IKK: IkB kinase; IkB: inhibitor kB; IRAK: IL-1 receptor-1 associated kinase; NEMO: NFkB essential modulator; NFkB: nuclear factor kB; NIK: NFkB inducing kinase; PI3K: phosphoinositol-3 kinase; PKC: protein kinase C; ROS: reactive oxygen species. Reprinted with permission from Kuldo JM, Ogawara KI, Werner N, Asgeirsdottir SA, Kamps JA, Kok RJ, Molena G. (2005) Molecular pathways of endothelial cell activation for (targeted) pharmacological intervention of chronic inflammatory diseases. Curr Vasc Pharmacol;3:11-39. © 2005 Bentham Science Publishers Ltd.

LPS-induced signaling pathways

Most pathogens are detected by the innate immune system via Toll-like receptors (TLRs). Currently, 13 different mammalian TLRs have been identified. TLRs are pattern recognition receptors, which recognize PAMPs. PAMPs are small molecular sequences unique to microorganisms that enable the host to recognize foreign pathogens. Toll-like receptors 1, 2, 4, 5 and 6 are expressed on the cell surface, where they specialize in the recognition of bacterial products, including bacterial lipoproteins and lipoteichoic acids (TLR-2) or LPS (TLR-4), which are unique to the cells walls of Gram-
positive and Gram-negative bacteria. In fact, bacterial components signal via a single TLR, but studies have shown that cell wall extracts from Gram-positive and Gram-negative organisms contain components that can activate both receptors\textsuperscript{189,190}. However, mice deficient in TLR-2 are more prone to infection with staphylococci \textsuperscript{191} and TLR-4 deficient mice are more prone to infections with Salmonella spp. \textsuperscript{192}. This suggests that Gram-positive infections have a TLR-2-dominant and Gram-negative infections a TLR-4-dominant signal.

LPS cell interaction involves the binding to LPS-binding protein (LBP) that transfers LPS to CD14. CD14 increases the LPS responses, but is not specific for LPS, because it enhances also immune responses to other pathogen products \textsuperscript{193}. In addition, LPS responses are not always dependent on CD14 \textsuperscript{194}. Formation of the complex between LPS and CD14 facilitates the transfer of LPS to the LPS receptor complex composed of TLR-4 and MD-2 \textsuperscript{195}. The TLR-4/MD-2 complex signals through adaptor molecules, myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain containing adaptor protein (TIRAP), Toll/IL-1 receptor domain containing adaptor inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM). These constitute two signaling pathways, one is MyD88-dependent and requires TIRAP, and the other is TRIF-dependent and requires TRAM (fig. 10) \textsuperscript{196-198}. Both adaptor proteins initiate a signaling cascade culminating in the activation of the transcription factors NF-κB, activator protein 1 (AP-1), and interferon regulatory factors (IRFs), subsequently leading to the induction of several inflammatory genes \textsuperscript{199}. 
Figure 10. Toll-like receptor (TLR) 4 signaling pathway. (A) The MyD88-dependent pathway. MyD88 activates IRAKs/ TRAF6 as well as the transcription factors NF-κB, AP-1 and IRF-5 further downstream. These transcription factors induce expression of pro-inflammatory cytokine genes. (B) The MyD88-independent pathway. TRIF signals the induction of Type I interferons by recruiting TRAF3 and RIP1 to activate transcription factor IRF3, as well as NF-κB and AP-1. Reprinted with permission from Lu YC, Yeh WC, Ohashi PS. (2008) LPS/TLR4 signal transduction pathway. Cytokine;42(2):145-51. © 2008 Elsevier Ltd.

In clinical studies, an enhanced TLR-2 and TLR-4 expression was observed in leukocytes from septic patients compared with healthy controls. In contrast, patients with septic shock were found to have decreased expression of TLR-2 and a trend to decreased expression of TLR-4. These findings correspond to the differing inflammatory responses in the clinical stages of sepsis with an up-regulation (sepsis) or down-regulation (severe sepsis/septic shock) of inflammatory cytokine production. Salomao et al. used polymerase chain reaction-array to evaluate the gene expression of genes related to TLR-mediated signal transduction in patients with sepsis, severe sepsis, and septic shock. The genes investigated included genes encoding for TLRs, adaptor
and interacting proteins, effectors, downstream pathways and target genes (NFκB, JNK/p38 pathway and IRF pathway). Their results showed that TLR-signaling pathway genes are differently regulated in peripheral blood mononuclear cells and neutrophils of septic patients, and that they are dynamically modulated across the different stages of sepsis. The gene expression of PBMCs revealed that the major differences between septic shock patients and healthy volunteers consisted of a down-regulation of genes in the septic shock group, mostly from the NFκB and JNK/p38 pathway. In contrast, the changes in gene expression observed in neutrophils comprised predominantly an up-regulation of all genes groups evaluated which persisted along the clinical spectrum of sepsis.

Evaluation of TLR expression and mapping of the intracellular signaling pathways are important aspects to understand the cell adaptation in their complex functions of sensors and effectors of host responses during sepsis.

The role of NFκB in sepsis
Several lines of evidence indicate a role of NFκB activation in the pathophysiology of sepsis, since a variety of pathogens known to cause sepsis and pro-inflammatory cytokine release during sepsis can activate NFκB. In peripheral mononuclear cells from septic patients NFκB activity is markedly increased, and the level of NFκB activity correlates with disease severity. In animal models of septic shock induced either by LPS or by CLP, NFκB inhibitors protected animals from septic lethality. Also, molecules proven to protect mice from lethal endotoxemia, such as IL-10, or to improve survival in severe sepsis patients, such as activated protein C, exert their protective effect by inhibiting NFκB activation. In models of endotoxin tolerance, cells or animals exhibited a down-regulated NFκB activity and reduced expression of NFκB-dependent genes, when subsequently exposed to endotoxin.

In the pathophysiology of sepsis transcriptional activation of multiple inflammatory genes marks an important characteristic. Studies have demonstrated that NFκB plays a crucial role in LPS- or cytokine-activated promoter activity of over 200 genes, many of which play important roles in the development of septic shock. These genes include cytokines (e.g., TNF-α, IL-1β, IL-6), chemokines (e.g., IL-8), adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin, P-selectin), enzymes (e.g., iNOS, COX-2) and acute-phase-proteins. In vivo inhibition of NFκB activation in animal models demonstrated reduced LPS-induced mRNA and protein expression of multiple pro-inflammatory cytokines and other molecules that play critical roles in the pathophysiology of sepsis.

Also, amelioration of the vascular derangement in both LPS and CLP models of septic shock could be demonstrated by inhibition of the NFκB pathway, which resulted in restored systemic hypotension, reversal of the depressed vascular contractile response and restoration of the impaired endothelium-dependent vasodilator.
response. Furthermore, NFκB is involved in multiple steps of the coagulation cascade, which shows abnormalities in sepsis including DIC. Inhibition of NFκB activation prevents coagulation, resulting in improved outcome of septic shock. The critical role of NFκB activation in septic pathophysiology and the effectiveness of inhibiting NFκB activation in correcting septic abnormalities indicate that targeting NFκB is a potential therapeutic strategy for the treatment of septic shock. However, NFκB activation controls not only the inflammatory response in the pathophysiology of sepsis, but also the bacterial clearance and normal cell homeostasis. Disruption of the NFκB signaling pathway impairs the host defense capacity to eliminate invading bacteria and leads to a worsened outcome in a bacterial infection model of sepsis, indicating that NFκB is also protective. NFκB inhibitors are not capable of differentiating between these processes so far. To be able to use inhibition of NFκB activation as a effective therapy option, strategies have to be developed to inhibit NFκB activation without interfering with the host-defense functions.

Just recently, an interesting study was published investigating the role of endothelial-intrinsic NFκB activity in multiple organ injury and host defense in response to sepsis using both LPS and CLP models of sepsis. To this end, double transgenic (TG) mice were generated that conditionally overexpressed a degradation-resistant form of IkBα (IkBαmt), a superior inhibitor of NFκB, selectively on endothelium. Until now, investigations into the role of NFκB activation in sepsis and other inflammatory conditions have been hampered by the fact that NFκB knockout mice are embryonically lethal. Also, conventional TG mice overexpressing IkBαmt selectively in endothelium were not qualified, since they have structural and functional defects in endothelium, as indicated by loss of endothelial tight junction, increased sensitivity to LPS-induced endothelial permeability, and enhanced susceptibility to tumor metastasis. Ye et al. overcame these problems by taking a conditional TG approach using a tetracycline-regulated gene expression system. Therefore, the mice did not express IkBαmt until induced by feeding with Dox, and had normal NFκB activity that is critically required for embryonic development. The NFκB inhibition was transient and restricted to endothelium, which had minimal effects on immune cell differentiation, development and function allowing the study of septic response under a physiological setting. When subjected to endotoxemia, TG mice showed endothelial-selective blockade of NFκB activation, repressed expression of multiple endothelial adhesion molecules, reduced neutrophil infiltration into multiple organs, decreased endothelial permeability, ameliorated multiple organ injury, reduced systemic hypotension, and abrogated intravascular coagulation. The TG mice also exhibited alleviated multiple organ injury and improved survival in the CLP sepsis model compared with wild-type (WT) mice. Thus, selective blockade of the endothelial NFκB pathway is sufficient to reduce multiple organ inflammation, prevent organ injury and improve survival, indicating that endothelial NFκB plays a critical role in septic multiple organ inflammation and
injury. The study also demonstrated that WT and TG mice had comparable capacity to clear three different pathogenic bacteria, S. pneumoniae, L. monocytogenes and S. enterica \cite{228}, indicating that endothelial NFκB does not play an important role in the host defense response to eliminate those pathogenic bacteria. This can be explained by a different host defense mechanism involved in the clearance of the three pathogenic bacteria \cite{234}. Since NFκB activation is a key component of host immune response \cite{227}, and NFκB p50 knockout mice showed severely defective clearance of S. pneumoniae and L. monocytogenes \cite{224}, the lack of effect of endothelial-selective NFκB inhibition on bacterial clearance is surprising. Studies have to be conducted to investigate which cellular NFκB system plays a major role in the host defense response against bacterial pathogens. In summary, these results demonstrate that endothelial NFκB plays divergent roles in the inflammatory and host defense responses against bacterial infection. Therapeutical strategies have to be developed to selectively inhibit the endothelial NFκB pathway.

\subsection*{1.1.7 THE ROLE OF CARBON MONOXIDE IN SEPSIS}

As described in chapter 1.1.2, the inflammatory process involves a multitude of mediators and systems. Also, the heme oxygenase system (HO) has been demonstrated to be involved in the control of inflammatory processes \cite{235-238}, in addition to its role in oxidant-induced injury \cite{239-241}. HOs are the rate-limiting enzymes in degradation of heme into carbon monoxide (CO), Fe$^{2+}$ and biliverdin, the latter being subsequently converted to bilirubin \cite{242}. The HO system comprises several isoenzymes \cite{243,244}, of which the inducible HO-1 isoenzyme is particularly important as an anti-inflammatory mediator \cite{235-237,245}. Studies in HO-1-null mice and reports on human HO-1-deficiency have strengthened the evidence that HO-1 is an important molecule in host defense against oxidant stress, and have also emphasized the potent anti-inflammatory properties of HO-1 \cite{245,246}. In these studies both mice and humans deficient in HO-1 expression showed increased vulnerability to inflammation. Furthermore, exogenous administration of HO-1 by gene transfer into rat lung provided protection against hyperoxia-induced lung injury in vivo by modulation of neutrophil inflammation and lung apoptosis \cite{247}. The protective function of HO-1 has been attributed to several possible mechanisms. All three HO-reaction products (i.e., iron, biliverdin, and CO) have been extensively discussed as potentially contributing to HO-mediated cytoprotection and anti-inflammatory mediators. However, some of the published data are controversial. A number of studies describe a down-regulation of VCAM-1 and E-selectin expression by HO-1 via bilirubin and iron chelation with no apparent involvement of CO in HUVEC \cite{236,237}. Others clearly demonstrate the anti-inflammatory potential of HO-1 mediated CO production in macrophages and monocytes \cite{235} as well as in rat pulmonary artery endothelial cells \cite{246,249}. The salutary effect of CO has also been shown for organ
transplantation and ischemia reperfusion injury animal models. The increase in inflammatory mediators was markedly inhibited in CO-treated recipients compared to non-treated recipients, and the inhibition correlated with improved renal cortical blood flow. When looking at pro-inflammatory cytokines, CO significantly inhibited LPS-induced TNF-α production, but it did not completely prevent the production of this cytokine by monocytes in vitro and in vivo.

Carbon monoxide
Carbon monoxide (CO) is a low-molecular-weight gas molecule that arises in nature as the product of the combustion of organic matter, such as from the burning of fossil fuels or tobacco. Environmental CO represents a major air pollutant, which is generally regarded as an inhalation hazard. It is well known that high inspired concentrations of this gas are toxic. The binding of CO to hemoglobin (Hb) inhibits O₂ transport and delivery to tissues. Thus, at elevated concentrations, CO acts as an asphyxiant, which causes tissue hypoxia that is associated with a number of clinical symptoms, including dizziness, loss of consciousness, and death upon prolonged or excessive exposure. Symptoms of CO poisoning in humans appear at carboxyhemoglobin (CO-Hb) levels of 20%, whereas loss of consciousness (coma) leading to death occurs in the range of 50–80% CO-Hb.

However, CO has recently emerged as a potential therapy for sepsis, based on its vasodilatory, anti-ischemic and anti-inflammatory activities. Observations of dramatic tissue protection from the application of low concentrations of CO in animal models of inflammation, sepsis, oxidative stress, and ischemia/reperfusion injury raised the possibility to use this gas clinically.

Dosage and application form
The dose of CO that has been applied experimentally in animal studies ranges from 10 to 1,000 parts per million (ppm). Most investigators have applied CO by inhalation of 250 ppm, corresponding to an inspiratory fraction of 0.025%. CO diffuses from the alveolar space into the capillaries and subsequently binds Hb for transport to the different tissues in the body. The extent of CO-Hb formation depends on the dose and the time of application. For cell culture experiments, also 5% CO₂ was present for buffering requirements.

As an alternative to inhalation for delivery of CO, molecules have recently been developed that are composed of transition metal carbonyls and capable of liberating CO in dose- and time-dependent fashion. These are referred to as carbon monoxide–releasing molecules (CORMs). Water-soluble forms of CORMs allow intravenous administration. In particular, CORM-3 (tricarbonylchloro(glyconato) ruthenium(II)) and CORM-A1 (sodium boranocarbonate),
which are both fully water-soluble, rapidly liberate CO when dissolved in physiological solutions. Interestingly, and in contrast to inhaled CO, CORMs appear to deliver CO directly to the tissues without significant formation of CO-Hb. These molecules might therefore, besides serving as research tools, also be of therapeutically interest to modulate ongoing inflammatory reactions by delivering CO in a controllable fashion.

The role of CO in sepsis
Sepsis can affect the synthesis of CO in humans. HO-1 expression and consequent CO production are up-regulated in aortic smooth muscle cells and polymorphonuclear cells of septic patients. Furthermore, the levels of CO measured on the exhaled breath were higher in patients suffering from severe sepsis as compared with critically ill patients without sepsis. Sepsis survivors in the same study showed higher levels of exhaled CO than nonsurvivors. Similar findings were demonstrated for pediatric patients. CO-Hb levels in the blood of septic newborn infants and septic children were elevated and more pronounced in septic shock compared to healthy controls. Based on these observations, it was hypothesized that up-regulation of endogenous CO synthesis might act as a protective mechanism mediating the anti-inflammatory response under septic conditions. Many studies have been conducted to investigate whether exogenous CO application might improve outcome and survival in animal models of sepsis. These studies demonstrated a protective effect of CO administration associated with prolonged survival and reduced mortality. The exact mechanisms by which CO exerts these effect are still unknown, yet multiple effects on the inflammatory response have been proposed.

Anti-inflammatory effects of CO in sepsis
CO can exert direct effects on immune competent cells, such as inhibiting the activation of monocytes, macrophages and leukocytes in vitro and in vivo. Furthermore, it suppresses the ability of T-cells to proliferate, and reduces the adhesion and migration of leukocytes in rats.

In vitro, CO inhibits the LPS-induced production of pro-inflammatory cytokines such as TNF-α, IL1-β and macrophage inflammatory protein-1β (MIP-1β) in cultured macrophages. Also, the application of CORM (e.g., CORM-3) has been demonstrated to reduce the level of TNF-α in murine macrophages. Furthermore, CO treatment promoted an increased production of the anti-inflammatory cytokine IL-10 during LPS challenge in cultured macrophages. Interestingly, the anti-inflammatory effect of IL-10 itself appeared to be mediated by HO activity and specifically required CO.

In vivo, these general anti-inflammatory effects of CO could also be observed. In murine endotoxemia models, CO preconditioning resulted in reduced production of serum TNF-α, IL-1β, and IL-6, reduced organ injury and prolonged survival after LPS challenge. And in line with the in vitro-studies, CO also increased dose-
dependently LPS-inducible IL-10 production \(^{235}\). In a pig study, CO reduced the development of DIC and completely suppressed serum LPS-induced IL-1β levels, while amplifying LPS-induced IL-10 production \(^{277}\). IL-10 is not absolutely essential for the anti-inflammatory effect of CO as in IL-10\(^{-/-}\) mice, CO inhibited LPS-induced TNF-α levels within the first hour to a similar extent as in wild-type mice \(^{235}\).

The application of CO does not only affect cytokine release, but also the activation of MAPK. In RAW 264.7 murine macrophages, CO application reduced the production of LPS-induced cytokine release on one side, and increased p38 MAPK activation on the other side. Of the MAP kinase kinases (M KK3, MKK4, and MKK6) that activate p38 MAPK, CO enhanced the LPS-mediated stimulation of MKK3 in RAW 264.7 and epithelial cells \(^{235, 278-280}\). In contrast, the CO-triggered down-regulation of LPS-induced IL-6 production in macrophages was mediated via the JNK-pathway, which regulates several transcription factors including AP-1 \(^{269}\). The IL-17-induced IL-6 production in pulmonary epithelial cells on the other hand, was inhibited by CO via the ERK1/2-dependent pathway without altering p38 MAPK or JNK \(^{281}\). In rat pulmonary artery endothelial cells treated with TNF-α, the exposure to CO specifically reduced ERK1/2 activation and increased p38 MAPK activation \(^{248}\). Taken together, CO exerts differential effects on p38 MAPK, JNK, and ERK1/2, which are dependent on cell type and stimulus. In vivo, the responsiveness of TNF-α to LPS treatment, as well as the inhibitory effects of CO, appeared down-regulated in MKK3\(^{-/-}\) mice compared with wild-type mice, suggesting that the MKK3/p38 MAPK pathway might play an important role in CO-mediated anti-inflammatory signaling \(^{235}\).

Downstream of p38 MAPK, heat-shock proteins appear to play an important contributory role in the anti-inflammatory effects of CO. Increased expression of heat-shock protein 70 (Hsp70) was involved in the protective effects of CO in murine lung endothelial cells and fibroblasts \(^{262}\). Suppression of Hsp70 expression and/or genetic deletion of heat shock factor-1, the principle transcriptional regulator of Hsp70, attenuated the cytoprotective and immuno-modulatory effects of CO in mouse lung cells and in vivo. Recently, it was demonstrated in a CLP-model that HO-1-derived CO affects the antimicrobial process without inhibiting the inflammatory response \(^{283}\). Mice overexpressing HO-1 in smooth muscle cells (SMC) of blood vessels and bowel ameliorated sepsis-induced death associated with Enterococcus faecalis infection \(^{283}\). However, the increase in HO-1 expression did not suppress circulating inflammatory cells or their accumulation at the site of injury, but did enhance bacterial clearance by increasing phagocytosis and the endogenous antimicrobial response \(^{283}\). Furthermore, injection of CORM-3 into WT mice increased phagocytosis and rescued HO-1-deficient mice from sepsis-induced lethality \(^{283}\).

In an initial attempt to translate the anti-inflammatory effects of CO observed in rodents to humans, a preclinical trial was performed \(^{284}\). Humans were exposed to 500-ppm CO for 1 h by inhalation, a dose that increased CO-Hb levels to 7%, followed by LPS
injection. LPS infusion transiently increased plasma concentrations of TNF-α, IL-6, and IL-8, as well as IL-1α and IL-1β mRNA levels. CO inhalation did not influence the cytokine response to endotoxin treatment in humans. Since many sepsis models have shown protective characteristics of CO application, further studies with different designs have to be conducted to investigate the potential of CO for reducing inflammation in septic patients.
1.2 AIM OF THE THESIS

As summarized in Chapter 1.1, sepsis is a complex clinical syndrome and comprises multiple derangements involving several different organs and physiological systems. There is convincing evidence that in addition to monocytes, also endothelial cells play an important role in the clinical outcome of sepsis, but the actual role of the endothelium in the course of sepsis is not fully understood.

In this thesis we aimed to gain insight in the heterogenous response of macro- and microvascular endothelial cells to inflammatory stimuli, to study the anti-inflammatory effect of the signaling molecule carbon monoxide (CO) and the mechanisms involved, and to investigate whether bone marrow-derived endothelial progenitor cells are increasingly mobilized in the course of sepsis and if this mobilization is associated with clinical outcome.

In Chapter 2 we tested the hypothesis that the response of endothelial cells to lipopolysaccharide (LPS) can be classified into general phenotypes of cells with a low and a high pro-inflammatory potential. Based on previous findings that serum IL-8 concentration is associated with severity of sepsis, and the fact that endothelial cells are the main origin of IL-8, endothelial cells were grouped according to their IL-8 production. We investigated by gene expression profiling whether low IL-8 production is associated with low expression of other inflammatory genes and which molecular mechanism might be involved in this phenomenon.

