A Cell Culture Model of Lipopolysaccharide-induced Endothelial Activation

Comparison of THP-1 Cells & PBMC-derived Monocytes

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DIPLOMA THESIS

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realized at the
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Universität für Bodenkultur
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Vienna, Austria

Vienna, September 2010

Acknowledgement

The mere fact that I have the opportunity to write an acknowledgement for my diploma thesis marks the end of a very long way – the end of my studies. Dealing with a cell culture model for sepsis-related processes, I hope that I could add at least a tiny piece to the knowledge of this particular disease syndrome. A scientific work like this needs resources and support from various sides.

I am indebted and grateful to my father, my friends at IAESTE BOKU and the Austrian Red Cross/Youth Red Cross and especially to Astrid, Gabi, Renate and Daniel. They all have believed in my abilities, even when I myself was not so sure about them anymore and furthermore accompanied me in dark times and bright times. Finishing my diploma thesis is definitely a bright time!

With regard to this work, I would like to express my utmost gratitude to ...

... **Dr. Viktoria Weber**, Donau-Universität Krems, for her support and guidance during the process of writing this thesis. She did not just offer the opportunity to work in this very interesting field, but her willingness to discuss every question and idea – even under limited time resources – as well as her enormous amount of patience were very important for this work.

... **Dr. Anita Schildberger**, Donau-Universität Krems, has been first and foremost a true friend and enjoyable colleague during the duration of my practical work and still is. In addition, she initiated me to the secrets of cell culture techniques and tirelessly guided me through the depths of flowcytometry.

... **Eva Rossmanith, Mag. Ingrid Linsberger and Ute Fichtinger** as well as the rest of the interdisciplinary team at the Center of Biomedical Technology. Besides their enormous experience and knowledge, they showed me important insights into methods and a lot of practical tricks.

... **Karlheinz Kellner** and **Dr. Marion Ettenauer** should be mentioned here for many enjoyable coffee-breaks and "Torterl".

... **Many volunteers** from the Donau-Universität Krems showed their interest in science in the willingness to donate blood for my experiments. Without them, my thesis would not have been possible.

Abstract

Sepsis is a systemic inflammatory host response to infection and leading cause of death in non-coronary ICUs. This disease coincides with a high mortality rate ranging from 30% to 50% and more for patients with septic shock. It is expensive to treat and therefore an enormous economic burden for worldwide healthcare resources. 750,000 people are annually affected by sepsis in the U.S. and 7,000–9,000 in Austria. Therapeutic options to treat sepsis are limited, as pathogenic mechanisms involved are still not fully understood.

This thesis aims to characterize an existing cell culture model that mimics the initial events in gram-negative sepsis. The model is based on lipopolysaccharide (LPS)-induced activation of endothelial cells. The continuous monocytic cell line THP-1 was stimulated for 4 hours with 10 ng/ml LPS from *Pseudomonas aeruginosa* in media containing 10% human plasma. The culture supernatants containing LPS and factors secreted by the stimulated THP-1 cells were subsequently applied to primary Human Umbilical Vein Endothelial Cells (HUVEC). To compare the stimulation patterns of THP-1 cells to those of human monocytes, monocytes were isolated from human blood and used in the cell culture model. In the second part of this work, HUVEC were compared to the cell line Human Pulmonary Microvascular Endothelial Cells ST1.6R (HPMEC ST1.6R). For this purpose HPMEC ST1.6R were stimulated with supernatants containing LPS and secreted factors received from either monocytes or THP-1 cells.

Endothelial activation in the model was monitored by different read-out assays. Cytokine secretion [Interleukin-6 (IL-6), Interleukin-8 (IL-8) and Tumor necrosis factor-alpha (TNF-α)] was measured after 1 hour and 16 hours. Additionally, the expression of the adhesion molecules ICAM-1 and E-selectin as well as nuclear factor-κB (NF-κB) activity were quantified after 16 hours.

Different levels of cytokine release by stimulated THP-1 cells and monocytes could be observed. However, stimulated HUVEC and HPMEC ST1.6R showed comparable secreted amounts of IL-6, IL-8 and TNF-α for both stimulating media. The expression of ICAM-1 was comparable for HUVEC stimulated with supernatants from THP-1 cells and monocytes. HPMEC ST1.6R showed a slightly increased expression of ICAM-1 for either of the stimulation media. Generally, the upregulation of ICAM-1 could be observed more clearly on HUVEC than on HPMEC ST1.6R. The determination of E-selectin as well as the quantification of NF-κB concluded in ambiguous results, most probably due to the low amount of protein available for the experiments.

In conclusion, the cell culture model used in this diploma work simulates relevant processes of sepsis in a cell culture system and may serve as a useful tool for preclinical studies of gram-negative sepsis. Nevertheless, further studies to evaluate the model, especially regarding the LPS stimulation patterns of THP-1 and monocytes, are necessary.

Zusammenfassung

Sepsis ist eine systemische Entzündungsreaktion des Körpers auf Infektionen. Die Krankheit ist eine der führenden Todesursachen auf Intensivstationen und gekennzeichnet durch hohe Behandlungskosten und Sterblichkeitsraten im Bereich von 30% bis über 50%. Daraus resultiert eine enorme ökonomische Belastung für das weltweite Gesundheitswesen. 750.000 Erkrankungen treten jährlich in den USA auf, in Österreich sind es immerhin ca. 7.000–9.000 Fälle. Therapeutische Möglichkeiten sind limitiert, da die krankheitsverursachenden Mechanismen noch nicht gänzlich erforscht sind.

Ziel dieser Arbeit war es, ein bestehendes Zellkulturmodell hinsichtlich der verwendeten Zelltypen zu charakterizieren. Das Modell imitiert die anfänglichen Ereignisse in gram-negativer Sepsis und basiert auf Lipopolysaccharid (LPS)-induzierter Aktivierung von Endothelzellen. Die humane Monozytenzelllinie THP-1 wurde mit 10 ng/ml LPS von *Pseudomonas aeruginosa* für 4 Stunden in Medium mit 10% Humanplasma stimuliert. Der Zellkulturüberstand enthielt LPS sowie andere ausgeschüttete Faktoren und wurde auf humane Nabelschnurendothelzellen (HUVEC) aufgebracht. Um die Stimulationsmuster von THP-1 Zellen mit jenen von Monozyten zu vergleichen, wurden letztere aus Humanblut isoliert und gleichermaßen im Modell eingesetzt. In einem zweiten Schritt wurden HUVEC mit der Zelllinie HPMEC ST1.6R verglichen. Beide Zelllinien wurden mit Zellkulturüberständen sowohl von THP-1 Zellen als auch von Monozyten stimuliert.

Die endotheliale Aktivierung wurde durch verschiedene Untersuchungsmethoden quantifiziert. Die Zytokinausschüttung [Interleukin-6 (IL-6), Interleukin-8 (IL-8) und Tumornekrosisfaktor-α (TNF-α)] wurde nach einer und 16 Stunden gemessen. Zusätzlich wurde die Expression der Adhesionsmoleküle ICAM-1 und E-Selektin und die Aktivität des Transkriptionsfaktors NF-κB nach 16 Stunden bestimmt.

Es konnten Unterschiede in der Zytokinausschüttung von stimulierten THP-1 Zellen und Monozyten beobachtet werden. Allerdings zeigten stimulierte HUVEC und HPMEC ST1.6R vergleichbare Mengen an IL-6, IL-8 und TNF-α für beide Stimulationsmedien. Die Expression von ICAM-1 war vergleichbar für stimulierte HUVEC. HPMEC ST1.6R zeigten allerdings nur eine geringfügige Expression von ICAM-1 bezüglich beider Stimulationsmedien. Generell wurde die erhöhte Expression von ICAM-1 deutlicher bei HUVEC als bei HPMEC ST1.6R beobachtet. Die Bestimmung von E-Selektin lieferte keine eindeutigen Ergebnisse, genauso wie die Quantifizierung von NF-κB. Vermutlich ist die für die Experimente nur in geringem Ausmaß verfügbare Proteinmenge die Ursache.

Das in dieser Diplomarbeit verwendete Zellkulturmodell simuliert relevante Prozesse der Sepsis in einem Zellkultursystem und kann als nützliches Hilfsmittel für präklinische Studien verwendet werden. Speziell hinsichtlich der LPS Stimulationsmuster von THP-1 Zellen und Monozyten sind allerdings weitere Studien zur Evaluierung notwendig.

Contents

1 Introduction

1.1 Definition of Sepsis

Sepsis is defined as a systemic inflammatory host response to infection. Various continuous stages are recognized with progressively increased risk of death from multiple-organ failure.

In 1991, the North American Consensus Conference of the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) introduced the term 'systemic inflammatory response syndrome (SIRS)' and consecutively defined the terms sepsis, severe sepsis, septic shock, sepsis-induced hypotension and multiple organ dysfunction syndrome (MODS) (Bone et al., 1992; ACCP/SCCM, 1992).

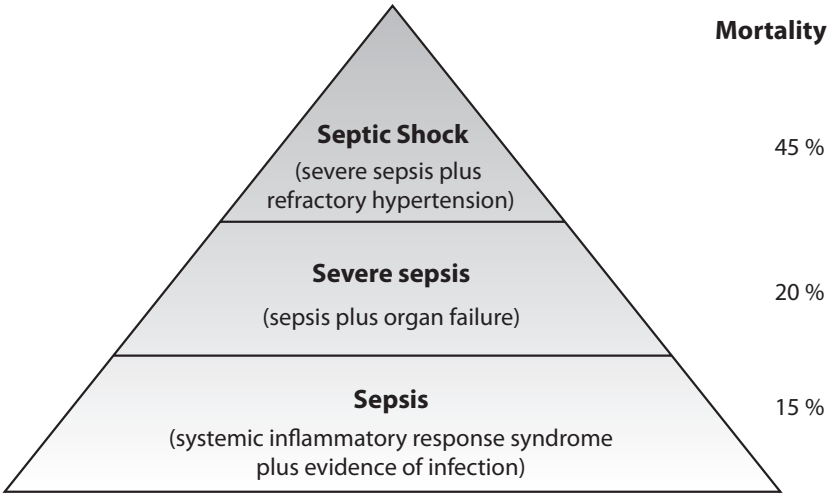

Figure 1.1: Various stages of the sepsis syndrome (adapted from Wenzel, 2002)

The term SIRS is used independently of its cause to describe the inflammatory process, including two or more of the clinical manifestations given in Table 1.1. If this inflammatory process is caused by infection, the term sepsis is applied. Sepsis with organ dysfunction is called severe sepsis. Septic shock is defined as severe sepsis with sepsis-induced refractory hypotension.

Revised knowledge about the pathophysiology and additional critical voices calling for a re-evaluation of the sepsis definitions (e.g., Vincent, 1997; Zahorec, 2000) were leading to another consensus conference of sepsis in 2001. Under the auspices of the SCMM, the ACCP, The European Society of Intensive Care Medicine (ESICM), The American Thoracic Society (ATS) and the Surgical Infection Society (SIS) the four criteria of SIRS have been expanded to a longer list of possible signs and symptoms. In addition, the PIRO-staging system for sepsis has been introduced (Levy et al., 2003b,a).

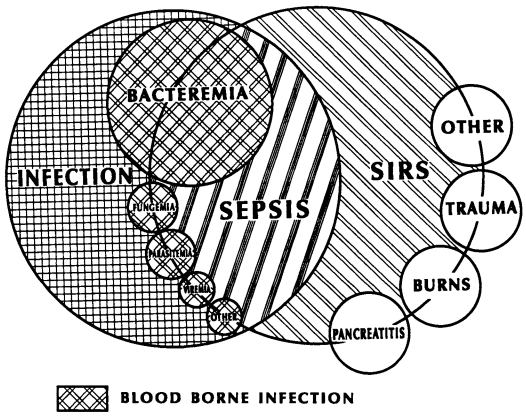

Figure 1.2: The interrelationship between infection, sepsis and SIRS (Bone et al., 1992)

The diagnostic criteria for sepsis agreed on in 2001 are a documented or suspected infection defined as a pathological process induced by a micro-organism as well as some of the non-sepsis-specific causes listed in Table 1.2.

The PIRO system is a classification scheme designed for sepsis using the **P**redisposition of the patient, the **I**nfection's nature, the **R**esponse of the host system and the **O**rgan dysfunctions for predicting the outcome in septic patients (see Levy et al., 2003a; Opal & Cross, 2005).

The PIRO staging system is at present a rather theoretical concept and thus not widely used in clinical practice so far. However, recent studies, e.g., by Moreno et al. (2008) and Rubulotta et al. (2009) rediscovered the PIRO system as a useful tool and showed attempts to apply the PIRO-system in practice. In contrast, the Acute Physiology and Chronic Health Evaluation (APACHE) score (Knaus et al., 1981) and its modifications as well as the Sepsis-related Organ Failure Assessment (SOFA) score (Vincent et al., 1996) are also widely applied in practice to predict survival probabilities for sepsis in Intensive-care Units (ICU). As the signs for SIRS are a helpful tool on bedside diagnostics, but none of these signs is specific for sepsis on its own, additional attempts are undertaken to find sepsis-markers for simplifying the bedside diagnosis process.

Systemic Inflammatory Response Syndrome (SIRS)
Body temperature > 38°C or < 36°C
Heart rate > 90 beats/min (tachycardia)
Respiratory rate > 20 breaths per minute or $PaCO_2$ < 32 mm Hg (Tachypnoe)
White blood cell (WBC) count > 12 x 10^9/l or < 4.0 x 10^9/l or 10% immature (band) forms

Table 1.1: The four main manifestations defining SIRS

Parameters	Symptoms
General	Fever (core temperature > 38.3°C) Hypothermia (core temperature < 36°C) Heart rate > 90 bpm or 2 SD above the normal value for age Tachypnea: > 30 bpm Altered mental status Significant edema or positive fluid balance (> 20 ml/kg over 24 hrs) Hyperglycemia (plasma glucose > 110 mg/dl or 7.7 mM/l) in the absence of diabetes
Inflammatory	Leukocytosis (WBC-count > 12,000/μl) Leukopenia (WBC-count < 4,000/μl) Normal WBC-count with > 10% immature forms Plasma C reactive protein > 2 SD above the normal value Plasma procalcitonin > 2 SD above the normal value
Hemodynamic	Arterial Hypotension (systolic blood pressure < 90 mm Hg, mean arterial pressure < 70, or a systolic blood pressure decrease > 40 mm Hg in adults or < 2 SD below normal for age Mixed or venous oxygen saturation > 70% Cardiac index > 3.5 l/minm^2
Organ dysfunction	Arterial hypoxemia (PaO_2/FiO_2 < 300) Acute oliguria (urine output < 0.5 ml/kgh or 45 mM/l for at least 2h) Creatinine increase ≥ 0.5 mg/dl Coagulation abnormalities (international normalized ratio > 1.5 or activated partial thromboplastin time > 60sec) Ileus (absent bowel sounds) Thrombocytopenia (platelet count > 100,000/μl) Hyperbilirubinemia (plasma total bilirubin > 4 mg/dl or 70 mmol/l)
Tissue perfusion	Hyperlactatemia (> 3 mmol/l) Decreased capillary refill or mottling

Table 1.2: Revised diagnostic criteria for SIRS from 2001 (Levy et al., 2003a)

Terminology	Consensus Definitions 1991
Infection	Microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue of microorganisms
Bacteremia	Presence of viable bacteria in the blood
SIRS	Clinical response arising from a non-specific insult, including two or more criteria from Table 1.1
Sepsis (= SIRS + evidence of infection)	Systemic response as a result to infection, including two or more criteria from Table 1.1
Severe Sepsis	Sepsis associated with acute organ dysfunction, hypoperfusion or hypotension
Septic Shock	Severe Sepsis with hypotension (despite adequate fluid resuscitation) along with the presence of perfusion abnormalities
Sepsis-induced Hypotension	Systolic blood pressure <90 mm Hg or a reduction of ≥ 40 mm Hg from the baseline in the absence of other causes for hypotension
Multiple Organ Dysfunction Syndrome (MODS)	Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention

Table 1.3: Definitions for Sepsis and Organ Failure (adapted from Bone et al., 1992)

1.2 Epidemiology

Severe sepsis affects the worldwide healthcare resources massively as it is a disease mainly treated in ICUs. It is the leading cause of death in the non-coronary ICU. Sepsis patients spend long time periods in ICUs, have a high mortality rate, are expensive to treat and cause additional costs through productivity losses due to premature death. In an international study, approximately 28% of infections in the ICU were associated with sepsis, 24% with severe sepsis and 30% with septic shock (Alberti et al., 2002).

Although the term "septicemia" is, due to its impreciseness, not recommended anymore to be applied for sepsis syndromes (Bone et al., 1992), the American Centers for Disease Control and Prevention (CDC) still uses this term in its regularly updated National Vital Statistics Reports. Septicemia was the 10^{th} leading cause of death for adults in the United States (U.S.) in 1999 and still was in 2005 (see Hoyert et al., 2001; Kung et al., 2008). Bacterial sepsis of newborns was even the 8^{th} leading cause for infant death.

Approximately 750,000 people are affected annually by sepsis and septic shock in the U.S., resulting in a mortality rate of approximately 28.6% or 215,000 deaths. Melamed & Sorvillo (2009) identified 6% sepsis-related deaths of all occurring cases of death in

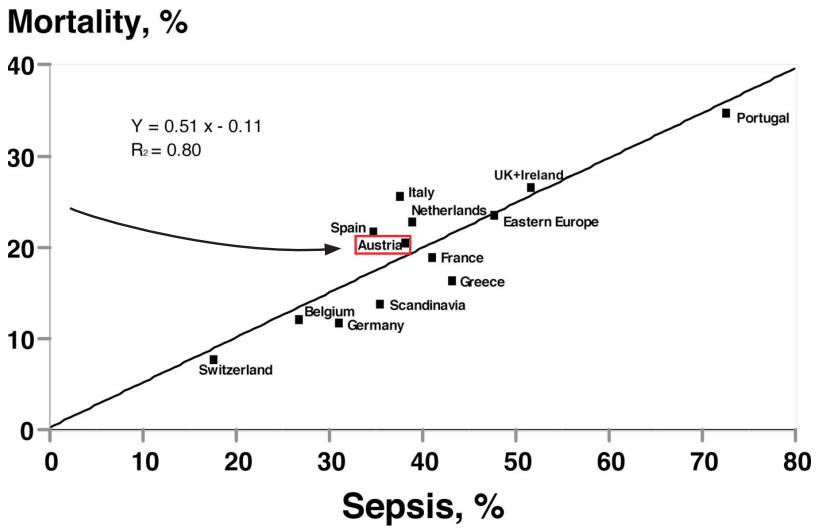

Figure 1.3: Relationship between mortality rates and frequency of sepsis in Intensive Care Units (ICU) in the SOAP-study (adapted from Vincent et al., 2006)

the U.S. from 1999 to 2005. Incidence and mortality of sepsis are increasing with the age of the infected patient. 22,100 USD average costs and an average length of stay of 19.6 days in the hospital per patient lead to general costs associated with severe sepsis of 16.7 billion USD annually (Angus et al., 2001). Between 1979 and 2000 an annualized increase of 8.7% of the incidence of sepsis was monitored by Martin et al. (2003) despite improved technical resources and better supportive treatment. The Emergency Departments (ED) are insofar affected, as patients with suspected severe sepsis are responsible for more than 500,000 visits in the ED per year (Wang et al., 2007). This means that the initial treatment of more than two-thirds of the annual U.S. sepsis cases is conducted in the ED. Rezende et al. (2008) pointed out that Brazilian ED teams were not able to diagnose more than one third of the overall incidences in the ED. As diagnosing sepsis as early as possible is crucial for treatment and apparently a high percentage of patients are initially presented to EDs, the proper training of staff should be a major concern of international health authorities.

In 2006 the Sepsis Occurrence in Acutely Ill Patients (SOAP) study was carried out in 22 European countries including Israel and observed an overall hospital mortality of 24.1%. More than 35% of the European patients had or developed sepsis during their stay in the ICU of which 27% died in the ICU, increasing to 32% and 54% for patients with severe sepsis and septic shock (Vincent et al., 2006). The wide range of hospital mortality of septic patients within the European countries is remarkable, varying from 14% in Switzerland to 41% in Portugal. The ICU mortality of sepsis patients could be identified in the same study with a range from 8% in Switzerland to 35% in Portugal (see Figure 1.3).

