Glucocorticoid receptors in severe inflammation

Experimental and clinical studies

MARIA BERGQUIST
Septic shock is one of the most common causes of mortality in intensive care, in spite of antibiotic treatment. Glucocorticoid treatment can be used to blunt an overwhelming immune response in severe inflammation. The varying effects of glucocorticoid treatment in sepsis are poorly understood, with consequences for the clinical guidelines for treatment. Glucocorticoids are potent anti-inflammatory mediators which exert their effects through the glucocorticoid receptor (GR). Deeper understanding about the mechanisms of GR signalling may help to guide and improve glucocorticoid treatment. The aim of this thesis was to analyse GR expression and binding capacity in experimental and human septic shock and severe inflammation with cellular specificity using flow cytometry. In the late phase of a murine sepsis model, we observed decreased GR expression in leukocytes. In a murine model of early endotoxic shock, we observed decreased GR binding capacity in spite of an increased expression, in neutrophils. Glucocorticoid treatment was beneficial only when administered early in both models. Compared to healthy subjects, GR expression was increased in leukocytes from patients during the initial sepsis phase, while GR binding capacity was only increased in lymphocytes and monocytes. In contrast, neutrophils and other leukocyte subsets displayed decreased GR binding capacity. Neutrophil numbers were increased in all patients with sepsis compared to healthy subjects. We also studied patients with burn injury after admission before any infectious event had likely occurred, and on day 7 post admission, when several of the patients had been diagnosed with sepsis. GR expression and binding capacity was increased in leukocytes on admission as compared to healthy subjects, and patients diagnosed with sepsis on day 7 had a further increased GR expression in T lymphocytes. GR binding capacity was decreased in proportion to the extent of the burn injury on day 14 post admission. In conclusion, sepsis and severe inflammation have significant impact on the expression and function of GR, likely to influence the efficiency of glucocorticoid treatment. In addition, glucocorticoid treatment is beneficial only when given early in these models of experimental sepsis.

Keywords: glucocorticoid receptor, sepsis, inflammation, flow cytometry

Maria Bergquist, Department of Medical Sciences, Clinical Physiology, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

© Maria Bergquist 2014

ISSN 1651-6206
ISBN 978-91-554-8994-6
urn:nbn:se:uu:diva-229119 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-229119)
Jag vet att bortom det jag dunkelt anar finns nya ting, mer sällsamt underbara än de jag höll förundrad i min hand.
Jag vet. Och jag är rik som ingen.
Jag håller i min hand de gåtfulla vissa tingen och deras bröder vänta mig i dolda land.

Pär Lagerkvist (1891-1974)
Istället för tro, 1919
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with permission from the publisher.
List of publications not included in this thesis


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>11β-HSD</td>
<td>11β hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adreno Corticotrophic Hormone</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>CARS</td>
<td>Compensatory Anti-inflammatory Response Syndrome</td>
</tr>
<tr>
<td>CBG</td>
<td>Cortisol Binding Globulin</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CIRCI</td>
<td>Critical Illness Related Corticosteroid Insufficiency</td>
</tr>
<tr>
<td>CORTICUS</td>
<td>Corticosteroid Therapy of Septic Shock</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-Releasing Hormone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>GCS</td>
<td>Glasgow Coma Scale</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid Response Element</td>
</tr>
<tr>
<td>HPA-axis</td>
<td>Hypothalamic-Pituitary-Adrenal axis</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid Receptor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor κ B</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural Killer cells</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RLS</td>
<td>Reaction Level Scale</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic Inflammatory Response Syndrome</td>
</tr>
<tr>
<td>TBSA</td>
<td>Total Body Surface Area</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Toxic Shock Syndrome Toxin</td>
</tr>
</tbody>
</table>
Introduction

Sepsis has plagued man since the dawn of time, described for the last 2000 years but the clinical definitions are recent (Bone et al. 1992, Vincent et al. 2013). It should be noted that patients dying of infectious diseases inevitably die of sepsis and sepsis related organ failure. As said by Lewis Thomas, it is not the infection itself that kill people but rather it is the hosts’ immune response attempting to fight the infection that ultimately causes the fatal outcome.

“They [microorganisms] will invade and replicate if given the chance, and some of them will get into our deepest tissues and set forth in the blood, but it is our response to their presence that makes the disease. Our arsenals for fighting off bacteria are so powerful, and involve so many different defence mechanisms, that we are in more danger from them than from the invaders. We live in the midst of explosive devices; we are mined.’
Lewis Thomas in The lives of a cell, 1974

The incidence of sepsis is increasing in all areas of the world where epidemiological studies have been conducted (Martin 2013). Gram-positive bacteria are currently the most common cause of sepsis (Annane et al. 2005). Despite falling proportional fatality rates with sepsis, the total number of people dying with sepsis each year continues to increase due to the growing number of cases/year (Gaieski et al. 2013). In spite of adequate antibiotic treatment, mortality remains high, and glucocorticoid treatment can be used with the rationale to blunt an overwhelming immune response. However, the use of glucocorticoid treatment in patients with sepsis and septic shock remains controversial in spite of a long history of trials (Moran et al. 2010). Outcomes from numerous studies published during the last decades range from demonstrating improved survival (Annane et al. 2002) to failing to show any mortality benefit and even indicating harmful effects (Sprung et al. 2008). Although not showing any difference in survival, the Corticosteroid Therapy of Septic Shock (CORTICUS) study showed a shorter time to
shock reversal in the hydrocortisone treated group as compared to placebo (Sprung et al. 2008). Nevertheless, a meta-regression analysing randomised trials testing low steroid doses for a week or more report a reduction in mortality, even when taking the CORTICUS study into account (Annane et al. 2009). The varying results can in part be explained by methodological differences between the trials, such as choice of drug, dose, duration and severity of illness. Most importantly, however, the variations illustrate the unpredictable outcome of the clinical application of a biological substance for which the mechanisms of action are not sufficiently known (Jaeschke & Angus 2009). As a consequence, there is no clear consensus for glucocorticoid treatment in sepsis, a fact that translates into discrepancies in treatment strategies between intensive care units. In particular, the high incidence of acquired adrenal insufficiency in septic patients has provided a strong rationale for the prolonged administration of low-dose corticosteroids (Annane et al. 2002), but the difficulties in assessing functional adrenal status are evident from the present literature (Venkatesh & Cohen 2011). In spite of these controversies, half of all patients in the intensive care units receive glucocorticoid therapy (Beale et al. 2010). Deeper understanding of the mechanisms behind the attenuated glucocorticoid response during sepsis may allow a more rational and individualised approach to glucocorticoid treatment. The focus of this thesis is therefore to investigate the glucocorticoid receptor in sepsis and severe inflammation.

Septic shock

Septic shock is one of the most common causes of death in intensive care, in spite effective antibiotics. It is projected to represent one million cases per year before the year 2020 in the United States alone (Dombrovskiy et al. 2007). The early high mortality is held to be caused by the overwhelming infection induced, the pro-inflammatory response consisting of both cellular and humoral components, which can progress into systemic disease and multi-organ failure. The host response is complex and varies both inter- and intra-individually with pro-inflammatory elements, referred to as the systemic inflammatory response syndrome (SIRS), and anti-inflammatory components, called the compensatory anti-inflammatory response syndrome (CARS) (Bone 1996, Munford & Pugin 2001). The inflammatory status of the patient is traditionally characterised by plasma concentrations of either pro-inflammatory cytokines such as tumour necrosis factor (TNF) and inter-
leukin 6 (IL-6) or anti-inflammatory cytokines such as interleukin 1 receptor antagonist (IL-1RA) or interleukin 10 (IL-10). More recently it has been demonstrated that the early phase of lethal sepsis is characterized by an overexpression of both pro- and anti-inflammatory cytokines simultaneously (Munford & Pugin 2001).

Although these processes are thought to happen simultaneously to some degree, in general there seems to be an initial predominance of hyperinflammation, driven by ‘cytokine storms’ causing fever, refractory shock, acidosis and multiple organ failure (Figure 1).

**Figure 1. Potential host immune response to sepsis.** Recent studies demonstrated that the early phase of lethal sepsis is characterized by an overexpression of both pro- and anti-inflammatory cytokines simultaneously. The host response is complex and highly variable depending on several factors such as patient’s age, comorbidities and pathogen virulence. Early mortality in septic shock is usually caused by a ‘cytokine storm’ causing high fever, shock and organ failure (upper red line). In case the pathogen is cleared, the patient recovers and the inflammatory balance is restored to a state of homeostasis (horizontal line). Late mortality in sepsis may be due to an insufficient or absent hyperinflammatory phase leading to an immunosuppressive state with high risk of secondary infection (lower red line). Adapted from (Hotchkiss et al. 2013b)

Early mortality in such patients is usually caused by cardiovascular collapse due to the hyperinflammatory response, as in examples of toxic shock syndrome or meningococcemia (Hotchkiss et al. 2013a). As the population is getting older, 75% of patients who die of sepsis in modern intensive care units are 65 years or older (Martin et al. 2006). These patients are often immunosuppressed and display less obvious signs of sepsis, such as hypoten-
sion, hypothermia and confusion, and may not have a detectable immunologic response to sepsis in either hyperinflammatory or immunosuppressive direction. Indeed, as treatments are improved and early mortality can be averted, immunosuppression may follow hyperinflammation even in previously healthy patients, in cases of prolonged disease with late mortality (Figure 1).

Impact of sepsis on the immune system

Sepsis affects all cells of the immune system, directly or indirectly. Leukocyte production in the bone marrow is increased and immature or newly differentiated cells are being released to travel with the circulation and migrate into inflammatory tissues (Geissmann et al. 2003). Cell apoptosis is variable between leukocyte types, and while apoptotic mechanisms related to sepsis have been linked to glucocorticoids (Ayala et al. 1995), TNF (Bogdan et al. 1997) and Fas-ligand (Ayala et al. 1998) in experimental animal models, the mechanisms behind sepsis induced apoptosis in humans remain elusive.

Neutrophils

Neutrophils belong to the innate immune system and are essential for early control of invading microorganisms and survival in sepsis (Marshall 2005). Neutrophils have a normal lifespan of around 24 hours after release from the bone marrow in a healthy subject, but develop a resistance to apoptosis in sepsis (Tamayo et al. 2012). A systemic neutrophil activation as in sepsis is also a high risk for the host as it is known to cause tissue damage and may lead to multi-organ failure (Brealey & Singer 2000, Thijs & Thijs 1998). Because of their delayed apoptosis, and that new neutrophils are continuously being released from the bone marrow during sepsis, patients often have excessive numbers of circulating neutrophils of different degrees of maturation (Drifte et al. 2013). Documented abnormalities in neutrophil function during sepsis include reduced production of reactive oxygen species (ROS), clearance of bacteria and loss of chemotactic activity (Alves-Filho et al. 2010, Kovach & Standiford 2012, Cummings et al. 1999). Moreover, in spite of not being known to release large quantities of cytokines, during sepsis they can produce vast amounts of IL-10 (Kasten et al. 2010) which may contribute to or worsen late phase immune paralysis and secondary infection related mortality.
**Monocytes**

Monocytes circulate in blood for less than 24 hours before migrating into tissues, where they differentiate into macrophages or dendritic cells, both during homeostasis and inflammation (Auffray et al. 2009). In addition, they are recruited to sites of infection to mediate direct antimicrobial activities (Serbina et al. 2008) and draining lymph nodes to promote adaptive immune responses (Shi & Pamer 2011). In sepsis, mediated by increased levels of IL-10 (Fumeaux & Pugin 2002) and cortisol (Le Tulzo et al. 2004), the reduced human leukocyte antigen (HLA) DR expression seen in monocytes is correlated to increased mortality (Tschaikowsky et al. 2002), possibly because of an impaired ability to present antigens to T lymphocytes. Monocyte production of pro-inflammatory cytokines, e.g. TNF, IL-1 and IL-6 is decreased in sepsis, although anti-inflammatory cytokine production, e.g. IL-10 and IL-1RA, is unaltered or increased (Cavaillon & Adib-Conquy 2006, Biswas & Lopez-Collazo 2009). These alterations occurring during sepsis may, if progressed, lead to immune paralysis.