In Chapter 3 we studied whether high concentrations of CO releasing molecules (CORM-3), which release CO in excess of endogenously produced CO, are able to modulate the expression of adhesion molecules by endothelial cells, and if this was mediated by similar mechanisms as have been reported for heme oxygenase (HO)-1. In Chapter 4 we set out to investigate the molecular mechanism involved in the CO-mediated down-regulation of VCAM-1 on endothelial cells.

In Chapter 5 we tested the hypothesis that clinical outcome in septic patients is largely dependent on the ability to reconstitute damaged endothelium. We used flow cytometry to identify and detect circulating endothelial progenitor cells in the peripheral blood of septic patients and studied whether this mobilization was associated with clinical outcome.

Chapter 6 provides a summary of the generated data and discusses some important issues raised during this research, highlighting future perspectives of potential therapeutic interventions, including CO-mediated interference with activation of
endothelial cells in sepsis and application of endothelial progenitor cells to maintain the integrity of the endothelial layer.
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GENERAL INTRODUCTION


GENERAL INTRODUCTION


GENERAL INTRODUCTION


GENERAL INTRODUCTION


CHAPTER 2

HETEROGENEITY IN LIPOPOLYSACCHARIDE RESPONSIVENESS OF ENDOTHELIAL CELLS IDENTIFIED BY GENE EXPRESSION PROFILING: ROLE OF TRANSCRIPTION FACTORS

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SUMMARY

Interindividual differences of endothelial cells in response to endotoxins might contribute to the diversity in clinical outcome among septic patients. The present study was conducted to test the hypothesis that endothelial cells (EC) with high and low pro-inflammatory potential exist and to dissect the molecular basis underlying this phenomenon. Thirty human umbilical vein endothelial cell (HUVEC) lines were stimulated for 24 h with lipopolysaccharide (LPS) and screened for interleukin (IL)-8 production. Based on IL-8 production five low and five high producers, tentatively called types I and II responders, respectively, were selected for genome-wide gene expression profiling. From the 74 genes that were modulated by LPS in all type II responders, 33 genes were not influenced in type I responders. Among the 41 genes that were increased in both responders, 17 were expressed significantly stronger in type II responders. Apart from IL-8, significant differences in the expression of pro-inflammatory related genes between types I and II responders were found for adhesion molecules [intercellular adhesion molecule (ICAM-1), E-selectin], chemokines [monocyte chemoattractant protein (MCP-1), granulocyte chemotactic protein (GCP-2)], cytokines (IL-6) and the transcription factor CCAAT/enhancer binding protein-delta (C/EBP-δ). Type I responders also displayed a low response towards tumour necrosis factor (TNF)-α. In general, maximal activation of nuclear factor κB (NFκB) was achieved in type I responders at higher concentrations of LPS compared to type II responders. In the present study we demonstrate that LPS-mediated gene expression differs quantitatively and qualitatively in types I and II responders. Our results suggest a pivotal role for common transcription factors as a low inflammatory response was also observed after TNF-α stimulation. Further studies are required to elucidate the relevance of these findings in terms of clinical outcome in septic patients.
INTRODUCTION

Infections with gram-negative bacteria are considered to be the most frequent cause in the onset of organ dysfunction, although other mechanisms might also be involved [1]. Despite considerable effort that has been given over the past decade to understand the systemic inflammatory response and characteristics of severe sepsis, mortality in septic patients remains high. It is clear that mortality is highly dependent on individual patient factors, e.g. pre-existing disease, hospitalization and age [2,3]. However, the reason why the course of sepsis develops more vigorously in some patients than in others has not yet been solved. Apart from health status, age and hospitalization it is believed that genetic variations within promoter, intron or exon sequences of inflammatory genes may, in part, determine the clinical course in septic patients [4].

Genetic polymorphisms have been identified in genes encoding inflammatory molecules, e.g. interleukin (IL)-1, IL-1 receptor antagonist (IL-RA), tumour necrosis factor (TNF)-α or Toll-like receptors (TLR) [5,6]. In septic patients mortality seems to be associated only with the TNFB2 allele [7–10], which is also associated with high TNF-α production by mononuclear cells. This suggests that TNF-α might play a pivotal role in mortality caused by sepsis. In clinical studies anti-TNF-α antibodies have nevertheless failed to demonstrate any significance on mortality in septic patients [11,12]. These findings therefore point towards the involvement of other factors that might, in concert with TNF-α, influence mortality in these patients.

There is compelling evidence that in addition to monocytes [13,14], endothelial cells also play an important role in the clinical outcome of sepsis. In septic patients a dysfunction in macro- and microcirculation is observed frequently. Moreover, endothelial cells have the propensity to produce high amounts of a variety of pro-inflammatory mediators, e.g. cytokines, chemokines and eicosanoids. As a consequence of increased expression of adhesion molecules on these cells, leukocytes of septic patients adhere much more strongly and migrate subsequently along a chemotactic gradient into the subendothelial interstitial tissue [15].

As the amount of inflammatory mediators, produced by endothelial cells, is highly heterogenous [16,17], severity of inflammation and thus mortality in sepsis could be dependent on interindividual variations of endothelial cells to respond to bacterial toxins.

In the present study, we therefore tested the hypothesis that the response of endothelial cells to lipopolysaccharide (LPS) can be classified into general phenotypes of cells with a low and a high pro-inflammatory potential. Based on previous findings that serum IL-8 concentration is associated with severity of sepsis [16,18,19], and the fact that IL-8 is produced strongly by endothelial cells [20,21], endothelial cells were grouped according to their IL-8 production. The following questions were then raised: (1) is low
HETEROGENEITY IN LPS RESPONSIVENESS

IL-8 production associated with low production of other mediators, (2) if so, what are these mediators and (3) what is the molecular basis for this phenomenon?

MATERIALS AND METHODS

Cell isolation and culture
Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords, as has been described previously [22]. The cells were cultured in essential growth medium for endothelial cells (Promocell, Heidelberg, Germany) in gelatine (1%) coated culture flasks (Greiner, Frickenhausen, Germany). Confluent monolayers were subcultured by trypsin 0.025 vol%/ethylenediamine tetraacetic acid (EDTA) 0.01 vol% (Promocell, Heidelberg, Germany). Characterization of endothelial cells was performed on the basis of uptake of acetylated low-density lipoprotein (LDL), a positive staining for factor VIII-related antigen and platelet-endothelial cell adhesion molecule (PECAM), and a negative staining for alpha smooth muscle actin.

Chemokine production
HUVEC (1×10^5 cells/ml) were seeded in 24-well plates and grown until confluence. The cells were stimulated for 24 h with 1µ g/ml of LPS (Sigma, Deisenhofen, Germany). In each experiment control HUVEC were included to determine basal expression of chemokines. Supernatants were collected and assessed for IL-8 production by enzyme-linked immunosorbent assay (ELISA) (R&D Systems GmbH, Wiesbaden, Germany). ELISA was performed according to the manufacturer’s instructions. Each experimental condition was performed in triplicate and each experiment was confirmed at least three times.

DNA-isolation and IL-8 genotyping
DNA was isolated from HUVEC using the Wizard genomic DNA purification Kit (Promega Corporation, Madison, WI, USA). IL-8 genotyping was performed by polymerase chain reaction (PCR), as described previously [23]. In brief, 40 ng of genomic DNA was added to a 25µl reaction mixture containing 0.2 mM deoxyribonucleoside triphosphate (dNTPs) (Gibco BRL, Eggenstein, Germany), 12.5 pmol of primer (Perkin Elmer Applied Biosystems, Weiterstadt, Germany), 0.5 units of Taq-DNA-polymerase (Invitrogen GmbH, Karlsruhe, Germany) and 1.5 mM MgCl2. After 5 min of denaturation at 94°C, amplification was performed in 30 cycles, each consisting of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C. After the last cycle primer extension was performed for 5 min at 72°C. T/A alleles were assessed by Mfe I restriction enzyme digestion of the PCR products (Roche, Basel, Switzerland) and visualized by ethidium bromide in 2% agarose gels.
RNA isolation, cRNA and array hybridization
Sample preparation and processing was performed according to the Affymetrix GeneChip Expression Analysis Manual (http://www.Affymetrix.com). Briefly, endothelial cell monolayers were stimulated with LPS (1μg/ml) or left in normal medium for 24 h. Total RNA was isolated from these cultures using Trizol®-Reagent (Gibco BRL). Hereafter, DNase treatment was carried out, using RNase free DNase I (Ambion, Woodward, Austin, TX, USA). RNA concentration and quality were assessed by RNA 6000 nano assays on a Bioanalyser 2100 system (Agilent, Waldbronn, Germany). Five μg of RNA was converted into cDNA using T7-(dT) 24 primers and the SuperScript Choice system for cDNA synthesis (Life Technologies, Inc., Rockville, MD, USA). Biotinlabelled cRNA was prepared by *in vitro* transcription using the BioArray high yield RNA transcript labelling kit (Enzo Diagnostics, Farmingdale, NY, USA). The resulting cRNA was purified, fragmented and hybridized to U133A gene chips (Affymetrix, Santa Clara, CA, USA). After hybridization the chips were stained with streptavidin–phycoerythrin (MoBiTec, Goettingen, Germany) and analysed on a GeneArray scanner (Hewlett Packard Corporation, Palo Alto, CA, USA).

Reverse transcription–polymerase chain reaction (RT-PCR)
One μg of total RNA was reverse transcribed into cDNA using the SuperScript TM II Preamplification System (Life Technologies, Karlsruhe Germany) according to the manufacturer’s instructions. The sequences of the primers used for amplification are listed in Table 1. Primers were purchased from Perkin Elmer. PCR was performed in a volume of 25μl containing 10 mM Tris HCl, 75 mM KCl, 1.5 mM MgCl₂, 200μM dNTPs, 20 pmol of each primer, 0.5 μl cDNA and 2.5 U of TaqDNA polymerase (Gibco BRL). A control containing no cDNA was always included. The PCR reactions were initiated at 94°C for 3 min and amplification was performed in 28–32 cycles, each consisting of 1 min at 94°C, 1 min at the annealing temperature and 2 min at 72°C, followed by final extension for 10 min at 72°C. PCR products were subjected to electrophoresis in 1% agarose (Serva, Boehringer Ingelheim, Heidelberg, Germany).
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Table 1: List of oligonucleotides used for cDNA amplification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size of PCR product (bp)</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GTCTTCACCACCATGGAGAA-3'</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>5'-ATCCACAGTCTTCTGGGTGG-3</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-CGATGTCATGCATAAAGACA-3'</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>5'-TGAATTCTCAGCCTCTCCAAA-3</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-TCAATGAGGAGACTTGCTGCT-3'</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>5'-ACAGCTCTGTTGTCTTCTAC-3'</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-TGTCCTGTCTCAGTCATAG-3'</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>5'-GAATCCCTGAACCCACTTCTG-3'</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5'-GCAAATGTGAGAGATGAGCCA-3'</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>5'-ACCCGTTCGGAGTCCAGTCA-3'</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>5'-TGTGAGCTCCACTGAGT-3'</td>
<td>307</td>
</tr>
<tr>
<td></td>
<td>5'-TCTGGCATAGTGGAAGTACA-3'</td>
<td></td>
</tr>
</tbody>
</table>

bp: Base pairs; GAPDH: glyceraldehyde-3-phosphate-dehydrogenase; ICAM: intercellular adhesion molecule; IL: interleukin; MCP: monocyte chemoattractant protein; PCR: polymerase chain reaction

Flow cytometry
Confluent endothelial cell monolayers were harvested by T/E and washed three times in PBS. Hereafter, monoclonal antibodies directed against intercellular adhesion molecule (ICAM-1) and E-selectin, both from Dako, Glostrup, Denmark, were added to the cells in dilutions of 1 : 20. Isotypematched anti-idiotypic antibodies were used as negative control. After 30 min at 4°C, the cells were washed and incubated with an anti-mouse F(ab')2, fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 30 min at 4°C. Finally the cells were washed three times with PBS before analysis was performed on a FACSscalibur (Becton Dickinson, Heidelberg, Germany).

Electrophorese mobility shift assay (EMSA)
Confluent monolayers of HUVEC were incubated overnight in 0.01% bovine serum albumin (BSA) containing culture medium without growth factors or fetal calf serum (FCS). The cells were stimulated for 4 h with different concentrations of LPS. Nuclear extracts and EMSA were performed essentially as described previously [24]. Briefly, nuclear factor-kappa B (NFκB) consensus oligonucleotides (Promega, Mannheim, Germany) were labelled to a specific activity > 5 ×10⁷ counts per min (cpm)/μg DNA. The labelled oligonucleotide was added to 10μg of nuclear extracts in a total volume of 20μl containing 10 mM HEPES (pH=7.5), 0.5 mM EDTA, 70 mM KCl, 2 mM dithiothreitol (DTT), 2% glycerol, 0.025% NP-40, 4% Ficoll, 0.1 M phenylmethyl-
sulphonyl fluoride (PMSF), 1 mg/ml BSA and 0.1μg/μl poly di/dc. In each experiment specificity of binding was demonstrated by adding cold consensus or mutated NFκB oligonucleotides to the nuclear extracts. In addition supershifts were performed by adding anti-p65 and -p50 antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) to the samples. DNA–protein complexes were separated on 5% non-denaturing polyacrylamide gels in low ionic strength buffer and visualized by autoradiography.

Statistical analysis
Normalization and data analysis of the chips were performed according to instructions provided by Affymetrix. Pairwise comparisons were made by using unstimulated probe arrays as baseline and LPS-stimulated probe arrays of the same cell line as the experiment. The baseline corrected data were imported into the Affymetrix Data Mining tool (dmt version 4.0) using the publishing tool (mdb version 3.0). Subsequently, the genes were filtered using Affymetrix statistical data analysis software (Affymetrix Microarray Suite version 5.0). Probe sets were excluded when the detection call was absent, when the change call gave no change (NC) in comparison analysis, or when the signal log ratio (SLR) between unstimulated and stimulated cells was between −1 and 1. SLR was used to describe the change between a target and a reference array. The change was expressed as log₂ ratio. Thus, a signal log ratio of 1 equals a fold change (FC) of 2. Only genes that fulfilled the filtering criteria were used for further analysis. Functional categorization of genes was based on ontological designations in the NetAffx Analysis Center (http://www.affymetrix.com), the AmiGO gene ontology database (http://www.godatabase.org) and gene descriptions in Online Mendelian Inheritance in Man (OMIM). Statistical analysis of SLRs was performed using Stata statistical software (Mann–Whitney test). A P-value < 0.05 was considered significant.

RESULTS

Heterogeneity in IL-8 production
A total of 30 primary HUVEC cultures was analysed for IL-8 production. Among these, strong differences in basal and LPS-mediated IL-8 production were detected. In general, basal IL-8 production was low and varied from 0.225 to 4.13 ng/ml/10⁶ cells (data not shown). Upon stimulation with LPS for 24 h IL-8 production was up-regulated, varying from 10 to 85 ng/ml/10⁶ cells (Fig. 1A). Low basal IL-8 production was not associated with low IL-8 production after LPS stimulation. IL-8 production was not associated under basal nor under stimulatory conditions with the −251 T→A polymorphism in the IL-8 promoter (Fig. 1B).
Gene expression profiling
Based on LPS-mediated IL-8 production, 10 HUVEC cultures were selected for further analysis, i.e. low (n=5) and high (n=5) IL-8 producing HUVEC. These were tentatively called types I and II responders, respectively. All genotypes with respect to the −251 A→T polymorphism in the IL-8 promoter were present in our selection (Table 2). In the next step we analysed if low IL-8 production was associated with low production of other mediators. Genome-wide gene expression profiling was performed and gene expression patterns were compared between types I and II responders using two algorithms, i.e. SLR and change P-value. From all genes present on the chip 74 genes were found to be modulated significantly by LPS in all type II responders (SLR > 1 or SLR < −1, change P-value increase ≤ 0.0045 < marginal increase ≤ 0.006; 0.006 < no change < 0.994; 0.94 ≤ marginal decrease ≤ 0.9955 ≤ decrease). Whereas the majority of these genes were up-regulated, only three were down-regulated. In contrast, only 41 genes were significantly up-regulated in all type I responders and these were also found up-regulated in type II responders. Thus 33 genes were modulated specifically only in all type II responders. Among these, genes encoding for cytoskeleton proteins, immune response genes and genes encoding for transcription factors were found (Table 3). The genes that were significantly down-regulated by LPS type II responders were thrombomodulin, the intercellular adhesion molecule connexin and bone morphogenetic protein (BMP)-4.

In order to compare whether the modulation of gene expression was quantitatively different for the 41 genes that were up-regulated in type I and type II responders the mean SLR for these genes was calculated in all five type I and type II responders. Comparisons between type I and type II responders revealed that 17 of 41 genes were more strongly up-regulated significantly in type II responders (Table 3). Significant differences between type I and type II responders for genes that might be implicated in inflammatory processes are depicted in Fig. 2A and include adhesion molecules (ICAM-1 and E-selectin), chemokines (MCP-1, IL-8, GCP-2), cytokines (IL-6), co-agglutination-related genes (thrombin receptor) and transcription factor (C/EBP-δ). Validation of the gene profiling data for IL-8, IL-6, MCP-1, E-selectin and ICAM-1 was performed by RT-PCR in all types I and II responders. Figure 2B shows the results of a representative experiment using two different types I and II responders.
### Table 2: Characteristics of the selected cell lines

<table>
<thead>
<tr>
<th>HUVEC line</th>
<th>Responder type</th>
<th>Basal IL-8 production (ng/ml/10⁶ cells)</th>
<th>LPS mediated IL-8 production (ng/ml/10⁶ cells)</th>
<th>IL-8 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>0.61 ± 0.12</td>
<td>10.87 ± 2.74</td>
<td>TA</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>0.92 ± 0.23</td>
<td>16.44 ± 2.67</td>
<td>TA</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>0.95 ± 0.19</td>
<td>16.38 ± 4.63</td>
<td>TA</td>
</tr>
<tr>
<td>13</td>
<td>I</td>
<td>0.54 ± 0.34</td>
<td>16.58 ± 2.09</td>
<td>TT</td>
</tr>
<tr>
<td>14</td>
<td>I</td>
<td>0.46 ± 0.33</td>
<td>18.70 ± 1.57</td>
<td>AA</td>
</tr>
<tr>
<td>5</td>
<td>II</td>
<td>0.38 ± 0.12</td>
<td>75.45 ± 7.98</td>
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</tr>
<tr>
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<td>II</td>
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<td>58.45 ± 4.50</td>
<td>TT</td>
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<tr>
<td>26</td>
<td>II</td>
<td>1.12 ± 0.11</td>
<td>66.50 ± 9.76</td>
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<tr>
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<td>II</td>
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<tr>
<td>29</td>
<td>II</td>
<td>1.88 ± 0.56</td>
<td>70.34 ± 12.0</td>
<td>AA</td>
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</tbody>
</table>

HUVEC: human umbilical vein endothelial cell; IL: interleukin; LPS: lipopolysaccharide
Figure 1. Heterogeneity in LPS-mediated IL-8 production amongst 30 HUVEC lines. (A) Endothelial cells were stimulated for 24 hours with 1 µg/ml of LPS. Supernatants were collected and assessed for IL-8 production. The results are expressed as mean IL-8 production ± SD of triplicate cultures. (B) Mean IL-8 production was calculated from the data set obtained in A for each of the different genotypes. The results are expressed as mean IL-8 production ± SD.
Table 3: Comparison of LPS mediated gene expression between type I and type II responders. Genes are grouped based on known or putative function as determined by GeneOntology classifications

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>GOMF</th>
<th>Typ II</th>
<th>Typ I</th>
<th>P-value</th>
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<td>transcription factor binding // activity</td>
<td>3,1</td>
<td>1,94</td>
<td>0.0068</td>
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<td>201502_s_at</td>
<td>CCAAT/enhancer binding protein (C/EBP), delta</td>
<td>2,06</td>
<td>1,6</td>
<td>0,24</td>
</tr>
<tr>
<td>209636_at</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
<td>1,93</td>
<td>1,65</td>
<td>0,68</td>
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**Table 3. Continued**

Bold type: enlisted are all the genes that were significantly up-regulated by lipoysaccharide (LPS) in all type I and all type II responders. Italic bold type: enlisted are all the genes that were expressed in both type I and II responders, but were expressed significantly more strongly expressed in type II responders. Note that the P-value refers to the comparison between type I and II responders. Plain text: enlisted are genes that were significantly modulated by LPS in all type II responders only. The results are expressed as mean signal log ratio (SLR).
HETEROGENEITY IN LPS RESPONSIVENESS

A

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SLR

B

Figure 2. Differences in gene expression between type I and II responders. (A) Significant differences in lipopolysaccharide (LPS)-mediated gene expression between type I and type II responders for genes implicated in inflammatory processes. Filled bars: type II responders, open bars: type I responders. The results are expressed as mean signal log ratio (SLR) (n=5 for each responder type) ± SD. (B) Validation of gene expression profiling was performed by reverse transcription-reverse polymerase chain reaction (RT-PCR) for interleukin (IL)-8, IL-6, monocyte chemoattractant protein (MCP)-1, E-selectin and intercellular adhesion molecule (ICAM)-1. Total RNA was isolated from unstimulated (-) or LPS (1 μg/ml, 24 h)-stimulated (+) cells. A representative experiment using two different types I and II responders is depicted. Similar findings were found in all types I and II responders.
Differences between types I and II responders are not restricted to LPS stimulation

In order to study if the differences between types I and II responders were specific for LPS stimulation, the production of IL-8 and the expression of ICAM was also investigated after TNF-α stimulation. As described for LPS, we found that TNF-α-mediated IL-8 production was significantly higher in type II than in type I responders. In fact, stimulation with 500 U/ml of TNF-α in type I compared to 5 U/ml in type II responders was required for the production of an equal amount of IL-8 (Fig. 3A). Similarly, TNF-α-mediated ICAM-1 expression was clearly higher in all type II responders (Fig. 3B).