A German study (Engel et al., 2007) monitored a hospital mortality of 55% for severe sepsis which is higher than observed in other studies. The authors explained this fact with the higher median age in their study population which has been shown to be related with increased mortality rates. The extrapolated population based incidence of sepsis in Germany was 76 to 85 cases per 100,000 according to this study. Statistical data for deaths occurring in Austria is unfortunately not available as "Statistik Austria" is subsuming death causes according to the "International Statistical Classification of Diseases and Related Health Problems", 10^{th} revision, (ICD-10 codes) and sepsis is not explicitly stated as a cause of death. The "Wiener Klinische Wochenschrift" published an article by Schmid et al. (2002b) with an estimation of 6,700 to 9,500 patients with severe sepsis annually in Austria. The ICU mortality was 43.2% with a length of stay of 18.1 days and no gender differences. Non-survivors were equally expensive as survivors although their length of stay was shorter. This reflects the attempt to save lives with all possible means. Direct costs of 192 million EUR to 272 million EUR and indirect costs caused by productivity losses due to premature death of 484 million EUR to 686 million EUR were estimated. This would result in total costs of 676 to 958 million EUR annually for the Austrian health care system. However, it is questionable whether a sample size of only 74 patient records is sufficient to justify these assumptions. This objection has not kept these authors from conducting two similar surveys prone to the same criticisms in Germany and Switzerland (Schmid et al., 2004, 2002a). The sample size used in the Swiss study was 61 patient records and resulted in an assumption of 3,500 to 8,500 cases of severe sepsis per year. The Swiss severe sepsis patient costs 3,244 CHF (approximately 2,150 EUR) daily which results in an estimated burden of illness from approximately 326.4 to 793.8 million EUR per year.

In contrast to the sample size in Austria and Switzerland, the sample size of 385 cases in Germany seems relatively high. However, put in relation to the total population, this still yields the same questionable sample ratio. Their findings imply annual costs for severe sepsis of 3,647 to 7,874 million EUR to the German society. The mean age of German sepsis patients is 58 years, overall mortality in the entire patient population 42.6%. Table 1.4 gives an overview of the studies carried out by Schmid et al. As already mentioned, the data should be interpreted with care.

Sepsis rates could be proven to be highest in winter (Danai et al., 2007), and mortality rates are higher for men than for women (Schroeder et al., 1998). Most likely, this is due to the assumption that sex steroids influence the inflammatory response depending on genetical predispositions (Trentzsch et al., 2003). The major sites for infection are the respiratory tract followed by the abdomen, blood and the urinary tract (Vincent et al., 2006). Additionally, a slight pre-dominance of gram-positive organisms can be seen. According to Annane et al. (2005) gram-positive bacteria are responsible for 30–50% of infections, whereas just 25–30% are gram-negatively induced. It is worth to

Countries	Austria	Switzerland	Germany
Author: Schmid et al.	2002b	2004	2002a
Incidence/year (est.)	6,700–9,500	3,500–8,500	44,000–95,000
lenght of stay (d)	18.1 (±17.1)	12.9 (±9.9)	16.6 (±14.4)
Mortality (in %)	43.2	49.2	42.6
Direct costs			
daily (EUR)	1,617	2,148	1,318
total (mio EUR)	192–272	97–235	1,025–2,214
per patient (EUR)	28,582	27,665	23,297
Indirect costs			
total (mio EUR)	484–686	230–845	2,622–5,660
per patient (EUR)	72,240	65,714	59,584

Table 1.4: Overview of sepsis data from an economic perspective in Austria, Germany and Switzerland (adapted from Moerer & Burchardi, 2006)

draw attention to the circumstance that a growing number of infections are caused by multidrug-resistant or polymicrobial infections.

In general, an innumerable amount of studies on the epidemiology of sepsis have been carried out in recent years, emphasizing many different aspects of the syndrome. However, comparability is difficult as the research approaches, the methods and the outcome differ considerably. A useful overview is given in Angus & Wax (2001).

1.3 Pathophysiology of Sepsis

It is agreed that the host response to invading pathogens rather than the pathogen itself is responsible for the severity of the sepsis syndrome (Aird, 2003). A brief look into the pathophysiology of sepsis including the involvement of the coagulation cascade, the complement system and various cell types like monocytes and endothelial cells shall be given below.

1.3.1 Endotoxin

The term endotoxin is often used interchangeably with the term lipopolysaccharide (LPS) as the principal component of the outer membrane of gram-negative bacteria. It is the bacterial component involved in the initiation of the septic syndrome in gram-negative infections (Bosshart & Heinzelmann, 2007). LPS consists of three structural units: the O-specific chain, the core region and the lipid A component. Each of these regions bears immunogenic properties (Peters et al., 2003; Bone, 1993).

Gram-positive bacteria do not contain endotoxins, but structures as peptidoglycan (PGN) and lipoteichoic acid (LTA), which can also bind to surface receptors (Bosshart & Heinzelmann, 2007; Sriskandan & Altmann, 2008; Cohen, 2002).

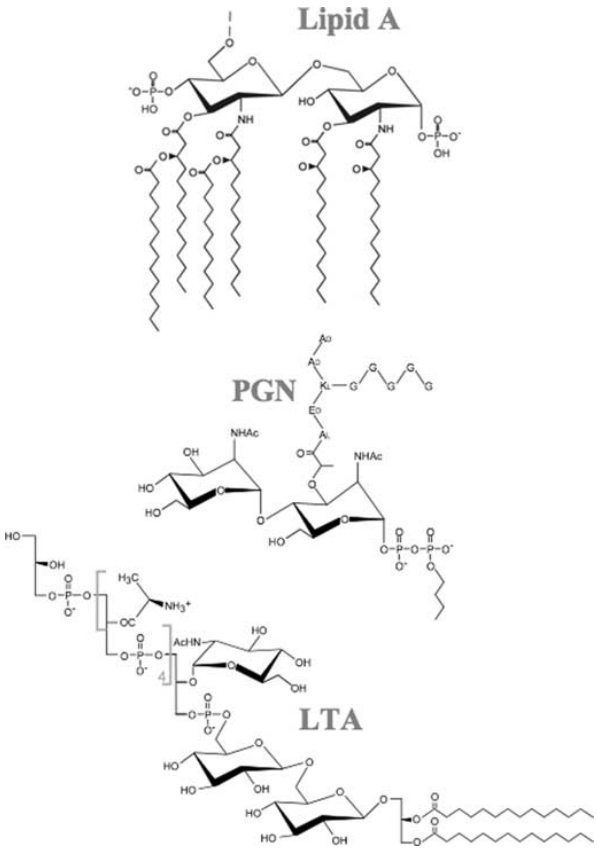

Figure 1.4: Structures of the Lipid-A component of LPS, Peptidoglykan and Lipoteichoic Acid (Bosshart & Heinzelmann, 2007)

1.3.2 Initiation of the Inflammatory Response

Innate immunity is based on receptors for recognizing danger-associated molecular patterns (DAMPs). These patterns can either be distinct molecular motifs of microbes, so called pathogen-associated molecular patterns (PAMPs), or alarmins, endogenous molecules produced by stressed or damaged cells involved in trauma or other tissue injuries (Bianchi, 2007; Opitz et al., 2007; Castellheim et al., 2009; Akira & Sato, 2003).

The receptors recognizing DAMPs are known as pattern recognition receptors (PRRs) and act as transmembrane or cytosolic receptors on the endothelium and immune competent cells like monocytes, macrophages, lymphocytes and neutrophils (Opitz et al., 2007; Castellheim et al., 2009). By binding of ligands to PRRs, signal transduction pathways are initiated. This leads to nuclear transcription factor activation which in turn up-regulates the expression of pro-inflammatory mediators. TLRs further amplify the inflammatory response by interaction with alarmins like heat-shock proteins (HSP) released after sepsis induced tissue injury (van der Poll & Opal, 2008).

Toll-like receptors (TLRs) are transmembrane cell-surface PRRs able to recognize bacterial, fungal, protozoal or viral structures. Another PRR family are the NOD-like

receptors (NLRs), which are in contrast to TLRs cytosolic molecules (Opitz et al., 2007; Takeda & Akira, 2005). Ten TLRs are currently known in humans with different ligand specificity, expression patterns and signalling pathways. Signalling of TLRs is in general very complex and involves different adaptor proteins (Warren, 2005).

TLR4 is the Toll-like receptor involved in sepsis events as it is an essential receptor for LPS recognition. Through degradation of bacterial cell walls, LPS starts to circulate in the bloodstream where it is able to associate with an acute-phase protein produced by the liver, the LPS-binding protein (LBP). The so formed complex binds with high affinity to CD14, a cell surface receptor on monocytes and macrophages. TLR4, as signal-transducing membrane protein, in turn senses CD14 (Bauer, 2002; Peters et al., 2003; Bosshart & Heinzelmann, 2007; Remick, 1995; Salomao et al., 2008; Glauser, 2000). This causes the subsequent stimulation of the adaptor protein myeloid differentiation primary-response protein 88 (MyD88) which results through a number of intermediate stages in activation of the IκB kinase complex (IKK). This complex phosphorylates and thereafter degrades IκB, the inhibitor of nuclear factor-κB (NF-κB). (Takeda & Akira, 2005; Castellheim et al., 2009; Sabroe et al., 2008; Biswas & Lopez-Collazo, 2009; van der Poll & Opal, 2008).

TLR2 is another Toll-like receptor involved in LPS-signalling. In addition, TLR2 is also necessary for the recognition of gram-positive structures like PNG and LTA (Akira & Sato, 2003; Sriskandan & Altmann, 2008).

1.3.3 The Transcription Factor NF-κB

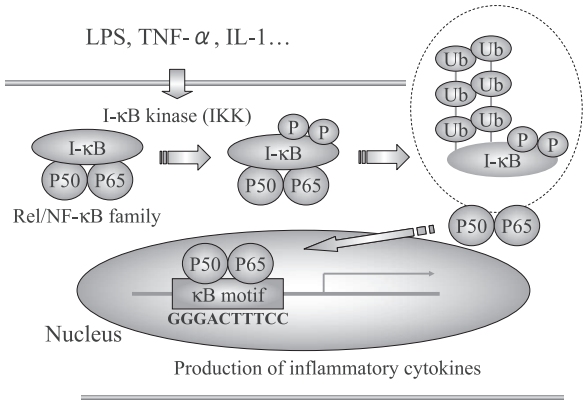

Figure 1.5: The NF-κB Signalling Pathway (Matsuda & Hattori, 2006)

The transcription factor NF-κB belongs to a family of factors playing an important part in the initiation of adaptive and innate immune response. Various stimuli can activate the transcription of more than 200 genes associated with inflammation and apoptosis (Castellheim et al., 2009).

NF-κB exists in an inactive heterodimeric form in the cytoplasm, where it is inhibited by IκB. If IκB is degraded, the translocation of NF-κB into the nucleus can take place which subsequently results in the binding to a specific consensus sequence and transcriptional activation of numerous pro-inflammatory mediators (O'Brien et al., 2007; Marshall, 2008; Matsuda & Hattori, 2006).

The regulation of NF-κB is a complex process involving various mediators. In addition, there are other pathways existent which can activate NF-κB without the involvement of IKK and IκB. NF-κB also mediates its own inhibitor IκB in a feedback loop (Castellheim et al., 2009; Remick, 1995).

1.3.4 Inflammation and Anti-Inflammation

Upon activation of NF-κB, a complex network of multiple pro-inflammatory cytokines, chemokines, adhesion molecules and acute phase proteins are secreted. This includes Tumor-necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), IL-2, IL-3, IL-5, IL-12, IL-18, Interferon-γ (IFN-γ), IL-6, IL-8, E-selectin, P-selectin, ICAM-1 and VCAM-1 to name just some.

This secretion causes activation of target cells including endothelial cells and leukocytes which enhance the pro-inflammatory reaction and further amplify the inflammatory process. During the secretion of pro-inflammatory mediators a compensatory anti-inflammatory response develops. This response involves the production of anti-inflammatory cytokines like IL-10 which are able to downregulate the production of the main pro-inflammatory cytokines TNF-α and IL-1. Ideally, these two responses are balanced and coordinated to conquer the invading infection. Imbalanced or uncontrolled regulatory mechanisms result in tissue damage and other host damaging processes (Rittirsch et al., 2008; Aird, 2003). Although anti-inflammatory events are necessary for controlling the systemic inflammation, a dominating anti-inflammatory response can also lead to immune system depression of the host (Rudiger et al., 2008; Adib-Conquy & Cavaillon, 2007). This is commonly described by the terms anergy, immunodepression or immunoparalysis (Annane et al., 2005). Cavaillon & Adib-Conquy suggest the term "leukocyte reprogramming" instead. However, the black and white picture of antagonists in inflammation is a huge oversimplification as several anti-inflammatory cytokines show additionally pro-inflammatory properties and some pro-inflammatory cytokines have anti-inflammatory characteristics as well.

Cytokines are small immunoregulatory proteins and constitute an important group of mediators in the pathogenesis of sepsis. All cytokines act through highly specific cell-surface receptors and are divided into pro-inflammatory and anti-inflammatory cytokines, although – as stated above – this strict separation is by no means in accordance with the complex relationships in the pathogenesis of the sepsis syndrome (Vincent & Abraham, 2006; Matsuda & Hattori, 2006). Cytokines are humoral components of the

innate immune system and act either as messengers between cells or organs or directly on invading pathogens (Rudiger et al., 2008).

Pro-inflammatory cytokines include TNF-α and IL-1 – the primarily produced inflammatory cytokines – as well as IL-2, IL-6, IL-8, IL-12 and IL-17. IL-1 and TNF-α as well as TNF-α and IFN-γ may act synergistically to trigger the inflammatory response (Tsiotou et al., 2005; Bone, 1993). The nature of IL-6 is janus-faced and controversial, as there is no evidence that it has distinct pro-inflammatory properties. Further, it is often mentioned as a cytokine which acts for dampening the inflammatory events (Dinarello, 1997; Opal & DePalo, 2000).

TNF-α is a primary mediator produced by activated macrophages. It amplifies and prolongs the inflammatory response by activating other cells to release cytokines and other mediators such as IL-1 and High-mobility group Box 1-protein (HMGB1) to promote further inflammation and tissue injury (Tracey, 2002). Macrophage migration inhibitory factor (MIF) is another pro-inflammatory cytokine produced by most immune competent cells. Unlike other cytokines, MIF has an individual receptor and protein structure and is stored intracellularly (Rittirsch et al., 2008). HMGB1, in contrast, is a cytokine-like nuclear binding-protein, which functions as a late-acting mediator in the inflammatory response. It is expressed by almost all nucleated cell types including monocytes, macrophages and neutrophils as well as being released by necrotic cells (van der Poll & Opal, 2008; Adib-Conquy & Cavaillon, 2007; Abraham & Singer, 2007; Cavaillon & Adib-Conquy, 2007; Cohen, 2002; Ulloa & Tracey, 2005).

Major anti-inflammatory cytokines include IL-4, IL-10, IL-11, IL-13, Transforming growth factor-β (TGF-β) and IL-1 receptor antagonist. They suppress gene expression and subsequent synthesis of IL-1, TNF-α and other pro-inflammatory cytokines (Dinarello, 1997; Hotchkiss & Karl, 2003; Tsiotou et al., 2005). With the exception of IL-1 receptor antagonist, all other anti-inflammatory cytokines have at least some pro-inflammatory properties as well (Opal & DePalo, 2000).

1.3.5 Monocytes and Macrophages

Monocytes are mononuclear phagocytes which circulate in the bloodstream. After one to three days they adhere to the endothelium and migrate into tissue where they differentiate into macrophages (see Section 1.3.7). Monocytes and macrophages have an important role in adaptive and innate immunity. They are able to ingest microorganisms through phagocytosis, produce pro- and anti-inflammatory mediators and macrophages additionally act as antigen-presenting cells to induce T-cell activation (Cavaillon & Adib-Conquy, 2005).

1.3.6 The Role of Endothelial Cells in Sepsis

Endothelial cells perform key functions in the innate immune response. They are highly metabolically active and consequentially play an important role in the pathogenesis of sepsis and other diseases.

The inner cellular lining of all blood vessels is formed by the endothelium, a single layer of flat endothelial cells. This results in a huge surface, a motive for seeing the endothelium as an immunological organ (Valbuena & Walker, 2006). Endothelial cells are in close and intimate contact with blood, blood cells and plasma proteins and connect circulating blood and the surrounding tissue (Aird, 2008; Dauphinee & Karsan, 2006; Hack & Zeerleder, 2001; Boos et al., 2006; Opitz et al., 2007).

They are functionally and phenotypically very heterogeneous according to their origin from different organs or species. Even cells from the same individual can respond differently to the same stimulus if the cells are located in different parts of the body. This phenomenon is referred to as endothelial cell heterogeneity or vascular diversity, resulting in differences in receptor and adhesion molecule expression, permeability and responses in different organs and organ regions to stimuli (Aird, 2004; Galley & Webster, 2004).

Their unique location allows endothelial cells to control several body functions. The regulation of blood flow and pressure, the control of coagulation and inflammation, the control of cell and fluid migration into tissues, the expression of adhesion molecules and the capacity to secrete chemokines and cytokines belong to the various functions of the endothelium. Several other functions in maintaining and regulating the hemostasis of organs, including vascular regulation and permeability, the growth of new blood vessels and regulating functions in angiogenesis are also part of the broad spectrum of endothelial functions (Hack & Zeerleder, 2001; Bauer, 2002).

Under physiological conditions, endothelial cells characteristically act anti-inflammatory, anti-coagulant, anti-adhesive and anti-aggregatory in their interaction with platelets and anti-adhesive in their interaction with blood leukocytes. Regarding the control of the coagulation process, endothelial cells can either release substances inhibiting coagulation (thrombomodulin, antithrombin, prostacyclin, nitric oxide) or substances for the promotion of the coagulation process (tissue factor, endothelin-1, plasminogen-activator-inhibitor-1-protein) (Becker et al., 2000; Vincent, 2001).

Under pathological conditions these characteristics can change rapidly, leading to a state referred to as “endothelial cell activation” or just “activation”, which describes the expression of an inflammatory phenotype of the endothelial cells. The endothelial activation can be caused by various mediators of inflammation like pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6. It is followed by the stimulation of pro-coagulant and pro-inflammatory pathways, which cause tissue damage up to organ failure in the host (Volk & Kox, 2000; Dauphinee & Karsan, 2006; Vallet & Wiel, 2001). Activated endo-

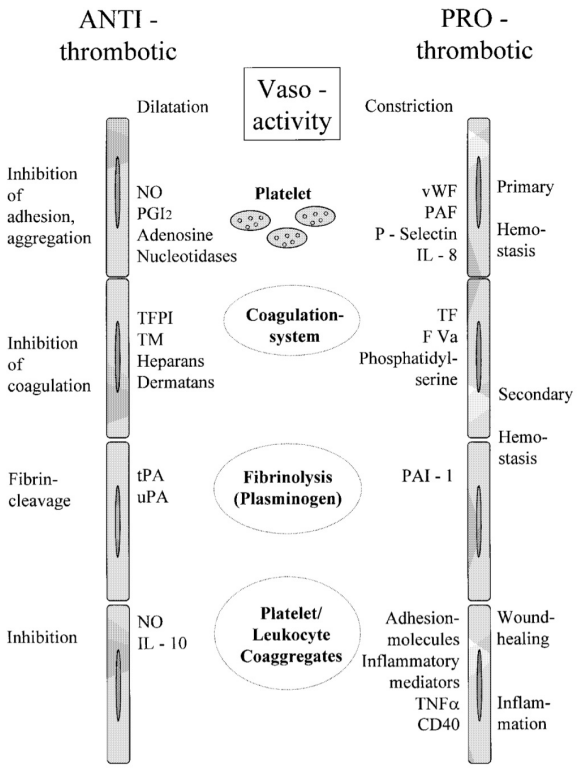

Figure 1.6: Anti-thrombotic and Pro-thrombotic functions of the endothelium (Becker et al., 2000)

thelial cells further release inflammatory mediators themselves, amplifying the immune response (Bauer, 2002; Hack & Zeerleder, 2001).

Aird (2004) considers the view of the activated endothelium as pro-coagulant and pro-adhesive as oversimplified and rather to be exchanged through the perception of activation as a spectrum of responses occurring under physiologic as well as pathophysiologic circumstances.

The endothelium in combination with monocytes plays a central role in the initiation of the host response to infection. Endothelial cells are among the first cells in the body that are exposed to circulating endogenous substances such as TNF-α and LPS in the bloodstream. They possess mechanisms that recognize structural patterns of bacterial pathogens and subsequently initiate the expression of transmembrane molecules such as Toll-like receptors. This process in turn activates various transcription factors including NF-κB, which regulates the expression of inflammatory mediators like cytokines, chemokines, and adhesion molecules (Valbuena & Walker, 2006; Opitz et al., 2007; Dauphinee & Karsan, 2006) (see Chapter 1.3.2).

The endothelium is a major target of sepsis-induced events and endothelial cell damage accounts for much of the pathology of septic shock. Severe sepsis frequently shows signs of excessive, unregulated and sustained generalized activation of the endothelium,

further leading to endothelial dysfunction and damage. Extensive cross-talk, feedback and overlapping between inflammation and coagulation can also be observed in sepsis (Boos et al., 2006; Peters et al., 2003; Aird, 2003).