**Natural Killer Cells**

Little is known about NK cells in human sepsis, probably due to their preferential location being in tissue under normal conditions, with circulating numbers decreasing even further during sepsis (Chiche et al. 2011, Venet et al. 2010, Forel et al. 2012). However, patients who do not survive sepsis exhibit less NK cell depletion than survivors and the remaining cells were very early activated and rapidly differentiated (Andaluz-Ojeda et al. 2011). NK cells have also been demonstrated to have decreased function in burn injury (Blazar et al. 1986, Bender et al. 1988).

**Lymphocytes**

T lymphocytes are classically considered as major players of the adaptive immune system, but are also crucial for the host response to several micro-organism derived exotoxins, e.g. toxic shock syndrome toxin (TSST-1). Sepsis readily triggers apoptosis in T and B lymphocytes, which are found to be decreased in both number and function (Hotchkiss et al. 2001, Hotchkiss et al. 1997), leaving a lymphopenic environment. CD4+ T lymphocytes are essential for the regulation of monocyte and macrophage function, hence severe ramifications for the immune response after their rapid and extensive loss to apoptosis. CD8+ T lymphocytes play a critical role in the control and elimination of intracellular pathogens (Harty et al. 2000). Upon recognition of microbial antigens, naive CD8+ T lymphocytes differentiate into antigen
specific effector cells which undergo extensive clonal expansion. After the invading pathogen is eliminated, CD8+ lymphocytes retrocede to normal levels and the remaining cells initiate the immunological antigen specific memory (Duong et al. 2014). Thus, apoptosis of CD8+ T lymphocyte numbers could seriously affect the host capacity to efficiently mount the immune response.

It is of essential importance for recovery to sustain the balance between effector and suppressor forces during immune response. Regulatory T lymphocytes are less prone to sepsis induced apoptosis (Monneret et al. 2003), leading to an immunosuppressive phenotype as a consequence of the other lymphocyte populations decreasing in number. Prolonged durations of sepsis with high pathogen load can exhaust the surviving T lymphocytes, with profound suppression of cytokine production and surface receptor expression (Boomer et al. 2011). Glucocorticoids have also been demonstrated to tip the equilibrium by having strong inhibitory effects on the proliferation of T effector lymphocytes and inducing differential apoptosis of regulatory T lymphocytes (Pandolfi et al. 2013).

B lymphocytes play a pivotal role in the adaptive immune response, including producing antibodies and presenting antigens to T lymphocytes (Vaughan et al. 2011), but have recently been found to be a relevant participant of innate immunity as well (Kelly-Scumpia et al. 2011). The role of B lymphocytes in sepsis is to the main part elusive, but a newly discovered subset of B lymphocytes (innate response activator B, IRA-B) has been shown to be critical in the immediate sepsis response (Rauch et al. 2012). It has also been suggested that B lymphocytes are contributors to the shift towards immunosuppression in sepsis (Shubin et al. 2011).

**Eosinophils**

Eosinophils are normally found in low numbers in circulation, with approximately 1-3% of total number of leukocytes (Rothenberg 1998). The further reduction of eosinophils in sepsis is still an unexplained phenomenon, but may be caused by cytokines such as TNF, acute phase reactants, adrenaline or glucocorticoid levels (Bass et al. 1980). The apoptotic effect of glucocorticoids may be mediated via inhibition of interleukins stimulating eosinophil growth and differentiation (i.e. IL-3, IL-5, and granulocyte macrophage colony stimulating factor, GM-CSF) (Druilhe et al. 2003). Further supporting this hypothesis, an increased level of circulating eosinophils is associated
with clinical signs of relative adrenal insufficiency (Beishuizen et al. 1999). In the beginning of the 1900s eosinopenia functioned as a marker for sepsis (Shaaban et al. 2010), and more recently it was observed that non-survivors have lower eosinophil numbers than survivors in sepsis (Merino et al. 2012).

**Cytokines and chemokines**

During exposure to pathogens during sepsis, toll-like receptors and intracellular pattern recognition receptors act as sensors of the pathogen-associated molecular patterns (PAMPs), recognized as danger signals. Cytokines play a critical role in the microbicidal response in sepsis by contributing to leukocyte recruitment, induction of haematopoiesis and fever. Leukocytes as well as endothelial and epithelial cells contribute to cytokine production, which can be both pro- and anti-inflammatory, beneficial or deleterious (Cavaillon et al. 2003).

Vast amounts of TNF, IL-6 and IL-8, among others, are circulating in plasma during sepsis. These main orchestrators of inflammatory cascades induces the release of large amounts of other cytokines, as well as their own production, i.e. TNF induces more TNF (Descoteaux & Matlashewski 1990), IL-1 induces more IL-1 (Dinarello et al. 1987), and in contrast, IL-10 negatively regulates IL-10 via autocrine loops (de Waal Malefyt et al. 1993).

IL-8 has been correlated to poor outcome in sepsis, the occurrence of shock (Endo et al. 1995) and development of multi organ failure (Marty et al. 1994). Increased levels of monocyte chemoattractant protein-1 and 2 (MCP-1 and MCP-2) (Bossink et al. 1995), macrophage inflammatory protein-1a and 1b (MIP-1a and MIP-1b) (O'Grady et al. 1999) and interferon-induced peptide-10 (IP-10) (Olszyna et al. 1999) have been found in patients with sepsis and volunteers after lipopolysaccharide (LPS) injection. MCP-1 is correlated to lethal outcomes and shock (Bossink et al. 1995).

Some cytokines are strictly considered to be anti-inflammatory, such as IL-10 and IL-1RA, which are also higher in non-survivors than survivors of sepsis (Marchant et al. 1994, Goldie et al. 1995). Some can be viewed as having dual roles, with both pro- and anti-inflammatory actions, such as in the case of IFN-γ (Zhao et al. 1998) and even IL-6, which can protect against mortality from experimental endotoxemia (Yoshizawa et al. 1996).
Burn injury

Burn injury is one of the most severe traumas imaginable. It is usually caused by thermal energy, such as scalding, flame or contact with hot objects, but similar trauma can be caused by exposure to caustic chemicals or radiation. The stress factors after burn injury are many and continuous; large open wounds, dressing changes, mechanical ventilation, surgery and infection, all plausible triggers of severe inflammation. The injury itself causes immediate or subsequent cell damage or death, giving rise to an inflammatory cascade activation. In addition, a hypermetabolic state follows with an ebb state, characterized by reduced metabolism, cardiac output and oxygen consumption, where after a flow state implacably follows with increased metabolism, peripheral insulin resistance, extensive protein wasting, lean body mass loss, bone and muscle catabolism and even functional and structural alterations of essential organs (Pereira et al. 2005, Miller & Btaiche 2009, Finnerty et al. 2008). The altered homeostasis after burn injury inevitably leads to changes in the circulating levels of cytokines, glucagon, catecholamines and steroids (Gauglitz et al. 2009, Jeschke et al. 2008). It has also been established that injury significantly alters transcriptional activity of pro-inflammatory mediators such as NF-κB and AP-1 in T lymphocytes (O’Suilleabhain et al. 2001). Moreover, after severe and persistent immune activation, an endogenous immune paralysis may follow, increasing the risk of subsequent infection and sepsis. Criteria for diagnosis of infection and sepsis used in most patients cannot be applied in burn injured patients. Due to the continuous inflammatory reaction after burn injury and the constant exposure to environment after loss of the primary barrier to microorganisms, inflammatory mediators are chronically released. In addition, high temperature (>38.5°C), significantly altered white blood cell counts, tachycardia and tachyspnea are routinely found after burn, making the standardized definitions incomplete indicators of sepsis (Greenhalgh et al. 2007). Instead, sepsis can be diagnosed by experienced burn physicians based on other indications such as increased fluid requirements, low platelet count and declining pulmonary and renal function.

Major burn injury, defined as more than 20% of the total body surface area (TBSA) in adults (Garmel 2012) causes severe systemic inflammation with marked elevations in plasma and tissue cortisol and adrenocorticotropic hormone (ACTH) (Parker & Baxter 1985, Vaughan et al. 1982, Cohen et al. 2009, Wilson et al. 1955) and Hypothalamic-Pituitary-Adrenal (HPA) axis
perturbations (Palmieri et al. 2006). It has also been confirmed that the risk of developing critical illness related corticosteroid insufficiency (CIRCI) after burn injury is increased with greater TBSA and older age, and is often missed (Reiff et al. 2007). With a disturbed HPA-axis leading to inadequate cortisol levels, exogenous glucocorticoid administration may be beneficial. However, due to the potential increased risk of infection and negative effects on wound healing, glucocorticoid treatment is not recommended in burn injury (Palmieri et al. 2006). The suggested inhibitory effects of exogenous glucocorticoid treatment on wound healing are believed to be due to anti-inflammatory actions, although endogenous glucocorticoids have been shown to have a regulatory role in wound repair in mice (Grose et al. 2002). This highlights the need for further investigation of GR and GC mechanisms in burn injury.

The HPA axis

Critical illness is associated with abnormal stress which activates the HPA axis, in turn stimulating the production of Corticotropin-Releasing Hormone (CRH) and ACTH to trigger cortisol secretion from the adrenal glands (Figure 2) (Chrousos 1995, Mastorakos et al. 1995). This hormone axis is firmly linked with the central nervous system and essential for survival (Tsigos & Chrousos 2002, McEwen 2007). Cortisol is the main endogenous glucocorticoid in humans (in mice, it is corticosterone). Under normal conditions, most of the circulating cortisol (~90%) is bound to the specific carrier protein cortisol binding globulin (CBG) (and a smaller fraction to albumin) (Gagliardi et al. 2010) and it thereby has restricted access to target cells. Only upon release from CBG, cortisol can migrate freely across cell membranes. Feedback regulation of the HPA-axis with inhibitory effects on CRH and glucocorticoid release is mediated by glucocorticoid and mineralocorticoid receptors (MR) located in the brain and anterior pituitary responsible for ACTH secretion. MR are also found in Na⁺ transporting epithelia, e.g. kidney and colon, and non-epithelial tissue, e.g. brain, heart and vessel wall (Funder 2005). MR binds cortisol with equal affinity as aldosterone and corticosterone in vitro (Arriza et al. 1987). In vivo, MR is protected from cortisol activation by 11β hydroxysteroid dehydrogenase (11β-HSD) conversion of excess cortisol into cortisone, inert to the receptor, and thereby allowing access to aldosterone (Edwards et al. 1988).
Figure 2. Schematic illustration of the Hypothalamic-Pituitary-Adrenal (HPA) axis feedback loop. Adrenal glucocorticoid production in sepsis is the result of the immune system pathogen-induced cytokine response. In turn, the immune response is modulated by the increased cortisol production. Adapted from (Webster & Sternberg 2004)

The HPA axis is a cornerstone in host protection during sepsis. Elevated levels of circulating plasma cortisol hallmarks critical illness (Boonen & Van den Berghe 2014), although inappropriately low levels of cortisol have also been linked to increased mortality (Widmer et al. 2005). Approximately half of the patients in septic shock have an inadequate glucocorticoid activity for the severity of illness, defined as CIRCI (Marik 2009, Annane et al. 2003, Annane 2008, Maxime et al. 2009). In spite of that the diagnostic criteria, originally based on a landmark study by Annane et al (Annane et al. 2000), are still under debate (Arafah 2006, Cohen et al. 2006), the concept is widely accepted – but whether or not these patients benefit from treatment with exogenous glucocorticoids remain a controversy.