To study if type I responders could be converted into type II responders, HUVEC were stimulated simultaneously with LPS and IFN-γ. This influenced neither the amount of IL-8 production (LPS versus LPS + IFN-γ) nor the type of responder (data not shown). Interestingly, however, pre-treatment of HUVEC with IFN-γ 24 h before stimulation with LPS induced a nearly twofold up-regulation in IL-8 production in both responder types (Fig. 3C).

To gain more insight into the mechanism underlying the differences between types I and II responders, activation of NFκB was investigated. In all types I and II responders NFκB was activated by LPS in a dose- and time-dependent fashion. In all type II responders maximal activation was achieved with lower concentrations of LPS (0.02 μg/ml), while maximal activation of NFκB in type I responders was observed at higher LPS concentrations (1 μg/ml) (Fig. 4A). No difference in the kinetic of NFκB activation between types I and II responders was observed (Fig. 4B). In line with this, no difference in the kinetic of IL-8 production was found between both responders, although type II responders produced significantly more IL-8 (Fig. 4C). Activated NFκB consisted of p65 and p50 subunits as demonstrated by supershift analysis (Fig. 4D). No differences between types I and II responders were found in this regard (data not shown). To demonstrate the functional relevance of NFκB activation by low concentrations of LPS, the expression of ICAM-1 was determined in types I and II responders using suboptimal concentrations of LPS. While ICAM-1 was clearly up-regulated with 0.1 μg of LPS in all type II responders, this was not found in type I responders (Fig. 5).
Figure 3. Susceptibility to tumour necrosis factor (TNF-α) stimulation in type I and type II responders. (A) Type I and type II responder cell lines were stimulated for 24 h with different concentrations of TNF-α. The supernatants were collected and assessed for interleukin (IL)-8 production by enzyme-linked immunosorbent assay (ELISA). The results are expressed as mean IL-8 ± SD production for each responder type (n= 5 for both type I and II). Hatched bars: type II responders, open bars: type I responders, *P < 0.05 compared to type I. (B) Constitutive (thin lines) and TNF-α mediated (bold lines) intercellular adhesion molecule (ICAM)-1 expression in type I (histograms in black) and type II (histograms in grey) responders. The negative control is depicted as filled histogram. The results of a representative experiment of a type I and II responder is depicted. Similar findings were found in all type I and II responders. (C) Endothelial cells of type I and II responders were pretreated with 125 ng/ml of interferon (IFN)-γ (+) or left untreated (−) for 24 hrs. Hereafter the cells were washed and stimulated with lipopolysaccharide (LPS) (1 μg/ml) (+) or not (−). Twenty-four h hereafter supernatants were collected and assessed for IL-8 production. The results are expressed as mean IL-8 ± SD production for each responder type (n= 5 for both type I and II). Hatched bars: type II responders, open bars: type I responders, *P<0.05 compared to type I.
**Figure 4. Electrophoresis mobility shift assay (EMSA) for NFκB.** (A) Susceptibility towards lipopolysaccharide (LPS) stimulation as measured by NF-κB activation in type I and II responders. The data of representative experiment are depicted. Similar findings were observed in all type I and II responders. Endothelial cells were stimulated for 4 hrs using different concentrations of LPS. (B) Time response of NFκB activation in type I and II responders. Endothelial cells were stimulated with 1 μg/ml of LPS. At different time-points the cells were harvested for preparation of nuclear extracts. The data of representative experiment are depicted. Similar findings were observed in all type I and II responders. Endothelial cells were stimulated for 4 h using different concentrations of LPS. (C) Kinetics of LPS mediated interleukin (IL)-8 production in types I and II responders. Human umbilical vein endothelial cells (HUVEC) were stimulated for different time periods with 1 μg/ml of LPS. Hereafter the supernatants were collected and assessed for IL-8 production. The results are expressed as mean IL-8 ± SD production for each responder type (n= 5 for both type I and II). Dotted line: type II responders, bold line: type I responders, *P< 0.05 compared to type I. (D) The specificity of the shifted bands was demonstrated by incubating a positive sample either with an excess of cold consensus (cons.) or mutated (mut.) NFκB oligonucleotides before adding labeled consensus NFκB oligonucleotides. Note that the NFκB shifted band (arrow) consisted of both p50 and p65 as demonstrated by super-shift (dotted arrow).

![Graphs showing LPS (μg/ml) vs FL-1 Height](image)

**Type I**

**Type II**

**Figure 5. Lipopolysaccharide (LPS) mediated intercellular adhesion molecule (ICAM)-1 expression in type I and II responders.** Endothelial cells were stimulated for 24 h with different concentrations of LPS. ICAM-1 expression was measured by fluorescence activated cell sorter (FACS) analysis. The data of a representative experiment is depicted. Similar results were obtained for all type I and II responders. The negative control is depicted as grey dotted histogram.
DISCUSSION

Sepsis and septic shock are major causes of morbidity and mortality in patients admitted to intensive care units. The systemic response to infection encompasses both pro- and anti-inflammatory phases that are marked by sequential generation of pro- and anti-inflammatory cytokines [25]. Among the pro-inflammatory cytokines TNF-α and IL-1β play a pivotal role [26]. These cytokines can, either alone or in conjunction with bacterial toxins, activate the vascular endothelium finally resulting in inflammation [27,28]. Interindividual differences in the endothelial inflammatory response to bacterial toxins might be considered as an additional factor influencing the clinical course of septic patients. In the present study we investigated if and to what extent primary cultures of endothelial cells display a heterogeneous response to LPS stimulation, resulting in cells with low and high pro-inflammatory characteristics.

To test this hypothesis we have grouped endothelial cell cultures according to the amount of IL-8 produced upon LPS stimulation and questioned whether low IL-8 production was associated with low expression of other inflammatory genes. The main findings of this study are first, that endothelial cells with low and high pro-inflammatory characteristics as such do not exist, although some pro-inflammatory genes are expressed significantly lower in so-called type I responders; secondly, that this was not specific for LPS and also observed after TNF-α stimulation; thirdly, that gene expression after LPS stimulation in type I and type II responders differ qualitatively and quantitatively from each other. Fourthly, endothelial cells of type I responders required higher LPS concentrations for maximal NFκB activation.

The search for predictors of mortality in septic patients is of crucial clinical relevance in order to treat these patients interindividually. Although a variety of factors has been suggested to be associated with mortality in sepsis [29–32], improvements in clinical outcome have been sporadic and, with few exceptions, are related to improvements in supportive care rather than to specific therapies. While in some studies soluble adhesion molecules, i.e. sICAM-1 and sVCAM-1 in serum of septic patients, were associated with mortality [29,30], this was not confirmed by others [33,34]. The relevance of circulating pro- and anti-inflammatory cytokines seems to be more consistent in this regard [31,32,35].

Apart from monocytes, the vascular endothelium is a large source from which circulating cytokines can be derived. This is particularly true for IL-8 and IL-6 [36,37]. Although it is difficult to estimate to what extent endothelial cells contribute to the amount of these cytokines in septic patients, the severity of sepsis is clearly associated with both [38,39]. It also remains to be elucidated whether low IL-8 and IL-6 production in Gram-negative sepsis is an intrinsic patient factor or whether this merely reflects bacterial load. Our data are in line with the former. LPS stimulation of some endothelial
cells, i.e. type I responders, resulted in low IL-8 production while type II responders produced high amounts of IL-8. In addition low IL-8 producers also expressed low IL-6 mRNA. Interestingly, our data also revealed that in addition to LPS the up-regulation of IL-8 and ICAM-1 was less pronounced in type I responders when stimulated with TNF-α. Hence, differences between types I and II responders were unlikely, due to functional differences in TLR or their level of expression. Moreover, no differences, either at mRNA or protein levels, for TLR4 and TLR2 between types I and II responders were identified (data not shown).

A more likely explanation could be the expression of transcription factors. Among the transcription factors that were influenced by LPS only the up-regulation of C/EBP-δ was reduced significantly in type I compared to type II responders. C/EBP-δ has been implicated in the regulation of pro-inflammatory cytokines such as IL-6, IL-8 and MCP-1 [40–42] and is activated by both TNF-α and LPS [43]. Because gene analysis was performed 24 h after LPS stimulation, differences in mRNA expression for other transcription factors may also exist between type I and type II responders, e.g. NFκB [44] and activator protein (AP)-1 [45]. These transcription factors are known to be transcribed early after stimulation. In a study by Bohrer et al. [46] investigating 25 septic patients, non-survivors could be distinguished from survivors by an increased activation of NFκB in mononuclear cells. This might be related to our findings using endothelial cells. Although no differences in NFκB p65 or p50 mRNA expression were detected among the cell lines, activation of NFκB was different between types I and II responders. Maximal activation of NFκB was already obtained with low concentrations of LPS in type II responders. In contrast, type I responders required 500-fold higher concentrations of LPS for maximal NFκB activation.

The clinical relevance of our findings with respect to types I and II responders remains to be elucidated and further studies are planned. Nevertheless, our study demonstrates that in addition to peripheral blood monocytes [47] heterogeneity in LPS responsiveness also occurs at the level of endothelial cells and thus might influence severity of the inflammatory response in septic patients.

ACKNOWLEDGEMENTS
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HETEROGENEITY IN LPS RESPONSIVENESS

CHAPTER 3

CO RELEASING MOLECULES INHIBIT TNF-α MEDIATED EXPRESSION OF VCAM-1 AND E-SELECTIN OF HEME OXYGENASE 1

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ABSTRACT

Heme oxygenase-1 (HO-1) exerts anti-inflammatory effects via heme degradation, resulting in the generation of biliverdin, Fe^{2+} and carbon monoxide (CO). Since CO itself up-regulates HO-1 expression, perpetuation of the anti-inflammatory effect is expected. We investigated how CO releasing molecules (CORM), i.e. CORM-3, modulate the expression of adhesion molecules on endothelial cells and if HO-1 mediated perpetuation was involved.

CORM-3 consistently inhibited the expression of VCAM-1 and E-selectin on TNF-α stimulated human umbilical vein endothelial cells (HUVEC), while ICAM-1 expression was marginally affected and only in 3 out of 8 lines tested. Although CORM-3 did not influence initial NFκB-activation, this was significantly reduced at later time-points. Down-regulation of VCAM-1 and E-selectin expression by CORM-3 also occurred when CORM-3 was added 24 hrs after TNF-α stimulation or when TNF-α was removed. This was paralleled by deactivation of NFκB, concomitantly with a reduction in VCAM-1 mRNA. Although TNF-α removal was more effective in this regard, VCAM protein was down-regulated more rapidly when CORM-3 was added compared to TNF-α removal. Induction of HO-1 occurred in a dose- and time-dependent manner and was mediated by Nrf2. Neither in HO-1 nor in Nrf2 siRNA-transfected HUVEC the efficacy of CORM-3 to down-regulate VCAM-1 expression was lost. In Nrf2 siRNA-transfected HUVEC CORM-3 no longer induced HO-1.

In conclusion, our study demonstrates that CORM-3-mediated anti-inflammatory effects are independent of HO-1 up-regulation. Although CORM-3 most likely exerts its effect by inhibition of sustained NFκB activation, down-regulation of VCAM expression also seems to be regulated post-translationally.
INTRODUCTION

Leukocyte extravasation occurring at the onset of inflammation is a highly regulated process characterized by bidirectional communication between endothelial cells and leukocytes. Upon exposure of endothelial cells to inflammatory mediators, adhesion molecules and chemokines are rapidly up-regulated, which in turn facilitate leukocyte migration \(^1^4\). In addition, ligand binding to CXC chemokine receptor 1 (CXCR1) and CXCR2 stimulates neutrophils to release a number of factors including proteases, cytokines, chemokines, and other chemoattractants that amplify inflammation and extend duration of the latter \(^5\). Hence, understanding the mechanisms controlling down-regulation of pro-inflammatory cytokines and adhesion molecules is now being widely recognized as a prerequisite for the identification of novel drug targets for inflammatory disease \(^2,6,7\).

In addition to its unambiguous role in oxidant-induced injury \(^8^\text{-}^{10}\), unequivocal evidence demonstrates that the heme oxygenase system (HO) is involved in the control of inflammatory processes \(^11^\text{-}^{14}\). The HO system comprises several isoenzymes \(^15,16\), of which the inducible HO-1 isoenzyme appears to be particularly important as anti-inflammatory mediator \(^11^\text{-}^{13},17\). HOs are the rate-limiting enzymes in degradation of heme into carbon monoxide (CO), Fe\(^{2+}\) and biliverdin, the latter being subsequently converted to bilirubin \(^18,19\).

Although a number of studies have postulated putative mechanisms by which HO-1 exerts its anti-inflammatory effect, some of the published data are controversial. While Soares et al. have shown that HO-1 down-regulates VCAM and E-selectin expression via bilirubin and iron chelation with no apparent involvement of CO \(^12,13\), Otterbein et al. and Sethi et al. clearly demonstrate the anti-inflammatory potential of CO in macrophages and monocytes \(^11\) as well as in endothelial cells \(^20,21\). The salutary effect of CO has also been shown for organ transplantation and ischemia reperfusion injury \(^22,23\). Recently, a new class of molecules, termed CO-releasing molecules (CORM), has been described \(^24\) that are composed of transition metal carbonyls. They are capable of liberating CO under appropriate conditions. In particular, CORM-3 (tricarbonylchloro(glyconato)ruthenium(II)) and CORM-A1 (sodium boranocarbonate), which both are fully water-soluble, rapidly liberate CO when dissolved in physiological solutions. These molecules might therefore be of therapeutically interest to modulate ongoing inflammatory reactions by delivering CO in a controllable fashion \(^25\). In addition, these molecules have been widely used to increase our understanding of the biological function of CO \(^26,27\).

Interestingly, CO itself can induce the expression of HO-1 in an Nrf2-dependent fashion as was demonstrated in hepatoma cells \(^28\). In addition, CO stabilizes HIF-1\(\alpha\), a putative transcription factor for HO-1 expression, in macrophages \(^29\). Since induction of HO-1 by
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CO might perpetuate the anti-inflammatory effect of CO we now investigated whether (A) HO-1 is induced by CORM-3 in endothelial cells, (B) its expression is regulated by the transcription factors Nrf2 or HIF-1α, and (C) if the anti-inflammatory effect of CORM was perpetuated by HO-1.

MATERIALS AND METHODS

Reagents
Reagents were obtained from the sources as indicated: endothelial cell culture medium (Promocell, Heidelberg, Germany), PBS (GIBCO, invitrogen, NY), fetal bovine serum (FBS) Gold (PAA laboratories GmbH, Pasching, Austria), trypsin/EDTA solution, DMSO, Tween 20, DEAE-Dextran, chloroquine, Hepes, Triton X-100, DTT, sodium deoxycholate, Tris-base, EDTA, APS, SDS, TEMED, tricarbonyldichlororuthenium(II) dimer, glycine, sodium ethoxide, human recombinant TNF-α (Sigma, St. Louis, MO), bovine serum albumin (SERVA, Heidelberg, Germany), protease inhibitor cocktail, 1st strand cDNA synthesis Kit (Roche Diagnostic, Mannheim, Germany), NF-κB consensus oligonucleotides, Dual-Glo Luciferase Assay System (Promega, Mannheim, Germany), Coomassie protein assay reagent (Pierce, Rockford, IL), Trizol (Invitrogen, Carlsbad, CA), chloroform, isopropanol, tetrahydrofuran, β-mercaptoethanol (Merck, Darmstadt, Germany). Primers and all reagents were purchased for TaqMan PCR (ABI, Darmstadt, Germany). All antibodies for flow cytometric analysis were purchased from R&D System (Minneapolis, MN) and all FACS reagents from Becton Dickinson (Heidelberg, Germany). All antibodies used for Western blotting, including horseradish peroxidase (HRP) conjugates, antibodies for supershifts as well as siRNAs were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) with exception of the anti-HO-1 (Stressgen, Victoria, Canada) and the anti-GAPDH antibodies (Abcam Cambridge, UK). Chemiluminescence reagent was purchased from PerkinElmer LAS Inc. (Boston, MA).

Cell culture
Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords as described previously. The cells were grown in basal endothelial medium supplemented with 10% FBS and essential growth factors until they formed a confluent monolayer. Cells were stimulated with 50ng/ml of TNF-α in the presence or absence of different concentrations of CORM-3. Unstimulated cells served as control.

Synthesis of tricarbonyldichloro(glycinato)ruthenium(II)
Tricarbonyldichloro(glycinato)ruthenium(II) ([Ru(CO)₂Cl(glycinato)]) was synthesized starting from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ([Ru(CO)₂Cl₂]). Briefly, [Ru(CO)₂Cl₂] (0.5 g) and glycine (0.151 g) were placed
under nitrogen in a round-bottomed flask. Methanol (291 ml) and sodium ethoxide (0.132 g) were added and the reaction was allowed to continue under stirring for 18 hrs at room temperature. The solvent was then removed under pressure and the yellow residue redissolved in tetrahydrofuran (THF). The yellow solution was evaporated down to give a pale yellow solid (yield 92-96%) and was stored in closed vials at -20°C. For each experiment CORM-3 was dissolved freshly in PBS.

**FACS analysis**

HUVECs were cultured in medium supplemented with 50ng/ml TNF-α in the presence or absence of 1mM CORM-3. HUVECs cultured in endothelial medium were used as control. FACS analysis was performed with 2×10⁶ cells using the following FITC-conjugated monoclonal antibodies: anti-human ICAM-1 (BBIG-I1), anti-human VCAM-1 (BBIG-V3) and anti-human E-selectin (BBIG-E5). Antibodies were added for 40 min at 4°C followed by extensive washing with PBS. FACS analysis was performed on a FACScalibur equipped with the CELLQuest software. The data were analysed by Windows Multiple Document Interface (WinMDI) software (Version 2.8).

**Western blot analysis**

HUVECs were stimulated for different time periods with 50ng/ml TNF-α in the presence or absence of 1mM CORM. Hereafter, the cells were harvested and lysed in 50 μl lysis buffer containing 10mM Tris-base, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1μM DTT and proteinase inhibitor cocktail. In some experiments, nuclear proteins were isolated as previously described (32, 33). Protein concentrations were assessed using Coomassie reagents. SDS-PAGE and Western blotting were performed essentially similar as described 30. Briefly, samples were resolved on 10 % SDS-PAGE and transferred onto PVDF filters by semi-dry blotting. The filters were incubated with 5% non-fat dry milk powder in PBS for 1 hour at room temperature to block unspecific background staining and hereafter incubated overnight at 4°C with specific polyclonal antibodies, depending on the experiment that was performed. The following antibodies were used: polyclonal rabbit anti-HO-1 antibody, polyclonal rabbit anti-Nrf2 antibody, polyclonal rabbit anti-IκB alpha antibody, polyclonal goat anti-VCAM-1 antibody, polyclonal rabbit anti-NF-κB p50 antibody, polyclonal rabbit anti- NF-κB p65 antibody. After extensive washing in PBS/Tween/5 % non fat dry milk powder, the filters were incubated 60 minutes with appropriate horseradish peroxidase-conjugated polyclonal IgG, followed by 3 times washing in PBS/Tween. Visualization of immunoreactive bands was performed by chemiluminescence reagent according to the manufacturer’s instructions. The filters were re-probed with monoclonal anti-Histone H1 antibody or monoclonal anti-GAPDH antibody to demonstrate equal loading.
Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from HUVECs as described above. Protein concentrations were determined by Bradford assay. EMSA was performed essentially as previously described \(^{31, 32}\). Briefly, NFkB consensus oligonucleotides were labelled to a specific activity >5x10^7 cpm/μg DNA. 10 μg of nuclear extracts were added to 1 ng of labelled oligonucleotide in a total volume of 20 μl containing 10 mM HEPES (pH=7.5), 0.5 mM EDTA, 70 mM KCl, 2 mM DTT, 2% glycerol, 0.025% NP-40, 4% Ficoll, 0.1 M PMSF, 1 mg/ml BSA and 0.1 μg/μl poly di/dc. In each experiment specificity of binding was demonstrated by adding cold consensus and mutated NFkB oligonucleotides to the nuclear extracts. In addition, supershifts were performed by adding anti-p65 and p55 antibodies to the samples. DNA-protein complexes were separated on 5% non-denaturing polyacrylamide gels electrophoresed in low ionic strength buffer and visualized by autoradiography.

RNA isolation, reverse transcriptase PCR, quantitative TaqMan PCR

Total RNA was isolated from confluent endothelial cell monolayers using Trizol®-Reagent. Thereafter, DNase treatment was carried out, using RNase free DNase I (Ambion, Darmstadt, Germany). RNA concentration and quality were assessed by RNA 6000 Nano assays on a Bioanalyzer 2100 system (Agilent, Boeblingen, Germany). 500 ng of total RNA was reverse-transcribed into cDNA using the 1st Strand cDNA Synthesis Kit. cDNA was diluted in 20 μl DEPC-treated water and stored at -20°C until use.

For reverse transcriptase PCR 1μl of cDNA was amplified in a 50μl reaction mix containing 10mM dNTPs, 50pM of each primer, 2.5 units Taq polymerase and 1.5mM MgCl\(_2\). The primers used were as follows: HO-1 forward: 5’- GCT CAA CAT CCA GCT CTT TGA GG-3’ and reverse: 5’- GAC AAA GTT CAT GGC CCT GGG A-3’; VCAM forward: 5’- CGA TCA CAG TCA AGT GTT CAG TTG-3’ and reserve: 5’- GCA ATT CTT TTA CAG CCT GCC T-3’; GAPDH forward: 5’- GTC TTC ACC ACC ATG GAG AA-3’ and reserve: 5’- ATC CAC AGT CTT CTG GGT GG-3’. The cycling conditions used for various primers were as follows: 4 min of denaturation at 94°C, followed by 28 (VCAM-1) or 25 (HO-1 and GAPDH) cycles of amplification, each consisting of denaturation for 30s at 94°C, annealing for 30s at 59°C (VCAM-1), 62°C (HO-1) or 55°C (GAPDH) and extension for 45s at 72°C. After the last amplification a final extension for 10 min at 72°C was performed for each reaction. PCR products were analysed on a 1 % agarose gel containing ethidium bromide.