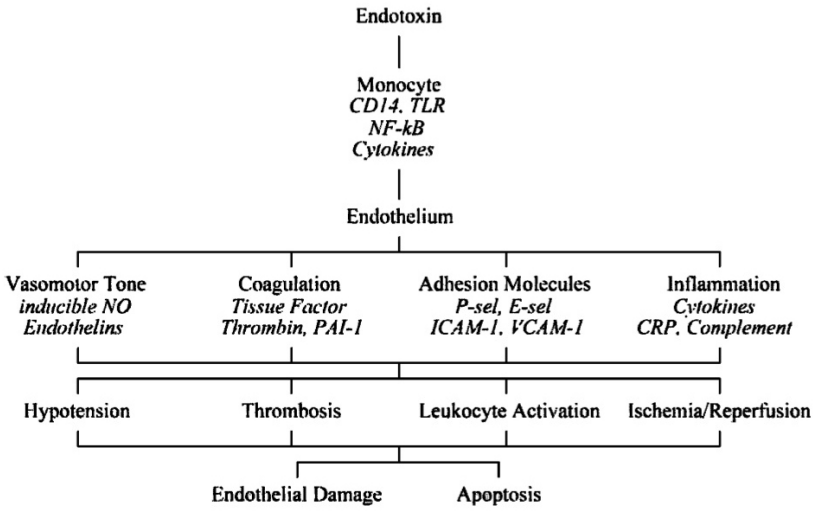

Figure 1.7: The endothelium as a pivotal point in Sepsis (Bauer, 2002)

The capacity of endothelial cells to adjust to local requirements is most likely the reason for the ability to change characteristics and phenotypes if they are cultured in vitro. This seems to be further influenced by isolation methods, culture conditions and purification protocols and is increasing with the number of cell passages (Volk & Kox, 2000). The most frequently used model cells, HUVEC, originate from the placenta, an organ not existent in a majority of patients. Therefore, comparison of experimental results from cell culture models to results from in vivo studies or other endothelial cell types, especially microvascular cells, is necessary for cross-checking (Valbuena & Walker, 2006).

1.3.7 Adhesion Molecules

A major step in inflammation is leukocyte recruitment by the endothelial surface designated as leukocyte transendothelial migration (TEM). It has been described as a multistep cascade involving soluble and membrane bound factors additionally to a variety of adhesion molecules. These molecules are hardly expressed under physiological conditions; but their surface expression and modulation is highly regulated by chemical mediators like cytokines. If inflammation is triggered by cytokines, endothelial cells start to produce various transmembrane receptors like selectins, intercellular adhesion molecules (ICAM), endothelial leukocyte adhesion molecules (ELAM) and vascular cell adhesion molecules (VCAM).

The process of leukocyte migration can be described in a three step sequence; the

first step is the interaction and "rolling" of leukocytes over the endothelial cells. The second step includes strong adherence to the endothelium and finally leads to the last step: migration into the tissue.

Selectins are adhesion molecules on endothelial cells (E-selectins), leukocytes (L-selectins) or platelets (P-selectins) and are receptors necessary and responsible for rolling on the endothelium. Consequentially, selectins play a role in the first step of leukocyte migration. The second step involves receptors from the integrin family, which are responsible for leukocyte arrest and strengthening of the adhesion. The expression of cell surface adhesion molecules including ICAM, ELAM, VCAM or platelet endothelial cell adhesion molecule, also takes place in this step. The final step comprises of the transmigration of activated leukocytes by platelet-endothelial cell adhesion molecule-1 (PECAM-1) and ICAM-1 (Hack & Zeerleder, 2001; Vallet & Wiel, 2001; Volk & Kox, 2000; Yang et al., 2005; Michiels, 2003). Once migrated into the tissue, leukocytes are drawn to microbes by chemotaxis. Through the recognition of PAMPs (see Chapter 1.3.2) they bind to the pathogens and subsequently phagocytosis takes place (Abraham & Singer, 2007).

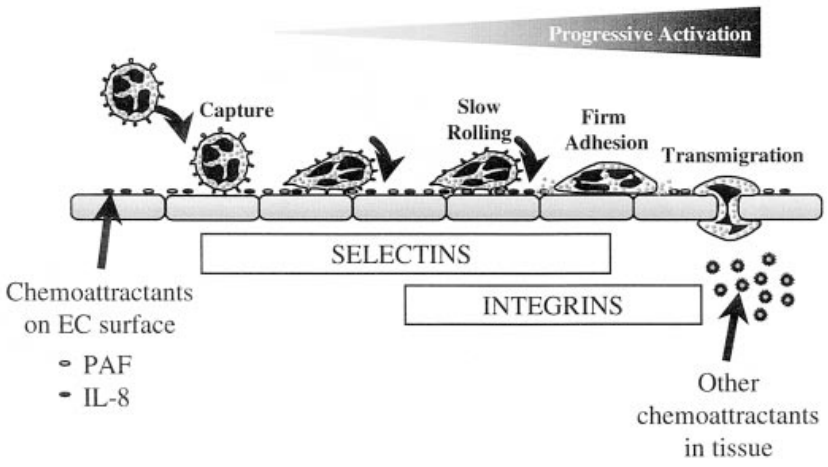

Figure 1.8: Leukocyte rolling on the endothelium (Jung et al., 1998)

Monocytes and neutrophils follow the same basic mechanisms for tissue migration, but have differences in adhesion molecule expression and response to cytokines and chemokines (Michiels, 2003).

1.3.8 Role of the Coagulation System

The human blood coagulation system proceeds through a series of proteolytic reactions of linked pre-enzymes culminating in the generation of fibrin. Serine proteases in the series are cleaved and activated in a sequential manner. The highly complex cascade can either be activated by the intrinsic pathway (contact activation pathway) or the

extrinsic pathway (tissue factor pathway). Both converge to a common pathway with the formation of thrombin and subsequently to insoluble fibrin. Together with cellular components like platelets, these fibrin molecules form a blot clot to arrest bleeding at the damaged blood vessel. All reactions within the clotting cascade occur on phospholipid membranes provided by endothelial cells, monocytes, platelets and neutrophils. Under normal physiological conditions, a constant generation of small amounts of coagulation proteases and natural anticoagulant proteins are in a delicate balance. During the pathological alterations during sepsis, this delicate state is out of control (Spronk et al., 2003; Esmon, 2000; Faust et al., 2001; Meijers & Bouma, 1999; Sidelmann et al., 2000).

The initiation of the coagulation process starts after a damage to the endothelial surface of a blood vessel which exposes coagulation factors like Tissue Factor (TF) in the blood to sub-endothelial proteins. This results in the formation of a complex with factor VIIa, down-streamed production of factor X and subsequently thrombin, which in turn activates platelets to the fullest and increases platelet adhesion. Subsequently, large amounts of thrombin are produced on the surface of activated platelets resulting in a stabilization of the blood clot. An alternative initiation of the coagulation cascade can take place when the contact system is activated by endotoxin or negatively charged particles inducing the synthesis of TF on leukocytes (Amaral et al., 2004; Esmon, 2004a).

Blood clots can be absorbed by fibrin degradation (fibrinolysis), which is a cascade itself. Plasmin is the main protease in this system. Under normal physiologic conditions, the balance between coagulation and fibrinolysis is maintained by regulatory mechanisms and prevents intravascular coagulation (Rittirsch et al., 2008; Amaral et al., 2004).

Extrinsic Pathway

The extrinsic pathway is the primary mechanism of thrombin generation in severe infections and sepsis. It provides a very rapid response to tissue injury, generating factor Xa almost instantly after vessel damage (Opal & Esmon, 2003; Opal, 2003; Amaral et al., 2004; Opal, 2004; van der Poll, 2008). Tissue factor (TF) is a membrane-bound glycoprotein expressed on various cells. Monocytes and endothelial cells can express TF upon induction by pro-inflammatory cytokines or endotoxin. After vessel trauma, TF becomes exposed to blood and complexes factor VII catalytically on phospholipid surfaces. The TF:factor VII complex subsequently converts factor IX and factor X into their active forms (IXa, Xa). Consecutively, factor Xa and its co-factor Va form the pro-thrombinase complex, which massively amplifies the conversion of pro-thrombin into thrombin on activated platelet membranes in presence of calcium as co-factor (Levi et al., 2003; Dahlbäck, 2000; Amaral et al., 2004; Gullo et al., 2005; Hoffman & Monroe, 2001; Levi & Poll, 2004; Schouten et al., 2008; Markiewski & Lambris, 2007; van der Poll, 2008; Choi et al., 2006; Meijers & Bouma, 1999; Esmon, 2004b).

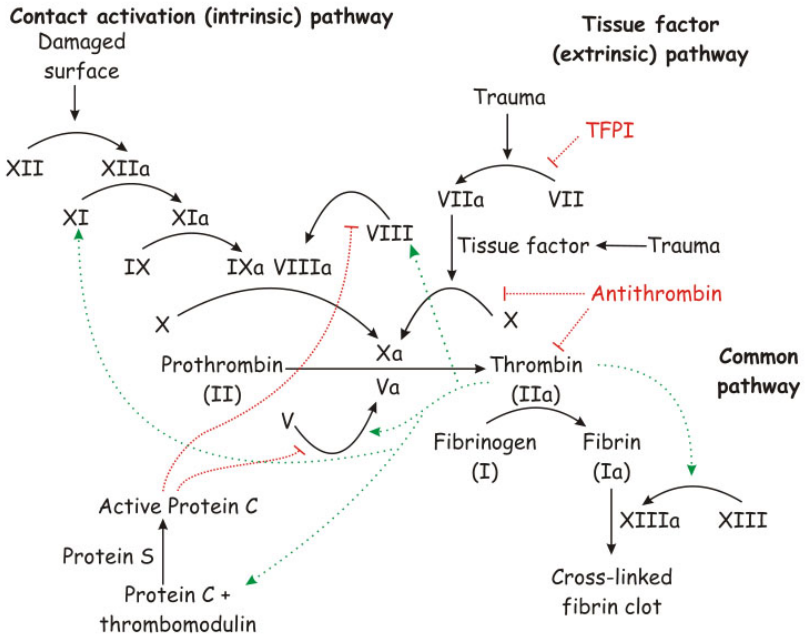

Figure 1.9: The Coagulation Cascade (This figure is taken from http://en.wikipedia.org/wiki/File:Coagulation_full.svg, 25.08.2010.)

Intrinsic Pathway

The intrinsic coagulation pathway is activated after plasma exposure to activating surfaces including phospholipids and glycosaminoglycans. The highly negative charged LPS is the prototype of microbial induction of the intrinsic pathway (O'Brien et al., 2007; Sidelmann et al., 2000; Levi et al., 2003; Opal & Esmon, 2003). Proteins involved in the contact reactions are pre-kallikrein, factor XII (Hageman factor), high-molecular weight kininogen (HMWK) and factor XI. If these components assemble on a negatively charged surface, pre-kallikrein is converted into kallikrein, the Hageman factor is activated and in turn activates factor XI. The activated factor XIa activates the tenase complex consisting of factor IXa, VIIIa and phospholipids. This complex eventually activates factor X, the first molecule of the common pathway (Meijers & Bouma, 1999; Sidelmann et al., 2000; Amara et al., 2008). The initiation of the intrinsic pathway can take place without contact activation as factor IX can directly be activated by the TF:factor VIIa complex from the extrinsic pathway. Furthermore, factor XI can be directly activated by thrombin (Spronk et al., 2003).

Common Pathway

The extrinsic and the intrinsic pathway of the coagulation cascade start converging to a common pathway with the activation of factor X and pro-thrombin conversion into thrombin. Thrombin itself represents a central role in the coagulation process by

cleaving fibrinogen into fibrin, the main component of a hemostatic clot. In addition, it activates factor XIII, which is a transglutaminase necessary for the cross-linking and stabilization of fibrin to a solid blood clot. Thrombin has pro-coagulant effects on one hand by activating factor VIII and V; on the other hand, it exerts anticoagulant properties through protein C activation and the promotion of prostacyclin formation. Furthermore, thrombin can induce the activation of adhesion molecules and the platelet-activating factor, leading to cell proliferation and inflammation. Thrombin therefore maintains a pro-coagulant state by continued activation of the tenase-complex (factor VIIIa:IXa) until down-regulating effects of the likewise activated anticoagulant pathways occur (Vincent, 2001; Amaral et al., 2004; Faust et al., 2001; Meijers & Bouma, 1999; Sidelmann et al., 2000).

Anticoagulant Pathways

Since undiminished generation of thrombin would lead to uncontrolled clotting, three major anticoagulant proteins are controlling the blot clot formation: Tissue-factor pathway inhibitor (TFPI), antithrombin (AT) and activated protein C (APC) (van der Poll, 2008; Sidelmann et al., 2000). The fibrinolytic system acts as an additional mechanism for the limitation of fibrin generation (Opal, 2003, 2004). These regulatory systems are continuously active under physiological conditions. As the endothelium is a key factor in maintaining the anticoagulant network, endothelial dysfunction as observed in severe infection is leading to a defect of all three pathways (Levi & Poll, 2004; Schouten et al., 2008). This further enhances the inflammatory process, as the anticoagulant pathways not just limit the coagulation response but also display a broad spectrum of anti-inflammatory activities themselves. These activities include suppression of apoptosis, inhibition of NF-κB signaling resulting in reduction of cytokine expression, minimization of endothelial cell interactions as well as minimizing leukocyte chemotaxis and modulation of diverse complement components (Opal, 2004; Esmon, 2004a).

Antithrombin (AT) is a hepatic plasma glycoprotein acting as a direct protease inhibitor. It is the predominant inhibitor of thrombin and factor Xa but inactivates a number of more serine proteases including the factors VIIa, IXa and XIIa (Levi & Poll, 2004; Choi et al., 2006; Amaral et al., 2004; Schouten et al., 2008). The rate of inactivation thereby is catalyzed by glycosaminoglycans (GAGs) like heparin or related heparan sulphate located at the endothelial cell surface. Specific pentasaccharide-fragments present in GAGs are binding to a central domain of AT resulting in a conformational change. This links AT covalently to the active binding site of the serine protease and thereby accelerates its inactivation. The use of heparin as a therapeutical anticoagulant rests on this molecular mechanism (Dahlbäck, 2000; Spronk et al., 2003; van der Poll, 2008; Levi et al., 2003; Opal, 2004, 2003). The AT-heparin mechanisms can also contribute to anti-inflammatory properties in addition to its anticoagulant activities. It reduces the

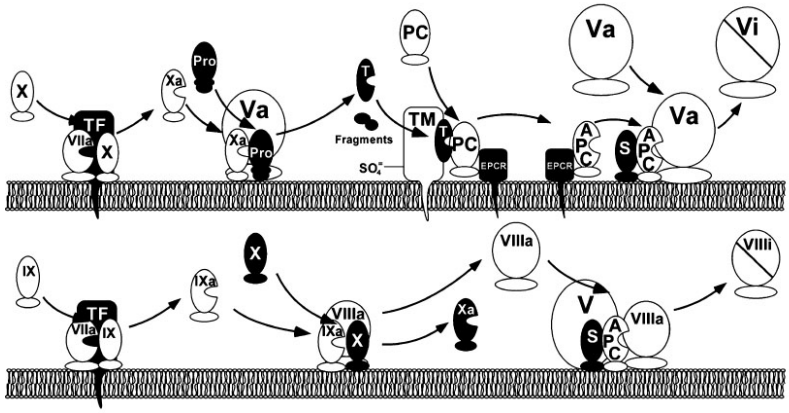

Figure 1.10: Model of the protein C anticoagulant pathway (Esmon, 2004b)

expression of IL-6 and TNF-α as well as leukocyte rolling on the endothelium. It further inhibits activation of NF-κB (Schouten et al., 2008; Opal & Esmon, 2003; Opal, 2003; Esmon, 2004a). During severe sepsis, a decrease of AT to less than 50% of normal levels could be observed (Esmon, 2004b). Reasons are impairment of the synthesis process, consumption and degradation through activated neutrophils (Levi & Poll, 2004; Esmon, 2004a). Pro-inflammatory cytokines active in severe infection reduce the synthesis rate of GAGs on the endothelial surface, which further reduces the AT function (Schouten et al., 2008; Levi & Poll, 2004).

Tissue factor pathway inhibitor (TFPI) is a protease inhibitor released by stimulated and damaged endothelial cells. Under normal conditions, it is endothelial cell-associated via GAGs but it additionally exists bound to lipoproteins in plasma (Schouten et al., 2008; Levi & Poll, 2004; van der Poll, 2008). TFPI specifically blocks factor X by complexing it. The originated TFPI:factor Xa complex subsequently binds to the TF:factor VIIa complex resulting in a quaternary compound and inactivation of TF:factor VIIa. This inhibition mechanism rapidly starts once factor Xa molecules are formed, deactivating the generation of thrombin (Spronk et al., 2003; van der Poll, 2008; Faust et al., 2001; Schouten et al., 2008; Choi et al., 2006; Meijers & Bouma, 1999; Esmon, 2004a; Levi & Poll, 2004; Opal & Esmon, 2003; Amaral et al., 2004; Opal, 2003).

The protein C pathway is the most dominant anticoagulation system and acts in a classic negative feedback loop. It controls the coagulation system by the ability of activated protein C (APC) to inactivate the cofactors Va and VIIIa (Schouten et al., 2008; Choi et al., 2006; Dahlbäck, 2000). The pathway is initiated through the binding of thrombin to thrombomodulin (TM) on the endothelial surface. Therefore, TM acts as a molecular switch for turning thrombin from a pro-coagulant mediator into an anti-coagulant activator (Schouten et al., 2008; Dahlbäck, 2000).

The activation step of protein C is catalyzed by thrombin. As mentioned above, it

binds to TM, a specific, membrane-associated protein receptor expressed on endothelial cells as well as monocytic surfaces. The formation of this complex converts the inactive vitamin-k dependent protein C into activated protein C. The presence of the endothelial protein C receptor (EPCR-1) augments this activation. If present, protein C binds to the EPCR-1 and this complex is activated by the thrombin:TM complex. If not present, the protein C is directly activated by the thrombin:TM complex. Once activated, APC dissociates from the EPCR-1 and interacts with its cofactor protein S to a membrane-bound complex. This proteolytically inactivates the two critical coagulation cofactors Va and VIIIa by cleavage resulting in a slowdown of thrombin generation and inhibition of clot formation (Esmon, 2000; Hoffman & Monroe, 2001; Vincent, 2001; Spronk et al., 2003; McGilvray & Rotstein, 1998; Faust et al., 2001; Levi et al., 2003; Dahlbäck, 2000; Faust et al., 2001; Esmon, 2004b; Opal & Esmon, 2003; Amaral et al., 2004; Levi & Poll, 2004; Esmon, 2004a; Opal, 2003).

Furthermore, APC has important anti-inflammatory and anti-apoptotic properties: it inhibits the generation of IL-1, TNF-α and TF as well as E-selectin mediated cell adhesion (Vincent, 2001; Rittirsch et al., 2008). In addition, it decreases the apoptosis of endothelial cells and lymphocytes (Rittirsch et al., 2008), dampens NF-κB translocation and cytokine signaling (Schouten et al., 2008; Esmon, 2004b; Levi & Poll, 2004). The anti-inflammatory effects of APC combined with its effects as an endogenous anticoagulant is likely responsible for the success of recombinant APC for the treatment of human sepsis (Opal, 2004). During sepsis, the APC system is impaired by the inflammatory process. This is the result of a decrease in the production of protein C in the liver as well as a down-regulation of TM expression at the endothelium. Furthermore, there is an enhanced consumption of protein S and protein C during sepsis (Schouten et al., 2008; Levi & Poll, 2004; Esmon, 2004a; Choi et al., 2006).

The fibrinolytic system is an additional modulation process for hemostasis. It limits clotting and regulates degradation of fibrin under physiological conditions. Initiation starts when plasminogen is converted by various proteases (e.g., tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA)) into plasmin. Plasmin itself degrades fibrin, fibrinogen, factor V and factor VIII. As inhibition factors of the plasminogen activators (PA) act the plasmin inhibitor and plasminogen activator inhibitors (PAI). PAI-1 is generated by endothelial cells as well as the liver and inactivates tPA and uPA through binding. It is the main down-regulator of plasminogen and is simultaneously increased when plasmin is generated (Amara et al., 2008; Opal & Esmon, 2003; Schouten et al., 2008; Opal, 2003; Sidelmann et al., 2000). During sepsis, the fibrinolytic system is rapidly activated by pro-inflammatory reactions. PAI-1 expression is up-regulated, whereas PA synthesis is decreased by TNF-α and IL-1 (Levi & Poll, 2004; Schouten et al., 2008; O'Brien et al., 2007).

Coagulation and Sepsis

Coagulation as well as its complex interaction with the inflammatory system is pivotal in the host defense system and subsequently in sepsis (Vincent, 2001; Choi et al., 2006). Septic patients, experimental and clinical ones respectively, show in most cases signs of activated coagulation processes, promoting thrombus and clot formation. Of primary importance here is the extrinsic pathway via TF and thrombin activation. Due to circulating pro-inflammatory cytokines during severe sepsis, monocytes and endothelial cells are activated and the expression of TF on their surface is up-regulated. In turn, TF activates various proteolytic cascades resulting in thrombin formation which generates fibrin from fibrinogen. This causes a systemic activation of the coagulation system and results almost always in major complications ranging from insignificant levels to the appearance of disseminated intravascular coagulation (DIC) (van der Poll, 2008; Sriskandan & Altmann, 2008; Rittirsch et al., 2008; Cohen, 2002; Opal, 2003). DIC is defined as a pathological activation of the coagulation cascade associated with increased mortality and resulting in consumption of coagulation factors, platelet dysfunction and inhibition of fibrinolysis. Consequentially thrombosis occurs throughout the body, which leads to prolonged clotting times and abnormal bleeding up to severe complications (Rittirsch et al., 2008; O'Brien et al., 2007; Tsiotou et al., 2005; Remick, 2007; Meijers & Bouma, 1999).