It is generally inferred that increased circulating cortisol during critical illness is a consequence of increased HPA-axis activity and increased levels of ACTH, although ACTH levels have been found at similar levels or even
below those of healthy controls (Vermes et al. 1995, Polito et al. 2011, Boonen et al. 2013). These findings suggest that rather than an increased production of cortisol, elevated cortisol levels are a consequence of a dysfunctional cortisol clearance from circulation which results in the supranormal cortisol levels found in the critically ill. Indeed, the expression and activity of A-ring reductases (the principal route of cortisol breakdown in humans) and 11β-HSD type 2 (converting cortisol to cortisone, inert to cells) were found reduced in liver but not in adipose tissue (Boonen et al. 2013). This raises the question whether the glucocorticoid receptor, the main target of cortisol, also has a decreased expression or function during critical illness. Previous quantitative studies of GR in critical illness investigated mRNA expression levels (Guerrero et al. 2013, Ledderose et al. 2012, van den Akker et al. 2009), which precludes conclusions about the protein expression and subsequent receptor function.

In addition to being the main humoral mediators of stress, cortisol is also an important integrator of normo-physiological functions, such as the circadian rhythm. These robust rhythms are maintained by the daily light-dark cycle and bi-directionally connected to eating and sleeping cycles of the organism (Riedemann et al. 2010). In spite of supranormal cortisol levels frequently found in sepsis, the diurnal rhythm is lost (Hardin 2009).

Glucocorticoids have been found to induce apoptosis in several cells and tissues, such as T lymphocytes, eosinophils and osteoblasts, and impair apoptosis in others, such as neutrophils, erythrocytes and liver cells (Schmidt et al. 2004). The increased endogenous cortisol levels in sepsis may thereby be the cause of the alterations in relative and absolute leukocyte numbers.

**Glucocorticoid Receptor**

Glucocorticoids are potent anti-inflammatory mediators commonly used in the treatment of a variety of inflammatory diseases. Their anti-inflammatory effects were first demonstrated to alleviate symptoms in rheumatoid arthritis in the 1940s (Hench 1949) and were the topic of the Nobel Prize in physiology 1950. Glucocorticoids act via the glucocorticoid receptor (GR) which is localized in the cytoplasm in its inactive state, stabilized by heat-shock proteins (Hsp 70 and Hsp 90) (Figure 3).
Figure 3. Transactivation. GRα resides in its inactive state in the cytoplasm, stabilized by heat shock proteins (Hsp). Upon activation by ligand binding, heat shock proteins dissociate from the GR, which then can translocate into the nucleus. There, it forms homodimers and binds to glucocorticoid response elements (GRE) on DNA, where it can interact with transcription complexes to enhance transcription of target genes. Adapted from (Leung & Bloom 2003).

Once activated by ligand binding, GR translocates into the cell nucleus where it can act either by induction of transcription (transactivation) as a homodimer or by interfering with expression of pro-inflammatory genes, e.g. NF-κB or Activator protein 1 (AP-1) (transrepression) (Figure 4) (Barnes & Larin 1997). Transrepression by GR monomers is generally held to mediate the majority of the anti-inflammatory effects of the GR-Glucocorticoid complex (Reichardt et al. 2001). Recent studies with GR dimerization deficient mice have shown that regulation of GRE is required for survival of septic shock (Kleiman et al. 2012), suggesting that transrepression alone, i.e. downregulation of NFκB and others, is not sufficient for management of sepsis.

The human GR has several isoforms, the predominant function belonging to GRα. The isoforms are expressed from the same gene (NR3C1) consisting of nine exons, but by alternative splicing of exon 9, the less abundant GRβ is expressed instead (Bamberger et al. 1995). GRβ has no known ligand binding activity but has been shown to form heterodimers with GRα and compete for glucocorticoid response elements (GRE) on DNA, and may thus inhibit the function of GRα (Figure 5). It has been proposed that an overexpression
NFκB consists of two subunits (p50 and p65) and resides in its inactive state in the cytoplasm, stabilized by IkBα. Upon activation by cytokines through membrane receptors, IkB kinase (IKK) phosphorylates IkBα, which then is degraded by proteases. NFκB translocates into the nucleus, where it binds to the κB motif on DNA, to promote transcription of pro-inflammatory target genes. Activated GR can bind to NFκB and impair its transcription by protein-protein interaction. Adapted from (Leung & Bloom 2003).

of GRβ, or imbalance between the two isoforms, could cause glucocorticoid resistance (Guerrero et al. 2013), but so far little is known about the GRα/GRβ ratio in sepsis and the significance of increased GRβ concentration for glucocorticoid treatment response is controversial (Yudt et al. 2003, Torrego et al. 2004, Goecke & Guerrero 2006, Kino et al. 2009).

Studies of human monocytes revealed that GC treatment in addition to suppressing the inflammatory functions of monocytes, also shifted them into an activated phenotype with anti-inflammatory effects including phagocytotic properties, which is less prone to apoptosis (Ehrchen et al. 2007). Furthermore, GC treatment has been shown to stimulate resolution of inflammation by monocytes and macrophages by increasing their clearance of pro-inflammatory complexes and dying neutrophils (Yona & Gordon 2007). GCs are commonly viewed as strictly immunosuppressive, although they have been shown to facilitate and assist in maintaining of immunity (Tischner & Reichardt 2007). Patients with primary adrenal insufficiency, Addison’s disease, lack endogenous GCs and despite of rigorous GC
replacement, this patient group are more susceptible to infection (Ruzek et al. 1999) and exhibit a more than doubled expected mortality rate (Bergthorsdottir et al. 2006). Within the normal physiologic range of HPA-axis activity, GCs can be immune stimulatory (Galon et al. 2002, Diefenbacher et al. 2008), while supraphysiologic doses result in anti-inflammatory effects. These findings strongly suggest that the effects of GCs are highly dose dependent.

In addition to the genomic effects of GCs mediated by GR acting as transcription factors, GCs can have immediate (within minutes) effects which seem to be independent of genomic regulation (Tasker et al. 2006). Growing evidence suggests that immediate (non-genomic) effects are mediated by a membrane bound GR and include cell response modulations, cardiovascular effects including myocardial inotropic activity, endothelium integrity, capillary permeability and smooth muscle interactions maintaining vascular tone (Sun et al. 2006). Also, GCs interact with noradrenaline and angiotensin II (Annane 2005). Although membrane bound GR are still relatively unexplored, advanced biomolecular techniques offers promise for further knowledge about these receptors and their therapeutic potential in the near future (Strehl et al. 2011, Vernocchi et al. 2013).
Modelling human sepsis in the mouse

One major drawback of studying sepsis in humans is the many insistent uncertainties, such as disease onset and the ethical problems of manipulating treatment in critically ill patients. Today, it is not ethically accepted to perform clinical interventional studies on unconscious subjects within the European Union. In addition, in clinical studies there is an inevitable variety within the study population due to existing comorbidities, associated injuries and varying time from onset of symptoms. The extensive assortment of pharmaceuticals used in clinics today further encumbers the analysis of patient groups. By studying sepsis in mice, several of these confounding influences and uncertainties can be overcome. Because the mouse is genetically and immunologically well characterized, it allows for disregard of the minimal genetic variance between individuals, and in-depth analysis of immune responses often translatable to human sepsis. Timing and choice of treatment can be strictly controlled, and due to the multitude of previous studies in mice, many pharmaceuticals and doses are already validated in mouse models.

A major advantage of using *Staphylococcus aureus* for modelling gram-positive sepsis is that mice, like humans, can be spontaneously infected by *S. aureus* (Bremell et al. 1991). A considerable difference in between the species is that the mouse is less sensitive to both bacteria and endotoxin (Warren et al. 2010, Schaedler & Dubos 1961), and therefore requires much higher doses to mirror a human response. The difference is likely an evolutionary effect caused by the contrasting environmental and antigen pressure on mice and humans in their respective habitats.

*Staphylococcus aureus*: gram positive sepsis

More than a hundred years ago, Ogston described his clinical observations of staphylococcal disease and its role in sepsis (Ogston 1882). Still today, *S. aureus* remains a versatile threat to humans, with an increasing frequency of infection but with little change in overall mortality (Lowy 1998, Laupland 2013). *S. aureus* can colonize human skin and mucosal membranes without causing harm, but it is also well known for causing infections, ranging from superficial skin infections to invasive sepsis and endocarditis. Among patients suffering from infections in the intensive care units, *S. aureus* remains one of the most common causative organisms (up to 20%) (Vincent et al. 2009).
S. aureus is, compared to other clinically relevant bacteria, unique in the sense that it is an increasing pathogen in both hospital and community settings, it continues to develop resistance to antimicrobial treatment, and it is a highly virulent pathogen. The many virulence factors which contribute to the success of the bacterium as a pathogen are, among others, avoiding phagocytosis, production of enzymes allowing tissue invasion and the release of superantigens like TSST-1. Superantigens are potent polyclonal T lymphocyte activators which form ternary complexes with MHC II and T-cell receptors, causing a profound non-specific T lymphocyte activation and systemic release of cytokines, such as IL-2, TNF and IFN-γ (Uchiyama et al. 1987, Papageorgiou & Acharya 2000).

LPS-induced endotoxic shock

Among the gram negative bacteria, Escherichia coli (E. coli) is the main pathogen in septic shock with an estimated frequency of 9-27% (Annane et al. 2005). Endotoxin, a major component from the outer membrane of gram-negative bacteria, e.g. LPS, can be used to model the systemic immune response seen in SIRS and septic shock, and activates the HPA axis in a similar way (Beishuizen & Thijs 2003). Infections by gram-negative bacteria are first sensed by the host via surface recognition of pathogen-related antigens (such as LPS and peptidoglycan) by Toll-like receptors (TLR) (Aderem & Ulevitch 2000). This pathway activates innate immune signalling by transcription factors NFκB, AP-1 and others, triggering the induction of proinflammatory genes transcribing e.g. TNF, IL-1 and IL-6, with major implications in the pathogenesis of sepsis (Salomao et al. 2008). The LPS induced shock model cannot fully replicate the clinical situation of severe systemic gram-negative infection. The main caveats are the relatively condensed temporal resolution, compared to the extended time from actual infection to manifest sepsis, and the absence of live bacteria for the immune system to eradicate. In spite of these limitations, LPS induced shock is useful in modelling SIRS and to modulate factors, which is not possible in the clinical setting.
Aims

Glucocorticoid treatment has variable effects in patients with sepsis, and the underlying biological mechanisms are not sufficiently understood. A deeper understanding of glucocorticoid receptor function during severe inflammation may help to explain these variations. The main aim of this thesis was to investigate GR expression and function in experimental and clinical sepsis and severe inflammation.

The specific aims were;

- To investigate if glucocorticoid receptor expression and/or binding capacity is altered in sepsis and/or severe inflammation (Paper I-IV)

- To investigate whether GR has an altered nuclear translocation in experimental sepsis and/or severe inflammation (Paper I-II)

- To investigate if timing is an important factor for glucocorticoid treatment in experimental sepsis and endotoxic shock (Paper I-II)
Methods

Animal models
Male C57BL/6J mice were obtained from Charles River Laboratories (Wilmington, MA) 6 to 8 weeks old, and maintained in the animal facility at the Department of Rheumatology and Inflammation Research at Gothenburg University under standard light and temperature conditions. Mice were fed soy-free laboratory chow and tap water ad libitum. Permission from the local animal research ethics committee, in accordance with national animal welfare legislation, was obtained for all experiments. All animals were weighed daily and systemic inflammation was visually graded by inspection of activity, fur condition and breathing frequency. Weight development was used to monitor general health and dehydration, with weight loss as a strong indicator of the animals’ failing to maintain a fluid balance by ceasing to eat and drink. If a mouse was judged too ill to survive for another 12 hours or if it was neurologically affected (mono- or hemiplegia), it was euthanized and defined as dead due to septic shock. The animals were anaesthetised with a mixture of Ketalar (Pfizer AB, Sollentuna, Sweden) and Dormitor Vet (Orion Pharma, Espoo, Finland) before they were culled.