Quantitative real-time RT-PCR was performed on the ABI-Prism 7700 sequence detection system with the TaqMan universal PCR master mix No AmpErase UNG (part no. 4324018). Taqman probes for VCAM-1 (part No. HS00174239_m1) and β-actin (part No. HS99999903_m1). Samples were run under the following conditions: initial denaturation for 10 minutes at 95°C followed by 40 cycles of 15 s at 95°C and 1 minute at 60°C. The levels of gene expression in each sample were determined with the
comparative cycle threshold method. PCR efficiency was assessed from the slopes of the standard curves and was found to be between 90% and 100%. Linearity of the assay could be demonstrated by serial dilution of all standards and cDNA. All samples were normalized for an equal expression of β-actin. Each experiment was repeated 3 times with similar results.

**Reporter assays**
HUVECs were transfected with a reporter construct containing 3 hypoxia response elements (3HRE.luc)\(^{33}\) or a construct containing an anti-oxidant response element from the HO-1 promoter (HO-ARE.luc)\(^{34}\). All transfections were performed using DEAE-dextran as described previously\(^{35}\) and in each case the ubiquitin-dependent Renilla luciferase reporter was co-transfected. Two days after transfection, cells were stimulated for 24 hrs with CORM-3. Luciferase activity was measured using the Dual-Glo Luciferase Assay System. All experiments were performed in triplicate and ARE- or HRE-dependent luciferase activities were normalized for luciferase activity generated by the Renilla luciferase control reporter. The results are expressed as fold increase compared to unstimulated controls.

**Cell transfection with siRNA**
HUVECs were seeded in 12-well plate at a density of 0.5-2×10^5 one day before transfection with HO-1 siRNA, Nrf2 siRNA or control siRNA. Transfection was performed according to the manufacturer’s instructions. Briefly, cells were incubated for 6 hrs in transfection medium supplemented with siRNA and transfection reagent. Hereafter, endothelial cell culture medium containing 20% FBS was added without removing the transfection solution and the cells were allowed to grow for an additional 24hrs. For each experiment the efficacy of siRNA was demonstrated by disappearance of the specific band in Western blot analysis.

**Statistical analysis**
Data are presented as mean ± SD for the indicated number of separate experiments. All analyses were based on more than three separate experiments. Differences between groups were determined by Student’s\(^ t \) test. A \( p \)-value of less than 0.05 was considered statistically significant.
RESULTS

Inhibition of adhesion molecules by CORM
CORM-3 inhibited TNF-α-mediated induction of VCAM-1 and E-selectin in all cell lines tested (n=8), while inhibition of ICAM-1 only occurred in 3 of these (figure 1A). Inhibition of adhesion molecule expression was mediated by the release of CO since a degassed solution of CORM-3 was ineffective (figure 1A, lower panel). To exclude that loss of adhesion molecule expression was due to proteolytic cleavage from the cell membrane, Western blot analysis with whole cell lysates was performed. As demonstrated for VCAM-1, induction by TNF-α was significantly attenuated by CORM-3 and was completely absent when endothelial cells were stimulated for 24 hrs in the presence of CORM-3 (figure 1B).
adhesion molecules is also depicted (dotted line). While limited TNF-a-induced expression of ICAM-1 occurred only in 3 out of 8 cell lines exposed to CORM-3 (upper panel), a weaker induction of VCAM-1 and E-selectin by TNF-a was consistently observed. Degassed CORM-3 solution revealed no effect (lower right panel). Characteristic flow cytometry profiles of 8 independent experiments are shown. **(B):** Time-dependent modulation of VCAM-1 expression by CORM-3. HUVEC were stimulated for different time periods with 50 ng/ml of TNF-α in the absence (-) or presence of 1 mM of CORM-3 (+). Hereafter, cell lysates were prepared and subjected to Western blotting. Membranes were incubated with anti-VCAM-1 antibody and hereafter reprobed with anti-GAPDH antibody to control for equal loading. A total of 3 independent experiments were performed, the result of a representative experiment is shown.

**CORM-3 acts through the NFκB pathway**

Since up-regulation of the adhesion molecules studied depends on activation of NFκB, we next assessed if CORM-3 interferes with this process. Within one hour of TNF-α stimulation NFκB-binding activity was detected in nuclear extracts of endothelial cells. However, neither at this time point nor after 2 hrs of TNF-α stimulation CORM-3 significantly influenced NFκB binding activity (figure 2A upper panel). In line with this, no influence of CORM-3 on the degradation of IκBα was observed (figure 2A, lower panel). In contrast to these early time points, NFκB binding activity was significantly reduced after 4 hrs of TNF-α stimulation in CORM-3-treated cells and gradually declined (figure 2B). This was reflected by a decrease in the nuclear expression of NFκB p65. Nuclear expression of NFκB p50, however, was not changed by CORM-3 (figure 2C).
Figure 2. Influence of CORM-3 on TNF-α-mediated NFκB activation. (A): Endothelial cells were stimulated for 1 or 2 hrs with 50 ng/ml of TNF-α in the absence (-) or presence of 1 mM of CORM-3 (+). Endothelial cells exposed to culture medium (M) only served as control. Nuclear extracts were prepared and used for EMSA as described in Materials and Methods (upper
panel). To assess the influence of CORM-3 on IκBα degradation, cells were stimulated for different time periods using the same concentrations of TNF-α and CORM-3. Cell lysates were analysed by Western blot for IκBα and GAPDH to demonstrate equal sample loading (lower panel). (B): Influence of CORM-3 on NFκB activation was also analysed at later time points. HUVEC were stimulated as in (A) and nuclear extracts were prepared at 0, 4, 12 and 24 hrs after TNF-α stimulation. Specificity of the shifted bands was demonstrated by addition of an excess of cold consensus (CC) or cold mutant (CM) oligonucleotides to the samples. (C): Nuclear expression of NFκB p65 and NFκB p50 in TNF-α-stimulated HUVEC. Cells were exposed to TNF-α as above in the presence (+) or absence (-) of 1 mM of CORM-3. At the time intervals indicated nuclear extracts were obtained and studied for expression of p65 and p50. To confirm equal loading of lanes, membranes were stained with anti-histone antibodies. In A, B and C, the results of 1 out of 4 individual experiments are shown.

The presence of TNF-α was required to maintain VCAM-1 and E-selectin expression on endothelial cells, since an almost complete down-regulation of these adhesion molecules occurred within 24 hrs after TNF-α removal. Although the expression of ICAM was also down-regulated when TNF-α was removed from the culture medium, it was still increased when compared to basal expression levels. Interestingly, when CORM-3 was added after 24 hrs to the TNF-α-containing culture medium, this also resulted in a complete loss of VCAM-1 and E-selectin expression (figure 3A). In contrast, the expression of ICAM-1 was not influenced. Hence, CORM-3 is able to down-regulate VCAM-1 and E-selectin even in the continued presence of TNF-α.

While TNF-α removal resulted in a rapid decrease in nuclear NFκB binding activity evident already 3 hrs after removal, NFκB binding activity was attenuated at early time-points after CORM-3 addition. Twelve hrs after addition of CORM-3, however, NFκB binding activity was also decreased in these cells (figure 3B). In line with these observations, steady-state VCAM-1 mRNA expression decreased much faster when TNF-α was removed compared to addition of CORM-3 (figure 3C). Nevertheless, in time response experiments we repeatedly observed that disappearance of VCAM-1 protein occurred much faster upon addition of CORM-3 than after TNF-α removal (figure 3D). CORM-3 did not influence stabilization of VCAM-1 mRNA since steady-state VCAM-1 mRNA decreased to a similar extent in actinomycin D-treated HUVEC both in the absence or presence of CORM-3 (data not shown).
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A

TNF-α removal  Addition of CORM-3

B

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Figure 3. Influence of CORM-3 or TNF-α removal on (A) surface expression of adhesion molecules, (B) NFκB activation, (C) expression of VCAM-1 mRNA and (D) expression of VCAM-1 protein. HUVEC were stimulated for 24 hrs with 50 ng/ml of TNF-α. Hereafter, the cells were washed to remove TNF-α or CORM-3 was added in the continued presence of TNF-α. Cells that were kept in the presence of TNF-α alone for similar time periods served as control. (A): Surface expression of ICAM-1, VCAM-1 and E-selectin after TNF-α stimulation (normal line), after addition of CORM-3 or removal of TNF-α (bold line). Basal expression of these adhesion molecules is also depicted (dotted line). (B): Nuclear extracts were prepared for electrophoretic mobility shift assays at 3, 6 and 12 hrs after TNF-α removal or after addition of CORM-3. Nuclear extracts were also prepared directly after 24 hrs of TNF-α stimulation (TNF-α) or from cells that were kept in the presence of TNF-α but not exposed to CORM-3 (no TNF-α removal). Cells that were kept in culture medium (M) were included to demonstrate TNF-α-mediated NFκB activation. (C): Steady-state VCAM-1 mRNA expression. HUVEC were stimulated with TNF-α for 24 hrs. Hereafter, cells were kept in the presence of TNF-α (open bars) or washed (filled bars) or CORM-3 was added to the cells (hatched bars). Subsequently, the cells were cultured for various time periods and total RNA was isolated. Results are expressed as fold increase compared to basal VCAM-1 mRNA expression. Significant differences were
found between TNF-α removal and CORM-3 addition at T=3 hrs, T=6 hrs (p<0.01 for both time points) and T=12 hrs (p<0.05). (D): HUVEC were treated as in C. At various time periods after TNF-α removal or CORM-3 addition, cell extracts were prepared. VCAM-1 protein was analysed by Western blotting. In A, B, C and D the results of representative experiments are shown. At least 3 different experiments were performed.

HO-1 is induced by CORM-3 in an Nrf2-dependent fashion
CORM-3 induced the expression of HO-1 in a time- and dose-dependent manner (figure 4A). While HO-1 was already slightly up-regulated at a concentration of 100 μM, this was much more pronounced when 1 mM was used (figure 4A, upper panel). At the latter concentration up-regulation of HO-1 protein was apparent already 3 hrs after addition of CORM-3 (figure 4A, middle panel). Degassing of the CORM containing solution did not completely abrogate up-regulation of HO-1, suggesting that this was partly mediated by the tricarbonylchlororuthenium(II) derivative itself (figure 4A, upper panel). PCR analysis revealed that up-regulation of HO-1 mRNA occurred at similar concentrations as was observed for HO-1 protein (figure 4A, right panel).

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Figure 4. Induction of HO-1 by CORM-3. (A): HUVEC were incubated for 24 hrs with different concentrations of CORM-3 or degassed CORM-3. Hereafter, cell lysates were studied by Western blot analysis for HO-1 and GAPDH expression (upper panel). In a second set of experiments, HUVEC were incubated for different time periods with 1 mM of CORM-3. The expression of HO-1 was assessed by Western blotting (middle panel). To demonstrate that HO-1 expression was regulated at the transcriptional level, total RNA was isolated from HUVEC that were incubated with different concentrations of CORM-3. RT-PCR for HO-1 and GAPDH was subsequently performed (right panel). (B): Nuclear translocation of Nrf2 was assessed by Western blot analysis of nuclear protein isolated from HUVEC that were stimulated for different time periods with 1 mM of CORM-3. (C): HUVEC were transfected with reporter constructs containing either the ARE consensus sequence of the HO-1 promoter (HO-ARE) or with a reporter construct containing 3 hypoxia responsive elements (3HRE). In each case cotransfection was performed with the ubiquitin-dependent Renilla luciferase reporter. Luciferase activities were normalized for luciferase activity generated by the Renilla luciferase control reporter. The results are expressed as fold increase compared to unstimulated controls. Significance was only found for the HO-ARE reporter, p<0.01 untreated vs. CORM-3 treated. In A, B and C the results of representative experiments are shown. At least 4 different experiments were performed.

Since these data suggested that CORM-3-mediated HO-1 expression was transcriptionally regulated, we next investigated the involvement of three transcription factors that have been shown to influence HO-1 transcription. Neither NFκB activation nor HIF-1α accumulation occurred after CORM treatment (data not shown). In contrast, nuclear translocation of Nrf2 was clearly evident 3 hrs after CORM-3 treatment (figure 4B). CORM-3 did not induce expression of a hypoxia-responsive element-dependent luciferase reporter construct, while this was the case in cells transfected with an Nrf2 reporter (figure 4C).
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*CORM-3 induced HO-1 expression does not contribute to inhibition of VCAM-1 expression*

Since CORM-3 induces HO-1 expression, this might further increase intracellular CO concentrations and hence may perpetuate down-regulation of adhesion molecules in the presence of TNF-α. To address this issue, we first stimulated endothelial cells with 1 mM of CORM-3 to induce HO-1 followed by a challenge with TNF-α. Despite an increase in HO-1 expression, induction of VCAM-1 by TNF-α was not influenced in these cells (figure 5A). To formally exclude a role for HO-1 in down-regulation of VCAM-1 expression we also used a siRNA approach to deplete HO-1 or Nrf2. The expression of both HO-1 and Nrf2 was almost completely blocked by this approach (figure 5B). Nevertheless, neither TNF-α-induced VCAM-1 expression, nor inhibition hereof by CORM-3 was significantly changed in HO-1- and Nrf2-depleted endothelial cells, respectively (figure 5C upper panel). Compatible with the pivotal role of Nrf2 in HO-1 induction, Nrf2 siRNA treatment abolished the up-regulation of HO-1 by CORM-3 (figure 5C, lower panel).
Figure 5. Induction of HO-1 is not involved in CORM-3 mediated down-regulation of VCAM-1 expression. (A): HUVECs were incubated for 24 hrs with CORM-3 (bold line) or left untreated (normal line). Hereafter, the cells were washed and stimulated for 24 hrs with 50 ng/ml of TNF-α. Basal expression of VCAM-1 is depicted as dotted histogram. (B): HUVECs were transfected with Nrf2, HO-1 or control siRNA. Two days after transfection the expression of HO-1 and Nrf2 was assessed by Western blot analysis. (C): Transfected cells were subsequently stimulated for 24 hrs with TNF-α (50ng/ml) or TNF-α plus CORM-3 (1 mM). The expression of VCAM-1 (upper panel) and HO-1 (lower panel) was assessed by Western blotting. The results of representative experiments are shown. At least 3 different experiments were performed.

DISCUSSION

Based on current evidence, the HO-1 pathway is of undisputable importance for the control of inflammation. Overexpression of HO-1 modulates pro-inflammatory processes in a number of cells including macrophages and endothelial cells, yet, the mechanisms by which HO-1 exerts its salutary effects are not completely delineated. In the present study we investigated the role of HO-1 in perpetuation of the anti-inflammatory effect of CORM-3. The main findings of this study were the following. First, CORM-3 consistently inhibited the up-regulation of VCAM-1 and E-selectin after TNF-α stimulation, but the influence on ICAM-1 was only marginal. Second, initial
activation of the NFκB pathway by TNF-α is not influenced by CORM-3. Instead, CORM-3 deactivates sustained activation of this pathway. Third, HO-1 is induced by CORM-3 in an Nrf2 dependent fashion. Fourth, induction of HO-1 does not contribute to down-regulation of VCAM-1 and E-selectin expression by CORM-3. There is some controversy on the main mediators through which HO-1 acts as a modulator of inflammation. Clearly, this might be related to the different cell types, i.e. macrophages 11, 36 or endothelial cells 12, 37, that were used in different studies. While in vitro 11, 38 and in vivo 39, 40 studies have demonstrated that CO acts as an anti-inflammatory mediator, more recent studies have questioned this paradigm by showing that bilirubin and/or Fe²⁺ chelation, both of which are increased as a consequence of HO-1 activity, play a more important role in down-regulation of adhesion molecules under inflammatory conditions 12, 13. Our own findings do not corroborate with the latter studies, as we demonstrate that CORM-3 has the propensity to inhibit VCAM-1 and E-selectin expression through the release of CO. Still, it can be argued that while CORM-3 strongly up-regulates HO-1, down-regulation of these adhesion molecules could occur though generation of bilirubin and ferritin-mediated Fe²⁺ chelation. Two lines of evidence are, however, against this assumption. First, pre-treatment of endothelial cells with CORM-3 did not largely influence VCAM-1 and E-selectin expression upon a subsequent challenge with TNF-α in the absence of CORM-3. Second, when induction of HO-1 was prevented by means of siRNA CORM-3 was still effective.

It must be emphasized that this study does not argue against the important role of the HO-1 system in control of inflammation, as it inherently demonstrates its anti-inflammatory potential through generation of CO, but our data do suggest that up-regulation of HO-1 per se not necessarily exerts anti-inflammatory properties. This notion is compatible with in vitro studies of Foresti et al. using cardiomyocytes 41. They showed that although hypoxia increased the expression of HO-1, cardiomyocytes were still vulnerable to damage during reoxygenation unless, hemin was added during the hypoxic phase. Therefore, substrate availability seems to be an important factor that influences the anti-inflammatory effect of HO-1. Production of cellular heme is a multistep process that includes mitochondrial and cytoplasmic elements. The rate-limiting step is the production of δ-aminolevulinic acid from succinyl CoA and glycine which is catalyzed by δ-aminolevulinic synthase (ALAS) 42, 43. ALAS is tightly regulated, in a negative feedback manner by heme, and positively by an increase in intracellular Ca²⁺ or protein kinase C activation 44. Taking into account that CO has a high affinity for Fe²⁺ and acts as ligand to complete the coordination shell of this atom, it is likely that CO-Fe complexes will form 45, 46. Moreover, CORM-3 behaved similar as cells stably transfected with HO-1 in regard to Fe homeostasis, as demonstrated by Watts et al. 47. Hence, if Fe²⁺ chelation can inhibit TNF-α mediated expression of adhesion molecules, not surprisingly, similar effects can be expected by the addition of CORM-3.
There is consistency amongst the in vitro studies that the major adhesion molecules that are affected by HO-1 are VCAM-1 and E-selectin. Nevertheless, in vivo studies also demonstrate down-regulation of ICAM-1, as a consequence of HO-1 expression. In addition, both basal and TNF-α induced expression of ICAM-1 are increased in Hmox1−/− compared to Hmox1+/+ endothelial cells. Our findings are in agreement with these studies in that VCAM-1 and E-selectin were consistently down-regulated by CORM-3, while ICAM-1 was marginally influenced and only in three out of eight HUVEC lines tested. In comparison, to VCAM-1 and E-selectin, ICAM-1 also behaved differently with respect to TNF-α removal. The expression of the former two adhesion molecules was completely lost 24 hrs after TNF-α removal but ICAM-1 expression was still increased at this time point.

The involvement of the NFκB pathway as a target by which HO-1 modulates the expression of adhesion molecules seems consistent throughout the different studies. If the NFκB pathway plays a pivotal role in HO-1-mediated modulation of adhesion molecules, then the question that arises is why CORM-3 not consistently affects ICAM-1 expression, although the role of NFκB activation in the regulation of ICAM-1 has been clearly demonstrated. Our finding that TNF-α removal results in deactivation of the NFκB pathway indicates the importance of sustained NFκB activation for maintaining high levels of all three adhesion molecules. Yet, deactivation of NFκB through the addition of CORM-3 to TNF-α-stimulated endothelial cells occurred much slower and did not affect ICAM-1 expression. It thus seems that, although deactivation of NFκB, and hence reduction in mRNA transcription, have a major effect on the expression of VCAM-1 and E-selectin, post-translational mechanisms might contribute to down-regulation of these molecules by CORM-3. In line with this, it was found that down-regulation in VCAM-1 protein by TNF-α removal was much slower compared to addition of CORM-3, although NFκB deactivation occurred much faster under the former condition.

Induction of HO-1 by CORM-3 occurred in an Nrf2-dependent manner. This is compatible with previous findings in hepatoma cells. Although HIF1α stabilization by CO has been described in macrophages, it was not involved in the induction of HO-1 in our study using endothelial cells. A number of molecules, mostly anti-oxidants, have the propensity to down-regulate adhesion molecules. HO-1 might be a common denominator for the anti-inflammatory effects mediated by these anti-oxidants as most of these affect the keap1/Nrf2 pathway and result in an increased HO-1 expression.

Our study however showed that neither Nrf2 nor HO-1 was involved in down-regulation of VCAM-1 expression by CORM-3.

In conclusion, our study demonstrates that CORM-3 can down-regulate VCAM-1 and E-selectin even in the continued presence of TNF-α. Although HO-1 was induced by CORM-3, it did not influence the expression of adhesion molecules. We speculate that the down-regulation of adhesion molecules is mediated by deactivation of the NFκB
pathway, concomitantly with an increased turn-over of these proteins. How CORM-3 influences protein turn-over is at present unclear. Our data also suggest that CORM-3 is of potential therapeutic importance in sepsis, ischemia/reperfusion injury and transplant rejection as it might resolve ongoing inflammation.
REFERENCES

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CHAPTER 4

MOLECULAR MECHANISMS INVOLVED IN THE CO-MEDIATED DOWN-REGULATION OF VCAM-1 ON ENDOTHELIAL CELLS IDENTIFIED BY GENE EXPRESSION PROFILING

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*Work in progress*
ABSTRACT

Experimental evidence has demonstrated that carbon monoxide (CO) exerts anti-inflammatory effects under a number of pathological conditions. We have previously shown that the effect of CO is not completely mediated via deactivation of the NFκB pathway. In the present study we hypothesize that CO can modulate a variety of pro-inflammatory pathways, thereby making CO very efficient to down-regulate inflammation. To this end, we used genome wide gene expression profiling, hierarchical analysis of significance of particular pathways and studied the relevance of these pathways for the anti-inflammatory effect.