An additional cause of the pro-coagulant state in sepsis is the impairment and down-regulation of the anti-coagulation pathways antithrombin, TFPI and the protein C system in the majority of severe septic patients (van der Poll, 2008; Sriskandan & Altmann, 2008; Cohen, 2002). This is coupled with elevated levels of PAI-1 in bloodstream and tissue and further enhances the DIC. In addition to their effect on thrombin generation, these natural anticoagulants have anti-inflammatory properties by inhibition of NF-κB, which dampens the release of monocyte-derived TNF-α (Rittirsch et al., 2008; O'Brien et al., 2007; Cohen, 2002).

Coagulation and Complement

The complement and the coagulation cascades are both proteolytic systems composed of serine proteases with similar activation patterns and structural characteristics. These cascades are linked to a complex cross-talking network by many connections. During sepsis the coagulation system is activated and enhances the inflammatory response which in turn activates the complement cascade (Choi et al., 2006; Esmon, 2004a). To name but a few examples, the classical pathway of the complement system is activated through the complement factor C1 as well as through the coagulation-factor XIIa. Furthermore, thrombin can activate C5 independently from C3 and is able to promote the activation of pro-inflammatory events including cytokine production and C5a generation. In turn, these cytokines have stimulatory effects to the coagulation cascade. The complement

system can amplify the coagulation response by modifying phospholipid-membranes, activating platelets and inducing the expression of TF by leukocytes. If anti-coagulant mechanisms are inhibited, pro-coagulant properties of the complement are increased. To summarize, the complement and the coagulation system are – together with the fibrinolysis system – closely linked through various direct interactions of the involved serine proteases. With focus to sepsis, this crosstalk is of special importance as the uncontrolled activation of either system is an essential contributor to the syndromes' pathology (Rittirsch et al., 2008).

1.3.9 Role of the Complement System

As an important part of the innate immune system, the complement is a set of over 30 different soluble serum proteins able to eliminate extracellular pathogens. The proteins are primarily produced in the liver and circulate as inactive precursors in blood and extracellular fluids. Their nomenclature is historically grown and discerns "components" (C) from "factors" (F) (Haeney, 1998).

Three different pathways for activating the complement are known so far: the classical, the alternative and the lectin pathway. In all three pathways activation of proenzymes implements an amplifying cascade, culminating in the activation of C3 followed by formation of the membrane attack complex (MAC). This complex is built out of different complement proteins (C5/C6/C7/C8/C9) and perforates the membrane of the pathogen, which results in lysis and death of the target cell. Lower doses of MAC can additionally activate endothelial cells to release pro-inflammatory chemokines (Ward, 2008b).

Binding of IgG or IgM antibodies to the pathogen surface – i.e. the classical antigen-antibody complex – activates the classical pathway. The alternative pathway, in contrast, is directly activated by recognition of exogenous surfaces. More specifically, the discrimination between self and non-self is recognized by repeating patterns of molecular structures on pathogenic surfaces called pathogen-associated molecular patterns (PAMPs) (Markiewski et al., 2007). In addition, the alternative pathway can also be activated through the classical or lectin pathway, reinforcing their impact. The recognition of bacterial surface sugars through mannose-binding lectin initiates the lectin pathway (Walport, 2001a; Goldfarb & Parrillo, 2005).

Besides the three established complement activation pathways, serine proteases belonging to the coagulation system can activate the complement cascade as well (Amara et al., 2008; Walport, 2001b). Thrombin, for example, represents a connection between coagulation and complement system. It has been demonstrated in rabbits to have the ability to indirectly activate the complement via the classical pathway (Ward, 2008a). In addition, acute phase proteins, which are increased during sepsis, activate the complement also in a pathogen-independent manner (Markiewski et al., 2008).

Another aspect of the complement system is the opsonisation, where invading microbes

are coated with complement products, resulting in neutralization by neutrophils and macrophages. A special complement protein (C5a) additionally acts chemotactic for phagocytic cells, meaning it causes enzyme secretion from neutrophils and macrophages to destroy cells nearby (Ward, 2008b).

The role of the complement system in the pathophysiology of sepsis is still not fully understood. Sepsis is definitely associated with a strong activation of the complement system. Furthermore, complement is assumed to be an important factor in inflammation in general, as the complement activation products C3a and C5a have pro-inflammatory effects (Markiewski & Lambris, 2007). It appears also possible that the complement has additional anti-inflammatory characteristics. A further indication for complement involvement in the pathophysiology of sepsis is the increase of complement activation products like C3a, C4a and C5a in plasma and serum in humans and animals with septic events (Goldfarb & Parrillo, 2005; Ward, 2008a,b; Guo & Ward, 2006). Interestingly, mice and rabbits deficient in certain complement proteins show increased susceptibility to LPS-induced sepsis and endotoxin shock (Czermak et al., 1999; Flierl et al., 2008). On the other hand, therapeutical potential is believed to be in the blockade of C5a and its receptor. First experiments showed decreased mortality rates in a rat sepsis model (CLP) for this therapy (Goldfarb et al., 2005; Ward, 2008a; Guo & Ward, 2006).

Markiewski et al. consider the role of the complement system nowadays as follows: *"Today, complement should rather be viewed as a system that orchestrates and connects various responses during immune and inflammatory reactions and not merely as a killer of bacteria."*

1.4 Therapeutic Options

During the past decades an enormous amount of studies in the field of sepsis treatment have been carried out. Despite that, therapeutic options to target and treat sepsis are limited as very few promising compounds made the transition from pre-clinical trials to valid therapeutic means. To date the only approved drug for treatment is recombinant activated protein C, also known as drotrecogin alfa. Generally, targeting the host response rather than the infecting pathogen has become the strategy of choice for the current sepsis therapies (Wheeler et al., 2009).

Sepsis patients require intensive treatment in the ICU. In addition to basic standard care and individual organ support, four key principles should be considered: *infection control* by removal of the infectious cause and antimicrobial therapy, *hemodynamic support* by fluid administration, *immunomodulatory interventions* and *metabolic or endocrine support*, respectively, by corticostereoids. The latter has been a controversial subject for several years. These guidelines have been published in 2002 in a concerted effort of professional societies and leading experts in the field of sepsis research and are

known under the terminus "Surviving Sepsis Campaign" (SSC) (Vincent, 2008; Dellinger et al., 2004, 2008).

Early detection and diagnosis of sepsis as well as rapid and aggressive surgical eradication of the infectious source are beneficial in the early state of sepsis. In combination with antimicrobial therapy it has to be performed as soon as possible for improving survival rates. Nevertheless, polymicrobial infections and rare or resistant microorganisms complicate infection control (Vincent, 2008; Wheeler & Bernard, 1999).

The field of corticosteroid administration in order to support the metabolic body functions is still controversial. As a result of their anti-inflammatory effects, low-dose glucocorticoids seemed a promising tool to improve survival rates and reduce organ failure (Minneci et al., 2009; Goodman & Sprung, 2002). However, corticosteroid therapy did not reduce mortality rates in two meta-studies (Sligl et al., 2009; Annane et al., 2009) and is still object of discussions (Japiassú et al., 2009). Intensive insulin therapy to combat hyperglycaemia in the septic patient slightly improved survival rates. The controversy surrounding this therapy is due to difficulties in the implementation of tight glucose control and questionable benefits (van den Berghe et al., 2001; Hirasawa et al., 2009).

As mentioned above, only recombinant activated protein C (activated drotrecogin alfa) has been verified to improve survival rates in septic patients. It has been approved by the Food & Drug Administration (FDA) in the U.S. as well as European pharmaceutical regulatory authorities and is currently marketed as XIGRIS© by Eli Lilly. Recombinant APC, a natural anticoagulant (see Section 1.3.8), is associated with increased risk of bleeding and is furthermore a very cost-intensive drug. In turn it reduced mortality dramatically over 28 days in a large multicenter study (PROWESS - Protein C Worldwide Evaluation of Severe Sepsis). Guidelines of the SSC suggest limiting the use of APC for severe sepsis cases with high risk of death and no bleeding disorders. Studies showed that APC is not effective in children or patients with a low risk of death (Bernard et al., 2001; Marshall, 2003; Vincent, 2008; Wheeler et al., 2009). Other immunomodulatory strategies focussing on endogenous anticoagulants include recombinant antithrombin and tissue factor pathway inhibitor. Both substances showed no increase in survival rates (Riedemann et al., 2003; Vincent, 2001; Wittebole et al., 2008; Warren et al., 2001).

Using the massive amount of circulating cytokines in the pathophysiologic process of the sepsis syndrome seemed a promising idea for therapeutic targets. However, immunological studies of mediator-specific agents targeting inflammatory mediators including TNF-α and IL-1 have not fulfilled the high expectations. Only afelimomab, a monoclonal antibody fragment directed against TNF-α showed significant reduction in mortality of high-risk patients in the MONARCS study. Despite that, the relatively small treatment effect demonstrated in the MONARCS could not be replicated. Unfortunately, anti-endotoxin strategies and anti-cytokine antibodies equally failed in clinical trials.

Reasons can be various, but include inappropriate dose, wrong timing and/or combination and heterogeneity respectively complexity of patient phenotypes (Riedemann et al., 2003; Wheeler et al., 2009; Vincent et al., 2002; Marshall, 2003).

To accommodate the complexity of the inflammation process, a new approach modulates the concentrations of inflammatory mediators rather than blocking them. Extracorporeal blood purification techniques – including plasma exchange, hemofiltration and plasmapheresis – are non-specific methods to remove toxins and inflammatory mediators. Combined with non-specific or specific adsorbents, these extracorporeal blood purification techniques seem to be a promising tool for increased survival rates in sepsis (Vincent et al., 2002; Glück & Opal, 2004; Kellum & Venkataraman, 2002; Ronco et al., 2003; Bellomo et al., 2003).

Buras et al. state that *"Sepsis has been viewed as potentially too complex for monotherapy"*, one very probable explanation for the complex and difficult task to control the sepsis syndrome. Or as Wheeler et al. say: *"Given the inherent complexity and redundancy of the host inflammatory response, future management strategies will likely encompass the use of multiple, synergistic agents acting upon different steps in the mediator cascade."*

1.5 Sepsis Models

It is beyond the scope of this diploma thesis to list, describe and discuss all pre-clinical sepsis models currently in use, taking into account that literally dozens of models have been published in the past years. Therefore just an introduction in the broad spectrum of sepsis models is given here.

Pre-clinical studies of sepsis can be categorized into several model types. There are various approaches to administer the sepsis-causing agents into the model organism including intravascular infusion of endotoxin or viable bacteria, soft tissue infection, cecal ligation and perforation (CLP), colon ascendens stent peritonitis (CASP), bacterial peritonitis, pneumonia or meningitis model. Model organisms can be of human, animal or cell culture origin. Last but not least, different animal species are used such as rats, mice, rabbits, guinea-pigs, dogs, pigs, sheep and non-human primates, each with various advantages and disadvantages (Poli-de Figueiredo et al., 2008; Marshall & Creery, 1998).

Rodents are a popular test organism as they are relatively cheap to maintain and can be kept in a pathogen-free environment. But in addition to a limited blood volume, rodents also differ in their reaction to endotoxin as they are quite resistant compared to humans (Poli-de Figueiredo et al., 2008). Non-human primates and pigs gained popularity in recent years, as their endotoxin sensitivity and tissue antigenicity is similar to humans (Goldfarb et al., 2005; Redl & Bahrami, 2005).

1.5.1 Viable Organism Infusion Models

A common and simple tool of mimicking sepsis is the inoculation of humans or animals with pure or mixed microbial flora. Besides gram-negative and gram-positive bacteria, various *Candida* strains are in use. However, these models are rather models of intoxication than a true description of infection (Buras et al., 2005), but their simplicity leads to frequent use in research. *Escherichia coli* is the most commonly used organism, injected either as a bolus or a continuous infusion with a wide variability of infusion duration and quantity. Heterogeneity can occur from virulence of the injected species and/or the site of application (Marshall & Creery, 1998). The consistency between the model and clinical sepsis is not fully given, as in the clinical disease bacteria spread from the site of infection constantly in contrast to the direct injection into the circulation (Schultz & van der Poll, 2002).

1.5.2 Endotoxemia Models

The endotoxemia model can be performed by intravenous endotoxin infusion either in animals or in humans. Generally, this model is characterized by its simplicity, high reproducibility and inexpensiveness. The normal dosage of highly purified *Escherichia coli* endotoxin for healthy volunteers is 2–4 ng/kg body weight. This dose causes symptoms of headache, fever, chills, pain, nausea, photophobia and an increased heart rate within one hour after administration (Marshall & Creery, 1998; Lowry, 2005; Fink & Heard, 1990; Lin & Lowry, 1998). Low dose infusion of endotoxin can induce an innate immune response comparable to that in sepsis patients, releasing proinflammatory sepsis mediators like TNF-α and various other cytokines (Buras et al., 2005; Remick & Ward, 2005).

The major disadvantage of this kind of model is a lack of infection focus and the disability to replicate the complex conditions in humans and therefore similar to the disadvantages of infusion models with viable organisms (Remick & Ward, 2005). According to Wichterman et al. (1980), endotoxin animal models are not able to suitable reproduce the metabolic modifications seen in human sepsis. Nonetheless, these models are useful for studying inflammatory processes (Lin & Lowry, 1998). Furthermore, the sensitivity to endotoxin differs clearly according to the species used. Whereas the resistance of rodents, cats and dogs to LPS is relatively high, rabbits, sheep, humans and non-human primates react intensively to endotoxin administration (Poli-de Figueiredo et al., 2008; Buras et al., 2005). The use of different adjuvants can increase the sensitivity of the model organism (Marshall & Creery, 1998). A noteworthy fact is that killed *Escherichia coli* appear to be more lethal than endotoxin itself (Poli-de Figueiredo et al., 2008).

1.5.3 Complex Models of Local Infection (Pneumonia and Peritonitis)

Pneumonia or peritonitis models try to mimic the typical situation of a septic patient where bacteria disseminate constantly from an infection focus. Most commonly this focus is located in the lung or abdominal cavity causing pneumonia or peritonitis (Schultz & van der Poll, 2002).

Experimental pneumonia is usually induced by *Streptococcus pneumoniae*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* by direct injection of bacteria into nose or trachea of the animal (Schultz & van der Poll, 2002).

Peritonitis models can be divided into cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP). Another, but already widely replaced model is the fecal pellet peritonitis model, where a fecal pellet in combination with a fibrin clot is implanted in the abdomen to induce sepsis (Rittirsch et al., 2007).

CLP was originally described in rats by Wichterman et al. (1980) almost three decades ago. The surgical procedure mimics the symptoms of a perforated appendicitis or diverticulitis and is considered by many researchers (Buras et al., 2005; Rittirsch et al., 2007) as the gold standard in experimental sepsis models. It is definitely a well described and commonly used model of polymicrobial sepsis with a similar cytokine response to that observed in clinical practice (Wang & Chaudry, 1998) and covers a wide range of sepsis occurrences from acute to chronic sepsis (Hubbard et al., 2005).

The model is said to be easy, generally reproducible, inexpensive and similar to human sepsis (Buras et al., 2005). Although it was originally described in rats, an adaption to many other animals such as mice, pigs, dogs and sheep has followed in the meantime (Fink & Heard, 1990)

Through a laparatomic procedure the cecum is ligated and then punctured once or twice with a needle. According to the size of the needle and number of punctures the severity of the septic outcome can be varied (Marshall & Creery, 1998; Hubbard et al., 2005). Wichterman et al. describe that 16 hours after CLP most rats show signs of illness and can be tested positive for enterobacteria in blood cultures. The septic reaction can furthermore be influenced by diet, as rats fed with meat instead of grain have a less lethal outcome (Wang & Chaudry, 1998).

The CASP-model is a slight modification of the CLP-model. The infection with intestinal bacteria is induced by a stent implanted into the ascending colon leaking faecal contents in the peritoneum. Thus polymicrobial peritonitis is mimicked (Buras et al., 2005; Rittirsch et al., 2007). Maier et al. (2004) suppose that CASP and CLP cover different varieties of sepsis given the fact that the CLP model forms an intra-abdominal abscess and the CASP model diffuse peritonitis.

1.5.4 Models of Immune Supression

The typical patient of clinical sepsis has a wide range of comorbidities. This is in contradiction to the common practice of performing animal studies with healthy, young and genetically similar animals under laboratory conditions to minimize environmental influences. Thus, immunosupressed animals started to be widely used in sepsis research. Defects in the animal's immune system can be induced by immunosuppressive agents including glucocorticoids, alcohol and specific inhibitors of certain immune system components (Opal & Cross, 1998, 2005).

1.5.5 Non-human Primate Models

Redl et al. (1998; 2005) use large animal models for testing new sepsis therapies. Primates, especially baboons, offer a variety of advantages compared to other animal models. Almost all physiological and immunological characteristics found in humans can be observed in primates too. Besides, crossreactivity with human diagnostic and therapeutic agents allow circumstances similar to clinical practice. The animal is treated in an ICU and instrumented, monitored and supported identically to humans.

1.5.6 The Deficiency of Animal Models to Simulate Sepsis

As mentioned before the predominantly majority of animal models are performed in young, uniform, healthy animals opposed to the clinical practice with aged patients with hypertension, cancer, atherosclerosis, diabetes, etc. Additionally, various other reasons can be observed for the failure to mimic sepsis in animal models. Whereas the septic study animal receives interventional drugs at the very beginning of the syndrome, this is usually not possible for the septic patient. This is in contrast to means of supportive therapy which are barely used in animals, but are standard therapy in human patients. Most clinical patients die of multiple organ failure (MOF) evolved days to weeks after the initial septic insult. The relevance of results from an animal model with significant

Experimental Sepsis	Septic Patient
Usually young adults	Often neonates or elderly
Endotoxin or defined bacterial/ fungal organism	Unknown/often multiple organisms
Usually blood borne	Often in tissue
Antibiotics +/−	Antibiotics often ineffective
Treatment usually early	Treatment usually late
Onset usually rapid	Onset usually slow
Comorbidities – seldom	Comorbidities – common

Table 1.5: Differences between animal models and septic patients (Esmon, 2004c)

Experimental animals	Species Genetic background Gender
Insult	Organism(s) Adjuvants Local vs. Disseminated challenge Site of challenge
Timing of intervention	Before insult Concomitant with insult After insult
Cointerventions	Immune compromise Systemic illness Previous acute insult Resuscitation Antibiotics Surgical source control
Dose	Magnitude of experimental insult Dose of treatment
Outcomes	Survival (short-term vs. long-term) Change in physiologic responses Change in biomarkers Change in inflammatory parameters

Table 1.6: Sources of variability in pre-clinical models of sepsis (Marshall et al., 2005)

mortality in the first 6–12 hours can thus be questioned (Poli-de Figueiredo et al., 2008). Biological heterogeneity can also interfere with the experimental practice as there is a different response known between species and strains within a species, as well as gender, age and nutritional status of the animal (Michie, 1998; Esmon, 2004c). According to Marshall et al. (2005) even the time of the day, where the experiment is performed (morning vs. afternoon) can affect certain physiologic responses.

Besides the strong ethical reasons to replace animal models with other non animal-based systems, techniques of molecular and cell biology improved significantly over the past decade. However, after initial studies in an in vitro cell culture system the need to confirm the results in more complex in vivo systems will remain (Marshall et al., 2005; Griffin, 1998). To avoid as much variability as possible, genetically similar inbreed animals of the same strain, sex, age, weight and nutritional status are normally used (Schultz & van der Poll, 2002; Marshall & Creery, 1998; Poli-de Figueiredo et al., 2008). This is again in contradiction with the clinical reality of old patients with many comorbidities.

Marshall & Creery (1998) describe the future direction of sepsis research in the following way: *"The future aim [of animal models] will be to understand how human sepsis reflects the animal models, rather than to design animal models of a poorly understood human condition"*.

1.6 Setup of the Cell Culture Model for Sepsis

The cell culture model for gram-negative sepsis used in this work was established at the Center for Biomedical Technology, Danube University Krems (Schildberger et al., 2010).

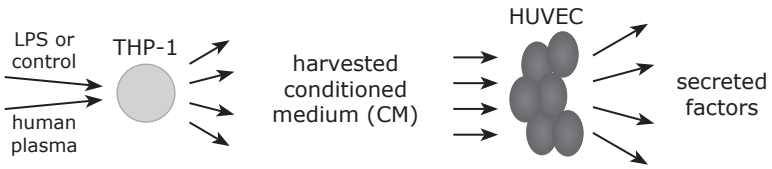

Figure 1.11: Cell Culture Model, Center for Biomedical Technology, Krems, Austria

The model consists of two steps. In the first step the continuous monocytic cell line THP-1 is stimulated with LPS for 4 hours with 10 ng/ml LPS from *Pseudomonas aeruginosa* in medium containing 10% human plasma. The culture supernatant (=conditioned medium, CM) contains LPS and various factors secreted by the THP-1 cells in response to stimulation.