Experimental S. aureus sepsis and LPS-induced shock
The TSST-1 producing S. aureus strain LS-1 was used for induction of sepsis by intravenous inoculation, as previously described (Gjertsson et al. 2012b). In short, bacteria were grown over night, harvested and resuspended in phosphate buffered saline (PBS) containing 5% bovine serum albumin and 10% dimethylsulphoxide (DMSO) and kept in aliquots at -20°C until use. The number of colony forming units (CFUs) was determined by repeated viable counts. Before inoculation, the bacteria were thawed, washed in PBS and diluted to an appropriate concentration based on the previously determined CFUs. Viable counts of inoculates were performed to determine the actual number of viable bacteria given in each experiment. Mice were inoculated intravenously with $3 \times 10^8$ CFU/mouse of S. aureus in 200 μL PBS in one of the tail veins.
For induction of endotoxic shock, mice were administered 10 mg/kg LPS from *E. coli* (O111:B4) in 200 μL PBS by intraperitoneal (i.p.) injection. The LPS dose was chosen from an initial dose finding experiment with three doses of 5, 10 and 25 mg/kg (n=18, data not shown).

**Dexamethasone Treatment Experiments**

To investigate whether the timepoint for treatment start is an important factor for dexamethasone treatment outcome, mice were administered Dexadrenson (Intervet AB, Sollentuna, Sweden) 0.05 mg/kg i.p. once daily for three (Paper I, SI Figure 2) or four (Paper II, SI Figure 3) consecutive days. Dexamethasone was chosen mainly for its longer duration of action and lack of mineralocorticoid effects, relative to hydrocortisone (Table 1).

| Table 1. Comparison of natural and synthetic hydrocortisone and dexamethasone |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| GR potency | MR potency | Shock reversing potency | Duration of action | Plasma Half-life | Equivalent dose |
| Hydrocortisone | 1 | 1 | High | 8-12 h | 90 min | 20 mg |
| Dexamethasone | 25 | 0 | Low | 36-72 h | 200 min | 0.75 mg |


The dexamethasone dose was chosen from recent literature where dexamethasone doses were titrated in male C57BL/6 mice after cecal ligation and puncture (van den Berg *et al.* 2011). In this experiment, the lowest dose (0.05 mg/kg) was associated with reduced mortality, but limited effect on immune response. The higher doses (0.25-2.5 mg/kg)powerfully reduced cytokine levels but did not affect mortality. As a decrease in mortality, not inflammation, is the main purpose for clinical treatment, we chose the low dose for experiments.

**Patients and healthy subjects**

**Patients with Septic Shock (Paper III)**

Twenty patients with sepsis were recruited between February 2012 and May 2013 in the general intensive care units of the Sahlgrenska University Hospital and the Kungälv Hospital. Informed consent was initially obtained via next of kin and later directly from survivors. The selection of patients was influenced in a non-systematic way over time by the availability of laboratory resources and not by patient characteristics. This multicenter study was
approved by the Ethical Committee for Human Research in Uppsala, Sweden.

**Inclusion criteria**

(i) age over 18  
(ii) sepsis according to the American-European consensus criteria (Levy et al. 2003)  
(iii) treatment with at least one vasopressor

**Exclusion criteria**

(i) known infection with human immunodeficiency virus or hepatitis B or C  
(ii) chronic treatment with GCs  
(iii) participation in any investigational drug study within four weeks preceding the study period,  
(iv) survival expected to be shorter than three days

**Blood and clinical data were collected on five occasions:**

(i) Within 24 hours of admission (T0)  
(ii) 24 hours after T0 (T1)  
(iii) 48-120 hours after T0 depending on logistics (T2)  
(iv) 4-19 days after ICU admission when the acute inflammatory phase was considered to be over and the patient was stable without support to vital functions (T3)  
(v) 5-13 months after the patient had been discharged from the intensive care unit (T4)

Clinical variables relevant for the degree of septic shock and organ dysfunction (blood pressure, heart rate, lactate, ScvO2, temperature, a-pH, base excess, serum creatinine, serum bilirubin, Glasgow Coma Scale (GCS) score, type and dose of vasoactive/inotropic agent) were registered for all timepoints where applicable. Blood gas values and clinical laboratory data (CRP, leukocyte and platelet counts and microbiological cultures) were obtained from laboratory records.

**Patients with Burn Injury (Paper IV)**

Thirteen patients were recruited between March 2012 and March 2013 from a larger study cohort in the Burn Center (BC) of Uppsala University Hospital, Sweden. Informed consent was initially obtained via a next of kin and later directly from survivors. Patient selection was defined by the availability
of laboratory analysis resources which varied in a non-systematic way independent of patient characteristics. This study was approved by the Ethical Committee for Human Research in Uppsala, Sweden.

**Inclusion criteria**

(i) age over 18 regardless of type of burn injury

**Exclusion criteria**

(i) known malignancy or immune deficiency
(ii) known infection with human immunodeficiency virus or hepatitis B or C
(iii) treatment with GCs, cytostatic drugs, tetracyclines or certain bisphosphonates
(iv) participation in any investigational drug study within four weeks preceding the study period

**Blood and clinical data were collected on five occasions:**

(i) Within 24 hours of admission (A)
(ii) 7 days after admission (Day 7)
(iii) 14 days after admission (Day 14)
(iv) 21 days after admission (Day 21)

For analysis of the impact of initial inflammation, the patients were categorized according to the extent of the burned surface, here defined as severely injured with >20% TBSA or moderately injured with <20% TBSA. For analysis of the impact of sepsis, patients were grouped according to its presence on day 7. Sepsis was considered present if there were laboratory signs of infection (increased CRP and PCT, increased or reduced leukocytes, reduced platelets) along with clinical signs of infection (body temperature >39 or <36 degrees, obvious wound infection, signs of pneumonia) and positive bacterial cultures from blood, airways or wounds. Newly developed circulatory instability with reduced blood pressure, increasing lactate levels and need for intravenous fluid and inotropic support were also included when presence of sepsis was evaluated. Clinical data representative of vital organ dysfunction (lowest platelet count, highest serum creatinine, highest serum bilirubin, lowest Reaction Level Scale (RLS 85) (Starmark et al. 1988) peak/maximal vasoactive/inotrope dose, lowest mean arterial pressure and
lowest PaO2/FiO2) during preceding 24 hours from sampling were registered and additional laboratory data were obtained from routine analyses (CRP, leukocytes, procalcitonin, and microbiological cultures when indicated). Patient weight, daily cumulative fluid administration and net fluid balance were registered on the days of sampling.

**Healthy subjects (Paper III and IV)**

Consenting healthy subjects were continuously recruited during the studies among non-smoking laboratory and hospital staff without any chronic or acute illness and with no medication. Samples were taken once from each healthy subject and served as comparison to patient samples in both studies (Paper III and IV).

**Flow cytometry**

Flow cytometry is a laser-based analytical technology commonly employed in cell biology as well as routine clinical chemistry and clinical research. By suspending cells and letting them pass a laser beam one by one in a stream of fluid, they can be biophysically separated by their characteristics, such as their relative size and granularity. The instrument measures these characteristics using an optical-electronic coupling system which records the laser scatter of each individual cell. Using fluorescent labels such as conjugated antibodies against distinct proteins, it is also possible to further separate cells by relative fluorescence intensity.

Flow cytometry was used in Paper I-IV to measure immune cell frequencies and to relatively quantify GR expression and bound FITC-dexamethasone by mean fluorescence intensity (MFI). Single cell suspensions were made of whole blood (Paper I-IV), spleen (Paper I and II) and lymph nodes (Paper I), and stained with surface markers for immune cell separation. The samples were then divided for intracellular staining of GR (Figure 6) and a FITC-dexamethasone binding assay. The antibody used for detection of GR was a monoclonal pre-conjugated antibody raised against a conserved region of the human GR with a high homology with the mouse GR. The specificity for mouse cells as well as the optimal concentration resulting in the highest signal of GR positive population and lowest signal of negative population was established. To control for unspecific binding, an isotype control was used for all sample types. The isotype control is an antibody of the same class (isotype, in this case human IgG1) raised against an antigen which is pre-
sumed to not be present in the studied cells. The isotype control is matched to the GR antibody by the same fluorochrome conjugate and the same sample preparation, i.e. buffer concentrations, permeabilisation, fixation and incubation times, etc. This process can be used to exclude potential undesirable antibody binding to Fc-receptors or unspecific fluorochrome binding (Figure 6).

**Figure 6.** Flow cytometry analysis of glucocorticoid receptor expression in CD8+ lymphocytes. a) Forward (FSC) and Side scatter (SSC) separates cell population based on their size and granularity. b) Lymphocytes divided by expression of CD4+ and CD8+. c) GR-FITC expression in CD8+ population. d) GR-FITC (red) compared to the isotype control (IgG1-FITC, white) expression in CD8+ population as histogram showing mean fluorescence intensity (MFI).

For analysis of GR binding capacity, FITC-labelled dexamethasone was used. The optimal binding assay of FITC-labelled dexamethasone was obtained by comparing different dilutions and incubation times. By pre-incubating samples with the equal concentration of unlabelled dexamethasone, the unspecific binding of FITC-labelled dexamethasone was determined to be approximately 7%. As an internal negative control unlabelled dexamethasone was used under the exact same conditions as the studied populations.
Although flow cytometry is an excellent technique to perform quantitative measurements on individual cells with speed, accuracy and precision, flow cytometers must be calibrated frequently to ensure reliability. The stability, uniformity and reproducibility of the instrument may vary over time due to temperature changes, laser drift, optics performance and stream flow etc. In addition, as sample preparation of each individual sample is performed on different days, variability in quantum yields of bound fluorescent dyes, as well as contingent instrument or operator errors must be eliminated. In the analysis of all patient samples, an internal standard was stained and analysed together with each sample on every occasion to control for day to day variation. The internal standard consisted of frozen aliquots of peripheral blood mononuclear cells (PBMCs) from a healthy blood donor. By normalising each sample to the internal standard sample, containing the exact same number of epitopes each time, the absolute number of GR/dexamethasone per cell in a sample is unknown, but it ensures an accurate and reproducible relative quantification, overcoming sample preparation or instrument variations over time.

Imaging flow cytometry

The ImageStream platform (Amnis Corp., Seattle, WA, USA) is a novel technology which combines the separating strength of flow cytometry with high resolution imaging. By merging the ability to acquire statistically robust cytometry data with the special resolution of detailed digital microscopy, it is possible to collect more features of cells, such as morphology and fluorescent signal location.

In Paper I and II, the ImageStream technique was used to analyse co-localization of splenocytes from mice with sepsis (Paper I) or LPS-induced endotoxic shock (Paper II) and healthy control mice. Subcellular compartments (nucleus and cytoplasm) can be measured using a DNA dye, and by combining this with the fluorescently labelled GR antibody and FITC-labelled dexamethasone, it is possible to study co-localization and determine the relative amount of fluorescent molecules that have been translocated into the nucleus.