When human umbilical vein endothelial cells (HUVEC) were stimulated with 1 mM of CO-releasing molecules-3 (CORM-3), the most significant changes were found in the proteasome and porphyrine pathways. In the former pathway 11 out 29 genes were changed in expression \((P=7.5E-14)\), all encoded for proteasome subunits. In the porphyrin pathway 5 out of 22 genes \((P=1.06E-5)\) were significantly changed and included: ALAS-1, FECH, HMOX1, FTH1 and BLVRB. In TNF-α stimulated HUVEC, CORM-3 inhibited the expression of a variety of inflammatory genes, e.g. chemokines/cytokines and adhesion molecules. Although the proteasomal activity was significantly increased by CORM-3, neither lactacystein nor MG132 were able to abolish down-regulation of VCAM-1 by CORM-3. The MAPK p42/p44 was activated by CORM-3, while p38 was inhibited. No influence on JNK was observed. Similar as found for CORM-3, inhibition of p38 resulted in down-regulation of VCAM-1 expression. Inhibition of p42/p44 could not overcome down-regulation of VCAM-1 by CORM-3.

In conclusion, our study demonstrates that CORM-3 inhibits the expression of a variety of pro-inflammatory molecules. At the transcriptional level this can be achieved through the inhibitory effect on the NFκB and p38 pathways. Although CORM-3 might also modulate the expression of these genes post-transcriptional, further evidence is required to demonstrate the functional role of activation of the proteasome pathway in this regard.
INTRODUCTION

The vascular endothelium is critically involved in the regulation of inflammation, including the coordination of leukocyte trafficking to specific tissues \(^1\). Upon exposure of endothelial cells (EC) to inflammatory mediators, such as lipopolysaccharide (LPS) or tumor necrosis factor (TNF)-\(\alpha\), EC modify their phenotype and become activated, promoting adhesion, activation and transmigration of leukocytes \(^1\). This is primarily accomplished by the up-regulation of a sequence of pro-inflammatory genes, including the adhesion molecules VCAM-1 \(^2\), ICAM-1 \(^3\) and E-Selectin \(^4\). Not surprisingly, these genes have been considered as potential targets for therapeutic intervention for inflammatory diseases \(^5\)–\(^7\).

Septic shock or sepsis is a systematic inflammatory response to infection, and is the leading cause of mortality for patients on the intensive care unit. So far, therapeutic approaches have failed to reduce significantly sepsis related mortality. Pro-inflammatory cytokines such as TNF-\(\alpha\) and interleukins (IL-1\(\beta\), IL-6, IL-8) released from macrophages exert direct effects on the organs or activate a multitude of secondary inflammatory mediators that in turn exert overlapping effects on endothelial cell function, vascular function, coagulation and hemodynamics. Although anti-inflammatory mediators such as IL-10 might limit the inflammatory process, they are also potentially harmful because of their involvement in so-called immune paralysis \(^8\).

Temporal spatial regulation of the inflammatory response is therefore of utmost importance to eliminate bacterial infectious and preventing mononuclear cell infiltration in organs through systemic immune activation.

In addition to its role in oxidant-induced injury \(^9\), the heme oxygenase system (HO) is a genuine system through which inflammatory processes are controlled \(^10\)–\(^13\). There are several genes encoding for distinct HO isoenzymes \(^14\)–\(^15\). The inducible HO-1 isoenzyme however, appears to be particularly important as an anti-inflammatory mediator \(^10\)–\(^12\), \(^16\). HOs are the rate-limiting enzymes in degradation of heme into carbon monoxide (CO), Fe\(^{2+}\) and biliverdin, the latter being subsequently converted to bilirubin \(^17\), \(^18\). It is believed that the anti-inflammatory properties of HO-1 are at large mediated by iron release and biliverdin \(^11\), \(^12\). Although CO, generated by HO-1 mediated heme degradation, most likely does not result in down-regulation of adhesion molecules \(^11\), \(^12\), we have recently demonstrated that at supra-physiological concentration CO is capable to down-regulate VCAM-1 and E-selectin, even in the continued presence of TNF-\(\alpha\) (see chapter 3). Therefore CO application might be of potential therapeutic relevance to down-regulate tissue inflammation. CO can be applied through inhalation however, the major disadvantage of this modality is that it directly competes with oxygen uptake in the lung. Carbon monoxide–releasing molecules (CORMs) have the propensity to liberate CO in a controllable fashion when dissolved in physiological solution \(^19\)–\(^23\). In contrast to inhaled CO, CORMs appear to deliver CO directly to the tissues without
significant formation of CO-Hb. In particular, CORM-3 (tricarbonylchloro(glycinato) ruthenium(II)) and CORM-A1 (sodium boranocarbonate), both of which are fully water-soluble and allow intravenous administration, rapidly liberate CO. This offers the possibility of applying higher non-toxic concentrations during inflammatory conditions.

In the present study, we hypothesized that the anti-inflammatory effect of CORM-3 is not restricted to its influence on the NFκB pathway. The hypothesis was tested by means of genome wide gene expression profiling, pathway analysis and by testing the relevance of significant changes in a particular pathway for the anti-inflammatory effect mediated by CORM-3.

MATERIALS AND METHODS

Synthesis of tricarbonylchloro(glycinato) ruthenium(II)
Tricarbonylchloro(glycinato) ruthenium(II) ([Ru(CO)3Cl(glycinato)]+) was synthesized starting from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ([Ru(CO)3Cl]2) (Sigma, St. Louis, MO) as previously described. Briefly, [Ru(CO)3Cl]2 (0.5 g) and glycine (0.151 g) (Sigma, St. Louis, MO) were placed under nitrogen in a round-bottomed flask. Methanol (291 ml) and sodium ethoxide (0.132 g) (Sigma, St. Louis, MO) were added and the reaction was allowed to continue under stirring for 18 hrs at room temperature. The solvent was then removed under pressure and the yellow residue redissolved in tetrahydrofuran (THF) (Merck, Darmstadt, Germany). The yellow solution was evaporated down to give a pale yellow solid (yield 92-96%) and was stored in closed vials at -20°C. For each experiment CORM-3 was dissolved freshly in PBS (GIBCO, Invitrogen, NY).

Cell culture
Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as described previously. The cells were grown in basal endothelial medium (Promocell, Heidelberg, Germany) supplemented with 10% FBS (PAA laboratories GmbH, Pasching, Austria) and essential growth factors until they formed a confluent monolayer. Cells were stimulated with 50ng/ml (1000×) of TNF-α in the presence or absence of 1 mM of CORM-3. This concentration was chosen on the basis of dose response studies and was not toxic to HUVEC. Unstimulated cells served as control.

RNA isolation, cRNA and array hybridization
Sample preparation and processing was performed according to the Affymetrix GeneChip Expression Analysis Manual (http://www.Affymetrix.com). Briefly, endothelial cell monolayers were stimulated with 50ng/ml (1000×) of TNF-α or left
unstimulated in the presence or absence of 1 mM of CORM-3 or left in normal medium for 24 h. Total RNA was isolated from these cultures using Trizol®-Reagent (Invitrogen, Carlsbad, CA). Hereafter DNase treatment was carried out, using RNase free DNase I (Ambion, Woodward, Austin, TX, USA). RNA concentration and quality were assessed by RNA 6000 nano assays on a Bioanalyzer 2100 system (Agilent, Waldbronn, Germany). Five μg of RNA was converted into cDNA using T7-(dT) 24 primers and the SuperScript Choice system for cDNA synthesis (Life Technologies, Inc., Rockville, MD, USA). Biotinlabelled cRNA was prepared by \textit{in vitro} transcription using the BioArray high yield RNA transcript labelling kit (Enzo Diagnostics, Farmingdale, NY, USA). The resulting cRNA was purified, fragmented and hybridized to U133A gene chips (Affymetrix, Santa Clara, CA, USA). After hybridization the chips were stained with streptavidin–phycoerythrin (MoBiTec, Goettingen, Germany) and analysed on a GeneArray scanner (Hewlett Packard Corporation, Palo Alto, CA, USA).

\textbf{Microarray processing and statistical analysis}

Gene expression profiling was performed using arrays of human U133A -type from Affymetrix. A Custom CDF Version 11 with Entrez based gene definitions was used to annotate the arrays. The Raw fluorescence intensity values were normalized applying quantile normalization. Differential gene expression was analysed based on loglinear mixed model ANOVA \textsuperscript{27,28}, using a commercial software package SAS JMP7 Genomics, version 3.1, from SAS (SAS Institute, Cary, NC, USA). A false positive rate of $\alpha=0.05$ with Holm correction was taken as the level of significance. The overrepresentation analysis (ORA) is a microarray data analysis that uses predefined gene sets to identify a significant overrepresentation of genes in data sets \textsuperscript{29,30}. Pathways belonging to various cell functions such as cell cycle or apoptosis were obtained from public external databases (KEGG, http://www.genome.jp/kegg/). A Fisher’s exact test was performed to detect the significantly regulated pathways.

\textbf{Preparation of cytosolic lysates}

HUVECs were stimulated for different time periods with 50ng/ml (1000\textsuperscript{a}) TNF-α in the presence or absence of 1mM CORM-3. Cells were lysed by addition of 50 mL of ice-cold distilled water. The lysed fraction was shock-frozen and defrosted several times, and then centrifuged at 4°C for 30 min at 40,000g to remove particulate and membranous material. The supernatants (cytosolic lysates) were freshly used for further analysis.

\textbf{Ubiquitin Immunoblotting analyses}

Immunoblotting to ubiquitin was performed as described in detail previously \textsuperscript{31}. In brief, lysate proteins (20 μg per lane) were separated by SDS-PAGE. Membranes were probed for ubiquitin with rabbit anti-ubiquitin antiserum (1:500, v/v; Sigma, St. Louis, MO) and
a corresponding horseradish peroxidase-labeled secondary antirabbit antibody (1:20,000, v/v; Jackson ImmunoResearch, Baltimore, MD). Standard was electrophoresed on each gel containing 100 ng of ubiquitin for quantification of free and conjugated ubiquitin. Enhanced chemiluminescence detection (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology, Rockford, IL) provided visualization of endogenous ubiquitin in cell lysates. Densitometric analyses and quantification was accomplished by imaging software (Image-Master TotalLab 1.11; Amersham Biosource). Protein loading control was performed by reprobing with a rabbit anti-mitogen-activated protein kinase (MAPK) p44/42 antibody (Cell Signaling Technology, Beverly, MA) and additional Ponceau S dye staining.

Ubiquitin protein ligation rates
The total ubiquitylation rate of cytosolic proteins, as a result of the total ubiquitin-protein ligase activity \(^{31}\), was measured as incorporation of biotinylated ubiquitin (ubiquitinb) into the sum of cytosolic proteins. Incubation mixtures contained 25 mM Tris/HCl, 5 mM MgCl\(_2\), 10 mM NaCl, 1 mM dithiothreitol, 1 mM ATP, 200 mg/mL ubiquitinb (Biomol, Plymouth Meeting, PA). Mixtures were incubated for 3 min time intervals at 37°C and separated by SDS-PAGE. On each gel, a lane containing 100 ng of ubiquitinb was electrophoresed, as a standard for quantification. Immunoblotting and densitometric analysis were performed using a monoclonal antibiotin antibody conjugated to horseradish peroxidase (New England Biolabs, Beverly, MA). The ubiquitylation rate is given in katal (kat). 1 kat was defined as 1 mol of ubiquitinb incorporated into cytosolic proteins with a molecular mass of greater than 5.5 kDa per s. For activity calculations from progress curves, the initial data points plus the origin were analyzed by linear regression analysis.

Proteasome activity assay
Fluorogenic substrates (Calbiochem/Merck, Darmstadt, Germany), carbobenzoxy-leu-leu-glutamic-acid-α-7-amido-4-methylcoumarin (Z-LLE-AMC), succinyl-leu-leu-val-tyr-AMC (Suc-LLVY-AMC) and carbobenzoxy-ala-arg-arg-AMC (Z-ARR-AMC) were used to determine main hydrolytic activities (LLE – caspase-like, LLVY – chymotrypsin, ARR – trypsin-like). Reactions were performed as microtiter plate assay (Greiner Bio-one, Frickenhausen, Germany) in 50 µl/well volume containing 15 µg protein extract and assay buffer (50 mM TrisHCl, 1 mM DTT, 5 mM ATP, 5 mM MgCl\(_2\); pH 8.0). To differentiate peptidase- and proteasome-specific activities inhibition by Epoxomicin [10 µM] and Ada-(Ahx)\(_3\)-(Leu)\(_3\)-vinyl sulphone [10 µM] (Biromol International LP, Plymouth Meeting, PA) were included. Towards addition of substrate Z-LLE-AMC [100µM], Suc-LLVY-AMC [100µM] respectively Z-ARR-AMC [200 µM] fluorescence (excitation/emission - 360/465 nm) was detected in time intervals of 15 min for 1h at
30°C (Genios, Tecan, Crailsheim, Germany). Proteolytic activity was calculated as pkat/mg total protein from the mean of three identical tests.

**FACS analysis**

HUVECs were pre-treated with the p42/44-inhibitor U0126 (Alexis Biochemicals, Lôrrach, Germany) for 2h or left untreated. Hereafter, cells were stimulated with 50ng/ml (1000") TNF-α in the presence or absence of 1mM CORM-3. HUVECs cultured in endothelial medium were used as control. FACS analysis was performed with 2x10⁶ cells using the FITC-conjugated monoclonal anti-human VCAM-1 (BBIG-V3) antibody (R&D System, Minneapolis, MN). Antibodies were added for 40 min at 4°C followed by extensive washing with PBS. FACS analysis was performed on a FACScalibur equipped with the CELLQuest software. The data were analysed by Windows Multiple Document Interface (WinMDI) software (Version 2.8).

**Western blot analysis**

HUVECs were pre-treated with or without the proteasome inhibitors lactacystein (Merck Biosciences, Darmstadt, Germany) and MG132 (Merck Biosciences, Darmstadt, Germany), or with or without the p38-inhibitor SB203580 (Alexis Biochemicals, Lôrrach, Germany) for 2h. Hereafter, cells were stimulated with 50ng/ml (1000") TNF-α in the presence or absence of 1mM CORM-3. HUVECs cultured in endothelial medium were used as control. After this, cells were harvested and lysed in 50 µl lysis buffer containing 10mM Tris-base, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1µM DTT (all from Sigma, St. Louis, MO) and proteinase inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). In some experiments, nuclear proteins were isolated as previously described 32,33. Protein concentrations were assessed using Coomassie reagents (Pierce, Rockford, IL). SDS-PAGE and Western blotting were performed essentially similar as described 26. Briefly, samples were resolved on 10 % SDS-PAGE (Sigma, St. Louis, MO ) and transferred onto PVDF filters by semi-dry blotting. The filters were incubated with 5% non-fat dry milk powder in PBS for 1 hour at room temperature to block unspecific background staining and hereafter incubated overnight at 4°C with specific polyclonal antibodies, depending on the experiment that was performed. The following antibodies were used: polyclonal goat anti-VCAM-1 antibody, polyclonal rabbit anti-ERK (p42/44) antibody, monoclonal mouse anti-phospho-ERK antibody (all antibodies, including horseradish peroxidase (HRP) conjugates from Santa Cruz Biotechnology, Heidelberg, Germany), polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada), polyclonal rabbit anti-p38 antibody, monoclonal rabbit anti-phospho-p38 antibody, monoclonal rabbit anti-JNK antibody and polyclonal rabbit anti-phospho-JNK antibody (all antibodies from Cell Signaling/New England Biolabs GmbH, Frankfurt, Germany) and anti-GAPDH antibodies (Abcam, Cambridge, UK). After extensive washing in PBS/Tween/5 % non fat
dry milk powder, the filters were incubated 60 minutes with appropriate horseradish peroxidase-conjugated polyclonal IgG, followed by 3 times washing in PBS/Tween. Visualization of immunoreactive bands was performed by chemiluminescence reagent (PerkinElmer LAS Inc., Boston, MA) according to the manufacturer’s instructions. The filters were re-probed with monoclonal anti-Histone H1 antibody or monoclonal anti-GAPDH antibody to demonstrate equal loading.

Quantitative TaqMan PCR
Total RNA was isolated from confluent endothelial cell monolayers using Trizol®-Reagent. Thereafter, DNase treatment was carried out, using RNase free DNase I (Ambion, Darmstadt, Germany). RNA concentration and quality were assessed by RNA 6000 Nano assays on a Bioanalyzer 2100 system (Agilent, Boeblingen, Germany). 500 ng of total RNA was reverse-transcribed into cDNA using the 1st Strand cDNA Synthesis Kit. cDNA was diluted in 20 μl DEPC-treated water and stored at −20°C until use. Quantitative real-time RT-PCR was performed on the ABI-Prism 7700 sequence detection system with the TaqMan universal PCR master mix No AmpErase UNG (part no. 4324018). Taqman probes for HO-1 (part No. Hs01110250_m1), Ferritin, heavy polypeptide 1 (part No. Hs01000477_g1), Biliverdin reductase B (Flavin reductase) (part No. Hs01106480_m1) and GAPDH (part No. Hs00266705_g1). Primers and all reagents were purchased from (Applied Biosystems, Darmstadt, Germany). Samples were run under the following conditions: initial denaturation for 10 minutes at 95°C followed by 40 cycles of 15 s at 95°C and 1 minute at 60°C. The levels of gene expression in each sample were determined with the comparative cycle threshold method. PCR efficiency was assessed from the slopes of the standard curves and was found to be between 90% and 100%. Linearity of the assay could be demonstrated by serial dilution of all standards and cDNA. All samples were normalized for an equal expression of GAPDH. Each experiment was repeated 3 times with similar results.

Cell culture for retroviral infection
Primary HUVEC were purchased from Clonetics (via Lonza). Cells were used between passages 3 and 5 in all experiments and cultured in a 1:2 mixture of endothelial growth medium (Lonza) and M199 medium (PAA) supplemented with 6.7% FBS, gentamicin, amphotericin, and Liquemien N5000 (Roche).

Retroviral Vectors and Stable Producer Cell Lines
The human HO-1 cDNA encoding the full open reading frame was amplified by PCR and cloned into the Ncol/BamHI site of pBabe. The primers used for amplification contained at the 5’ and 3’ flanking sites the Ncol or BamHI sequence. After amplification, the product was TA cloned according to the manufacturer’s instruction (Roche Applied Science, Mannheim Germany). The HO-1 containing vector was herafter digested with
NcoI and BamHI and the HO-fragment was isolated from the agarose gel. Fragment and
pBabe vector were ligated with the Rapid DNA-Ligation kit (Roche Applied Science,
Mannheim Germany).

Retroviral Infection of HUVEC with supernatant from ΦNX Producer Cells
Retroviral infections of HUVEC were essentially performed as described earlier.  
Briefly, ΦNX producer cells were stably transfected with 7 μg pBabe puro HO-1, 16 μg
GAG-POL and 5 μg VSVG using Lipofectamine 2000 (Invitrogen Life Technologies).
After a selection period of 14 days in DMEM medium containing 2 μg/ml Puromycin the
obtained polynucle HO-1 ΦNX producer cell line was seeded at a density of 35000
cells/cm² (e.g. 2x10⁶/10 cm dish) 3 days before transfection. Two days before infection,
HUVEC were seeded in 10cm dishes at a density of 3 x 10⁵ cells/well in a 1:2 mixture of
endothelial growth medium (EGM) and M199. Twenty-four h before infection, the
culture medium of the producer cells was changed from Dulbecco’s modified Eagle’s
medium to the EGM/M199 mixture. At the day of infection, ΦNX cell supernatant was
obtained, filtered through a 0.8-μm filter, and 5 μg/ml polybrene (Sigma) was added to
the filtrate. Thereafter, EGM was removed from endothelial cell cultures and replaced by
the ΦNX cell supernatant containing the retrovirus in three consecutive rounds. After
selection for puromycin resistance conferred by a gene expressed from the retroviral
backbone using 2 μg/ml puromycin, stably transduced HUVEC were incubated in
conventional endothelial growth medium.

RESULTS

CORM-3 mediated changes in gene expression profile of HUVEC
We used a genome wide gene expression profiling approach to study the influence of
CORM-3 stimulation on gene expression in HUVEC. The most significant changes in the
number of regulated genes were found in the proteasome pathway (11 out of 29, P=7.5E-14)
and the porphyrin pathway (5 out of 22, P=1.06E-5). All genes that were significantly
up-regulated in the former pathway encoded for proteasome sub-units (table 1), while
significant up-regulation of gene expression in the later pathway included genes
encoding for 5-Aminolaevulenic acid synthase-1 (ALAS-1), Ferrochelatase (FECH),
Heme oxygenase-1 (HMox1), Ferritin heavy chain 1 (FTH1) and biliverdin beta
reductase (BLVRB) (table 1). Down-regulation in gene expression by CORM-3 was not
observed in both pathways.
**MOLECULAR MECHANISMS INVOLVED IN CORM EFFECT**

Table 1. CORM-3 mediated changes in gene expression profile of HUVEC in the proteasome pathway (A) and the porphyrine pathway (B). (Change are expressed as increase in expression compared to Medium)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Medium vs CORM</th>
<th>(P)-Log10</th>
<th>change (Log2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteasome pathway</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSMA4</td>
<td>7.95</td>
<td>0.62</td>
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**Influence of CORM-3 on TNF-α induced expression of pro-inflammatory genes**

To assess to what extent CORM-3 was able to inhibit TNF-α mediated expression of pro-inflammatory genes, we compared change in gene expression between unstimulated vs TNF-α stimulated HUVEC, and unstimulated vs TNF-α plus CORM-3 stimulated HUVEC (table 2). Because of the relative small number of replica (n=3/group) and because principal component analysis (PCA) revealed heterogeneity amongst the different groups (fig 1), a stringent cut-off for significance was chosen, i.e. $P(-\text{Log10}) \geq 6$. We focused on genes that are associated with inflammation, e.g. signaling molecules, transcription factors, adhesion molecules, apoptosis and cyto/chemokines. TNF-α stimulation resulted in the up-regulation of a variety of signaling molecules, e.g. MAP3K, NFKB and DUSP related genes. With exception of MAP3K8 and DUSP4, CORM-3 did not influence gene expression in the majority of TNF-α regulated signaling molecules. In contrast, TNF-α regulated genes encoding for adhesion molecules (VCAM-1, ICAM-1 E-selectin, Integrin αV), colony-stimulating factors (GM-CSF (CSF2) and G-CSF (CSF3)) and CXCL or CX3CL chemokines (CXCL1, 2, 5, 11 and CX3CL1) were strongly suppressed when HUVEC were stimulated with TNF-α in the presence of CORM-3 (table 2).
Table 2. CORM-3 mediated changes in gene expression profile of HUVEC in inflammatory genes. *(Change are expressed as increase in expression compared to Medium)*

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<th>Genes</th>
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Figure 1. Principal component analysis showing distinct clustering of the groups: untreated (green), CORM-3 treated (red), TNF-α treated (blue) and TNF-α + CORM-3 treated (brown). Each dot represents the data set of 1 individual HUVEC culture.