In a second step human umbilical vein endothelial cells (HUVEC) are stimulated with the CM and the resulting endothelial activation is monitored with various readout assays including cytokine secretion, expression of adhesion molecules and nuclear factor-κB (NF-κB) activity.

1.7 Modifications of the Cell Culture Model

To further characterize the existing cell culture model, THP-1 cells were compared to monocytes isolated from human blood. Likewise, macrovascular endothelial cells (HUVEC) were compared to microvascular endothelial cells (HMPEC ST1.6R).

For a detailed description, see Chapter 3, Materials and Methods.

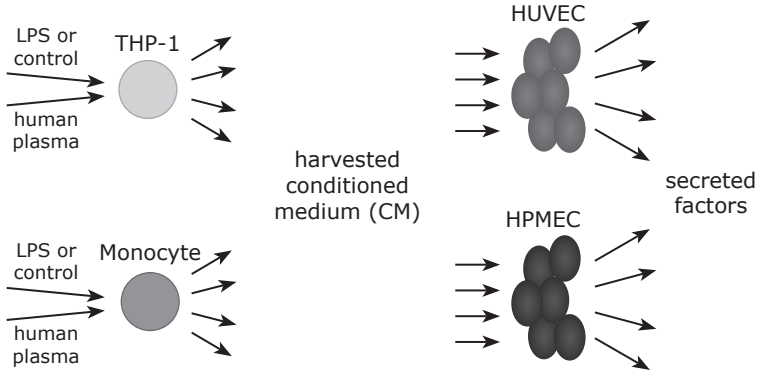

Figure 1.12: Modified Cell Culture Model, Center for Biomedical Technology, Krems, Austria

2 Objective of the diploma work

The aim of this thesis was to further characterize a cell culture model established at the Center for Biomedical Technology/Donau-Universität Krems that mimics the initial events in gram-negative sepsis.

In the model, monocytic THP-1 cells are stimulated for 4 hours with LPS in media containing 10% human plasma. Culture supernatants are applied to Human Umbilical Vein Endothelial Cells (HUVEC) for monitoring the endothelial activation.

The question of interest was whether peripheral blood mononuclear cell (PBMC)-derived monocytes from human blood behave comparably to THP-1 cells concerning their stimulation characteristics or if qualitative or quantitative differences between PBMC-derived monocytes and THP-1 cells can be demonstrated. For this purpose monocytes from blood donors were enriched by gradient centrifugation and further purified with a monocyte negative isolation kit. The purified monocytes were processed analogously to THP-1 cells in the model.

In addition, the behavior of the currently used HUVEC was compared to microvascular endothelial cells (HPMEC ST1.6R) as it is known from the literature that microvascular cells play an important part in sepsis. Parameters used to monitor cell activation were cytokine release, expression of adhesion molecules and determination of NF-κB activity, as the activation of this pathway is involved in regulating the transcription of many of the immunomodulatory mediators participating in the development of sepsis.

3 Materials and Methods

3.1 Cell Culture

3.1.1 Human Umbilical Vein Endothelial Cells

Isolation of Human Umbilical Vein Endothelial Cells

Human Umbilical Vein Endothelial Cells (HUVEC) are primary macrovascular endothelial cell lines obtained from the main vein in human umbilical cords. The cords were provided by the gynaecological department of the hospital in Krems, Austria, according to ethical standards (informed consent and comprehensive information of the donors).

Endothelial cells were isolated by an adaptation of the method of Jaffe (Jaffe et al., 1973) within 10 hours after delivery. The umbilical veins were transferred into sterile, cooled Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) by the medical personnel and kept cooled until processing. The cord was inspected to find possible puncture marks and both ends were cut off with a sterile scalpel. After cannulation the umbilical vein was rinsed with Medium 199 (M199, Sigma-Aldrich) supplemented with HEPES (0.02 M, Sigma-Aldrich) and Penicillin-Streptomycin (Pen/Strep, PS, 100 µM, Sigma-Aldrich) – further designated as M199/HEPES/PS – to wash out remaining blood and blood clots. Pre-warmed (37°C) dispase solution (BD Biosciences) was then infused into the vein and the vein was incubated at 37°C for 15 min. The dispase solution containing endothelial cells was rinsed out of the vein with M199/HEPES/PS with 20% foetal bovine serum (FBS, HyClone), then centrifuged at 500xg for 5 min at room temperature (RT).

The cells were suspended in M199/HEPES/PS with 20% FBS, supplemented with sodium heparin (15 IU/ml, Baxter) and Endothelial Growth Factor Supplement (ECGS, 10 µg/ml, BD Biosciences). Incubation was carried out at 37°C with 5% CO_2. The following day, the culture medium was discarded, the cells were carefully washed with M199/HEPES/PS, and the medium changed to new M199/HEPES/PS with 20% FBS supplemented with heparin and ECGS.

Culture Conditions for HUVEC

HUVEC were cultured in T 75 cm^2 or T 175 cm^2 cell culture flasks (nunc) in M199/HEPES/PS with 20% FBS supplemented with heparin and ECGS. The cells reached confluence within four to six days depending on the different growth rates of various cell batches. When confluent they were split at a ratio of 1:2 by detaching the HUVECs with pre-warmed 0.02% EDTA solution (Sigma-Aldrich) and incubated for 3 to 5 min at 37°C. The EDTA-activity was stopped with 10 ml M199/HEPES/PS and the suspension was centrifuged at 500xg for 5 min at RT. The cells were resuspended in M199/HEPES/PS

with 20% FBS supplemented with heparin and ECGS.

Cryopreservation of HUVEC

The detachment of HUVEC originating from two T 75 cm^2 or one T 175 cm^2 flasks was performed as described above. The cell pellets were resuspended in M199/HEPES/PS with 20% FBS containing 10% Dimethyl sulfoxide (DMSO, Sigma). 1 ml of the suspension was filled into a cryo vial (Greiner) and placed in a 4°C prechilled StrataCooler© Cryo Preservation Module, which is specially designed for controlled freezing rates of cells (0.4 – 0.6°C/min). The StrataCooler© Cryo Preservation Module was deposited in a −80°C freezer for a minimum of 12 hours before transferring the cryo vials into liquid nitrogen for long-term storage.

For thawing, the frozen cryovials were immediately placed in a 37°C water bath. M199/HEPES/PS with 20% FBS was added just before the whole cell suspension was thawed completely. After centrifugation of the suspension at 500xg for 5 min at RT, the cell pellet was resuspended in M199/HEPES/PS with 20% FBS & heparin and ECGS-supplement, seeded out into two T 75 cm^2 flasks or one T 175 cm^2 flask and finally incubated at 37°C under 5% CO_2.

3.1.2 Human Pulmonary Microvascular Endothelial Cells

Origin of the Cell Line HPMEC ST1.6R

Human Pulmonary Microvascular Endothelial Cells (HPMEC) are primary microvascular endothelial cells obtained from lung tissue. The use of primary cells implicates certain disadvantages such as the limited lifespan of diploid cells in culture, the contamination with other cell types and the difficulties to obtain large cell numbers, just to name a few. To avoid such problems, immortalized cell lines have been established and are commonly used.

The cell line HPMEC ST1.6R was established in the laboratory of Prof. C. James Kirkpatrick at the Johannes-Gutenberg-University in Mainz. The immortalized cells were gained after co-transfection of lung cells resected from adult patients with malignant tumors with plasmids encoding the catalytic component of telomerase (hTERT) and the simian virus 40 (SV40) large T antigen (Krump-Konvalinkova et al., 2001). The HPMEC ST1.6R were obtained directly from the laboratory of Prof. Kirkpatrick. The batch was designated as passage number 0, as no information on the passage number was available.

Culture Conditions for HPMEC ST1.6R

HPMEC ST1.6R were cultured in T 75 cm^2 or T 175 cm^2 flasks in M199/HEPES/PS supplemented with 20% FBS, heparin and ECGS. The only difference to the maintenance of HUVEC was the higher amount of ECGS added (30 µg/ml). The cells reached

confluence within two to three days and were split at a ratio of 1:3 by detaching the cells with pre-warmed 0.02% EDTA solution and incubating for 3 to 5 min at 37°C. The EDTA-activity was stopped by dilution with M199/HEPES/PS and the suspension was pelleted at 500xg for 5 min at RT. The cells were resuspended in M199/HEPES/PS supplemented with 20% FBS, heparin (15 IU/ml) and ECGS (30 µg/ml). The cryopreservation and thawing of HPMEC ST1.6R was carried out as described above for the HUVEC.

3.1.3 THP-1 Cells

THP-1 is a human leukaemic cell line originally derived from the peripheral blood of a one-year-old male infant with acute monocytic leukemia (Tsuchiya et al., 1980) and was purchased from the American Type Culture Collection (ATCC, TIB-202). As no information on the passage number was available, the acquired batch was designated as passage number 0.

Culture Conditions for THP-1 Cells

THP-1 cells were cultured in cell culture flasks with RPMI 1640-medium (Sigma-Aldrich) with 10% FBS, HEPES and PS. The splitting ratio was 1:3 or 1:4 every three to four days by centrifuging the cell suspension at 120xg for 7 min at RT. The cells were resuspended at a concentration of approximately $0.2x10^6$ cells/ml in RPMI 1640/HEPES/PS supplemented with 10% FBS.

Cryopreservation of THP-1 Cells

Cells were pelleted at 120xg for 7 min at RT and resuspended at a cell concentration of $2–6x10^6$ cells/ml in RPMI 1640-medium with 10% FBS, HEPES and Pen/Strep, enriched with 10% DMSO. The cryovials were stored in the StrataCooler© Cryo Preservation Module as described above.

For thawing, the frozen cryovials were immediately placed in a 37°C water bath. Before the cell suspension was thawed completely, RPMI-1640 with 10%FBS, HEPES and Pen/Strep was added. The suspension was centrifuged at 120xg for 7 min at RT, the cell pellet resuspended in RPMI-1640 with 10% FBS, HEPES and Pen/Strep and the cell solution was seeded out in cell culture flasks. Incubation was carried out at 37°C under 5% CO_2.

3.2 Isolation of Monocytes from Human Blood

The isolation of monocytes from human blood was performed by density gradient centrifugation with "Ficoll-paque^{TM} plus" followed by "Dynabeads© MyPure^{TM} Monocyte Kit 2 for untouched cells" (Invitrogen). The kit processed up to 1x10^8 cells/ml supplied beads.

3.2.1 Counting of Blood Cells

Prior to processing, blood obtained from healthy volunteer donors was measured with a Sysmex hematology analyzer. A complete blood count was performed. The principle of the method is counting of the blood cells by electric impedance. The amount of leukocytes as well as the amount of monocytes according to the Sysmex measuring was used for calculating the yield of monocytes.

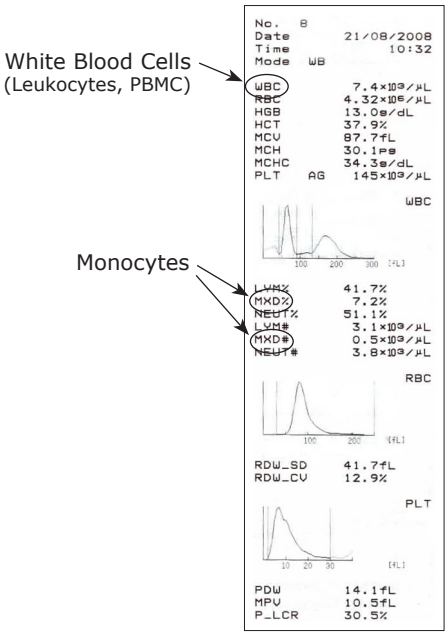

Figure 3.1: Example for a typical Sysmex complete blood count print

PBMCs and monocytes were counted with a Neubauer-hemocytometer (Marienfeld) according to standard procedures during the monocyte isolation process.

3.2.2 Buffer Preparation

Buffers for the isolation of monocytes from human blood were phosphate buffered saline (PBS) and PBS containing 0.1% w/v bovine serum albumin (BSA) and 2mM ETDA. All buffers and materials were produced sterile or sterilised before use.

3.2.3 Preparation of Material

Parts of the monocyte isolation were carried out in glass tubes for easier handling. To prevent activation or loss of monocytes, the glass tubes were coated with Sigmacote© (Sigma-Aldrich) according to the manufacturer's recommendations. Thereafter, the tubes were sterilized for 90 min at 121°C/2 bar.

3.2.4 Isolation of Monocytes

Density Gradient Centrifugation with Ficoll-paque™ plus

Ficoll-paque™ plus (GE healthcare) is a medium for gradient centrifugation and for instance used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood. The density, viscosity and osmotic characteristics of the solution result in the formation of layers with different cell types after centrifugation. Erythrocytes sediment completely through the Ficoll-paque and can be collected at the bottom of the tube. Directly above the erythrocytes, granulocytes can be harvested. According to their lower density, lymphocytes, monocytes and platelets stay at the interface between Ficoll-paque and blood plasma.

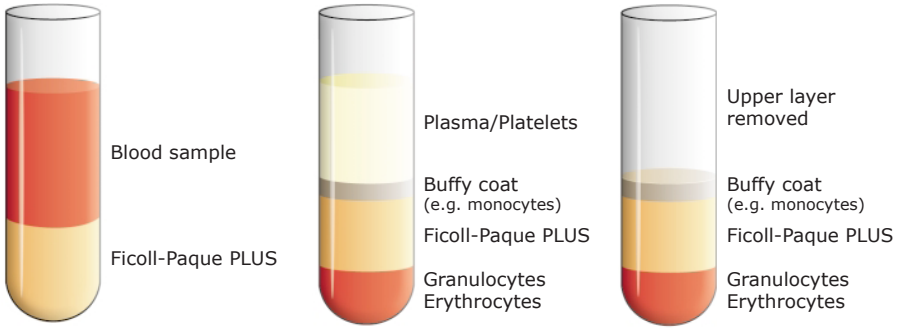

Figure 3.2: Ficoll procedure (adapted from GE healthcare):
(1) PBS/blood-dilution is layered on top of Ficoll-Paque PLUS
(2) after centrifugation without brake the various blood fractions can be seen
(3) after removing the upper plasma layer the lymphocytes can be harvested

Venous blood samples, anticoagulated with citrate (final sodium citrate 12.9 mmol/L, Mayerhofer Pharmazeutika), were taken from healthy volunteers. 150 or 30 ml blood, respectively, were mixed 1:1 with PBS and aliquots were layered carefully on top of Ficoll-paque. The mixing ratio was 1:3 and the procedure was carried out in 50 ml polypropylene (PP)-tubes (Greiner). This resulted in 37.5 ml PBS/blood-dilution layered on top of 16.6 ml Ficoll-paque per tube.

Optimization of Density Gradient Centrifugation with Ficoll-paque™ plus

Two different protocols were compared in order to optimize the yield of PBMCs. The first protocol was set up according to the Ficoll-paque™ plus manual. Citrate-anticoagulated blood was mixed with PBS and transferred to the PP-tubes as described above. The tubes were then centrifuged at 400xg for 35 min without using the brake. After carefully collecting the buffy coat, which is the fraction that contains most of the white blood cells and platelets, they were transferred to a clean tube and a minimum of 6 ml PBS was added for washing. Centrifugation at 80xg for 10 min and subsequent discarding of the supernatant followed. The remaining pellet was resuspended in 6–8 ml PBS and again centrifuged with 80xg for 10 min. After discarding the supernatant a pellet for further processing was received.

The second protocol was the protocol for Ficoll-paque plus centrifugations established at the Center for Biomedical Technology, Krems. As it turned out that the yield of PBMCs with this latter protocol was higher, it was used for all further experiments. Citrate-anticoagulated blood was mixed with PBS and transferred to the PP-tubes as mentioned above. The tubes were then centrifuged at 1180xg for 30 min without using the brake. The PBMCs were collected, transferred to a clean tube and the tube was filled up with PBS for washing. Centrifugation with 1180xg for 20 min followed. After discarding the supernatant a pellet for further processing was obtained.

Negative Isolation with Dynabeads©

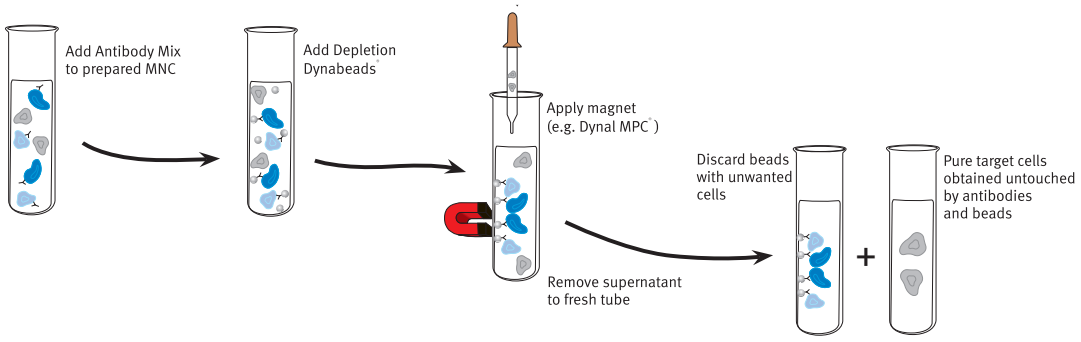

Figure 3.3: Negative isolation of PBMC using Dynabeads (adapted from Invitrogen): (1) Antibody-mix is added and binds to the undesired cells (2) added Dynabeads are binding to the antibodies (3) undesired cells can be separated with a magnet (4) target cells can be used for further procedures

The cell pellets isolated by gradient centrifugation were diluted in 30 ml PBS and according to the Dynabeads-manual a second gradient centrifugation step was carried out. For this purpose 6 ml of the diluted buffy coat were added on top of 3 ml Ficoll-paque™ plus in silanized glass tubes and centrifuged for 20 min at 160xg without brake.

The cells were washed three times with PBS containing BSA and EDTA (see Chapter 3.2.2) before the isolation of monocytes with Dynabeads, which was carried out strictly according to the manual of the manufacturer.

Dynabeads are uniform, superparamagnetic polystyrene beads, which can be used for positive or negative isolations of different cell populations. The Dynabeads supplied in the "Dynabeads© MyPure™ Monocyte Kit 2 for untouched cells" are coated with monoclonal human anti-mouse IgG antibodies, which are Fc specific and recognise all mouse IgG subclasses. For negative depletion, an antibody-mix specific for CD3, CD7, CD16, CD19, CD56, CDw123 and CD235a is added to the cell-suspension and the antibodies bind to undesired cell populations in the buffy coat, such as natural killer cells (CD7, CD16, CD56), T- (CD3, CD7, CD56) and B-lymphocytes (CD19), macrophages (CD16, CDw123), granulocytes (CD16, CDw123) and erythrocytes (CD235a). In the next step, anti Fc Dynabeads are added, bind to the antibody-tagged cells and are separated from the solution with a special magnet (Promega).

Assessment of Purity of Monocytes

The assessment of the monocyte purity was performed with flow cytometry as described under Chapter 3.4.2.

3.3 Experimental Cell Culture Model for Gram-negative Sepsis

3.3.1 Stimulation of THP-1 Cells and Monocytes with LPS

THP-1 cells between passages 8 and 13 were centrifuged at 120xg for 7 min at RT and resuspended in M199/HEPES/PS. Viability and cell count were determined by using 0.14% trypan blue (Sigma-Aldrich) and a Neubauer hemocytometer. Another centrifugation at 120xg for 7 min at RT followed. The pellet was resuspended in M199/HEPES/PS supplemented with 10% human plasma, heparin (6 IU/ml) to obtain a cell count of $1.0x10^6$ cells/ml. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_20$) was added to restore the correct Ca^{2+} concentration of the cell culture medium, as it was complexed by citrate contained in the plasma.

Monocytes obtained from the isolation step were pelleted at 800xg for 20 min at 4°C and resuspended in M199/HEPES/PS supplemented with 10% human plasma, sodium heparin (6 IU/ml) and calcium chloride dihydrate ($CaCl_2 \cdot 2H_20$) to obtain a cell concentration of $1.0x10^6$ cells/ml.

The cell suspensions were stimulated for 4 hours with 10 ng/ml LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich) in 6 well-tissue culture plates, 12 well-tissue culture plates, 24 well-tissue culture plates, T 75 cm^2 or T 175 cm^2 cell culture flasks (nunc) at 37°C and 5% CO_2. The cell suspensions were pelleted at 1000xg for 10 min at 4°C, the

supernatant (=conditioned medium) was harvested, aliquoted and stored at −80°C until further use.

3.3.2 Stimulation of HUVEC and HPMEC ST1.6R with Conditioned Medium obtained from Monocytes and THP-1 Cells

HUVEC in passage 4 or 5 and HPMEC ST1.6R between passage 16 and 21 were detached as decribed under Chapters 3.1.1 and 3.1.2, centrifuged at 500xg for 5 min or 120xg for 7 min at RT, respectively, and resuspended in M199/HEPES/PS. Determination of viability and cell count was carried out by using 0.14% trypan blue. After another centrifugation at RT and 500xg for 5 min or 120xg for 7 min, respectively, the obtained pellet was suspended in M199/HEPES/PS supplemented with sodium heparin (15 IU/ml) and ECGS (10 µg/ml) to obtain a cell concentration of $0.6x10^6$ cells/ml. 500 µl of this suspension were seeded into 12 well-tissue culture plates (Greiner) and incubated for 17 hours at 37°C in a humidified atmosphere (5% CO_2).