The major drawbacks of imaging flow cytometry are the limited throughput and number of colours, as compared to flow cytometry. Flow cytometers
normally analyse large numbers of different cell types due to fluorescent labels and high sensitivity because of their optics, while the fairly new imaging systems can still only analyse a few colours at once and it needs to acquire a large number of cells to achieve statistically robust data. While a flow cytometer can separate cells rather automatically based on defined characteristics such as size, granularity and fluorescent colour, imaging flow cytometry requires individual cell annotation to manually define classifications of nuclear translocation, etc. However, it is offering much deeper information compared to traditional cytoplasmic/nuclear fractionation techniques, which are limited to analysing an assortment of cell types. Also, traditional determination of subcellular localisation by compartment analysis would be performed using e.g. Western blot; a technique which is semi-quantitative at best and hardly can compete with the quantitative ImageStream analysis. Therefore, the combination of flow cytometry and imaging flow cytometry allows high resolution, statistically robust and high throughput analysis of leukocyte population frequencies, relative receptor density, binding capacity and translocation.

qPCR

Polymerase chain reaction (PCR) is a technology which mimics DNA replication in a test tube. Using a thermal cycler and one key enzyme, thermostable DNA polymerase, it amplifies specific DNA sequences from relatively small amounts of starting material. The process involves three steps which are repeated; melting (denaturing), annealing of primers to target DNA sequences and elongation (DNA polymerase extension of the primers).

Using fluorescence to label the amplified DNA, the amount of DNA formed after each cycle can be detected. The amount of initial DNA in the sample generates a proportionally faster increase in fluorescence. This method is called quantitative, or real time, PCR (qPCR), and is the method of choice in molecular biology for gene expression analysis, viral load and pathogen detection, among others.

The main advantages with qPCR is that it is fast and considered to be a high throughput technique with high repeatability. Its outstanding sensitivity is an obvious advantage, detecting down to a few copies of DNA. However, it is also a disadvantage, as it is extremely sensitive to errors and impurities in
sample preparation. Further disadvantages are costs of material and time consuming data analysis.

Statistics
Data have been analysed using Graphpad Prism for Windows (Graphpad Software Inc, La Jolla, CA, USA) and are presented as means and standard error of the mean (SEM) (Paper I) or mean and standard deviation (SD) (Paper II and IV). In Paper III, due to data distribution, raw data is presented as geometric means and confidence intervals. For statistical testing of not normally distributed data (Paper III and IV), the data were transformed using the natural logarithm followed by two-tailed Student’s t test, one-way ANOVA with either Bonferroni, Tukey or Dunnett’s corrections where appropriate.

Weight changes in Paper I was compared using linear regression in a mixed model in the Statistical Language R using the Ime4 package. Survival curves in Paper II were analysed using a Log Rank Mantel Cox test (Graphpad Prism). Multivariate analysis in Paper III was performed using SAS 9.3 (SAS Inst Inc, Cary, NC, USA).
Results and Comments

Glucocorticoid Receptor Expression

Experimental *Staphylococcus aureus* sepsis (Paper I)
To investigate the effect of *S. aureus* sepsis on GR expression and function, we analysed CD4+ and CD8+ T lymphocytes, B lymphocytes, monocytes and neutrophils in blood and spleen from infected and healthy control animals. The main finding was that GR expression progressively decreased throughout the experiment in all analysed leukocyte populations in blood and spleen during *S. aureus* sepsis (the decrease did not reach statistical significance in spleen monocytes) (Figure 7). In lymph nodes, GR expression tended to decrease in septic mice during the disease course, although not significantly decreased compared to healthy controls at the end of the experiment. To our knowledge, decreased GR expression over time and with cellular specificity has not been described previously during the course of sepsis. The changes were first evident in blood leukocytes exposed to initial bacteraemia, followed by a decrease in spleen and tentative decrease in lymph node cells.

Figure 7. Glucocorticoid receptor (GR) expression in CD8+ lymphocytes from blood and spleen of mice during *Staphylococcus aureus* sepsis analysed by flow cytometry and determined as mean fluorescence intensity (MFI). Data represent mean and standard error of the mean (SEM). n = 4-8 animals per group. *p<0.05, **p<0.01, ***p ≤ 0.001, ****p ≤ 0.0001, One-way ANOVA with Bonferroni correction.

That GR is essential for life is demonstrated by that new-born GR knock-out mice die due to a defect in lung maturation (Cole *et al.* 1995). A marked
decreased GR expression during progressing inflammation would most likely fail to meet the turbulent inflammatory response, which has detrimental effects for the host. In addition, as the corticosterone levels at this time are also decreased, one could speculate that the decrease in GR expression is maladaptive. In the case of adrenal dysfunction or exhaustion, it would be economic to upregulate GR to obtain the maximal effects of the little glucocorticoids which may be produced. However, as we have only analysed GR in circulating leukocytes and in lymphoid organs (spleen and lymph nodes), this study does not expand our understanding to other tissues, such as endothelium and central organs e.g. lungs, kidneys and liver. By downregulating GR in lymphoid cells and upregulating GR in vital organs which are not invaded by pathogens, the host could potentially save central organs from failing while not compromising the anti-inflammatory properties of leukocytes.

Another possible explanation for the decreased GR expression in blood could be an increased number of circulating immature cells with putative lower GR expression. However, this explanation is not likely to be the single cause of the decreased GR expression observed in spleen cells, as to our knowledge, immature leukocytes have not been demonstrated in organs during sepsis. Regardless of the reason for the decreased GR expression, the net result for the individual is most likely a decreased ability to respond to endogenous cortisol or glucocorticoid therapy.

**Experimental endotoxic shock (Paper II)**

The observed GR decrease in the late phase of *S. aureus* sepsis (Paper I) prompted us to investigate the GR expression and function in the early phase of shock. Using an endotoxic shock model in Paper II, we studied early regulations of GR expression and dexamethasone binding, and its correlations with initiation of glucocorticoid treatment. In this study we observed that neutrophils and B lymphocytes increased their GR expression in both blood and spleen. In the acute LPS-induced shock model, initial GR regulations were more distinct in spleen than in blood leukocytes, which may be a consequence of LPS being administered i.p. following that abdominal organs were exposed before and/or to a higher dose than peripheral blood leukocytes. Our finding of increased GR expression is consistent with a previous study where mice injected with Shiga toxin from gram-negative bacteria displayed an increased GR protein expression in neutrophils 24 hours after injection (Gomez *et al.* 2003). Several other experimental studies suggest
that GR expression is decreased after endotoxin exposure, although these studies have used radioligand competition assays with $^{3}$H-dexamethasone as a marker for GR (Stith & McCallum 1983, Li & Xu 1988), which does not allow conclusions about the GR density *per se*, as dexamethasone would quantify GR binding rather than GR expression.

A previous study in human peripheral blood mononuclear cells (PBMC) showed that bacterial superantigens increase the concentration of GR$\beta$ and suggest this causes T lymphocyte resistance to glucocorticoids (Hauk *et al.* 2000), although the questioned antigen specificity of polyclonal antibodies against GR$\beta$ makes this conclusion uncertain, as well as the use of immunohistochemistry for quantification. As the GR$\alpha$ and $\beta$ isoforms originate from alternative splicing of the same gene, the only accurate way of specifically quantifying the GR$\beta$ isoform to date is by primer design over the exon 8-exon 9 splice site. As this receptor isoform is expressed at a very low level, a primer design avoiding the contamination of genomic DNA is essential. In Paper II, we designed an assay for the relative quantification of mRNA expression of the two GR isoforms in mouse. The results show that both GR$\alpha$ and $\beta$ are decreased during LPS-induced shock (Figure 8). Hence, there is an inconsistency in between the GR protein and mRNA concentration, or in between the spleen and the kidney. In a hyperinflammatory situation, it is plausible that cells will generate new GR at the highest rate allowed by the rate limiting factor. In eukaryotes, transcription is estimated to be slower than translation because of transcription initiation, intron excision and post-transcriptional RNA processing. Additionally, one mRNA strand is translated by several ribosomes simultaneously (polysomes). It is thus credible that the GR mRNA is translated into protein faster than new mRNA can be transcribed.

![Figure 8](image-url)  
*Figure 8.* Expression levels of glucocorticoid receptor isoforms GR$\alpha$ and GR$\beta$ mRNA in kidney from healthy control mice and mice with endotoxic shock 12 and 36 hours after injection of lipopolysaccharide (LPS). Horizontal lines represent means. *$p \leq 0.05$, **$p \leq 0.01$, One-way ANOVA with Tukey correction.*
Patients with Septic Shock (Paper III)

With the background of early and late GR regulations during experimental sepsis and endotoxic shock, we wished to explore if similar regulations occur in human subjects. In this study, GR expression during and after sepsis requiring intensive care was studied. The kinetics of the GR response in individual patients was highly variable, reflecting different individual time spans from infection. Therefore, an average of the individual maximum and the individual minimum values of GR expression (GRmax and GRmin) during the treatment episode (T0-T3) were chosen for comparison to healthy subjects. The main finding of this study is that the maximum GR expression was increased in all analysed leukocytes and decreased at its minimum in neutrophils and eosinophils (Figure 9). The downregulation of GR observed in granulocytes occurred when patients were respiratory and circulatory stable, without support to vital functions.

This is, to our knowledge, the first study reporting GR protein expression over time during sepsis. Previous studies by other groups have analysed GR mRNA expression in either neutrophils (van den Akker et al. 2009) or T lymphocytes (Ledderose et al. 2012). As leukocytes of the innate and adaptive system carry different roles in the immune response to sepsis, and as all circulating cells are affected by the septic environment, no single subset of leukocytes can be distinguished as the most important one. Cells of the adaptive system, T and B lymphocytes, displayed a slower GR upregulation as compared to the innate immune cells (granulocytes and monocytes). This discrepancy is likely due to that the adaptive immune cells are classically dependent on other cells for their activation, whereas innate immune cells can react instantly to immunological triggers.
Figure 9. Individual maximum and minimum values of glucocorticoid receptor (GR) expression (GRmax and GRmin) in T lymphocytes and neutrophils from peripheral blood sampled during and shortly after the sepsis phase (T0-T3) and at one single occasion for healthy subjects (HS). Box plot bands represent the median and whiskers the range. Data were normalised using the natural logarithm and differences between patients and healthy subjects were tested using One-way ANOVA with Dunnett’s multiple comparisons test. **p<0.01, ****p<0.0001.

We observed no difference in GR expression in between patients treated or not treated with hydrocortisone. Traditionally, GR mRNA transcription is thought to be downregulated by glucocorticoids, a phenomenon which has been observed in vitro (Okret et al. 1986, Burnstein et al. 1994) and in rat tissue (Kalinyak et al. 1987, Dong et al. 1988). This effect is likely to be dose-dependent and perhaps also mediated by dexamethasone (used in the mentioned studies), but not necessarily by hydrocortisone.

Patients with Burn Injury (Paper IV)
The findings from septic shock prompted us to investigate GR in patients suffering from both non-infectious shock and sepsis in sequence, hence the choice to study burn injury. We investigated GR expression and binding capacity during the inflammatory and infectious phases of burn injury requiring specialized burn care. On admission, GR expression was significantly higher in the severe group as compared to healthy subjects in T lymphocytes, while higher in both patient groups as compared to healthy subjects in neutrophils before any infectious invasion (Figure 10).
In line with this, previous research in mice has also shown increased GR expression after burn injury (D'Elia et al. 2010). Increased GR expression may be a direct effect of the immediate inflammatory response following burn injury. We did not observe any significant difference in between the severe and moderate burn groups on admission. It has been suggested that the inflammatory and metabolic response is dependent on the size of the injury, but such conclusions have been drawn from persistent inflammation and changes in body compositions from admission to discharge (Jeschke et al. 2007). Hence, the inflammatory response may not differ noticeably between severe and moderate burn injury during the first 24 hours post injury.

The GR expression increased in T lymphocytes from admission to day 7 in all patients (n=5) who were diagnosed with sepsis, but decreased in neutrophils in most patients (n=4/5) (Figure 11). As most of the patients who were diagnosed with sepsis also had a significant burn injury, it is not possible to exclude the possibility that the GR alterations are due to the burn extent rather than the presence of sepsis. However, all patients with sepsis displayed an increased GR on day 7 in T lymphocytes and NK cells, in spite that all of them did not have an injury >20% TBSA, which makes sepsis a possible reason for the observed regulations.
Figure 11. Glucocorticoid receptor expression (GR) change from admission to day 7 in leukocytes from peripheral blood of patients with or without sepsis on day 7. Data points represent individual patients. GR expression increases from admission to day 7 in T lymphocytes from patients with sepsis and decreases in patients without sepsis (p=0.03). In neutrophils, no difference in between the groups was observed. Data were normalized using the natural logarithm and differences between the groups were tested using a paired Students t test.