*CORM-3 activates the ubiquitin proteasome system (UPS)*

We next investigated if up-regulation of proteasome sub-units by CORM-3 resulted in an increased proteasomal activity. As demonstrated in figure 2A two of the three known proteasome protease activities (caspase (C)- and trypsin (T)-like activity) were significantly increased by CORM-3 treatment (p<0.05). Although in the affymetrix analysis no differences were found in the expression ubiquitin ligases, we did observe an increase in ubiquitin conjugates, but not in free ubiquitin, in cell lysates obtained from CORM-3 treated cells (Fig. 2B and C).
Figure 2. Quantification of proteasome activity and endogenous ubiquitin in cell lysates. To investigate whether CORM-3 influences proteasome activity and endogenous ubiquitin levels, HUVEC were exposed to 1mM CORM-3 for 4 hours or left untreated, and cytosolic lysates were prepared. (A) The proteasome activity for each of the main proteasomal protease activities including caspase (C)-, chymotrypsin (CT)- and trypsin (T)-like activity was measured. The
results are expressed as mean simple linear regression ± SEM; *p<0.05 was considered to be statistically significant. *Significant differences between CORM-3 treated HUVEC and untreated HUVEC. (B) Cytosolic levels of free and conjugated ubiquitin were detected by immunoblotting. An exemplary immunoblotting experiment with cytosolic lysates from untreated (Lane 2) and CORM-3-treated (Lane 3) HUVEC is shown. The lane MM displays the protein marker. (C) Densitometric analysis of conjugated and free ubiquitin. In A and C the results of representative experiments are shown. A total number of three experiments were performed.

The UPS is not involved in CORM-3 mediated down-regulation of VCAM-1
Addition of CORM-3 to TNF-α stimulated HUVEC dose dependently inhibited VCAM-1 protein expression (Fig 3A). To study if proteasomal degradation of VCAM-1 was involved in this process, we stimulated HUVEC for 24 hrs with TNF-α to induce VCAM-1 and subsequently added CORM-3 to the cells in the presence or absence of lactacystin (20 μM) or MG132 (10 μM). As shown in figure 3B, neither lactacystin nor MG132 were able to rescue VCAM-1 expression.

Figure 3. CORM-3 mediated down-regulation of VCAM-1 expression not influenced by proteasome inhibition. (A) To investigate whether CORM-3 down-regulates VCAM-1 expression in a dose-dependent manner, HUVEC were exposed to TNF-α and different dosages of CORM-3. Proteins were isolated and Western blotting with antibodies against VCAM-1 was performed. (B) To assess whether proteasomal inhibiton influences VCAM-1 expression, HUVEC were pretreated either with lactacystin, MG132 or left untreated, and exposed to TNF-α and CORM-3. Proteins were isolated and Western blotting with antibodies against VCAM-1 was performed. In A and B the results of representative experiments are shown. A total number of three experiments were performed.
**HO-1 overexpression inhibits TNF-α mediated VCAM-1 expression**

Although affymetrix analysis already revealed a significant up-regulation of genes related to the porphyrin pathway, we performed independent experiments to confirm that HO-1, ferritin and biliverdin-reductase were significantly up-regulated by CORM-3 (Fig. 4). Because quantitative PCR confirmed the affymetrix findings, we next tested the role of HO-1 in down-regulation of VCAM-1. To this end, HO-1 was overexpressed in HUVEC using retroviral transfection. As control, cells were transfected with the empty pBABE vector. Overexpression of HO-1 clearly inhibited VCAM-1 when cells were stimulated with TNF-α (Fig 5).

![Graph showing relative quantity of HO-1, Ferritin, and Biliverdin-Reductase](image)

**Figure 4. Confirmation of microarray data by real-time RT-PCR.** To validate the gene profiling data, we performed quantitative real-time PCR of 3 genes of the HO-1 system, which were changed most significantly: heme oxygenase (HO-1), ferritin and biliverdin-reductase. The results are expressed as mean simple linear regression ± SEM; p<0.05 was considered to be statistically significant. *Significant differences between CORM-3 treated HUVEC and untreated HUVEC.
**MOLECULAR MECHANISMS INVOLVED IN CORM EFFECT**

Figure 5. Over-expression of HO-1 influences VCAM-1 expression. To assess whether HO-1 over-expression influences VCAM-1 expression, HUVEC were infected with an adenoviral HO-1 or empty-vector, and exposed to TNF-α. Proteins were isolated and Western blotting with antibodies against VCAM-1 was performed. In the figure, negative pBabe-HO-1 vector transfection refers to the transfection with an empty pBabe vector. The results of representative experiments are shown. A total number of three experiments were performed.

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**A**

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**B**

Time of TNF-α stimulation (minutes)

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**Figure 6. Influence of CORM-3 on MAPK.** (A) To investigate the effect of CORM-3 on MAPK, HUVEC were exposed to 1mM CORM-3 for the indicated time points. Proteins were isolated and Western blotting with antibodies against p42/p44 and pp42/44 performed. To investigate the effect of CORM-3 on MAPK in TNF-α stimulated cells, HUVEC were exposed to 50 ng/ml TNF-α with or without 1mM CORM-3 for the indicated time points. Proteins were isolated and Western blotting with antibodies against (B) p42/p44 and its phosphorylated form, (C) and against p38 and its phosphorylated form was performed. The results of representative experiments are shown. A total number of three experiments were performed.

**CORM-3 differentially influences p38 and p42/p44 activation**

We also studied if CORM-3 modulates mitogen activated protein kinases (MAPK) p38, p42/p44 and pJNK. CORM-3 clearly activated p42/p44 in a time dependent fashion (Fig. 6A) in unstimulated HUVEC. When HUVEC were stimulated with TNF-α with or without addition of CORM-3, activation of p42/p44 not only occurred faster, but was also more pronounced when CORM-3 was present during TNF-α stimulation (Fig. 6B). In contrast, activation of p38 was inhibited under the latter condition (Fig 6C). No influence of CORM-3 on pJNK activation was found in these studies (data not shown).

To test if CORM-3 mediated down-regulation of VCAM-1 might be caused either by inhibition of p38 or by activation of p42/p44, we tested the influence of the p38 inhibitor SB203580. As shown in figure 7A VCAM-1 expression was reduced when SB203580 was present during TNF-α stimulation. Inhibition of p42/p44 during CORM-3 treatment did not abrogate VCAM-1 down-regulation (Fig 7B).
Figure 7. The effect of MAPK inhibitors on VCAM-1 expression. (A) HUVEC were pretreated with a p38-inhibitor (SB203580) and exposed to 50 ng/ml TNF-α for the indicated time points. Proteins were isolated and Western blotting with antibodies against VCAM-1 performed. (B) HUVEC were pretreated with a p42/44-inhibitor (U0126) or left untreated, and exposed to 50 ng/ml TNF-α with or without CORM-3 for 24 hours. Cells were harvested and stained for VCAM-1, and FACS-analysis was performed. In A and B the results of representative experiments are shown. A total number of three experiments were performed.

DISCUSSION

There is unambiguous evidence that CO or CORM-3 have the propensity to modulate inflammation. In many of these studies inhibition of the NFκB pathway has been considered to be the main mechanism by which CO or CORM-3 control inflammation. Yet, we have described (previous chapter) that apart from the inhibitory effect on the
NF-κB pathway CORM-3 down-regulates VCAM-1 also post-transcriptionally. This prompted us to study putative pathways that could be involved in the anti-inflammatory effect of CORM-3 on endothelial cells in more detail. The most important findings were the following. First, CORM-3 activates the ubiquitin proteasome system (UPS), which is reflected by up-regulation in gene-expression of proteasome sub-units and functionally by an increased ubiquitination and proteasome activity. Second, CORM-3 up-regulates mRNA expression of genes encoding for heme synthesis (ALAS-1), heme degradation (HO-1 and biverdin reductase) and iron chelation (ferretin and ferrochelatase), all belonging to the porphyrin pathway. Third, CORM-3 down-regulates a number of TNF-α regulated genes encoding for signaling molecules, adhesion molecules and chemo/cytokines. Fourth, the UPS is not involved in CORM-3 mediated down-regulation of VCAM-1. Fifth, CORM-3 differentially influences MAPK activation. Inhibition of p38 might contribute to the down-regulation of VCAM-1, while activation of p42/p44 by CORM-3 is not involved.

A number of studies have suggested that proteasome inhibitors might be used therapeutically as they down-regulate inflammation and attenuate fibrosis. Because proteasome inhibitors interfere in the NFκB activation cascade by preventing IκB degradation, they also inhibit NFκB depended production of pro-inflammatory mediators. CORM-3 inhibits sustained NFκB activation (chapter 3), yet they also increase ubiquitination and proteasomal activity, eligible conditions for IκB degradation. Therefore, it seems that inhibition of sustained NFκB activation is downstream the UPS, which is compatible with the data shown in chapter 3. If activation of the UPS does not inhibit NFκB activation, than a logical question is how and to what extent activation of the UPS might contribute to the anti-inflammatory effects of CORM-3. This could be achieved either by proteolytic degradation of transcription factors, as has been shown for c-Fos and c-Jun or by tagging newly synthesized pro-inflammatory proteins for proteasomal degradation. With respect to the latter we could show that MG132 or lactacystein were not able to inhibit CORM-3 mediated down-regulation of VCAM-1. Whether proteasomal degradation of other pro-inflammatory proteins occurs under these conditions can not be excluded.

Although we did not observe an increased expression of ubiquitin ligases in affymetrix analysis, there was a significant increase in polyubiquitin conjugates after CORM-3 treatment. If the cut-off for significance was decreased to P(-log10)>3, than expression of the UBR2 gene, encoding a ubiquitin ligase E3, was slightly increased (23 %, P=0.0001, data not shown). Up-regulation of this ubiquitin ligase remains to be tested in independent experiments by qPCR.

Our study also shows that overexpression of HO-1 by means of retroviral transfection impairs TNF-α mediated up-regulation of VCAM-1. Although CORM-3 up-regulates HO-1 and inhibits TNF-α mediated VCAM-1 expression, we can exclude that induction of HO-1 by CORM-3 is underlying this effect (see previous chapter). As suggested in a
study of Nakao et al. 43, CO treatment is associated with a reduction in intracellular heme content. Therefore up-regulation of HO-1 may not have functional consequences in terms of immune modulation when available substrate is limited.
CORM-3 differentially influences MAPK activation. While p42/p44 is inhibited by CORM-3, at present it is unclear if this is directly mediated by release of CO or if this is mediated by the Ruthenium(II) (Ru(II)) compound itself. Lin et al. have shown that the heavy metal Pb(II) persistently stimulates p42/p44 without activating JNK, p38, and ERK5 in CL3 human lung cancer cells 44. This seems to be accomplished by MAPK phosphatase-1 degradation via the UPS 45. It remains to be elucidated whether CORM-3 uses a similar mechanism to activate p42/p44. Nevertheless activation of p42/p44 did not contribute to CORM-3 mediated down-regulation of VCAM-1. In contrast, CORM-3 inhibited p38 activation and inhibition hereof by SB203580 clearly decreased VCAM-1 expression. Hence, inhibition of p38 by CORM-3 might contribute to down-regulation of VCAM-1. We are aware that our data on p38 are in conflict with previous published studies showing that CO activates p38 10. Differences in concentration (1% in the study of Otterbein vs. 1mM CORM-3 in our studies) and application form (Gas vs. CORM-3) could explain this controversy. p38 might be involved in the regulation of sustained NF-kB activation 46 or might function as a co-regulator for a number of genes which are NFκB-dependent 47. In addition p38 is also involved in mRNA stability 48. This might occur at the level of AU rich elements in the 3'UTR of certain mRNA or alternatively by suppressing the expression of microRNA (miRNA). It must be noted that there is no experimental evidence for the later; this is currently under investigation. Pietersma et al. 49 have shown that inhibition of p38 inhibits VCAM-1 expression independent of mRNA stability. Recently an interesting study by Harris et al. 50 identified miRNA-126 as a putative miRNA that can bind to the 3’UTR of VCAM-1 mRNA. Binding of miRNA126 does not result in mRNA degradation but rather inhibits VCAM-1 mRNA translation. Further studies are warranted to elucidate the role of miRNA in CORM-3 mediated down-regulation of VCAM-1 or more generally in the anti-inflammatory effects of CORM-3.
REFERENCES

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CHAPTER 5

INCREASED CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN SEPTIC PATIENTS: CORRELATION WITH SURVIVAL

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*Critical Care Medicine 2007;35:1677–1684*
INCREASED CIRCULATING EPC IN SEPTIC PATIENTS

ABSTRACT

Objective: Endothelial damage and detachment of endothelial cells are known to occur in septic patients. Thus, recruitment of circulating endothelial progenitor cells (cEPCs) to these lesions might have a beneficial effect on the clinical course in septic patients. Therefore, we were interested in whether EPCs, detected by flow cytometry, are increasingly mobilized during sepsis and if this mobilization is associated with clinical outcome.

Design: Prospective, nonrandomized study.

Setting: Intensive care unit of a university hospital.

Patients: Patients with (n=32) and without (n=15) sepsis and healthy volunteers (n=15).

Interventions: Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation, and cEPCs were characterized by three-color fluorescence flow cytometry using antibodies against CD133, CD34, and vascular endothelial growth factor receptor-2. Serum concentrations of vascular endothelial growth factor, granulocyte macrophage-colony stimulating factor, and erythropoietin were determined by enzyme-linked immunosorbent assay. Severity of sepsis was assessed according to Acute Physiology and Chronic Health Evaluation II scoring.

Measurements and Main Results: In septic patients, the number of cEPCs was significantly higher than in nonseptic intensive care unit patients (p<0.05) and healthy controls (p<0.02). Nonsurvivors (n=8), defined as death within 28 days after onset of sepsis, had significantly lower numbers of cEPCs than survivors (n=24) (p<0.0001). The number of cEPCs was correlated with survival in septic patients. Serum vascular endothelial growth factor concentrations were significantly higher in septic patients compared with nonseptic intensive care unit patients and healthy controls (p<0.01) and correlated with the cEPC numbers (p<0.0001). Similar findings were observed for granulocyte macrophage-colony stimulating factor and erythropoietin.

Conclusions: Our data suggest that cEPC enumeration in peripheral blood of septic patients might be a valuable marker to assess the clinical outcome in these patients.
INTRODUCTION

Bacterial toxins and pro-inflammatory cytokines initiate a series of immunologic events that alter endothelial function in the macro- and microcirculation. Vasodilation, capillary leakage, endothelial swelling, leukocyte sequestration, thrombosis, and organ dysfunction are well documented in septic patients. Although initially these changes seem not to differ between survivors and nonsurvivors, capillary perfusion only increases in survivors over time, suggesting that altered endothelial function plays an important role in the development of multiple organ failure in sepsis.

Under normal conditions, endothelial cells (ECs) are firmly attached to the extracellular matrix via so-called focal adhesion contacts. In addition, adjacent ECs interact with each other via specialized structures (i.e., adherence and tight junctions), thereby forming a tight barrier to prevent vascular leakage and passive leukocyte migration into the interstitial tissue. However, under pathologic conditions, ECs can be detached from the vasculature and thus appear in the circulation. Inadequate formation of focal adhesion contacts, proteolysis of the endothelial basal membrane, apoptosis of ECs, and the production of anti-angiogenic factors are among other causes for the release of ECs into the circulation.

Simultaneous with these pathologic processes, reconstitution of the endothelial layer is initiated. This can obviously occur via migration and proliferation of surrounding mature ECs. However, terminally differentiated ECs have a low proliferative potential; hence, their capacity to substitute damaged endothelium is limited. Therefore, adequate vascular repair requires additional support.

Many studies have convincingly demonstrated that vascular maintenance, repair, angiogenesis, and neovascularization are partly mediated by recruitment of endothelial progenitor cells (EPCs). These cells are bone marrow-derived and have the propensity to differentiate into mature ECs. Their phenotype is characterized by the expression of specific hematopoietic marker CD34, the stem cell marker CD133, and the endothelial marker vascular endothelial growth factor receptor-2 (VEGFR-2). However, the identification of EPCs from mature ECs is complicated by the presence of CD34 on both cell types. Therefore, discrimination between circulating mature ECs and circulating EPCs (cEPCs) can be further made by the expression of CD146 and CD105 on mature cells. In healthy individuals, only a small number of cEPCs (~0.002% of total peripheral blood mononuclear cells [PBMCs]) can be found in peripheral blood, whereas this is substantially increased on physical stress and under pathophysiologic conditions.

Mobilization of cEPCs from the bone marrow critically depends on the activation of metalloproteinases and up-regulation of adhesion molecules. This is most likely mediated by soluble factors such as vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), and erythropoietin (EPO).
INCREASED CIRCULATING EPC IN SEPTIC PATIENTS

Serum concentrations of these factors dramatically increase under pathologic conditions, concomitantly with an increase in the number of cEPCs\textsuperscript{23-27}. While metallo-proteinases act to release cEPCs from the bone marrow stroma, adhesion molecules facilitate trafficking of these cells into the circulation\textsuperscript{28}. Recently, first evidence was provided that cEPCs play a pivotal role in reendothelialization not only after vascular damage but also after severe inflammation\textsuperscript{19, 22}. However, most of the investigators used the colony-forming assay for cEPC enumeration. Although this method could be used to identify and quantify cEPCs, it is time-consuming and likely insensitive for general application in a clinical setting. In the present study we performed a practicable multicolor FACS analysis to detect cEPCs in peripheral blood. Using this method we tested the hypothesis that clinical outcome in septic patients is largely dependent on the ability to reconstitute damaged endothelium. Serum concentrations of VEGF, GM-CSF, and EPO were determined in parallel to the number of cEPCs found in circulating blood and were correlated with Acute Physiology and Chronic Health Evaluation (APACHE) II scoring and mortality.

MATERIALS AND METHODS

Subjects
Patients with sepsis (n=32) were selected from the intensive care unit (ICU) of the University Hospital Mannheim within 48 hrs after sepsis onset or at admission to the ICU. Patients included were enrolled consecutively over a 2-yr period (2004–2005) and met the diagnostic criteria for sepsis of the American College of Chest Physicians/Society of Critical Care Medicine\textsuperscript{29}. The rate of bacterial evidence was 70%. Sepsis severity was assessed by the APACHE II score\textsuperscript{30}, and mortality was defined as death occurring within 28 days after diagnosis. Exclusion criteria were cardiogenic or hemorrhagic shock, chronic obstructive pulmonary disease, isolated acute respiratory distress syndrome, absence of mechanical ventilation, and use of statins, angiotensin-converting enzyme inhibitors, activated protein C, and hydrocortisone. Clinical data of each patient were recorded.
For controls we recruited 15 patients from the ICU who did require mechanical ventilation, hereafter referred to as ICU controls, and 15 healthy volunteers from our laboratory staff, hereafter referred to as healthy controls. ICU controls, mainly neurosurgical patients, did not meet the criteria for sepsis, septic shock, or systemic inflammatory response syndrome and were not treated with statins or angiotensin-converting enzyme inhibitors. This study was approved by the Ethics Committee of the University of Heidelberg.
**Blood Sampling**

Blood (25 mL) was obtained from the central venous catheter of septic patients within 48 hrs after sepsis onset or in the case of ICU controls within 24 hrs after admission to the ICU. A second blood sample was obtained from septic patients 5 days after the first. In healthy controls, 20 mL of blood was collected in tubes containing sodium citrate (0.105 M) as anticoagulant by insertion of a 20-gauge cannula intravenously. The initial 5 mL of blood was discarded to minimize EC contamination from the puncture wound of the vascular wall.

**Flow Cytometry**

All blood samples were processed within 1 hr after collection. PBMCs were prepared by gradient centrifugation using Ficoll-Hypaque (Amersham Biosciences, Freiburg, Germany). The expression of cell-surface antigens was determined by three-color immunofluorescence staining as described previously. One hundred microliters of PBMC (containing 1 x 10⁶ cells) was incubated with 10 µL of FcR-blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 10 mins to inhibit nonspecific bindings. Thereafter, the cells were incubated at 4°C for 30 mins with 10 µL of PE-conjugated anti-human CD133 monoclonal antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany), 10 µL of PerCP-conjugated anti-human CD34 monoclonal antibodies (BD Biosciences, Heidelberg, Germany), and 10 µL of APC-conjugated VEGFR-2 monoclonal antibodies (R&D Systems, Wiesbaden-Nordenstadt, Germany). Isotype-matched immunoglobulin G1 and immunoglobulin G2a antibodies (DakoCytomation, Hamburg, Germany) were used for each patient and measurement as negative controls. The cells were washed three times to remove unbound antibodies and finally resuspended in 400 µL of FACS solution (BD Biosciences, Heidelberg, Germany). FACS analysis was performed on a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using WinMDI 2.8 software (Scripps Research Institute, La Jolla, CA). A minimum of 500,000 events were collected. FACS analysis of each probe was performed in triplicate. The frequency of eEPCs in peripheral blood was determined by a twodimensional side-scatter/fluorescence dot-plot analysis of the samples after appropriate gating. EPC counts are expressed as percentage of total PBMC in each patient or control.