After 17 hours, the cells reached confluency, the medium was discarded and each well was washed with 1000 µl M199/HEPES/PS. After washing, the cells were stimulated with 500 µl conditioned medium or control medium from THP-1 cells or monocytes at 37°C in a humidified atmosphere (5% CO_2). Supernatants were harvested after one hour and 16 hours, aliquoted and frozen at −80°C. HUVEC and HPMEC ST1.6R were used to perform flow cytometry and preparation of nuclear and cytoplasmic extracts (see below).

Three independent experiments were carried out. In each, one batch of HUVEC was stimulated with a conditioned medium obtained from an independent monocyte isolation. HPMEC ST1.6R were in different passage numbers but origined from the same batch. They were stimulated in the same three experiments with the same conditioned media as HUVEC and also further treated as described for them.

3.4 Read-out Assays

3.4.1 NF-κB Activity

Nuclear and Cytoplasmic Extraction

Nuclear and cytoplasmic fractions were prepared with the Nuclear Extract Kit (Active Motif).

All extraction steps were performed on ice to prevent protein degradation. HUVEC and HPMEC were washed with pre-warmed PBS followed by harvesting with 0.25% trypsine-EDTA solution (Sigma-Aldrich). The suspensions were diluted with ice-cold PBS and centrifuged for 5 min at 500xg and 4°C. After discarding the supernatant 100 µl phosphatase inhibitor cocktail (supplied with the Nuclear Extract Kit) in PBS

were added and the solution was centrifuged again as described above. Cell counting with 0.14% trypan blue was performed to identify the needed amount of buffers. The pellets were resuspended in hypotonic buffer (supplied with the same kit) and incubated for 15 min on ice. After adding detergent, the pellets were vortexed 10 sec at highest speed and afterwards centrifuged at 14,000xg for 1 min. The obtained supernatant was the cytoplasmic fraction and was immediately frozen at −80°C.

The nuclear pellet was resolved in complete lysis buffer and vortexed for 10 sec at highest speed. Subsequently an incubation period on ice with shaking at 150 rpm for 30 min followed. The suspension was vortexed for 30 sec at highest speed and centrifuged at 14,000xg for 10 min. The supernatant (nuclear fraction) was immediately frozen at −80°C.

NF-κB Activity Assay

The determination of nuclear NF-κB activity was performed with the Trans AM^{TM} NF-κB p65 Chemi Activity Assay (Active Motif). The principle of this assay is an immobilized oligonucleotide containing an NF-κB consensus binding site which binds to the activated NF-κB from nuclear extracts. An antibody directed against the p65 subunit of NF-κB detects the bound NF-κB complexes. Luminescence quantification is possible due to a secondary antibody conjugated to horseradish peroxidase (HRP).

Buffers were components of the kit and prepared in accordance to the recommendations of the manufacturer and the test was carried out in duplicates. Nuclear extracts were diluted with lysis buffer to a protein content of 3 µg per 50 µL sample.

The 96-well plate was prepared by adding 30 µL complete binding buffer to each well used. Thereafter, 20 µL samples diluted in lysis buffer, HeLa control nuclear extract (positive control) or complete lysis buffer as blank were added. The plate was incubated for one hour at RT with mild agitation. Afterwards, the plate was washed three times with wash buffer, before 30 µL of NF-κB antibody were added. The plate was again incubated for one hour, but without agitation. A washing circle with 3 washing steps was performed. Subsequently, the HRP-conjugated antibody was pipetted to the plate and the plate was incubated for another hour without agitation at RT. The last washing circle included 4 washing steps, before 50 µL of chemiluminescent working solution were added to each well. The plate was incubated in the dark for 20 min, before the chemiluminescence was measured. A second measurement was performed after 30 min.

3.4.2 Flow cytometry

Expression of Adhesion Molecules

For the staining, HUVEC or HPMEC were washed with PBS pre-warmed to 37°C before the detachment with 0.02% EDTA. The wells were rinsed with 4°C PBS and the

suspensions were transferred to pre-cooled FACS tubes. To increase the volume of the suspension, 2 ml PBS were added and the tubes were centrifuged at 500xg for 5 min at 4°C. The supernatant was discarded and the rests of the fluid dripped off on paper. The same procedure was carried out with the positive controls.

Antibodies used in the staining:

- PE ICAM-1 (CD54):
  Phycoerythrin(PE) mouse anti-human CD 54 (#555511, BD Pharmigen)
- PE-Cy5 E-selectin (CD62):
  CD 62E PE-Cy5-conjugated mouse anti-human monoclonal antibody (#550040, BD Pharmigen)
- PE control:
  R-Phycoerythrin (R-PE)-conjugated mouse IgG1, κ monoclonal immunoglobulin isotype control (#555749, BD Pharmigen)
- PE-Cy5 control:
  PE-Cy5-conjugated mouse IgG1, κ monoclonal immunoglobulin isotype control (#555750, BD Pharmigen)

For the staining, a mastermix was prepared based on 6 µl of CD 54 antibody and 6 µl CD62E antibody per 100 µl PBS containing 2% FBS.

112 µl of mastermix was added to the samples and the solution was mixed by pipetting up and down and vortexing, respectively. For the positive controls, 100 µl PBS containing 2% FBS and 6 µl required antibody were added and the procedure was continued as described above. All tubes were incubated on ice for 30 min in the dark. After incubation, 2 ml PBS were added and the solutions were centrifuged at 500xg for 5 min at 4°C. The cells were washed with 2 ml PBS and centrifuged again. The supernatant was discarded and the stained cells were suspended in 300 µl PBS. Cells were analyzed on a BD FACScan flow cytometer (Becton Dickinson). 10,000 gated cells were presented in histograms using the Cell Quest Software (Becton Dickinson).

Assessment of the Purity of Monocytes

For the assessment of the purity of monocytes, the following antibodies were used:

- FITC CD14:
  Fluoresceinisothiocyanate (FITC) mouse anti-human CD 14 (#555397, BD Pharmigen)
- FITC control:
  FITC mouse IgG1, κ monoclonal immunoglobulin isotype control (#555748, BD Pharmigen)

20 µl of antibody solution were added to 1.0x10^6 monocytes in 100 µl PBS for each sample and the solution was mixed by pipettung up and down. All tubes were incubated on ice for 30 min in the dark. After incubation, 2 ml PBS were added and the solutions were centrifuged at 500xg for 5 min at 4°C. The cells were washed with 2 ml PBS and centrifuged again. The supernatant was discarded and the stained cells were dissolved in 300 µl PBS. Cells were analyzed on a BD FACScan flow cytometer (Becton Dickinson). 10,000 gated cells were presented in histograms using the Cell Quest Software (Becton Dickinson).

3.4.3 Enzyme-Linked Immunosorbent Assays (ELISA)

TNF-α-ELISA

For the determination of TNF-α concentrations, two different ELISA-kits were used: TNF-α Enzyme Amplified Sensitivity Imunnoassay (EASIA) KAP1751 (BioSource) and human TNF-α BMS223/4CE (Bender Medsystems).

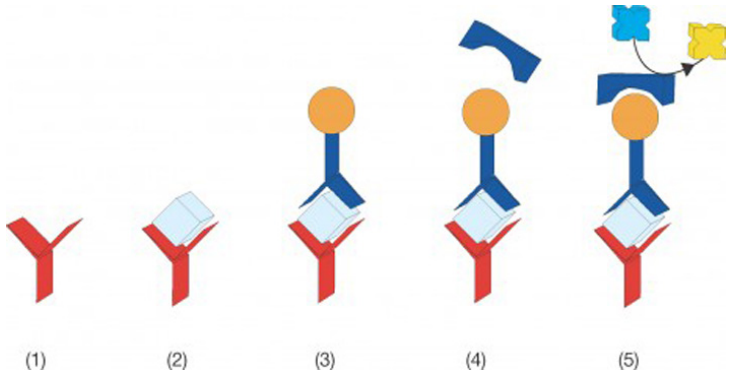

Figure 3.4: Principle of a Sandwich-ELISA (adapted from Bender MedSystems): (1) capture antibodies are attached to an ELISA-plate (2) sample-antigen binds to the capture antibody (3) biotin-conjugated detection antibody binds to the antigen captured by the first antibody (4) streptavidin-HRP is added and binds to the biotin conjugated detection antibody (5) coloured product is formed in proportion to the amount of antigen present in the sample; the reaction is terminated by addition of acid and the absorbance is measured at 450 nm

The principle of both ELISAs is a microwell plate coated with polyclonal or monoclonal TNF-α antibodies. Distinct epitopes of this anti-TNF-α antibody react with TNF-α present in samples, standards and controls. A biotin-conjugated monoclonal secondary antibody binds to the bound TNF-α. Streptavidin-HRP is added and binds to the secondary antibody. Upon addition of a substrate solution reactive with HRP a coloured product is formed proportional to the bound TNF-α. The reaction is stopped with acid and the absorbance can be measured at 450 nm. The procedures were performed according to the instructions of the manufacturers.

3.4.4 Bio-Plex© Cytokine-Assays

Quantification of TNF-α, IL-1β, IL-6, IL-8 and IL-10

Bio-Plex© cytokine-assays (Bio-Rad) are bead-based assays to quantify multiple cytokines in different matrices. The advantage of this system is that multiple analytes can be analysed at once with minimal sample volume (50 µl) required. The assay is designed in a capture sandwich immunoassay format with antibodies against the cytokine of interest covalently coupled to fluorescent, colour-coded polystyrene beads. Cytokines in sample or standard bind to the antibody-bead-couple and are recognized by a biotinylated detection antibody specific for an epitope on the surface of the cytokine. Streptavidin-phycoerythrin (PE) binds to the biotinylated detection antibody. By using a special flow cytometer with two lasers and associated optics the cytokines can be quantified.

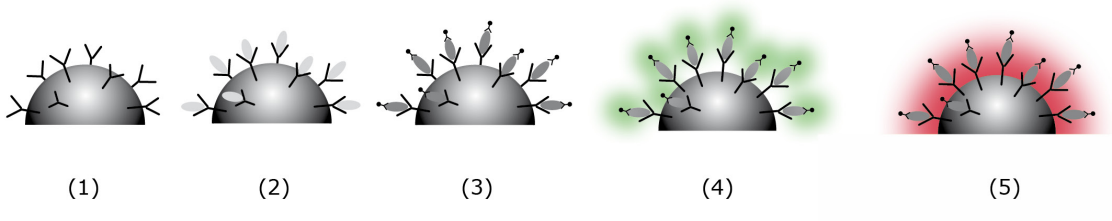

Figure 3.5: Bio-Plex© Cytokine-Assay procedure:
(1) color-coded microspheres are used as molecular carriers, probes are bound to the bead (2) the bound probes capture the target molecules in the sample (3) fluorescently-labeled reporter tags bind to the sample molecule (4) first laser excites molecular tags (5) second laser measures the fluorescence intensity of the bead

All samples as well as the cytokine standard were kept on ice until use. The cytokine standard contains a mixture of cytokines in known concentrations. It was reconstituted in 500 µl M199 with 10% FBS and – after gently vortexing – incubated on ice for 30 min. Subsequently, the standard series was prepared in M199 with 10% FBS in different ranges for each cytokine. Table 3.1 shows the concentration ranges for TNF-α, IL-6, IL-8, IL-1β and IL-10.

50 µl of the multiplex bead working solution were added to each well after vortexing the solution accurately and moistening all wells with 80 µl of assay buffer before. The buffer was removed by vacuum filtration. 80 µl wash buffer were applied and removed by vacuum filtration and the step was repeated. Thereafter, 50 µl of standard or sample were pipetted onto the multiwell plate in duplicates. An incubation step for 30 min with mild agitation and protection from sunlight followed. After three washing steps with 80 µl wash buffer each and vacuum filtration in between, 25 µl of previously prepared detection antibody solution were pipetted into the wells. The plate was incubated for

Std ID	TNF-α	IL-6	IL-8	IL-1β	IL-10
S1	91756	41825	23530	50509	33387
S2	22939	10456	5883	12627	8347
S3	5735	2614	1471	3157	2087
S4	1434	654	368	789	522
S5	358	163	92	197	130
S6	90	41	23	49	33
S7	22	10	6	12	8

Table 3.1: Concentration ranges of cytokine standards for the Bio-Plex analysis (pg/ml)

30 min with mild agitation and protection from sunlight, before three washing steps took place. 50 µl of freshly prepared streptavidin-PE were added to each well and the multiwell plate was incubated for 10 min with mild agitation and protection from sunlight. The last washing step with three times 80 µl wash buffer and vacuum filtration was carried out before resuspending the beads in 125 µl assay buffer per well. The plate was shaken for 30 sec at the highest speed possible without splashing the samples out of the wells and immediately measured in the Bio-Plex© System.

3.4.5 Protein Quantification

For protein quantification the ProStain™ Protein Quantification Kit (Active Motif) was used. The kit uses a dye which absorbs light at different wavelenghts (612nm/503nm) in free or protein-bound state. Background emerging from unbound dye is eliminated as the bound dye has a 50-fold greater emission compared to free dye.

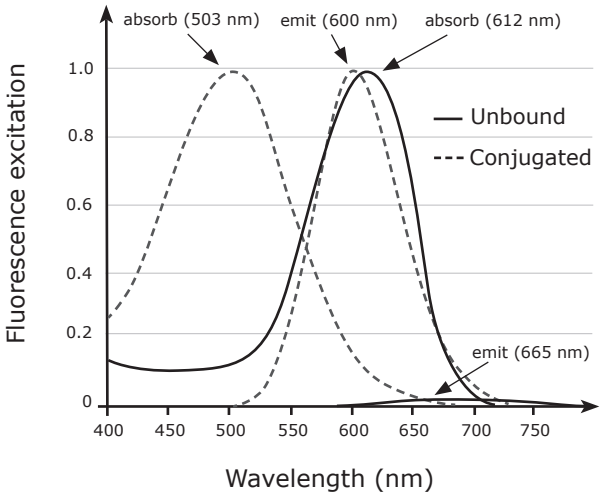

Figure 3.6: Absorption and emission spectra of free vs. bound dye of the ProStain™ Protein Quantification Kit (adapted from Active Motif)

Normalized absorption and emission spectra of free (solid lines) and conjugated dye (dotted lines) are shown in Fig 3.6.

The lyophilized dye reagent was resuspended in methanol prior to the first usage of the test. The stock bovine serum albumine (BSA) to prepare the standard curve was resuspended in aqua bidestillata to provide a concentration of 1 mg/ml, aliquoted and stored at −20°C.

Before use, all kit components were brought to RT. A black microtiter plate was preloaded with 100 µg dilution buffer for the blank. Thereafter, standards 1–7 (0.32, 0.63, 1.25, 2.50, 5.00, 7.50 and 10.00 µg/ml) were applied. All standards were performed in triplicates.

Controls and samples were applied in 1:200 dilutions in dilution buffer, 100 µl per well. 100 µl dye reagent working solution were added and all wells accurately mixed. The multiwell plate was incubated in the dark at RT for 30 min before the fluorescence was measured at 488/635nm.

4 Results

4.1 Isolation of Monocytes from Human Blood

4.1.1 Yield of Monocytes

The isolation of monocytes from human blood can be regarded as a procedure containing two stages. At the beginning PBMCs were enriched using the Ficoll-paque^{TM} plus density gradient centrifugation. The second step was the isolation of monocytes from buffy coat by negative isolation with "Dynabeads© MyPure^{TM} Monocyte Kit 2 for untouched cells".

	Protocol 1 (32 ml)	Protocol 2 (32 ml)	Protocol 2 (150 ml)
Initial amount of PBMC/µL (Sysmex)	5.4x10^3	4.3x10^3	8.5x10^3
Initial amount of PBMC/total (Sysmex)	1.7x10^8	1.4x10^8	1.3x10^9
Initial amount of monocytes/µL (Sysmex)	0.6x10^3	0.4x10^3	0.7x10^3
Initial amount of monocytes/total (Sysmex)	1.9x10^7	1.3x10^7	1.1x10^8
Amount of PBMC after Ficoll-paque procedure/total (hemocytometer)	1.3x10^6	8.7x10^6	1.50x10^8
Amount of PBMCs after Ficoll-paque procedure/total (Sysmex)	no detection	1.9x10^7	no detection
Yield of PBMCs after Ficoll-paque procedure	0.8%	6.2%/13.5%	11.5%

Table 4.1: Yield of PBMC after Ficoll-paque procedure; Protocol 1 & 2 are explained in Chapter 3.2.4

The results specified in the following section were obtained during the optimization of the procedure for the enrichment of PBMCs; they may not suffice to scientific standard of reporting, but are included here for completeness. As described in Chapter 3.2.4, two different protocols for density gradient centrifugation with Ficoll-paque were compared. The procedures specified in the original manual provided by the manufacturer did not result in satisfactory yields of monocytes after the Ficoll-paque step. While during the first centrifugation step of the blood sample, the yield of the PBMCs was still about 50%, the loss during the subsequent washing steps with centrifugation at 80xg for 10 min was much higher. At the end of the procedure, the yield was only 0.8% of the initial amount of PBMCs in the blood sample (see Table 4.1).

On the other hand, the yield obtained with the second protocol, was 6.2%/13.5% of the initial amount of PBMCs in the blood sample (see Table 4.1). As already stated in

	Exp 1	Exp 2	Exp 3
Initial amount of monocytes/µL (Sysmex)	$0.5x10^3$	$0.7x10^3$	$0.7x10^3$
Initial amount of monocytes/total (150 ml blood) (prior to Dynabeads/hemocytometer)	$7.5x10^7$	$10.5x10^7$	$10.5x10^7$
Amount of monocytes/total (150 ml blood) (after Dynabeads/hemocytometer)	$25.2x10^6$	$58.0x10^6$	$57.5x10^6$
Yield of monocytes	33.6%	55.2%	54.8%

Table 4.2: Yield of monocytes after negative isolation using Dynabeads

Chapter 3.2.4, this protocol was used as standard protocol for Ficoll-paque centrifugations in the laboratory where I conducted the research for my diploma thesis.

To determine the initial amount of PBMCs or monocytes, respectively, a complete blood cell count was performed using a Sysmex hematology analyzer (see Chapter 3.2.1). During the isolation process, monocytes and PBMCs were counted using a hemocytometer.

In the optimization experiments, the yield of monocytes ranged between 7.5% and 11.3% of the hypothetically possible yield and could be increased to an average of 48% in the main experiment.

To obtain a higher amount of monocytes in general, the processed blood volume was increased from about 40 ml at the beginning of the optimization experiments to 150 ml in the subsequent experiments. With the increase of the used blood amount and the use of the second protocol for the Ficoll-paque procedure, the yield of monocytes could be increased to 48% of the initial amount of monocytes (see Table 4.2). The use of glass tubes and coating those with Sigmacote© was undoubtedly helpful for the increase.

4.1.2 Purity of Monocytes

As described in Chapter 3.4.2, the purity of monocytes was assessed with flow cytometry. Monocytes after isolation with Ficoll-paque™ plus and Dynabeads© were stained with a FITC-coupled monocyte-specific antibody against CD14. A FITC-coupled IgG monoclonal antibody was used as control. The assessment of the purity of monocytes was performed just once. According to the technical service of Invitrogen the purity of monocytes after the purification procedure is normally >90%. In the single experiment we performed, the purity was 95% (Table 4.3).

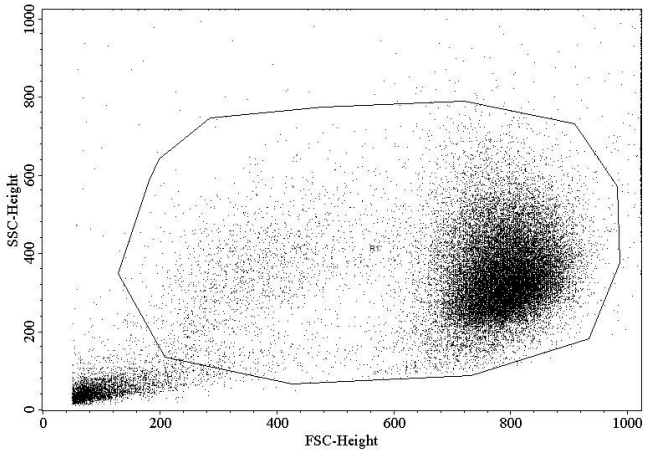

Figure 4.1: Dotplot Forward Scatter (FSC) vs. Side Scatter (SSC) of CD14 FITC-stained monocytes. The region of interest was gated.

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	0	0.00	0.00	***	***	***	***
UR	0	0.00	0.00	***	***	***	***
LL	1645	4.94	4.30	2.36	1.80	176.72	169.34
LR	31660	95.06	82.76	95.08	85.51	352.96	341.95

Table 4.3: Quandrant statistics of the Side Scatter (SSC) of CD14 FITC-stained monocytes

Figure 4.1 shows a dotplot of the Forward-Scatter (FSC), which corresponds to the size of the cells, versus the Side-Scatter (SSC), which corresponds to the granularity of the cells. The lower left cell population depicts small debris. The other area, including the monocytes, was gated.