Glucocorticoid Receptor Binding Capacity

GR binding capacity was assessed by \textit{ex vivo} binding of fluorescence-labelled dexamethasone, determined by flow cytometry as mean fluorescence intensity (MFI) and normalized to an internal standard.

Experimental \textit{Staphylococcus aureus} sepsis (Paper I)

As a measure of GR function, we analysed the \textit{ex vivo} binding of FITC-labelled dexamethasone in CD4+ and CD8+ T lymphocytes, B lymphocytes, monocytes and neutrophils in blood and spleen from infected and healthy control animals. GR binding capacity tended to decrease in T lymphocytes (CD4+ and CD8+) in blood, in opposite to spleen where a transient increase was observed for CD4+ lymphocytes. On the contrary, B lymphocytes demonstrated a doubling of FITC-labelled dexamethasone binding in both blood and spleen. A decreased binding capacity was seen in monocytes from blood, although this decrease was not consistent in monocytes from spleen. The binding capacity was not altered in neutrophils from neither blood nor spleen, or in lymphocytes originating from lymph nodes. As the GR expression was decreased, it may be a compensatory mechanism that its binding capacity is increased, as in the case of B lymphocytes. On the other hand, as lymphocytes are known to go into apoptosis during sepsis (Hotchkiss \textit{et al.} 2001), the biological consequence of an increased binding capacity in B lymphocytes may be negligible for the organism, in relation to the expanding neutrophil numbers with and intact GR binding capacity. B lymphocytes
are known to be responsive to glucocorticoids in all developmental subsets (Gruver-Yates et al. 2013) which may suggest that the increased GR binding capacity seen in B lymphocytes is the reason for their broad apoptosis in sepsis.

**Experimental endotoxic shock (Paper II)**

During experimental endotoxic shock, GR function, as illustrated by the FITC-labelled dexamethasone binding capacity, was increased only in B lymphocytes in both blood and spleen. The main finding was that neutrophils from spleen, in spite of showing an increased GR expression, had a decreased dexamethasone binding capacity (Figure 12). Other experimental studies have shown decreased GR binding capacity in hepatocytes from rats using $^3$H-dexamethasone radioligand competition assays, after endotoxin exposure (Stith & McCallum 1983, Li & Xu 1988). While GR regulations in tissues other than spleen were beyond the scope of our study, the results seem to illustrate the different properties of cells belonging to the innate and adaptive immune system.

![Figure 12](image-url)  
*Figure 12. Glucocorticoid receptor (GR) expression (a) and dexamethasone binding (b) in B lymphocytes and neutrophils from spleen during endotoxic shock analysed by flow cytometry and determined as mean fluorescence intensity (MFI). Data represent mean and standard deviation (SD) of 4 to 8 animals per group. * p < 0.05, *** p ≤ 0.001, **** p ≤ 0.0001, One-way ANOVA with Bonferroni correction. LPS; lipopolysaccharide*

The importance of an intact GR response is emphasized by that overexpression of GR in mice makes them resistant to endotoxic shock by LPS (Reichardt et al. 2000). Our study showed that neutrophils, in spite of their increased GR expression, have a decreased GR binding capacity. During the
first 36 hours after LPS exposure, neutrophils were the only leukocyte subset which increased in number, from less than 10% in blood in healthy mice to more than 50% of the total leukocyte count in mice with endotoxic shock. In combination with their increased number relative to other leukocyte populations, the decreased GR binding capacity may contribute to an absent or insufficient response to glucocorticoid treatment in critical illness.

**Patients with Septic Shock (Paper III)**

Individual maximum and minimum values of GR binding capacity were chosen in the same way as described for GR expression (above) and were compared to healthy subjects. Maximum GR binding capacity was increased in T lymphocytes and monocytes in patients as compared to healthy subjects. Minimum GR binding capacity was decreased in NK-cells, neutrophils, B lymphocytes and eosinophils (Figure 13). Moreover, GR binding capacity was higher in T lymphocytes from GC non-treated patients compared to GC treated patients (p<0.005) and healthy subjects (p<0.01) and it was lower in neutrophils, B lymphocytes and eosinophils from GC treated patients as compared to GC non-treated patients and healthy subjects (p<0.05).

![Figure 13](image)

**Figure 13.** Individual maximum and minimum values of glucocorticoid receptor binding capacity (DEXmax and DEXmin) in T lymphocytes and neutrophils from peripheral blood sampled during and shortly after the sepsis phase (T0-T3) and at one single occasion for healthy subjects (HS). Box plot bands represent the median and whiskers the range. Data were normalised using the natural logarithm and differences between patients and healthy subjects were tested using One-way ANOVA with Dunnett’s multiple comparisons test. **p<0.01, ****p<0.0001.

These findings are in line with previous studies which reported decreased GR binding capacity in peripheral blood mononuclear cells in sepsis (Molijn *et al.* 1995) and lung tissue in experimental acute lung injury (Liu *et al.* 1993). It is also consistent with our own findings in experimental endotoxic
shock (Paper II) where neutrophils displayed a decreased GR binding in spite of an increased GR expression. In this human study, the decreased binding capacity was also observed in NK cells, B lymphocytes and eosinophils. As neutrophils are increasing in number during sepsis, their impaired GR binding capacity may have severe consequences for the control of the immune regulation.

At the timepoint originally chosen to reflect the patients’ normal healthy state, 5-13 months after sepsis, three of the surviving patients displayed a marked increased GR binding capacity (Figure 14). Common for the three patients was hydrocortisone treatment during the initial phase of sepsis, although dosages, clinical parameters, cell counts, or GR expression/binding capacity did not differentiate them from other patients. At the time of sampling after recovery, no patient displayed any signs of ongoing inflammation.

![Figure 14](image)

**Figure 14.** Glucocorticoid receptor binding capacity (DEX) in T lymphocytes from peripheral blood sampled after recovery (T4) in patients initially treated with glucocorticoid (GC), patients not treated with glucocorticoid (no GC) and healthy subjects (HS).

In spite of this, the group initially treated with hydrocortisone also tended to have higher levels of some of the measured cytokines as compared to the untreated patients; in some cases (e.g. IL-1α) the levels after recovery were higher than at any previous timepoint (Figure 15). Possible reasons for the observed increased binding capacity long after sepsis recovery remain speculative at this time but pose an interesting question for further studies. At the same timepoint after recovery, survivors in our study still had reduced numbers of CD3+ and CD4+ T lymphocytes irrespective of hydrocortisone treatment. This is, to our knowledge, a novel observation. If a persistent
decrease in T lymphocytes after sepsis recovery is confirmed, this may explain the increased risk of developing subsequent infections (Wang et al. 2014) and death among survivors of sepsis after hospital discharge (Quartin et al. 1997).

**Figure 15.** Plasma cytokines during and after sepsis (T0-T4), and in healthy subjects (HS). Cytokines were analysed by flow cytometry. Plots represent geometric means with confidence intervals. GC: patients with glucocorticoid treatment, no GC: patients without glucocorticoid treatment HS: healthy subjects

**Patients with Burn Injury (Paper IV)**

On admission, GR binding capacity was higher in T lymphocytes from both the severe and moderate group, but in neutrophils significantly higher only in the moderate group. GR binding capacity was not altered in patients with sepsis on day 7 as compared to non-septic patients or healthy subjects. In addition, we observed a negative correlation between GR binding capacity and burn size in T lymphocytes and neutrophils on day 14 (Figure 16).

**Figure 16.** Glucocorticoid receptor binding capacity (DEX) in T lymphocytes and neutrophils from peripheral blood displayed a negative correlation to the area of burn injury (%TBSA) of patients 14 days after injury. TBSA; total body surface area.
The finding of increased GR binding capacity stands in contrast to experimental findings in burn injured rodents where scald and heat stress caused decreased GR function in liver 12 hours after burn (Liu et al. 2002, Wang et al. 2010). Differences in organ and species may explain the discrepancies in findings. In addition, most patients in our study were included and blood was sampled within the first 12 hours after burn, which may be critical as we observed a negative correlation between burn size and GR binding capacity in T lymphocytes and neutrophils 14 days after burn injury. Previous studies in mice have shown that burn injury induces perturbations in T lymphocyte homeostasis with a maximal effect ten days after burn injury, including activation of a hyperactive T lymphocyte phenotype characterized by overall unresponsiveness to antigen-induced stimulation and increased spontaneous apoptosis (Patenaude et al. 2005, D’Elia et al. 2010). Hence, it is plausible that while GR expression is initially increased to meet the inflammatory response to injury, GR binding capacity is compensatory decreased at a later state of inflammation to keep the balance between hyperinflammation and immunosuppression, and protect against putative secondary infection and sepsis, which extensive burn injury carries a high risk for.

Glucocorticoid Receptor Translocation

When GR becomes activated by ligand binding, it translocates into the cell nucleus where it binds to DNA to activate or repress transcription of target genes involved in regulation of inflammatory and immune responses (Glass & Saijo 2010). In the experimental studies, translocation was measured for both the GR itself and for binding of FITC-labelled dexamethasone. The two analyses are both important as the translocation of GR reflects the receptor translocation induced by endogenous ligand, a snapshot image of the fixed cellular process unaltered by exogenous ligand or stimulation. The FITC-labelled dexamethasone translocation experiment reflects if an exogenous ligand can be translocated into the cell nucleus, provided that it can first bind to free receptors in the cytoplasm.

Experimental Staphylococcus aureus sepsis (Paper I)

Translocation of GR and FITC-labelled dexamethasone into the cell nucleus was measured in CD3+ and CD3- splenocytes from septic and healthy control animals. GR translocation was remarkably increased two days after infection, followed by a decline to a control level on the last two days of the experiment (Paper I, Figure 4). While GR binding capacity was largely unaltered in most leukocytes (Paper I, Figure 3), FITC-labelled dexamethasone
translocation decreased on day three of the experiment. The translocation of FITC-labelled dexamethasone was not affected during the first two days, which is well in accordance with the culminating concentration of endogenous corticosterone at the same timepoint (Paper I, Figure 5). On the last two days of the experiment, GR translocation and corticosterone was decreased to a control level. This likely reflects an adrenal exhaustion rather than restoration, as the animals’ clinical condition was gradually worsening until the end of the experiment. Also, adrenal glands from mice in this late phase of *S. aureus* sepsis displayed marked increase of vitamin E, a strong indicator of oxidative stress, and extremely low levels of cholesterol, also pointing towards adrenal exhaustion (unpublished data). The decrease in FITC-labelled dexamethasone translocation seen on day three of the experiment is of particular interest as the endogenous ligand was decreased. Thus, it does not seem to be the cause of the inability of FITC-labelled dexamethasone to enter the cell nucleus.

**Experimental endotoxic shock (Paper II)**

To investigate whether LPS-induced shock affected GR and FITC-labelled dexamethasone translocation into the nucleus, we analysed the subcellular distribution of GR and FITC-labelled dexamethasone in T lymphocytes, B lymphocytes and neutrophils from LPS administered and healthy control mice. The results in Paper II showed a transiently decreased GR translocation at 12 hours after LPS in all analysed leukocytes, but retroceded to control levels at 36 hours (Figure 17a). In this model we observed a divergence in the FITC-labelled dexamethasone translocation between neutrophils and lymphocytes, as neutrophils had a decreased FITC-labelled dexamethasone translocation at 12 hours in opposite to both the T and B lymphocyte population, displaying an increased translocation (Figure 17b).
Figure 17. (a) Glucocorticoid receptor (GR) and (b) FITC-dexamethasone (DEXA) nuclear translocation in neutrophils and B lymphocytes from spleen of endotoxic shock animals and healthy controls. Values represent mean and standard deviations of translocation relative to control animals based on similarity dilate mean. n=5-6 animals per group. Students t test, *p<0.05, **p<0.01, ***p<0.001. (c) Representative images showing brightfield, nuclear stain Hoechst (violet), FITC-dexamethasone (green) and CD3+ (yellow).