**Enzyme-Linked Immunosorbent Assay**

The serum concentrations of VEGF, GM-CSF, and EPO were assessed using a highly sensitive enzyme-linked immunosorbent assay kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) in triplicate samples obtained from 5 mL of serum. Enzyme-linked immunosorbent assay was performed according to the manufacturer’s instructions.
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Statistical Methods
All data are presented as mean ± SEM. Both parametric and nonparametric methods were used. Analysis of variance and Kruskal-Wallis test were performed when comparing the three groups. Nonparametric statistical analysis (Kruskal-Wallis) was especially used to compare the three groups regarding GM-CSF, since the concentration in healthy controls was below the detection limit. Student’s t-test and U test were used to compare survival in the septic patient group. Receiver operating characteristic analysis was performed to predict survival probability from cEPC numbers. Correlation analyses (Pearson/Spearman) were considered for all target variables. We considered p<0.05 to be statistically significant. All analyses were performed using the SAS system (version 8.2, SAS Institute, Cary, NC).

RESULTS
There was no statistical difference in mean age between the groups: in the sepsis group 55.3 ± 13 yrs, (survivors 53 ± 12 and nonsurvivors 62 ± 16 yrs), in the ICU controls 57 ± 16 yrs, and in the healthy controls 58 ± 15 yrs. Of the 32 patients (18 male and 14 female) fulfilling the sepsis inclusion criteria, eight died within 28 days after diagnosis (i.e., 25% mortality rate). In the survivor group, three septic patients died after 28 days. No differences in leukocytes or C-reactive protein (CRP) were found between survivors and nonsurvivors (survivors, white blood cell count 16.1 ± 8.5 white blood cells/μL, CRP 178 ± 84 mg/L mg/L; nonsurvivors, white blood cell count 20.1 ± 7.2 10E9/L, CRP 166 ± 54 mg/L). Relevant characteristics of all septic patients included in this study are summarized in Table 1.
Table 1. Data of patients with sepsis, including gender, age, mortality, Acute Physiology and Chronic Health Evaluation (APACHE) II score, type of infection, white blood cell (WBC) counts and C-reactive protein (CRP) level at time of first blood sampling

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<th>Gender</th>
<th>Age</th>
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<th>Type of infection</th>
<th>WBC, $10^3$/mm³</th>
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The percentage of hematopoietic stem cells, defined as positive staining for CD34 and CD133, was low in healthy controls (0.15% ± 0.66%). While it was already significantly increased in ICU controls (0.24% ± 0.11%), there was still a significant increase of CD34+/CD133+ cells in septic patients—0.52% ± 0.4% (Fig. 1). Within the septic patients, survivors did not differ from nonsurvivors in this respect.

![Bar graph showing percentage of circulating hematopoietic progenitor cells in healthy controls, ICU controls, and sepsis patients.](image)

**Figure 1. Percentage of circulating hematopoietic progenitor cells.** FACS analysis from CD34/CD133-positive cells in the peripheral blood mononuclear cell (PBMC) fraction of healthy volunteers (n=15), nonseptic intensive care unit (ICU) patients (n=15), and septic patients (n=32). Significant differences were found between all three groups. The results are expressed as mean simple linear regression ± SEM; p<0.05 was considered to be statistically significant.

In addition, the percentage of VEGFR-2+ cells within the population of CD34+/CD133+ cells was measured. In healthy controls, only 25% ± 15% of CD34+/CD133+ cells stained positive for VEGFR-2. Both in ICU controls (35.5% ± 22%) and in septic patients (45.5% ± 18%), this was significantly higher (Fig. 2). This corresponds to a percentage of cEPCs (CD34+/CD133+/VEGFR-2+ cells) in the total PBMC fraction: 0.039% ± 0.002% in healthy controls, 0.087% ± 0.04% in ICU controls, and 0.162% ± 0.1% in septic patients (Fig. 3). During the course of sepsis, the percentage of cEPCs remained high; that is, 5 days after the first sampling, cEPC counts were not significantly altered in the survivor group (data not shown). In the nonsurvivor group, only two patients were still alive after 5 days.
Figure 2. Circulating endothelial progenitor cell quantification in peripheral blood mononuclear cell (PBMC) fraction. FACS analysis data representative for each investigated group: healthy volunteers, intensive care unit (ICU) controls, septic patients. A–C, dot plots show negative control; D–F, dot plots show PBMCs stained with anti-CD34 and anti-CD133 monoclonal antibodies; G–I, histograms show the percentage of vascular endothelial growth factor receptor-2 (FLK)-positive cells in the population of CD34/CD133-positive cells. APC, allophycocyanin.
We next investigated if changes in the number of cEPCs were associated with sepsis severity and survival. As shown in Figure 4, sepsis survivors had significantly higher numbers of cEPCs (0.170 ± 0.06) than nonsurvivors (0.06 ± 0.028). However, nonsurvivors had still significantly more cEPCs compared with healthy controls. The patients who died in the survivor group had a lower number of cEPCs than the remaining patients in this group but still a higher number (0.13 ± 0.03) than the nonsurvivor group. The logistic regression revealed a strong association between cEPC numbers and survival in the sepsis group. Septic patients with high cEPC numbers had a higher probability to survive than patients with low cEPC numbers (p<0.036; estimate = 92.03; area under curve = 0.97).

Severity of sepsis was assessed by APACHE II scoring. The mean APACHE II score was significantly higher in the nonsurvivor than in the survivor group (19.6 ± 5 vs. 13.08 ± 4, p<0.01). However, only in the survivor group could a significant correlation between APACHE II score and cEPC numbers (r = 0.44; p<0.03) be detected.
The measurement of the circulating growth factors showed the following findings. In healthy controls, the mean serum VEGF concentration was low (56.1 ± 29.8 pg/mL). In both patient groups—that is, nonseptic ICU patients (477 ± 275 pg/mL, \( p<0.01 \)) and septic patients (1351 ± 733 pg/mL, \( p<0.01 \))—there was a significant increase in serum VEGF concentrations compared with healthy controls. Serum VEGF concentrations were also significantly higher in septic patients compared with the ICU controls (\( p<0.01 \)). Similar to VEGF concentrations, also GM-CSF and EPO concentrations were significantly increased in septic patients compared with ICU and healthy controls (GM-CSF, 3.3 ± 2.2 pg/mL vs. 0.9 ± 0.7 pg/mL vs. 0.049 ± 0.3 pg/mL; EPO, 100.6 ± 75 mUI/mL vs. 19.9 ± 12 mUI/mL vs. 5.7 ± 3 mUI/mL) (Fig. 5). Within the group of septic patients, mediator production of survivors and nonsurvivors was not significantly different (VEGF \( p<0.48 \), GM-CSF \( p<0.50 \), EPO \( p<0.91 \)). A significant correlation between cEPC numbers and serum levels of VEGF (\( r = 0.65; p<0.0001 \)), GM-CSF (\( r = 0.58; p<0.0001 \)), and EPO (\( r = 0.57; p<0.0001 \)) was detected for all the groups. Consequently, high numbers of cEPCs were correlated to high levels of VEGF, GM-CSF, and EPO. Comparing the association between cEPC concentration and
mediator production in septic patients, a significant correlation was only observed for GM-CSF in the nonsurvivor group (r = 0.73, p<0.04).

Figure 5. Up-regulation of growth factors in serum. (A) Erythropoietin (EPO), (B) granulocyte macrophage-colony stimulating factor (GM-CSF), and (C) vascular endothelial growth factor (VEGF) concentrations in serum of healthy volunteers, nonseptic intensive care unit (ICU) patients, and septic patients. The results are expressed as mean simple linear regression ± SEM.
and were significantly different between all investigation groups; \( p<0.05 \) was considered to be statistically significant.

**DISCUSSION**

The present study demonstrated an increased mobilization of cEPCs in septic patients compared with nonseptic ICU patients and healthy individuals. Concomitantly with this, serum concentrations of VEGF, GM-CSF, and EPO were significantly increased. The percentage of cEPCs was correlated with sepsis survival; that is, septic patients with a high percentage of cEPCs had a higher probability of survival.

To our knowledge, this is one of the first studies using standard flow cytometry as a practicable method to detect cEPCs in peripheral blood. Different approaches have been used to quantify EPCs in a variety of patients and controls \(^\text{11, 19, 22, 32}\). Most of these assays, however, rely on cell separation and subsequent cell culturing, making it difficult to implement these assays in routine diagnostics. In the present study we have used standard FACS analysis to quantify EPC numbers in PBMCs \(^\text{33}\). Since a FACS apparatus is present in almost every routine lab, and monoclonal antibodies against EPC markers are commercially available, this method has the potential to be more widely used in the near future and find its place in routine diagnostics.

Although the exact phenotype of cEPCs is still controversially discussed, the presence of CD34, CD133, and VEGFR-2 seems to be proven \(^\text{19, 34}\) and therefore used in this study. Other potential endothelial markers (e.g., CD45, CD14, or VEGFR-1) are likely not specific for EPCs.

Detachment of ECs has been reported under various pathologic conditions that are associated with vascular injury \(^\text{4, 22}\). Also, microvascular alterations occur during sepsis, ultimately leading to breakdown of the endothelial barrier function \(^\text{35-38}\). Mutunga et al. \(^\text{4}\) observed an increase of circulating ECs during sepsis and concluded that endothelial damage occurs. The increased number of cEPCs found in this study might therefore be a consequence of the body’s attempt to limit vascular damage by inducing endogenous endothelial repair mechanisms.

EPCs also seem to play a role under inflammatory conditions, as was suggested in the study by Yamada et al. \(^\text{39}\). Those authors observed that inflammatory stimuli induced a rapid release of EPCs into the circulation in humans and concluded that a sufficient number of EPCs is required for proper repair of lung tissue following bacterial pneumonia. Moreover, an association between cEPC numbers and CRP concentrations in human serum \(^\text{21}\) also suggests a link between inflammation and EPC mobilization.

The importance of vascular repair under inflammatory conditions (e.g., sepsis) has thus far not been studied. Burnham et al. \(^\text{22}\) found for the first time an increased number of cEPCs in patients with acute lung injury. They used the colony-forming assay to determine EPC and observed an association between EPC concentration and survival.
Because in some of these patients acute lung injury-associated septic shock was present, we found herein a first confirmation of our results. We observed an increase in cEPC numbers in all investigated septic patients and a correlation between cEPC concentration and survival. The number of cEPCs, however, was still increased in nonsurvivors compared with nonseptic patients, which is in agreement with the observations of Sakr et al. In that study, it was shown that capillary perfusion in septic patients is increased over time only in survivors, but not in nonsurvivors, and might be due to the recruitment of EPCs and subsequent repair of vascular damage. The number of cEPCs was still increased during severe sepsis, even when clinical symptoms of sepsis had been normalized. In our study, the increase of cEPC concentration was found prolonged over an investigation period of 5 days. This suggests that the endothelial alteration and repair are still ongoing and effective in improving microvascular organ perfusion, particularly in sepsis survivors. Any dysfunction in EPC release by bone marrow exhaustion or failure could be excluded, since white blood cell counts were found normal or enhanced, but not suppressed.

Apart from the correlation between cEPC concentration and survival in general, we also found in the survivor group that there was a correlation between APACHE II scores and cEPC at time of admission. This score was chosen because it has become one of the most accepted among the general ICU severity of illness scoring systems. To risk-adjust patients with longer and very severe illnesses, several other models of organ dysfunction have become available, including the Sequential Organ Failure Assessment. Because on admission all patients with severe sepsis or septic shock were given an appropriate therapy and hence fulfilled the exclusion criteria, we did not use the Sequential Organ Failure Assessment.

The recruitment of cEPCs during inflammation is most likely mediated by cytokines. Van der Flier et al. reported a correlation between increased plasma VEGF levels and severity of multiple organ dysfunction during the course of sepsis. They found significantly higher VEGF levels in nonsurvivors than in survivors and concluded a potential role of this mediator in the development of sepsis-associated capillary leakage. We confirmed the increase in VEGF production in septic patients. However, we did not observe significant differences in VEGF levels between survivors and nonsurvivors.

The increase in EPC numbers after VEGF administration or endogenous VEGF up-regulation under septic conditions underlines the importance of this mediator in mobilization of EPCs. In addition to VEGF, EPO and GM-CSF are also able to mobilize EPCs with a similar potency. In line with the observations of Heeschen et al., our data show a correlation between the number of cEPCs and serum VEGF and EPO levels, but not with CRP concentrations. Our data also confirm the previous observation by Torre et al. that GM-CSF serum concentrations are increased in septic patients and patients with noninfectious systemic inflammatory response syndrome.
The clinical relevance of cEPCs in a variety of vascular disorders has already been studied. Hill et al. 11 reported that levels of cEPCs may be a surrogate biological marker for vascular function and cumulative cardiovascular risk. Werner et al. 32 found that increased levels of cEPCs were associated with a reduced risk of future cardiovascular events among patients with coronary artery disease. In our study, we observed a correlation between cEPC numbers and survival in septic patients. The level of cEPCs in patients with vascular disorders might have the potential to predict clinical outcome and may help to identify patients at increased risk.

CONCLUSION

EPCs may exert an important function as an endogenous repair mechanism to maintain the integrity of the endothelial layer by replacing denuded parts of the microcirculation. Our study suggests that an increased number of cEPCs during sepsis may predict survival in these patients. Future studies are required to examine the potential of cEPC enumeration in routine diagnostics.
REFERENCES

INCREASED CIRCULATING EPC IN SEPTIC PATIENTS


CHAPTER 6

SUMMARY,

GENERAL DISCUSSION

AND

PERSPECTIVES
6.1 SUMMARY

The endothelium plays an important role in the clinical outcome of sepsis. A dysfunction in macro- and microcirculation is frequently observed, and endothelial damage and detachment of endothelial cells can occur in septic patients. Moreover, endothelial cells (EC) have the propensity to produce high amounts of a variety of pro-inflammatory mediators. In this thesis we studied the inflammatory response of the endothelium under septic conditions and the modulating effect of CO releasing molecules on activated endothelial cells. Furthermore, we investigated the behaviour of bone marrow-derived endothelial progenitor cells in septic patients.

In chapter 2, we looked at the interindividual differences of endothelial cells in response to endotoxins, which might contribute to the diversity in clinical outcome among septic patients. We demonstrate that LPS-mediated gene expression differs quantitatively and qualitatively in different HUVEC isolates with either high or low pro-inflammatory potential, which was determined according to their IL-8 production. HUVEC with low pro-inflammatory potential also displayed a low response towards tumour necrosis factor (TNF)-α and needed higher concentrations of LPS for maximal activation of nuclear factor κB (NFκB) compared to HUVEC with high pro-inflammatory potential.

In chapter 3, we studied how CO releasing molecules (CORM)-3 modulate the expression of adhesion molecules by endothelial cells and whether HO-1 perpetuated the modulation. Our results demonstrated that CORM-3 consistently inhibited the up-regulation of VCAM-1 and E-selectin by TNF-α stimulated HUVEC, which was partly due to deactivation of NFκB. Interestingly, down-regulation of VCAM-1 and E-selectin expression by CORM-3 even occurred when CORM-3 was added 24 hrs after TNF-α stimulation. Continuous presence of TNF-α was required for sustained expression of VCAM-1 and TNF-α removal was more effective in reducing VCAM-1 mRNA level than CORM-3 addition. On the protein level, in contrast, VCAM-1 was down-regulated more rapidly when CORM-3 was added. This suggests that the modulation of VCAM-1 by CORM-3 partly occurred at the post-transcriptional level. CORM-3 itself up-regulated HO-1 in an Nrf dependent fashion, but HO-1 expression did not significantly contribute to CORM-3 mediated down-regulation of VCAM-1. The efficacy of CORM-3 to down-regulate VCAM-1 expression was neither lost in siRNA based HO-1 nor Nrf knock down in HUVEC. These data imply that CORM-3 mediated down-regulation of VCAM-1 is independent of HO-1 expression.
Since we showed in the previous chapter that CORM-3 are able to downregulate adhesion molecule expression by endothelial cells and that this effect is not completely mediated via deactivation of the NFκB pathway, we set out in chapter 4 to investigate the molecular mechanisms involved in the CO-mediated down-regulation of adhesion molecules by HUVEC. To this end, we used genome wide gene expression profiling and hierarchical analysis of particular pathways, and studied the relevance of these pathways for the anti-inflammatory effects of CORM-3. The most significant CORM-3 mediated changes were found in the proteasome and porphyrine pathways. In TNF-α stimulated HUVEC, CORM-3 inhibited the expression of a variety of inflammatory genes, including chemokines/cytokines and adhesion molecules. Although the proteasomal activity was significantly increased by CORM-3, proteasome inhibitors lactacysteine and MG132 were not able to abolish down-regulation of VCAM-1 by CORM-3. When looking at the involvement of MAPK, we found that p42/p44 was activated by CORM-3, while p38 was inhibited and JNK was unaffected. Similar to the CORM-3 effects, inhibition of p38 resulted in down-regulation of VCAM-1 expression, while inhibition of p42/p44 did not influence the CORM-3 mediated down-regulation of VCAM-1.

In chapter 5, we investigated the behaviour of endothelial progenitor cells (EPCs) and their association with clinical outcome in septic patients. To this end, we measured the levels of EPCs and EPC mobilizing factors (VEGF, GM-CSF and EPO) in the peripheral blood of septic patients and investigated whether mobilization is associated with clinical outcome. Our study demonstrated that the levels of EPCs in septic patients were significantly higher than in non-septic intensive care unit patients and healthy volunteers. EPC levels in septic patients correlated with survival, and sepsis nonsurvivors had significantly lower numbers of EPCs than survivors. Serum concentrations of EPC mobilizing factors were significantly higher in septic patients compared with non-septic intensive care unit patients and healthy controls and correlated with the cEPC numbers.

In summary, the results presented in this thesis demonstrate that septic conditions can severely affect endothelial cell function which may play an important in the pathophysiology and clinical outcome of sepsis. As such the endothelium might provide an important target for therapeutic interventions using either drug-based or cell-based approaches. We provided insight into heterogeneity in LPS responsiveness at the level of endothelial cells, which might influence severity of the inflammatory response in septic patients. Furthermore, we show the ability and some aspects of the mechanism of CORM-3 to modulate adhesion molecule expression by endothelial cells, making it an interesting potential drug for sepsis. Finally, we describe a potential role of EPCs to serve as a biomarker to predict clinical outcome and identify patients at increased risk.
Further studies are necessary to fully understand the interindividual endothelial heterogeneity in responsiveness to inflammatory mediators and the vascular-bed specific endothelial heterogeneity in sepsis, as well as the potential of the endothelium as a target for sepsis therapy.

6.2 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

As described in chapter 1 and also implied in chapter 5, several lines of evidence suggest the occurrence of endothelial dysfunction in sepsis. In the course of sepsis loss of endothelial barrier function occurs, which subsequently leads to sub-endothelial edema and leukocyte infiltration. Moreover, endothelial cells, besides leukocytes, also produce an array of inflammatory mediators that further deteriorate barrier function and promote leukocyte infiltration into the sub-endothelial tissue. Both deterioration of endothelial barrier function and leukocyte infiltration, contribute to a large extent to the development of multi-organ failure. In this thesis several aspects of the role of the endothelium in sepsis have been addressed.

Role of endothelial heterogeneity in sepsis

The endothelial inflammatory response may be genetically determined and polymorphisms in certain genes may be involved in heterogenous response to certain stimuli. Indeed we have demonstrated in chapter 2 that the general inflammatory response of endothelial cells to LPS varies largely among different individuals. Using a genome wide gene profiling approach on HUVEC, we have found two types of responders toward LPS that can be distinguished on the basis of a different expression profile in a set of inflammatory genes, including chemokines IL-8, IL-6 and MCP-1, adhesion molecules ICAM-1 and VCAM-1 and transcription-factors CEBP and NF-kB. Moreover, we could demonstrate a higher threshold for LPS-induced NF-kB activation in those HUVEC that expressed low mRNA amounts for these inflammatory genes.

Although we could clearly demonstrate functional and phenotypic heterogeneity amongst different HUVEC isolates, the clinical significance of this phenomenon is at present not known. We have to keep in mind that our study only employed HUVECs and that endothelial phenotypes vary from one site of the vasculature to the other. Even in endothelial cells from one vascular bed heterogeneity exists, as seen in our study. In sepsis, vascular bed-specific responses of endothelial cells were also found in in vivo-models. In a rat model of CLP, the diverse multi-organ transcriptional programs activated during systemic inflammation were explored. DNA microarray analyses of the various tissues demonstrated remarkable differences in the expression of genes, of which some were specific to the endothelium (e.g. VCAM) 1. In a mouse model of endotoxemia, organ specific differences in the expression of endothelial cell adhesion
molecules could be observed \(^2\). In a baboon model of E. coli bacteremia, expression of tissue factor by endothelial cells became detectable only in the splenic microvasculature, but not in other organs \(^5\). It is very likely that other responses of the activated endothelium such as inflammatory mediator production also display spatial heterogeneity.

In order to find out, whether the endothelial responder type, as found in chapter 2, determines clinical outcome during sepsis, we set out to investigate whether circulating endothelial cells (cEC), isolated from peripheral blood, could also be classified into these responder-types, and if a certain responder-phenotype of these cells correlated with clinical outcome in septic patients. Many studies have investigated cEC in a variety of vascular disorders, and only a few of these describe long-term cultures of such cells \(^6\)-\(^11\). Therefore, we first had to establish a reproducible method for isolation and culture of cEC. Although we were able to isolate cEC from peripheral blood of septic patients, only in less than 10% of these experiments endothelial cultures could be established. The numbers of cEC were very low and it was difficult to stimulate their proliferation to confluence in tissue flasks. Isolation and culture of cEC from healthy individuals as controls was impossible due to the extremely low numbers of cEC. Consequently, we were not able to test our hypothesis. To investigate whether the endothelial responder type largely determines clinical outcome during sepsis, we need to optimize our method for isolation and culture of cEC.