Settings of the fluorescence-intensity of the IgG-control were set to have 2.58% false positive cells. This is within the convention to set this level between 1–3% (Givan, 2001).

The histogram in figure 4.2 shows the increased fluorescence-intensity of gated CD14 stained monocytes compared to the IgG-control.

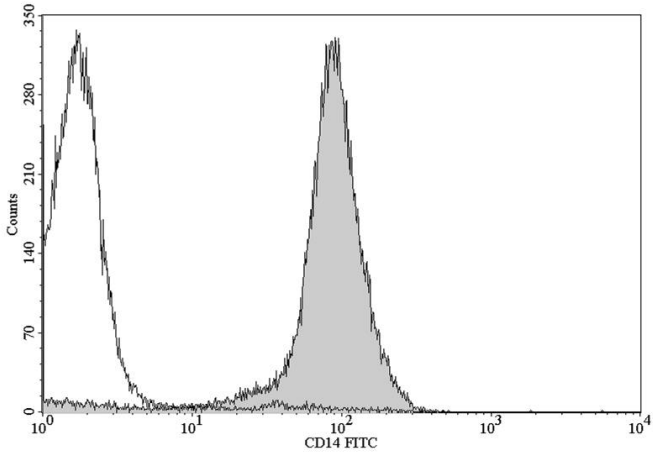

Figure 4.2: Histogram-Overlay of the gated FITC-control (white) and CD14-stained monocytes (grey).

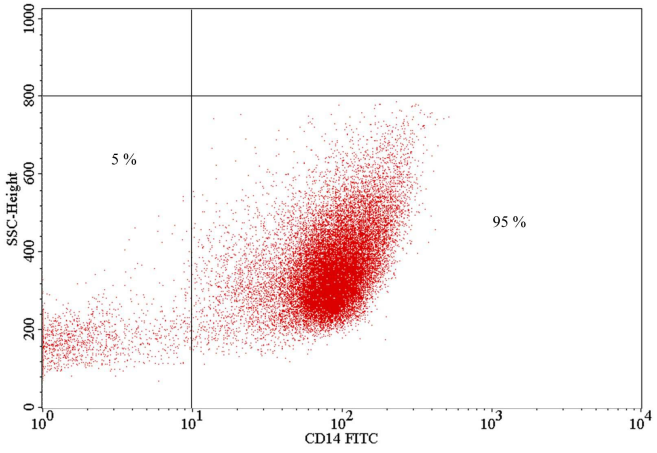

Figure 4.3: Dotplot SSC vs. the gated CD14-stained monocytes. The purity of the isolated monocytes is 95%.

4.2 Stimulation of THP-1 Cells and Monocytes with LPS

Prior to stimulation of the endothelial cells, the concentration of TNF-α in the conditioned media was quantified as a marker for the efficiency of stimulation. The TNF-α levels of conditioned media obtained from monocytes in the first two experiments were lower than in the third experiment. TNF-α levels obtained from monocytes were comparable to TNF-α levels obtained from THP-1 cells, except for experiment 3 where a difference of about 100 pg/ml between the conditioned medium obtained from monocytes and the conditioned medium obtained from THP-1 cells occurred.

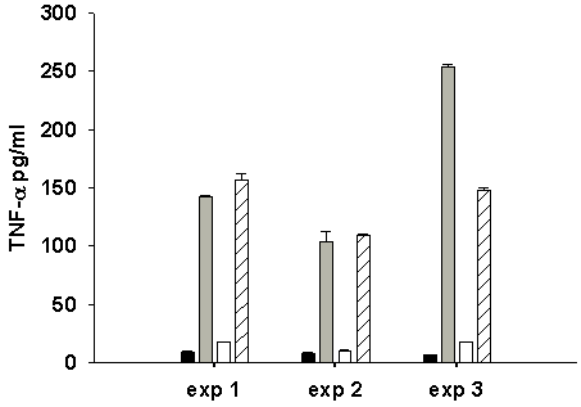

Figure 4.4: TNF-α levels after stimulation of THP-1 cells (□ THP-1 control, ▨ THP-1 + LPS) and monocytes (■ Monocyte control, ■ Monocytes + LPS) with 10 ng/ml LPS for 4 hours in stimulation media containing 10% human plasma. Measured with ELISA (Bender Medsystems).

	Monocyte control	*SD*	Monocytes + LPS	*SD*	THP-1 control	*SD*	THP-1 +LPS	*SD*
Exp 1	8.63	1.39	141.95	1.78	17.44	0.69	156.59	5.37
Exp 2	7.90	1.04	103.56	8.49	10.10	0.69	109.56	0.71
Exp 3	6.49	—	253.41	2.65	17.91	—	148.15	1.32

Table 4.4: TNF-α levels after stimulation of THP-1 cells and monocytes (pg/ml)

4.3 Stimulation of HUVEC and HPMEC ST1.6R with Conditioned Medium obtained from Monocytes and THP-1 Cells

As described in Chapter 3.4, release of cytokines, expression of adhesion molecules and NF-κB activity were used as read-out assays to measure the endothelial cell activation.

4.3.1 Cytokine Release after HUVEC and HPMEC ST1.6R Stimulation

TNF-α, IL-1β, IL-6, IL-8 and IL-10 were quantified with the Bio-Plex© Cytokine-Assay. Conditioned media were obtained from THP-1 cells and monocytes stimulated with 10 ng/ml LPS for 4 hours. Control medium was obtained from THP-1 cells and monocytes without addition of LPS.

IL-1β levels were generally very low and were only determined in the first experiment. In this experiment, HUVEC stimulated with monocytic conditioned medium produced less IL-1β (around 40 pg/ml) than HUVEC stimulated with THP-1 conditioned medium (around 70 pg/ml). IL-1β levels released from HUVEC with THP-1 conditioned medium decreased at 16 hours to about 4 pg/ml, whereas levels released from HUVEC with monocytic conditioned medium decreased just slightly (about 30 pg/ml). HPMEC ST1.6R stimulated with monocytic conditioned medium had a considerably higher production of IL-1β (around 30 pg/ml) compared to HPMEC ST1.6R stimulated with THP-1 conditioned medium, which had values in the range of the control media (around 3 pg/ml). IL-1β released from HPMEC ST1.6R after stimulation with monocytic conditioned medium did not decrease at 16 hours.

IL-10 levels were measured in all three experiments, but the secreted levels of IL-10 were below the detection limit of 8 pg/ml for all samples. The observed trend showed a higher IL-10 release from both HUVEC and HPMEC ST1.6R stimulated with monocytic conditioned medium than with THP-1 conditioned medium. The IL-10 release after stimulation with THP-1 conditioned medium was in the range of the control media. At 16 hours a decrease of the levels could be observed.

TNF-α secretion from HUVEC stimulated with conditioned medium obtained from monocytes was higher than secretion after stimulation with conditioned medium obtained from THP-1 cells. As shown in Figure 4.5, there was almost no difference between the TNF-α levels from the conditioned medium and the TNF-α levels after 1 hour of HUVEC-stimulation. After 16 hours, TNF-α levels were at the lower limit of detection. This implies that TNF-α was derived from monocytes & THP-1 cells and was furthermore degraded over 16 hours. In comparison, TNF-α secretion from HPMEC ST1.6R is illustrated in Figure 4.5, top right panel. Secretion after stimulation with conditioned medium obtained from monocytes was higher than secretion after stimulation with conditioned medium obtained from THP-1 cells.

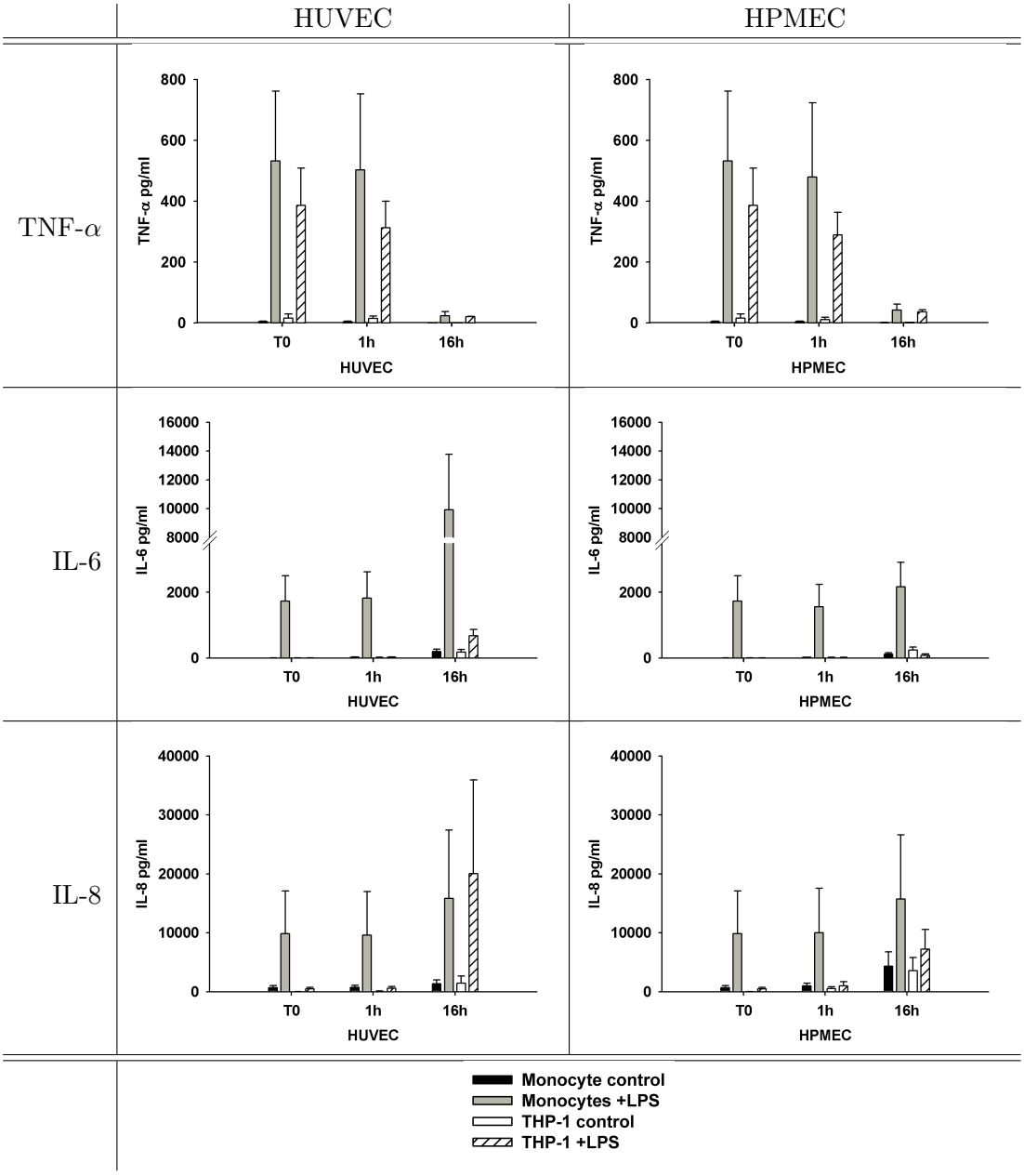

Figure 4.5: Cytokine Secretion for HUVEC/HPMEC ST1.6R after stimulation with conditioned media for 1h and 16 hours. Conditioned media were obtained from THP-1 cells and monocytes stimulated with 10 ng/ml LPS for 4 hours in stimulation media. Cytokine concentrations are expressed as mean ± SEM (n=3). T_0=cytokine concentration at the end of THP-1/monocyte stimulation (prior to application of the conditioned media to endothelial cells but after freezing two times). Measured with Bio-Plex Cytokine-Array (BioRad).

As explained above, there was almost no difference between TNF-α levels from the conditioned medium and TNF-α levels after 1 hour stimulation of HPMEC ST1.6R. At 16 hours no TNF-α could be detected anymore, similar to HUVEC as described earlier. Thus, regarding TNF-α secretion, there is no difference between HUVEC and HPMEC ST1.6R.

Conditioned medium obtained from monocytes had innately higher IL-6 levels than conditioned medium from THP-1 cells. Hence, it is not surprising that HUVEC stimulated with conditioned medium from monocytes had a higher IL-6 release than those stimulated with conditioned medium from THP-1 cells as depicted in Figure 4.5. There was almost no difference between IL-6 levels from the conditioned medium and after 1 hour stimulation of HUVEC. At 16 hours IL-6 levels were increased by a factor of 5 for HUVEC stimulated with conditioned medium from monocytes and by a factor of 24 for HUVEC stimulated with conditioned medium from THP-1 cells. This indicates that IL-6 is produced by the stimulated endothelial cells. Remarkably, the IL-6 level at 16 hours is lower for conditioned medium from THP-1 cells than the primary conditioned medium (T_0) obtained from monoctyes.

IL-6 secretion from HPMEC ST1.6R with conditioned medium from monocytes was higher than secretion with conditioned medium from THP-1 cells. As already mentioned for IL-6 secretion from HUVEC, medium obtained from monocytes had innately higher IL-6 levels than conditioned medium from THP-1 cells. There was almost no difference between the IL-6 levels from the conditioned medium (T_0) and after 1 hour stimulation of HPMEC ST1.6R. At 16 hours IL-6 levels were increased. Again can be said that the IL-6 levels at 16 hours are lower for conditioned medium from THP-1 cells than the T_0-level from monocytes. Comparing the two figures for IL-6 in Figure 4.5 reveals that IL-6 levels after 16 hours were lower for HPMEC ST1.6R stimulated with monocytic conditioned medium than for HUVEC.

As mentioned above for IL-6 levels, IL-8 was also innately higher for conditioned medium from monocytes than for THP-1 cells. This could be seen at HUVEC as well as HPMEC ST1.6R. IL-8 secretion from HUVEC with conditioned medium obtained from monocytes was higher than secretion with conditioned medium obtained from THP-1 cells. As stated above for TNF-α and IL-6, there was also almost no difference between the IL-8 levels from the conditioned medium and after 1 hour stimulation of HUVEC. At 16 hours IL-8 levels were increased, indicating that the HUVEC produced IL-8. Remarkably, the IL-8 increase from HUVEC stimulated with THP-1 conditioned medium was higher than the increase from HUVEC stimulated with monocytic conditioned medium.

In comparison, IL-8 secretion from HPMEC ST1.6R is depicted in the lower right panel of Figure 4.5. Secretion of IL-8 with conditioned medium from monocytes was higher than secretion with conditioned medium from THP-1 cells. As mentioned earlier, there was almost no difference between the IL-8 levels from the conditioned medium and

after 1 hour stimulation of HPMEC ST1.6R. At 16 hours, IL-8 levels were increased, similar to HUVEC as described above. This indicates again that the endothelial cells are producing IL-8.

4.3.2 Expression of Adhesion Molecules

The experiments described in Chapter 3.3.2 were also monitored with respect to the expression of the adhesion molecules ICAM-1 and E-selectin on HUVEC and HPMEC ST1.6R. The flow cytometric analyses were performed after 16 hours of stimulation with conditioned medium. In the first experiment an insufficient amount of cells was available to receive reliable results. However, the trend in this first experiment was comparable to the results received from the other two experiments. The determination of E-selectin also concluded in non-reliable results. Various reasons can be held responsible for that, e.g., a low amount of cells in general. Schildberger et al. (2010) showed that E-selectin was upregulated after 16 hours of HUVEC stimulation with conditioned medium obtained from THP-1 cells. The trend in experiment 3 was comparable to these findings, whereas experiment 2 showed no upregulation of E-selectin after HUVEC stimulation with THP-1 conditioned medium. However, HPMEC ST1.6R showed no upregulation at all for E-selectin in none of the three experiments.

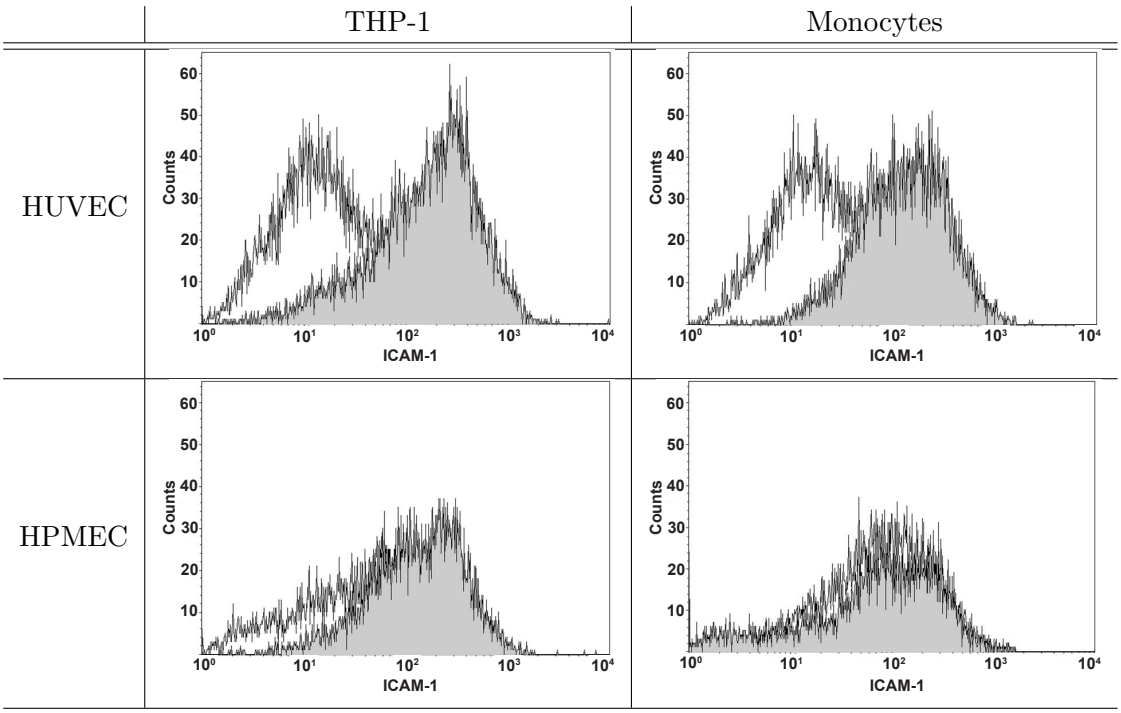

Figure 4.6: Expression of ICAM-1 on HUVEC/HPMEC ST1.6R stimulated with control medium (black) and monocytic conditioned medium/THP-1 conditioned medium (grey) from experiment 2

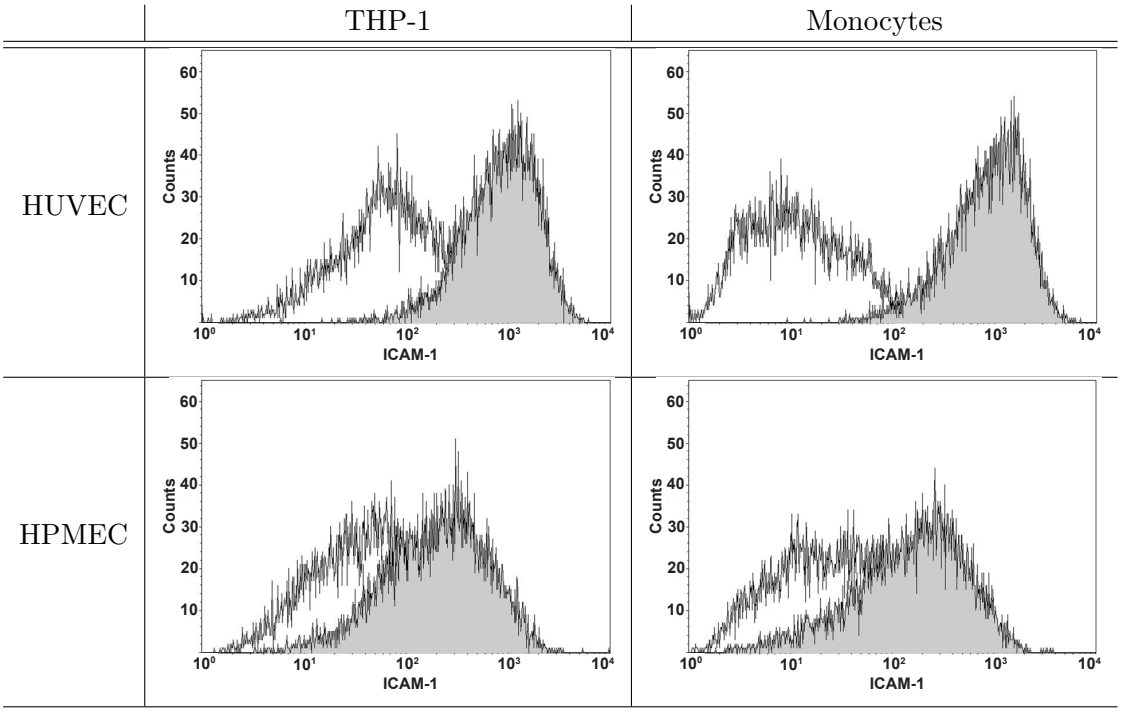

Figure 4.7: Expression of ICAM-1 on HUVEC/HPMEC ST1.6R stimulated with control medium (black) and monocytic conditioned medium/THP-1 conditioned medium (grey) from experiment 3

As shown in Figure 4.6, the second experiment resulted in upregulation of ICAM-1 on HUVEC. There is a slight difference between the shift after 16 hours stimulation with THP-1 conditioned medium and 16 hours stimulation with monocytic conditioned medium. Generally, a clear upregulation can be seen on HUVEC. In comparison, the basal expression of ICAM-1 on HPMEC ST1.6R is already relatively high. The stimulation with conditioned medium is not resulting in any upregulation of ICAM-1, no matter if the conditioned medium is obtained from THP-1 cells or monocytes.