This decrease may reflect an absence of endogenous ligand (corticosterone) during the first 12 hours after LPS administration, when the production of corticosterone was not yet adequate for the present inflammatory trauma. At 36 hours, the GR was translocated to the same level as healthy controls, likely due to endogenous ligand accessible to the cells. The observation that FITC-labelled dexamethasone was translocated to a higher extent 12 hours after LPS administration in T and B lymphocytes also supports this theory. This could be explained by that GR was free in absence of endogenous ligand, followed by a decrease in translocation at 36 hours, when GR was ‘blocked’ by endogenous ligand. However, the neutrophils displayed the opposite pattern, in the sense that they translocated FITC-labelled dexamethasone to a lesser extent at 12 hours after LPS administration, a decrease which is reversed by 36 hours. In light of that neutrophils from spleen displayed no change in GR expression at 12 hours after LPS-induced shock (Figure 12), it is interesting that they bound less dexamethasone, and had a decreased translocation of exogenous ligand. The recovered FITC-labelled
dexamethasone translocation at 36 hours may be an effect of the increased GR expression, as the free receptors may be in excess due to the expansion of neutrophils, even as the corticosterone release from the adrenal glands has caught up. In addition to ligand mediated GR activation, posttranslational modification through phosphorylation is also needed for GR function. It has been suggested that phosphorylation of the GR is necessary for nuclear translocation (Hu et al. 1997, Irusen et al. 2002, Munck et al. 1990). The reason for the decreased FITC-labelled dexamethasone translocation remains elusive. While being beyond the scope of this study, further investigations are warranted to answer this question.

Timing of Glucocorticoid Treatment

**Experimental Staphylococcus aureus sepsis (Paper I)**

To investigate whether timing of dexamethasone treatment start had any effect on the outcome in experimental *S. aureus* sepsis, dexamethasone was administered at different starting points from 22 to 96 hours after infection. When dexamethasone was administered early during the infection course (starting 22 or 26 hours after *S. aureus* inoculation) weight recovery was significantly improved compared to when dexamethasone treatment was started at 48, 72 or 96 hours after infection (*p*<0.001) (Figure 18). The early treatment groups also had an improved weight recovery compared to mice treated with antibiotics only (*p*≤0.05).
Figure 18. Effect of dexamethasone treatment on clinical course of experimental *Staphylococcus aureus* sepsis. Weight change (%) of mice intravenously inoculated with *S. aureus*. Data are shown as mean ± standard error of the mean (SEM) weight change in percent of 8-9 animals per group and was analysed using linear regression in a mixed model. The weight gain was estimated based on the slope of the regression line and differences between groups were tested using a z-test. *p ≤ 0.05, **p < 0.001.

The downregulation of GR was apparent late in the progression of sepsis, and may explain why glucocorticoids were effective if administered early, well before a receptor decrease. Recent literature suggests that glucocorticoid treatment can reduce mortality when initiated early (Annane 2010, Park et al. 2012), but as far as we know, an underlying mechanism like ours has not previously been proposed.

The main outcome in this study was weight change as an indication of general health. It has previously been shown in our mouse model of *S. aureus* sepsis, as well as in others sepsis models, that weight loss is a reliable marker of general health and is correlated to morbidity and mortality (Jonsson et al. 2008, Gjertsson et al. 2012a, Jonsson et al. 2004, Palmqvist et al. 2002, Tarkowski et al. 2001, Hultgren et al. 2001, Hultgren et al. 1999, Bremell & Tarkowski 1995, Granger et al. 2013, Ray et al. 2010). A body weight loss of 20% is a strong indication that the animal has not been eating or drinking, and is not maintaining a fluid balance. As mortality was not a desired outcome and we wished to mimic the clinical situation as far as possible, all animals were treated with antibiotics. Previous studies have also seen a beneficial effect of dexamethasone treatment in mice when it is given concomi-
tantly with antibiotics (Silverstein & Johnson 2003) or simultaneous with toxin injection (Gomez et al. 2003).

Bacterial load was investigated in kidneys, as our group have previously shown that this strain of *S. aureus* is cleared from circulation within 48 hours to settle down particularly in the kidneys (Bremell et al. 1991, Verba & Tarkowski 1996). Dexamethasone treatment did not significantly affect bacterial load in kidneys in our experiment, although the groups receiving dexamethasone late (48-96 hours after infection) had one order of magnitude higher bacterial load as compared to untreated animals or animals treated early with dexamethasone (22 and 26 hours after infection). The CORTICUS trial showed increased superinfections in the glucocorticoid treatment arm (Sprung et al. 2008), and experimental studies have shown impaired neutrophil function and increased bacterial overgrowth of wounds by increased endogenous glucocorticoids through stress prior to infection (Shurin et al. 1994, Rojas et al. 2002). In contrast to these data, we did not observe an increase in bacterial load in kidneys from animals treated early with dexamethasone. This suggests that endogenous and/or exogenous glucocorticoids can have contrasting effects depending on when it is released or administered in relation to infection, and that treatment start early after infection is optimal.

The results from this experiment showed that only the groups receiving dexamethasone treatment early during the disease course (22 and 26 hours after inoculation) had an improved weight recovery as compared to all other groups. The late treatment groups did not differ in weight recovery from the antibiotic control group without dexamethasone treatment. This result points to that time is important for glucocorticoid treatment, as it was only beneficial when administered early in this experimental sepsis model.

**Experimental endotoxic shock (Paper II)**

To investigate whether timing for start of dexamethasone treatment had any effect on survival in this model, dexamethasone was administered at different starting points from 2 to 36 hours after LPS administration. In addition, one group was administered dexamethasone starting 2 hours prior LPS. Only when dexamethasone was administered early after LPS (starting 2 hours after LPS administration) overall survival was significantly improved compared to endotoxic animals without dexamethasone treatment (Figure 19).
As the results from experimental *S. aureus* sepsis indicated that glucocorticoid treatment during sepsis was only beneficial when administered early, and with the background of many previous studies proving an effect only when glucocorticoid treatment was administered before LPS (Barber *et al.* 1993, Santos *et al.* 1993), in Paper II we included a group to receive treatment before LPS. However, this group did not have an increased survival rate as compared to other groups.

The group receiving dexamethasone at 2 hours prior to LPS had, unexpectedly, a mortality that was similar to the mice given LPS without dexamethasone treatment (p=0.52). In previous mice experiments by others, methylprednisolone improved survival only when administered before antibiotics in gram-negative infection (Fadel *et al.* 2008), and dexamethasone protected from a lethal injection of TNF when administered 30 min before TNF, but not 1, 4 or 8 hours after (Van Bogaert *et al.* 2011). The authors suggest that TNF causes a glucocorticoid resistance with compromised GR signalling. These data stand in contrast to ours, although differences in challenge (LPS versus TNF) and dexamethasone dose (500x higher; 0.05 mg/kg versus 25 mg/kg) make comparisons uncertain. Another possible explanation to that the group receiving dexamethasone 2 hours prior to LPS did not have an improved survival as compared to the LPS control group, could be that this low dose of dexamethasone to the main part was bound to receptors

**Figure 19.** Effect of dexamethasone treatment start in experimental endotoxic shock. Survival is improved in endotoxic mice where dexamethasone treatment was started 2 hours after LPS (n=9) compared to the LPS control group (p=0.028) (n=8). The group receiving dexamethasone treatment 2 hours before LPS (n=9) had decreased survival compared the healthy control group (p=0.01). Log Rank Mantel Cox test.
during the first two hours and thereby cleared from circulation, leaving an insufficient amount of dexamethasone available to abrogate the pro-inflammatory effects of the later injection of LPS. In line with our results, a few previous experimental studies have also shown benefit of dexamethasone treatment after LPS (Schuler et al. 1976, Johannes et al. 2009). In our experiment of endotoxic shock, dexamethasone treatment improved mortality only when given 2 hours after LPS. These experimental data add support to the theory that there is a time window when glucocorticoid treatment is beneficial in the treatment of septic shock.

Comparison of GR in sepsis and burn injury

Sepsis can be described as SIRS resulting from infection (Drifte et al. 2013). Severe burn injury, as well as many other traumas, also leads to SIRS, but at least initially, is not caused by infection. Sepsis and burn injury have many similarities, which also makes the sepsis diagnosis difficult in burn injury. For instance, the lymphocyte apoptosis observed in patients with septic shock, well known in sepsis (Hotchkiss et al. 2001), was also observed in our study of patients with burn injury.

It is uncertain if the observed GR regulations in sepsis are caused by infection itself or by the severe inflammation it gives rise to. Naturally, sepsis cannot be studied in a non-inflammatory context, but studying sterile inflammation may add information about what triggers GR regulations. Therefore, GR expression and binding capacity from patients with septic shock (Paper III) and patients with burn injury on admission, before any infection has likely occurred (Paper IV), is compared below.

Peripheral blood from patients with sepsis was sampled at several timepoints during the acute phase of sepsis. Since the true timepoint of infection is unknown, it would hardly be biologically relevant to select one timepoint (T0-T3) as more representative than another. Instead, the individual GR maximum and minimum were chosen for each individual, and a group mean was created. For this comparison, both the GR maximum and the GR minimum of the patients with sepsis have been compared to the patients with burn injury on admission. Thus, the mature sepsis is compared to the non-infectious inflammation of recent burn injury of both significant and moderate extent.
GRmax expression was found to be increased in most leukocyte subsets in patients with sepsis (Paper III) as well as burn injury (Paper IV), and in comparison, patients with sepsis displayed an increased GRmax expression in T lymphocytes (p<0.05, Figure 20) and NK cells (p<0.05) as compared to patients with burn injury. The same trend was observed in Paper IV, as T lymphocytes from burned patients diagnosed with sepsis on day 7 post admission increased their GR expression over time. It is therefore possible that infection further increases GR expression from, compared to healthy subjects, an already elevated level. The increased GR expression may be the underlying reason for lymphocyte apoptosis in the presence of elevated glucocorticoid levels (Liddicoat et al. 2014), which may be a mechanism for glucocorticoids to downregulate immunity.

In patients with sepsis, GR expression was found both increased at max and decreased at min in neutrophils as compared to healthy subjects in Paper III. In patients with burn injury, GR expression was increased in neutrophils both from patients with significant and moderate injury (Paper IV). In comparison, GRmax expression tended to be lower in sepsis patients as compared to patients with burn injury (near statistical significance, p=0.06).

In addition, GRmin expression was found to be lower in all leukocyte subsets in sepsis patients as compared to patients with burn injury on admission (p<0.0001).

Figure 20. Maximum expression levels of glucocorticoid receptor (GR) in T lymphocytes and neutrophils from individual patients with sepsis (T0-T3, Paper III) and from patients with burn injury on admission (Paper IV). Bar graphs represent the mean and standard deviation. Data were normalized using the natural logarithm and differences were tested using Students t test. *p<0.05
The maximum GR binding capacity (DEXmax) was found to be increased in T lymphocytes in patients with sepsis (Paper III) as well as in CD3+, and CD4+ T lymphocytes from patients with burn injury (Paper IV). In T lymphocytes, there was no difference in GR binding capacity between sepsis patients and patients with burn on admission (Figure 21).