**Endothelium as a therapeutic target in sepsis**

Since the endothelium plays an important role in mediating the sepsis phenotype, it represents an attractive therapeutic target for this disease \(^12\). But it is important to notice that endothelial cell activation is not the same as endothelial activity. As described in chapter 1.1.4, normal/healthy endothelium displays various kinds of different functional activities. Thus, therapies targeted towards the endothelium should not aim to switch off endothelial activity completely, but rather restore the normal state of activity. In this respect it is also fundamental to understand the nature of the normal state of activity, thereby taking into account endothelial heterogeneity throughout the vascular tree.

When looking at the progress in sepsis therapy in the last decade, five phase 3 clinical trials have demonstrated improved survival in critically ill patients or patients with severe sepsis. These include the use of low tidal volume ventilation \(^13\), activated protein C \(^14\), low dose glucocorticoids \(^15\), intensive insulin therapy \(^16\), and early goal-directed therapy \(^17\). It has been suggested that each of these regimens might exert its benefit also through a protective effect on the endothelium \(^18\). For example, low tidal volume ventilation might decrease barotrauma to pulmonary endothelium; activated recombinant human (rh) APC attenuates the endothelial response to inflammatory mediators, inhibits cell apoptosis and improves the barrier function \(^19\), \(^20\); low dose steroids might inhibit pro-inflammatory pathways in endothelial cells including NFkB;
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intensive insulin therapy reverses the harmful effect of hyperglycemia on endothelial cells; and early goal directed therapy might lead to favorable hemodynamics at the level of the endothelium. Several studies also discussed the role of statins in attenuating cell dysfunction by reducing NFκB activation and decreased release of inflammatory mediators 21, 22. Therefore, it has been suggested that rhAPC and statins might represent prototypes of a novel class of drugs that belong to the category of “attenuators of endothelial dysfunction” which may be advantageous for sepsis treatment.

In chapter 3, we have studied the effect of CORM-3 on endothelial cells, which might serve as “attenuators of endothelial activation”. We could show that CORM-3 consistently inhibited TNF-α mediated up-regulation of VCAM-1 and E-selectin by HUVEC and also deactivated sustained NFκB activation. Based on the observation that CORM-3 also down-regulated VCAM-1 expression post-transcriptionally, we set out in chapter 4 to study the putative pathways involved in the anti-inflammatory effect of CORM-3 on endothelial cells and found MAPK p38 contributing to CORM-3 mediated down-regulation of VCAM-1. By understanding the mechanisms involved in CORM-3 mediated anti-inflammatory effects, we will be able to identify the targets involved and grasp in more detail the effect of CORM-3 on the activity and function of cells. These factors are important when assessing its potential application in clinical use.

In chapter 1.1.7, the anti-inflammatory effects of HO-1 and CO and in vivo-studies with CO in animal models of sepsis have been described, which demonstrate an association of CO administration with prolonged survival and reduced mortality. Until recently, studies were lacking that use CORM instead of applying CO by inhalation in animal models of sepsis. CORMs allow intravenous administration and deliver CO directly to the tissues without significant formation of CO-Hb in contrast to inhaled CO 23. To evaluate the potential of CORM to have a beneficial effect on sepsis, its administration in animal models of sepsis, both endotoxemia and CLP, will be essential.

Recently, the effect of CORM-2 administration on the modulation of liver inflammation during sepsis was investigated in a murine CLP model 24. The CLP-induced increase of PMN tissue accumulation, ICAM-1 expression and NFκB activation in the liver of septic mice was significantly attenuated by systemic administration of CORM-2, suggesting its potential in clinical sepsis management. Similar observations were made for the lung 25, liver 26 and the small intestine 27 of thermally injured mice treated with CORM-2. More studies are required to consider also the response of other organs to CORM administration and the clinical outcome in animal models of sepsis and septic shock.

Clinical relevance of endothelial progenitor cells
The clinical importance of circulating endothelial progenitor cells (eEPCs) was studied in a variety of vascular diseases 28-31. Hill et al. 28 investigated in their study the EPC numbers in 45 men, who had various degrees of cardiovascular risk but no history of cardiovascular disease. They observed a strong correlation between the number of
cEPCs and the Framingham risk factor score of the patients. In addition, they found a significant relation between endothelial function, which was measured by flow-mediated brachial-artery reactivity, and EPC numbers. The cEPC levels were a better predictor of vascular reactivity than the presence or absence of conventional risk factors, suggesting that EPCs might be a surrogate biomarker for vascular function and cumulative cardiovascular risk. Werner et al. \(^{29}\) studied the EPC numbers in 519 patients with coronary artery disease confirmed by angiography and evaluated after 12 months the association between baseline EPC levels and clinical course. The clinical course included death from cardiovascular causes, the occurrence of a first major cardiovascular event (myocardial infarction, hospitalization, revascularization, or death from cardiovascular causes), revascularization, hospitalization, and death from all causes. Increased EPC levels were associated with a reduced risk of death from cardiovascular causes, a first major cardiovascular event, revascularization and hospitalization, but EPC levels were not predictive of myocardial infarction or of death from all causes. Based on this, cEPCs might predict the occurrence of cardiovascular events and death from cardiovascular causes and may serve as a biomarker to identify patients at increased cardiovascular risk.

In chapter 5, we describe a significant correlation between cEPC numbers and survival in septic patients, suggesting EPC levels to be a prognostic biomarker for the clinical course in sepsis. Thereby, we could identify patients at increased risk and adapt treatment accordingly. This finding was recently confirmed \(^{32}\).

In vascular diseases the ideal biomarker should be able to identify the degree of vascular injury and the regenerative capacity of the body, which makes EPCs a potential candidate. However, the existing variations in methodology of EPC characterization might weaken the diagnostic impact. As mentioned in chapter 1.1.5, different methods of quantification and functional assessment are currently available, such as flow cytometric analysis or matrigel assays. Recently, it has been suggested that EPC counts cannot be reliably used for the estimation of EPC number in peripheral blood or the bone marrow, and that flow cytometry may be the more optimal technique \(^{33}\). In order to utilize EPCs as a surrogate prognostic biomarker able to reflect the degree of endothelial damage and the regenerative capacity of the body, a reproducible and reliable laboratory test has to be established or agreed upon. To this end a widely accepted standardization of markers that define true EPCs are necessary.

The initial observation of circulating progenitor cells with endothelial properties was met with enthusiasm because it held the promise of cell-based therapies for all kinds of vascular diseases \(^{34}\). This has been complicated by several facts. The number of EPCs in the circulation of healthy controls (as well as animals) is extremely low, whereby isolation and culture is made difficult \(^{35}\). Also, as mentioned before, no consensus exists on the type or types of cell surface markers for EPCs. An important observation relevant to EPC investigations is the identification of two EPC subtypes with potentially different
roles in endothelial repair 36, 37. These two different cell types are obtained by culturing peripheral blood mononuclear cells (PBMC) and examining certain properties in vitro. PBMC that grow early (approximately 7 days) into colony-forming units on fibronectin-coated flasks are termed early EPCs. They possess cell surface antigens consistent with hematopoietic progenitors (i.e., CD34), along with endothelial-specific markers (i.e., CD31) and markers of monocytic lineage (i.e., CD14), among others. They have less potential to form true endothelium in tube-forming assays in three-dimensional cultures either in isolation or in co-culture with HUVEC. However, early EPCs have been reported to secrete significant amounts of pro-angiogenic growth factors 38. In contrast to this, late or outgrowth EPCs appear after 2 or more weeks in culture and do not secrete these factors to a measurable extent. Late EPCs do share some markers in common with early EPCs, such as CD31, but do not possess hematopoietic cell-specific surface antigens such as CD45 or CD14. These cells may be more operational in replacing damaged or destroyed endothelium, because in vitro, they will readily form endothelial tubes and functional blood vessels in animal models. Therefore, it may be that each of these cell types plays a unique role in proper endothelial repair 39. In sepsis, the subtypes of EPCs have not been investigated yet. Early EPCs might be involved in controlling endothelial damage by secreting soluble pro-angiogenic factors, which stimulate proliferation of existing endothelial cells. Late outgrowth EPCs might be involved in replacement of the damaged endothelial layer. In vitro culture studies are necessary to study the functional role of these subtypes of EPCs in sepsis.

In the past years several phase I and II clinical trials have been designed to restore blood flow into the ischemic heart and limbs using transplanted autologous progenitor cells (for a detailed list see 40). In randomized studies assessing the impact of intracoronary injection of progenitor cells on myocardial function and volumes in acute myocardial infarction (AMI) controversial results in terms of recovery of ventricular function have been reported 41-46. Even in the studies which show a significant beneficial effect of the treatment, the magnitude of change of left ventricular (LV) ejection fraction seems modest (+5.5% cells vs. + 3% controls 43), although benefits in terms of ventricular remodeling (reduction of infarct size/LV volumes) have been described. These effects have been attributed to a reduced apoptosis rate and increased neo-vasculogenesis in the peri-infarct myocardium 47. To increase the success of EPC transplantation treatments, advancement in understanding the biology of EPC subpopulations and the homing mechanisms into the target tissue is necessary.

In sepsis and sepsis-related organ dysfunction, the majority of in vivo studies regarding cell-based therapies have used bone marrow-derived mesenchymal stem cells (BMDMScs). In the lung, BMDMScs inhibited the acute systemic inflammatory response and protected in a murine model the occurrence of endotoxin-induced acute lung injury (ALI) 48-50, thus conferring a survival benefit 49. This coincided with significant histological improvement 48, 49. Recently, it was suggested that infusion of
autologous EPC ameliorates oleic acid-induced lung injury in a rabbit model highlighting the potential of cell-based therapies for treatment of ALI and ARDS. Circulating EPCs were obtained from rabbits using Ficoll density gradient centrifugation. One week after culturing, ALI was induced in rabbits by oleic acid, and autologous early EPCs were transplanted intravenously. When the animals were killed 48 h later, fluorescently labeled EPCs were detected in pulmonary arterioles of animals injected with EPCs. Western blotting analysis performed on pulmonary artery of these animals revealed that inducible nitric oxide synthase expression was suppressed in animals that had received EPCs. In addition, animals subjected to lung injury who received EPCs had a decreased wet-to-dry weight ratio, a decrease in hyaline membrane formation, decreased hemorrhage, and a lower percentage of neutrophils present within the lung. Although not endothelial specific, these findings suggest that EPC infusion had an effect on preservation of alveolar–capillary barrier integrity. Although injected EPCs appeared in the pulmonary vasculature, they were not observed elsewhere in the lung. Lungs of animals treated with EPCs appeared significantly more normal after injury, yet it is unclear exactly which role EPCs have in preserving and restoring lung architecture. Also, the relatively small number of infused EPCs (approximately $10^5$ cells) and the short time period between EPC infusion and animal death (48h) makes it less likely that EPCs themselves played a significant structural role in repair of damaged endothelium. However, it is feasible that these cells contribute in a paracrine manner. Another issue, which is also raised by the authors of the study, is the relevance of an autologous cell infusion model. In a clinical setting it is impossible to collect cells from patients before disease onset to use later on in illness.

A more feasible possibility might be to use therapies such as granulocyte–colony stimulating factor (G-CSF) that enhance endogenous EPC release and could be used after the detrimental process of sepsis (or ALI) has initiated. In animal studies of AMI, G-CSF stimulated circulating bone marrow-derived stem cells were attracted to injured myocardium and able to reverse cardiac dysfunction and remodeling through mechanisms of neo-vascularization and prevention of progressive cardiomyocyte apoptosis. Randomized controlled trials testing this hypothesis in humans reported mixed results without clear evidence that G-CSF administration may significantly accelerate recovery of ventricular function in the early or mid-term follow-up after AMI. This might be due to the differences in timing of G-CSF administration and patient’s selection among these studies.

Potential therapies targeted at repair or limiting of endothelial damage in sepsis have not been thoroughly explored and despite our lack of clear understanding of the purpose and function of EPCs, it seems reasonable that such therapy might benefit patients with sepsis. Nonetheless, additional investigations are clearly required to determine whether benefits of this type of therapy are clinically significant, and to ensure that there are no unanticipated adverse effects.
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In summary, endothelial cell function is severely affected in sepsis and plays an important role in the clinical outcome of septic patients. Therapeutic interventions using drug-based or cell-based approaches targeted at the endothelium might be potential treatment strategies for sepsis. Several issues need further attention, including the role of endothelial heterogeneity in sepsis, the mechanisms involved in CORM mediated anti-inflammatory effects on endothelial cells and its potential application in clinical use, as well as an increased understanding of EPC biology and the potential clinical use of EPC transplantation for septic patients. This understanding will contribute to develop improved therapies for sepsis.
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CHAPTER 7

SAMENVATTING
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Het endotheel speelt een belangrijke rol in het klinische beeld en het ziekteverloop bij sepsis. Hierbij wordt vaak dysfunctie van zowel de macro- als de microcirculatie waargenomen, met endotheelschade en verlies van bloedvatwandintegriteit als gevolg van het loslaten van de endotheelcellen. Verder hebben endotheelcellen de tendens om een grote verscheidenheid aan ontstekingsmediatoren te produceren die een bijdrage aan de pathologie van sepsis kunnen leveren.

In dit proefschrift hebben wij deze ontstekingsreactie van het endotheel in septische condities bestudeerd en de modulerende effecten van carbon-monoxide (CO) producerende moleculen op geactiveerde endotheelcellen. Verder hebben wij het gedrag van uit beenmerg afkomstige endotheel-voorlopers, zogenaamde endotheel progenitor cellen of EPCs, in septische patiënten onderzocht.

In hoofdstuk 2 hebben we gekeken naar interindividuele verschillen in reactie van endotheelcellen op endotoxine (LPS: lipopolysaccharide), welke zouden kunnen bijdragen aan de variatie in het klinische beeld en ziekteverloop dat wordt waargenomen bij sepsis patiënten. Wij hebben aangetoond dat de LPS-gemediaeerde genexpressie kwantitatief en kwalitatief verschilt in verschillende humane navelstrengendotheelcel-isolaten (HUVEC), met een hoog respectievelijk laag ontstekingspotentieel dat werd bepaald door de hoogte van hun IL-8 productie. HUVEC met een laag ontstekingspotentieel vertoonden ook een lage reactie op tumor necrosis factor-α (TNFα) en in vergelijking met HUVEC met een hoog ontstekingspotentieel was er een hogere concentratie van LPS nodig voor een maximale activering van de pro-inflammatoire transcriptiefactor NFαB.

In hoofdstuk 3 hebben we bestudeerd hoe het CO producerende molecuul CORM-3 (CO releasing molecule-3) de expressie van adhesiemoleculen door endotheelcellen onder invloed van inflammatoire stress moduleren en of het enzyme hemoxygeenase (HO)-1 effect heeft op deze modulatie. Onze resultaten toonden aan dat CORM-3 de TNFα gestimuleerde inductie van de expressie van VCAM-1 en E-selectine door HUVEC inhibeert, gedeeltelijk als gevolg van remming van de transcriptiefactor NFαB. Interessant is de bevinding dat deze inhibitie van VCAM-1 en E-selectin ook optrad wanneer CORM-3 24 uur na TNFα stimulatie werd toegevoegd. De ononderbroken aanwezigheid van TNFα was vereist voor een aanhoudende expressie van VCAM-1, en het verwijderen van TNFα was efficiënter in het verminderen van VCAM-1 mRNA expressie dan toevoeging van CORM-3. Op het eiwit niveau daarentegen nam de VCAM-1 expressie sneller af als CORM-3 werd toegevoegd. Deze data wijzen erop dat modulatie van VCAM-1 door CORM-3 gedeeltelijk op het posttranscriptionele niveau plaatsvindt. CORM-3 toevoeging leidde tot de inductie van HO-1 genexpressie in de
endotheelcellen op een Nrf afhankelijke manier, maar HO-1 expressie leidde niet tot een significant vermindering van CORM-3 gemedieerde inhibitie van expressie van VCAM-1. De efficiëntie van CORM-3 om VCAM-1 expressie te remmen was noch gebaseerd op HO-1 noch op Nrf, wat we aantoonden in cellen waarin we middels siRNA de respectievelijke genen hadden uitgeschakeld. Deze gegevens impliceren dat de CORM-3 gemedieerde remming van expressie van VCAM-1 onafhankelijk is van HO-1 expressie.

In het vorige hoofdstuk toonden we aan dat CORM-3 in staat is om de expressie van adhesiemoleculen te verminderen. Omdat dit effect niet volledig via de NFkB route verloopt, onderzochten we in hoofdstuk 4 nader de moleculaire mechanismen die betrokken zijn bij CO gemedieerde vermindering expressie van adhesiemoleculen in HUVEC. Hiervoor gebruikten wij genoombrede genexpressie profilerings en hiërarchische analyse van specifieke moleculaire routes die in een cel geactiveerd kunnen worden onder ontstekingsstress. We bestudeerden het belang van deze routes voor de ontstekingsremmende effecten van CORM-3. De meest significante CORM-3 gemedieerde veranderingen werden gevonden in de proteasoom en porphyrine routes. CORM-3 remde in HUVEC de TNFα geïnduceerde expressie van een verscheidenheid aan ontstekingsgerelateerde genen, met inbegrip van chemokines/cytokines en adhesiemoleculen. Hoewel de proteasoom-activiteit beduidend werd verhoogd door behandeling met CORM-3, toch konden proteasoomrremmers zoals lactacysteine en MG132 de vermindering expressie van VCAM-1 zoals bewerkstelligd door CORM-3 niet teniet doen. Kijkend naar de betrokkenheid van MAPK, vonden wij dat p42/p44 door CORM-3 werd geactiveerd, terwijl p38 werd geremd en JNK onveranderd bleef. Vergelijkbaar met de effecten van CORM-3 resulteerde het blokkeren van p38 tot een vermindering expressie van VCAM-1, terwijl de remming van p42/p44 geen invloed had op de CORM-3 gemedieerde afname van VCAM-1.

In hoofdstuk 5 onderzochten wij het gedrag van endotheel-voorloperscellen (EPCs) en hun associatie met het klinisch ziekteverloop in septische patiënten. Daartoe maten wij de concentraties van EPCs en van EPC mobiliserende factoren zoals VEGF, GM-CSF en EPO, in het perifere bloed van septische patiënten, en onderzochten we of de hoogte van deze waarden is geassocieerd met het klinische ziekteverloop. Onze studie toonde aan dat de bloedwaarden van EPCs in septische patiënten beduidend hoger waren dan die in gezonde vrijwilligers en niet-septische patiënten op de intensive care. Waarden van EPC in septische patiënten correleerden met de overleving, en sepsis non-survivors hadden beduidend lagere EPC waarden dan de overlevenden. Serum concentraties van VEGF, GM-CSF en EPO waren hoger in septische patiënten dan in gezonde vrijwilligers en niet-septische patiënten op de intensive care, en correleerden met de circulerende EPC aantallen.
SAMENVATTING

Samengevat tonen de resultaten in deze thesis dat sepsis de functie van het endotheel sterk kan beïnvloeden. Deze veranderde endotheelcelfunctie kan een belangrijke rol spelen in de pathofysiologie en het klinische verloop van sepsis. Op basis hiervan kan worden gezegd dat het endotheel een belangrijk doelwit kan zijn voor therapeutische interventies gebaseerd op conventionele medicijnen of op cellen. Wij hebben de heterogeniteit van endotheelcellen in reactie op LPS laten zien, dat een belangrijke relatie met, en invloed kan hebben op, de ernst van de ontstekingsreactie in septische patiënten. Tevens hebben we laten zien dat de CO-vrijmakende stof CORM-3 de capaciteit heeft om de expressie van de adhesiemoleculen door endotheelcellen te remmen, en hebben we enkele achterliggende mechanismen blootgelegd, wat CORM-3 tot een interessant geneesmiddel voor de behandeling van sepsis maakt. Tot slot beschrijven wij een potentiële rol van EPCs als biomarker om het klinisch verloop van sepsis te voorspellen en patiënten te identificeren met een verhoogd risico op een ernstige inflammatoire reactie. Verdere studies zijn noodzakelijk om verschillen in endotheelgedrag in reactie op sepsis tussen individuen beter te duiden, zowel waar het hun reactie op inflammatoire mediatoren betreft als ook de orgaanvatbed-specifieke verschillen in reactie. Tevens zal meer onderzoek nodig zijn om het potentieel van het endotheel om te fungeren als doelwit voor sepsistherapie te begrijpen.
APPENDIX

Acknowledgements

Publication list & Awards

Curriculum vitae

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APPENDIX

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K. Nowak', N. Rafat', S. Belle, C. Weiß, C. Hanusch, C. Manegold, P. Hohenberger, G. Ch. Beck. Circulating endothelial progenitor cells are increased in human small and non-small cell lung cancer patients and correlate with progression (in revision) (*These authors contributed equally to this work)


*Many abstracts for several national and international medical congresses and meetings.*

**AWARDS**

*Roger-Bone-Award* 2008 of the German Sepsis Association for an outstanding publication in the field of clinical sepsis research, presented at the *9th German Interdisciplinary Congress for Critical Care and Emergency Medicine* in Hamburg, 03rd – 06th December 2008

Poster Price of the German Sepsis Association at the *3rd International Congress Sepsis and Multiorgan Dysfunction – Consensus and Controversies* in Weimar, 5th – 8th September 2007

Winner of the session “Immunology” at the *15th European Student Conference* at the Charité in Berlin/Germany, 19th – 23rd October 2004

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GENERAL AND SCIENTIFIC EDUCATION

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<th>Date</th>
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<td>10/08</td>
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Figure 2. Dynamic time-course of the inflammatory response during sepsis.

Figure 3. Functions of resting endothelial cells.
Figure 4. The leukocyte adhesion cascade.

Figure 5. Electron microscopic analysis of normal mouse aortic endothelium and aortic endothelium of mice subjected to septic shock.
Figure 6. *In vitro* methods of EPC culture.
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Figure 8. EPC homing to activated endothelium.

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Figure 10. Toll-like receptor (TLR) 4 signaling pathway.
Figure 1. Principal component analysis showing distinct clustering of the groups: untreated (green), CORM-3 treated (red), TNF-α treated (blue) and TNF-α + CORM-3 treated (brown). Each dot represents the data set of 1 individual HUVEC culture.