Figure 4.7 shows the third experiment, which generally resulted in a clearer picture. Again it can be observed that ICAM-1 is upregulated on HUVEC after stimulation with conditioned medium. The upregulation of HUVEC stimulated with monocytic conditioned medium in this experiment is higher than with THP-1 conditioned medium. The basal expression of ICAM-1 on HPMEC ST1.6R is again relatively high as stated above. However, the stimulation with conditioned medium resulted in a slight upregulation, which was a little bit higher after stimulating with monocytic conditioned medium than after stimulating with THP-1 conditioned medium.

It was monitored that the upregulation of ICAM-1 generally can be observed more clearly on HUVEC than on HPMEC ST1.6R.

4.3.3 NF-κB activity

The three same experiments as described in Chapter 3.3.2 were monitored with respect to the NF-κB activity. The preparation of nuclear extracts was performed after 16 hours of stimulation with conditioned medium. However, from these three experiments (Chapter 4.8) just the last experiment (experiment 3) showed results as expected after previous experiments in our working group. There are several possible explanations: on one side there was a very low protein amount obtained after nuclear extraction, which could have resulted in non-reliable findings. On the other hand the time-point for measurement after 16 hours is already relatively late. As known from other experiments, the activity of NF-κB is already decreasing at this stage.

In the first experiment the NF-κB activity on HUVEC stimulated with monocytic conditioned medium was as high as the basal expression, whereas HUVEC stimulated with control medium obtained from monocytes showed even a lower NF-κB activity than the basal expression on HUVEC. THP-1 conditioned medium slightly upregulated the NF-κB activity compared to the basal expression, whereas the control medium obtained from THP-1 doubled the NF-κB activity compared with the basal expression. Regarding HPMEC ST1.6R, just the cells stimulated with the monocyte control showed a lower NF-κB activity than the basal expression. THP-1 control and both conditioned media resulted in a slight up-regulation of the NF-κB activity.

The second experiment depicts an up-regulation of NF-κB activity on HUVEC for THP-1 conditioned medium. The control was in the range of the basal expression as expected. NF-κB activity on HUVEC stimulated with monocytic conditioned medium or monocytic control medium were similar and showed in both cases a doubling of the NF-κB activity value of the basal expression. NF-κB activity values on HPMEC ST1.6R were all in the range of the basal expression, except for cells stimulated with monocytic conditioned medium, where it was slightly higher.

As shown in Figure 4.8, experiment 3 was the only experiment with a result consistent with previous experiments. The expression of NF-κB activity is increased for HUVEC stimulated with monocytic and THP-1 conditioned medium. The NF-κB activity after stimulating HUVEC with monocytic conditioned medium is 0.75-fold higher than for HUVEC stimulated with THP-1 conditioned medium. HPMEC ST1.6.R showed an increased NF-κB activity after stimulation with monocytic conditioned medium as well. Remarkably, it didn't show an increased NF-κB activity after stimulation with THP-1 conditioned medium as it was expected. As the control medium of THP-1 resulted in a double NF-κB activity on HPMEC ST1.6R compared to the basal expression, mistakes should be considered.

All in all, these experiments would have to be repeated using a higher number of cells to obtain a higher amount of protein in the nuclear extract.

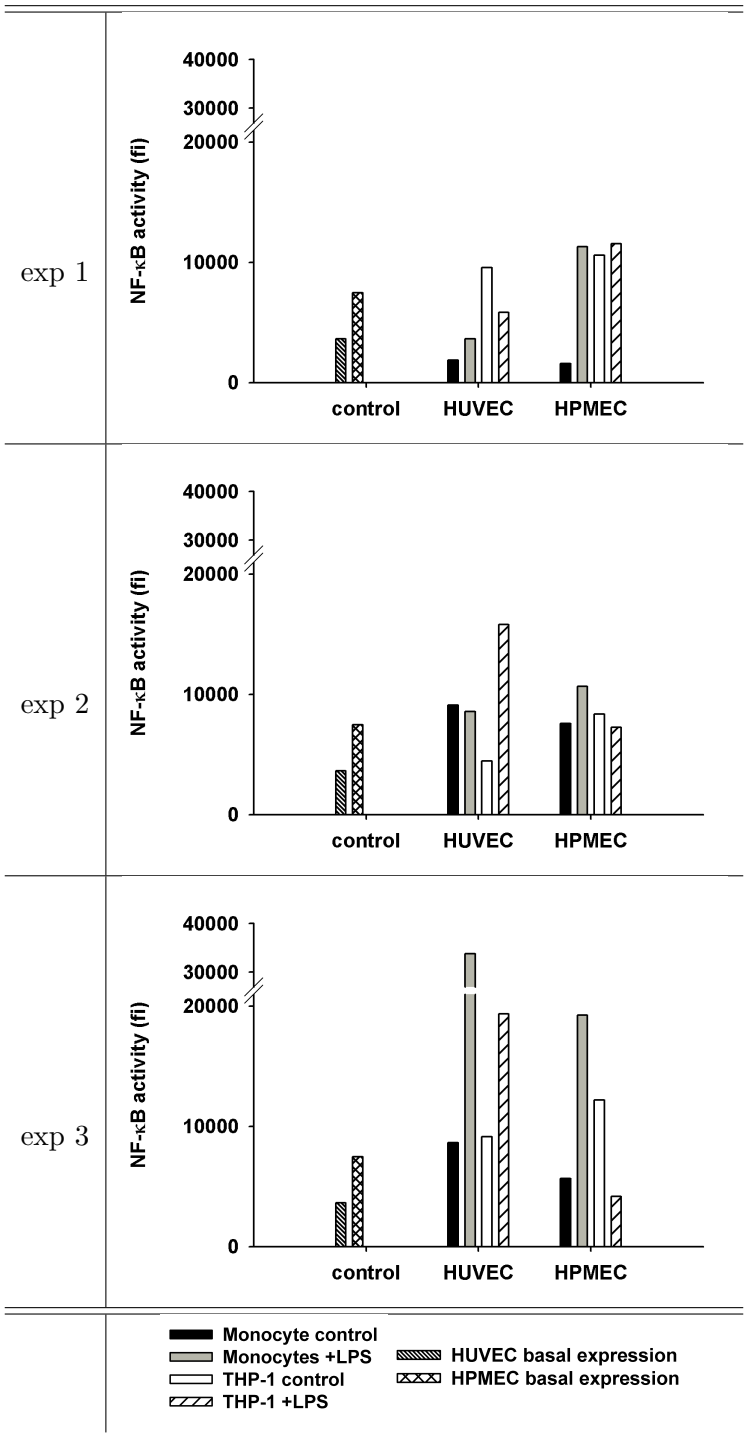

Figure 4.8: Expression of NF-κB activity from experiment 1–3. Nuclear extracts were obtained from HUVEC and HPMEC ST1.6R after 16 hours stimulation with conditioned media obtained from THP-1 cells and monocytes. Controls show the basal activity of NF-κB. NF-κB activity is expressed as mean ± std.dev in fluorescence activity (fi). Measured with Trans AM^{TM} NF-κB p65 Chemi Activity Assay (Active Motif).

5 Discussion

Sepsis is a complex syndrome of various clinical manifestations. Numerous sepsis models have been established, but the development of effective therapy still remains a challenge. In recent years, one approach to the treatment of sepsis was to target specific mediators of sepsis and blocking them with antibodies. However, these attempts turned out to be ineffective in reducing the mortality of this syndrome (Hotchkiss & Karl, 2003; Annane et al., 2005; Cohen, 2002). Reasons can be various; the selection of the proper target molecule (e.g. cytokines, endotoxines, mediators from coagulation or complement cascade, ...) and the proper timing of treatment seem crucial. This is due to the fact that inflammation in sepsis proceeds in different stages; after a hyperinflammatory phase, the so-called "cytokine-storm", where the levels of the mediators IL-1, IL-6, IL-8 and TNF-α rapidly rise, an anti-inflammtory phase follows which can easily processes into an immunoparalyzed status. This status is characterized by an inability of the immune system to react to infections properly and a massive apoptosis of immune cells.

The cell culture model characterized in this work was established to study the effect of selective adsorbents, which are developed for eliminating defined mediators in the inflammation cascade. In addition, it also allows to answer questions related to the pathophysiology of sepsis and to find new targets for adsorption or other therapeutic strategies. The processes of gram-negatively induced sepsis are simplified in this static model, which uses the continuous immortalized monocytic cell line THP-1 and primary Human Umbilical Vein Endothelial Cells (HUVEC). Reasons for the selection of a continuous cell line in general and THP-1 cells in particular were the ability to grow abundant quantities of cells in adequate time and the reduction of experimental variability. HUVEC, on the other hand, are a well characterized primary cell line and endothelial cells which are easy to acquire. The only caveats are the limited supply of cells due to slow growth rates and the laborious isolation process.

The aim of the characterization of the established cell culture model was to dispel doubts about the similarity of monocytic THP-1 cells and monocytes isolated from human blood. Sharif et al. (2007) demonstrated that the response to LPS stimulation of THP-1 cells and PBMC-derived macrophages is similar. According to this study, THP-1 cells are a more accurate model for LPS stimulation patterns than U937 cells, another monocytic continuous cell line. Nevertheless, a proof of THP-1 cells with the conditions and parameters of the established model was necessary.

In my work, I was able to demonstrate that THP-1 cells and PBMC-derived monocytes react similar, but not identical to LPS stimulation. For two out of three experiments, TNF-α levels after LPS stimulation of monocytes and THP-1 cells, respectively, were comparable.

A key factor in our model of gram-negatively induced sepsis is the stimulation process.

A combination of factors secreted by monocytic cells is used to stimulate the endothelial cells. This resembles the human *in vivo* situation in a better way than a single stimulus, but is different from other studies where a stimulus is given by single factors such as LPS or TNF-α. The concentrations of LPS or TNF-α used are often high and vary in the different models. Sharif et al. (2007) used 2 µg/ml LPS, Hashimoto et al. (2003) 1 µg/ml LPS, Heagy et al. (2003) 10 ng/ml LPS, Hoffmann et al. (2002) 10 ng/ml TNF-α, to mention just a few.

Furthermore, other cell culture models are often carried out as one-step-models. The two-step model used in this work, however, mimics the monocytic response to microbial patterns and the subsequent release of secreted factors as well as the induced activation of endothelial cells. The addition of 10% human plasma to the media instead of fetal bovine serum (FBS), which is the standard growth promoter in cell cultures, ensures an authentic response to the LPS stimulus. Human plasma contains coagulation factors and many other factors whose functions are not fully understood yet and which are not present in FBS.

Whereas HUVEC are macrosvascular endothelial cells which are easy to obtain and a widely used model for the endothelium, central events of sepsis take place in the microvasculature. Therefore, the second aim of this thesis was to compare macrovascular endothelial cells to or replacing them by microvascular endothelial cells, namely HPMEC ST1.6R.

In the established model, TNF-α was released from THP-1 cells or monocytes stimulated with LPS. TNF-α levels were not amplified by the endothelium, which can be seen from the nearly equal levels of TNF-α before stimulation of HUVEC and HPMEC ST1.6R and after stimulation for one hour. At 16 hours, TNF-α levels were reduced in both endothelial cell types for both conditioned media almost to the basal level, which indicates that TNF-α was degraded, a finding which is in line with the known short half-life time of TNF-α.

IL-6 and IL-8 showed a completely different picture. Both cytokines were primarily produced by the endothelium, which is reflected in an increase after 16 hours of HUVEC and HPEC ST1.6R stimulation. The increase was more significant on HUVEC and if monocytic conditioned medium was used. Remarkably, the IL-8 increase from HUVEC stimulated with THP-1 conditioned medium was higher than the increase from HUVEC stimulated with monocytic conditioned medium. To confirm this result, continuative experiments have to be carried out. IL-6, IL-8 and also TNF-α had generally higher levels if conditioned medium was obtained from monocytes compared to medium obtained from THP-1 cells.

During the detection of the expression of the adhesion molecules ICAM-1 and E-selectin on HUVEC and HPMEC ST1.6R, I faced various difficulties. For the first out of three experiments, the amount of cells available was too low to receive reliable results.

This was most probably also the reason for ambiguous results in the expression of E-selectin in general. In contrast to Schildberger et al. (2010) and Müller et al. (2002), I could not show clearly observable up-regulation of E-selectin after 16 hours of HUVEC stimulation with conditioned medium obtained from THP-1 cells. Results for HUVEC stimulated with conditioned medium obtained from monocytes were ambiguous, too. HPMEC ST1.6R showed no up-regulation for E-selectin in any of the three experiments. This is in contrast to Krump-Konvalinkova et al. (2001) and Unger et al. (2002), where an up-regulation of E-selectin, ICAM-1 and other cell adhesion molecules after stimulation with pro-inflammatory stimuli is described.

One possible explanation for this finding is that HPMEC ST1.6R in our laboratory were not the cells we thought them to be. As we obtained the cell line from the laboratory from Prof. Kirkpatrick in Mainz, many cells in the vial were found to be dead. Whether this was due to improper transport conditions or other reasons could not be determined. The cells we subsequently started to maintain could eventually represent the fittest cells, which survived from the original sample.

Up-regulation of ICAM-1 could be observed on HUVEC and on HPMEC ST1.6R from both conditioned media. Generally it can be said that the up-regulation was more clear on HUVEC than on HPMEC ST1.6R and it was slightly higher when the endothelial cells were stimulated with monocytic conditioned medium than with THP-1 conditioned medium.

Just the last out of three experiments for quantification of NF-κB activity showed results as expected from previous findings. In addition to a very low protein amount after nuclear extraction, the time-point of 16 hours was most likely in the decreasing phase of the oscillatory activity of NF-κB. López-Bojórquez et al. (2004) showed that HUVEC responded to conditioned medium obtained from LPS-activated macrophages with NF-κB activation. This activation was higher when stimulation was carried out with conditioned medium than with individual recombinant cytokines.

All in all, the NF-κB activity experiments would have to be repeated using a higher number of cells to obtain a higher amount of protein in the nuclear extract and a different time-curve. The miniaturization of the model from cell culture flasks as seen by Schildberger et al. (2010) to cell culture plates as used in this work is certainly a reason for the challenges with low cell numbers. However, just a minor amount of monocytes could be isolated with a justified investment of time and material, which also limited the scale of all following experiments. But miniaturization contains, on the other hand, various advantages, such as a lower use of reagents which results in lower costs and better statistical reproducibility due to a higher number of experiments.

Generally, it can be said that THP-1 cells and monocytes provided similar results in terms of cytokine release and expression of adhesion molecules. Monocytes result in slightly higher endothelial activation than THP-1 cells. The use of the cell line THP-1

instead of primary isolated monocytes in our two-step model of gram-negative sepsis is therefore fully justified for simplification reasons. Nevertheless, one should be aware of the fact that monocytes induce stronger activation compared to THP-1 cells.

Contrariwise, HUVEC and HPMEC ST1.6R could not be proven to exhibit similar behavior. If this is due to the questionable origin of the HPMEC ST1.6R or other reasons is to be the subject of future work.

The isolation process of monocytes was characterized by initially low yield. During the optimization process, two different protocols for density gradient centrifugation were compared. It is worth to point out that the protocol stated in the manufacturer's manual did not result in satisfactory yields. In addition, plastic tubes were substituted by glass tubes, whose surfaces were coated with Sigmacote© to avoid activation of the blood cells. The monocyte yield could be increased to an average of 48% during the optimization process. The purity of monocytes was determined with FACS-analysis and could be demonstrated to be 95%.

Further investigation of the LPS stimulation patterns of THP-1 cells and monocytes in the cell culture model used in this work seems worthwhile. In general, the model can be seen as a useful tool for testing new approaches to target specific mediators in sepsis.

List of Abbreviations

ACCP ..... American College of Chest Physicians

APACHE ..... Acute Physiology and Chronic Health Evaluation

APC ..... Activated Protein C

AT ..... Antithrombin

ATCC ..... American Type Culture Collection

ATS ..... American Thoracic Society

BSA ..... Bovine Serum Albumin

CASP ..... Colon Ascendens Stent Peritonitis

CD14 ..... Cluster of Differentiation-14

CDC ..... Centers for Disease Control and Prevention

CHF ..... Swiss Franc

CLP ..... Cecal Ligation and Perforation

CM ..... Conditioned Medium

DAMPs ..... Damage Associated Molecular Patterns

DIC ..... Disseminated Intravascular Coagulation

DMSO ..... Dimethyl Sulfoxide

EASIA ..... Enzyme Amplified Sensitivity Imunnoassay

ECGS ..... Endothelial Growth Factor Supplement

ED ..... Emergency Department

EDTA ..... Ethylenediaminetetraacetic Acid

ELAM-1 ..... Endothelial-Leukocyte Adhesion Molecule-1/E-selectin/CD62E

ELISA ..... Enzyme-Linked Immunosorbent Assay

EPCR-1 ..... Endothelial Protein C Receptor-1

ESICM ..... European Society of Intensive Care Medicine

EUR ..... Euro

FACS ..... Fluorescence Activated Cell Sorting

FBS ..... Fetal Bovine Serum

Fc ..... Fragment crystallizable

FDA ..... Food and Drug Administration, US Department of Health & Human Services

FITC ..... Fluoresceinisothiocyanat

FSC ..... Forward Scatter

GAG ..... Glycosaminoglycan

HBSS ..... Hank's Balanced Salt Solution

HEPES ..... 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

HMGB1 ..... High-mobility Group Protein-B1

HMWK ..... High-molecular-weight Kininogen

HPMEC ..... Human Pulmonary Microvascular Endothelial Cells

HRP ..... Horseradish Peroxidase

HSP ..... Heat Shock Protein

HUVEC ..... Human Umbilical Vein Endothelial Cells

ICAM-1 ..... Inter-Cellular Adhesion Molecule-1/CD54

ICD-10 ..... International Statistical Classification of Diseases and Related Health Problems, 10^{th} Revision

ICU ..... Intensive-care Unit

IFN ..... Interferon

IgG ..... Immunoglobulin G

IKK ..... IκB Kinase

IL ..... Interleukin

LBP ..... Lipopolysaccharide-binding Protein

LPS ..... Lipopolysaccharide

LTA ..... Lipoteichoic Acid

M199 ..... Medium 199 (Sigma-Aldrich)

MAC ..... Membrane Attack Complex

MIF ..... Macrophage Migration Inhibitory Factor

MODS ..... Multiple Organ Dysfunction Syndrome

MOF ..... Multiple Organ Failure

MyD88 ..... Myeloid Differentiation Primary Response Gene-88

NF-κB ..... Nuclear Factor kappa-light-chain-enhancer of activated B Cells

NLR ..... NOD-like Receptor

PA ..... Plasminogen Activator

PAI ..... Plasminogen Activator Inhibitor

PAMPs ..... Pathogen-associated Molecular Patterns

PBMC ..... Peripheral Blood Mononuclear Cells

PBS ..... Phosphate Buffered Saline

PE ..... Phycoerythrin

PECAM-1 .... Platelet-Endothelial Cell Adhesion Molecule-1/CD31

PGN ..... Peptidoglycan

PIRO ..... Sepsis Staging System focused on **P**redisposition, **I**nsult, **R**esponse & **O**rgan failure

PP ..... Polypropylene

PROWESS .... Protein C Worldwide Evaluation in Severe Sepsis Study

PRR ..... Pattern Recognition Receptor

PS, Pen/Strep . Penicillin-Streptomycin

SCCM ..... Society of Critical Care Medicine

SIRS ..... Systemic Inflammatory Response Syndrome

SIS ..... Surgical Infection Society

SOAP ..... Sepsis Occurrence in Acutely Ill Patients

SOFA ..... Sepsis-related Organ Failure Assessment

SSC ..... Side Scatter

SSC ..... Surviving Sepsis Campaign

SV40 ..... Simian Virus 40

TEM ..... Leukocyte Transendothelial Migration

TF ..... Tissue Factor

TFPI ..... Tissue Factor Pathway Inhibitor

TGF ..... Transforming Growth Factor

THP-1 ..... Human acute monocytic Leukemia Cell Line THP-1

TLR ..... Toll-like Receptor

TM ..... Thrombomodulin

TNF-α ..... Tumor Necrosis Factor-alpha

tPA ..... Tissue-type Plasminogen Activator

U.S. ..... United States of America

uPA ..... Urokinase-type Plasminogen Activator

USD ..... United States Dollar

VCAM-1 ..... Vascular Cell Adhesion Molecule-1/CD106

List of Tables

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