In patients with sepsis, the GR binding capacity (DEXmax) was found decreased in neutrophils (Paper III). In patients with burn injury, GR binding capacity (DEX) was found increased in the patients with moderate burn, but not significantly increased in patients with severe burn (Paper IV). In comparison, patients with sepsis displayed a decreased GR binding capacity (DEXmax) in neutrophils (p<0.01, Figure 21) as compared to patients with burn injury on admission. The same trend was observed in Paper IV, as neutrophils from burned patients diagnosed with sepsis on day 7 post admission decreased their GR expression over time (all but one). It is therefore possible that infection decreases GR binding capacity in neutrophils while inflammation (in moderate burn injury) increases it.

![Figure 21](image_url)

**Figure 21.** Maximum binding capacity of glucocorticoid receptor (GR) in T lymphocytes and neutrophils from individual patients with sepsis (T0-T3, Paper III) and from patients with burn injury on admission (Paper IV). Bar graphs represent the mean and standard deviation. Data were normalized using the natural logarithm and differences were tested using Students t test. **p<0.01.

As neutrophils expand in number both in sepsis and after burn injury while other leukocyte subsets seem to retract, regulation of neutrophils may be of additional interest in both conditions. The difference in GR binding capacity is not likely caused by glucocorticoids, as cortisol levels are similar in between non-GC treated sepsis patients and burned patients on admission (p=0.92). However, as time may be an important confounder, these comparisons preclude conclusions about whether sepsis or severe inflammation causes the GR increase.
General Discussion

In spite of the variable results from clinical studies in severe sepsis and septic shock, half of the patients receive glucocorticoid treatment (Beale et al. 2010). Many factors may influence response to glucocorticoid treatment, including the expression and function of GR. In this thesis we explored GR expression and function in experimental and clinical sepsis and severe inflammation. One of the main findings was decreased GR binding capacity in neutrophils both in experimental endotoxic shock and in patients with septic shock (Paper II and III). Considering this as well as the expanding number of neutrophils in sepsis, the inability of neutrophils to respond to endogenous or exogenous glucocorticoids may have severe ramifications for the host. Another main finding was an increased GR expression in most of the circulating leukocyte subsets during the initial phase of sepsis in patients. An increased GR expression was also observed in patients with burn injury on admission, but here in combination with an intact or increased GR binding capacity. An upregulation of GR and increased binding capacity may be an adaptive response to dampen an aggressive inflammation in the absence of an invading pathogen. In the presence of infection, while T lymphocytes display an increased GR expression and binding capacity, neutrophils appear to become resistant to glucocorticoid induced apoptosis through decreased GR binding capacity. This is likely protecting the host from immunosuppression which would give way to the present infection. A study in children with sepsis showed that neutrophils had a transiently decreased mRNA expression of GR isoforms, which may explain glucocorticoid resistance in this leukocyte subset (van den Akker et al. 2009). A more recent study in adults with sepsis showed a decreased GR mRNA expression in T lymphocytes (Ledderose et al. 2012). These results stand in contrast to ours, as we observed an increased GR expression during the same time frame (first 24 hours after sepsis diagnosis). This discrepancy may be explained by differences in mRNA and protein expression. During the early hyperinflammatory phase of progressing sepsis, GR protein may be upregulated at a high speed in preparation of halting the immune response as soon as the pathogen is cleared. Increased translation rate resulting in increased GR protein may be observed as simultaneous exhaustion of GR mRNA. GR binding capacity may be more readily controlled by the cell for its optimal purposes through a simple phosphorylation step, in comparison to a costly turnover of new receptors. The results from these studies highlights the importance of both GR
expression and function, which both are likely to affect the response to glu-
corticoids.

In the late phase of progressed experimental sepsis, we observed a decreased
GR expression in leukocytes from blood and spleen (Paper I). A decreased
expression or function of GR during severe inflammation could have a func-
tion to preserve energy for the organism when energy supplies are low or
needed for other critical processes, such as evading an invasive pathogen.
Cortisol productions in the adrenal glands is energy consuming, and have a
limited rate by the active transfer of free cholesterol to the outer membrane
of the mitochondria (Nussey & Whitehead 2001). It would be resource effi-
cient for the organism to recycle the already produced cortisol by allowing
multiple signalling instead of clearing it, and thereby conserving energy for
essential metabolism and immunity. In addition, by moderating the binding
of GR, the cells may avoid an overwhelming anti-inflammatory response by
the high circulating cortisol, at the same time as keeping the vital balance
with the pro-inflammatory response. A sufficiently high concentration of
circulating cortisol also allows for local regulation of effects by up- or
downregulation of GR expression or function in target tissues or cells. This
could protect the host against secondary infection, which may arise from a
hypothetical systemic and prolonged activation of GR. There is increasing
support for that GR is regulated tissue-specifically during critical illness
(Goodwin et al. 2013, Takigawa et al. 2013, Indyk et al. 2013). Our studies
of GR regulations were restricted to circulating leukocytes and lymphoid
tissue from mice. Clearly, many other tissues are involved in the processes
we aim to describe in part, and it would be highly relevant to investigate GR
regulations in for instance endothelium and all central organs which are of-
ten failing in septic shock. We chose to analyse GR regulations using flow
cytometry for studying immune cells, which are of obvious interest in sepsis.
This method allows analysis of cells in peripheral blood, relatively easily
accessible and non-invasive. However, it requires all cells to be fresh, intact,
and free in suspension. Studying endothelium or organ tissue (except for
spleen and lymph nodes which can easily be suspended) would require other
techniques, such as immunohistochemistry or Western blot, which are semi-
quantitative at best. Similarly, quantitative PCR, which was employed in
Paper II, provides information on GR regulations at mRNA level, but pre-
cludes information about the result of translation; the GR protein. Future
studies could aim at developing sensitive and specific methods to accurately
To quantify GR protein and its isoforms in tissue, to gain a more comprehensive picture of GR regulations in severe inflammation.

**Methodological considerations**

Natural glucocorticoids (cortisol and hydrocortisone) quickly dissociate from GR after signalling, as compared to synthetic glucocorticoids with higher affinity for the receptor (Stavreva et al. 2009). However, FITC-conjugated dexamethasone is likely to have a decreased affinity as compared to the unlabelled steroid, and could thereby be closer to the affinity of corticosterone. Thus, decreased dexamethasone binding capacity is not likely caused by competitive binding of cortisol, corticosterone or hydrocortisone. Also, at the time of dexamethasone incubation in the binding assay, the numerous previous washing steps would have eliminated any unbound endogenous glucocorticoids originally present in the sample, as these small, non-polar steroid hormones freely cross the cell membrane and would have diffused into extracellular buffer during washing steps. Therefore, one interpretation of the decreased GR binding of dexamethasone is that the severe inflammation itself decreases binding capacity of the present receptors in circulating leukocytes. This may be an adaptive mechanism when GR are up-regulated and cortisol levels high in response to severe inflammation, protecting the organism from an inordinate or premature anti-inflammatory response.

On the other hand, it has been suggested that activated GR can re-bind to DNA and signal again, reusing the same ligand (Stavreva et al. 2009). If this recycling of signalling receptors takes place in leukocytes, a decreased dexamethasone binding capacity would simply reflect increased binding and signalling of endogenous glucocorticoids or hydrocortisone treatment, and the factual GR binding capacity would not be possible to detect. However, if this occurs, GR binding capacity should display an opposite pattern as compared to the endogenous corticosterone levels, which was not observed in experimental sepsis (Paper I).

Total serum/plasma levels of cortisol (and corticosterone) represent both the free, active fraction of cortisol as well as the cortisol bound to CBG. Previous studies have suggested that measuring free levels of cortisol would more accurately reflect the true cortisol activity than total levels (Aardal-Eriksson et al. 1998). Especially after burn injury, as a consequence of increased vascular permeability, many binding proteins such as CBG and albumin de-
crease (Garrel 1996, Palmieri et al. 2006). Thus, it is possible that the free fraction of cortisol is increased during critical illness, making a relevant comparison between total cortisol concentration in between patients and healthy subjects difficult.

It is common in experimental as well as clinical and observational studies to compare a study group to a control group. In experimental studies, it does not pose a problem to unbiasedly select individuals from the same litter to serve as healthy controls. For human studies, selection of controls is more challenging. For our studies, healthy subjects were recruited among hospital and laboratory staff from both Sahlgrenska and Uppsala University Hospital. As a consequence, the mean age of the healthy subjects was significantly lower than the both patient populations. As immunological competence is known to decrease with age (Inoue et al. 2013), this difference must be considered a limitation. In addition, more women than men were recruited as healthy subjects as compared to the patient populations. When dividing the healthy subjects into two groups according to age or gender, no difference was found between the groups for any of the measured parameters; GR expression, GR binding capacity, leukocyte number, cytokines or cortisol. This observation does not preclude that age or gender has effects on the immune response, although it does not seem to affect our primary measured parameters in healthy subjects.

For future studies, it would add more information to design the comparator group closer to the study population. For instance, it would be interesting to compare sepsis or burned patients to another group of critically ill requiring similar intensive care treatment. Due to the lack of previous knowledge about human GR regulations on protein level, we did not have sufficient information to select another comparator group or to make an informed statistical power calculation to guide sample size, but instead based the power analysis on estimation. Future study designs may benefit from the acquired data from these studies, to model a closer comparator group and to predict the required sample size.

Of mice and/or not men?
While humans and mice have many similarities, it is worth considering their differences when interpreting preclinical data. Mice have been essential for the vast progress made in medical research the last century, and will most likely continue to be the preferred experimental model and dominate the
scientific literature as they mirror human biology well in many respects. Recent literature has shown that many inflammatory mouse models correlate poorly both to the human disease they attempt to mimic, and also, to other mouse models (Seok et al. 2013). Reasons for these discrepancies are suggested to be the evolutionary distance between mice and humans and the single mechanistic models versus the complex nature of human disease. To date, we know that approximately 300 genes are unique to the species (Waterston et al. 2002), but they have evolved in very different ecological niches and have been exposed to different pathogenic challenges. For example, while healthy human blood is rich in neutrophils (30-50%), mouse blood has less neutrophils (10-25%) but a has a strong prevalence of lymphocytes (75-90%) (Doeing et al. 2003). Also, humans are less resilient to endotoxin than mice; whereas a dose of 30 ng/kg endotoxin causes shock in humans, the lethal dose in mice is one million fold higher (5-25 mg/kg) (Sauter & Wolfensberger 1980). Another difference is that B lymphocytes from mice express TLR4, recognising LPS, while human B lymphocytes do not (Garraud et al. 2012). While cortisol is the main endogenous glucocorticoid in humans, through convergent evolution, corticosterone is predominant in rodents. When comparing results from burn patients to experimental results from rodent models, it should be noted that no existing mouse model can allow intensive care support and consistent survival of mice with a burn injury greater than 20-25% TBSA. Comparing experimental models to human burn injured patients with significantly more severe injury should thus be done with caution. 

With these caveats in mind, it is difficult to directly extrapolate data from mice models to humans. The species differ hugely in size and lifespan, and a time window for glucocorticoid treatment in mice may be significantly longer in humans. Also, the veritable pharmacopeia of drugs that human patients are exposed to may affect their pathophysiological and genomic responses. This may result in that immunological responses do not occur in exactly the same way even in different humans. In addition, glucocorticoid receptors have been identified in the sea lamprey Petromyzon marinus, which diverged from the jawed vertebrates (gnathostomes) about 450 million years ago (Thornton 2001), whereas mice and humans diverged in evolution somewhere only between 65 and 75 million years ago (Mestas & Hughes 2004), supporting that the mouse is a relevant model for studying glucocorticoid receptors. Notwithstanding, a complex disease such as sepsis involv-
ing the entire organism cannot be discerned in *in vitro* or tissue models, and alas, an *in vivo* model in mammals is still unmatched.

**A treatment window for glucocorticoid therapy in sepsis?**

The results from our experimental sepsis models as well as patient studies by others (Annane 2010, Park et al. 2012) indicate that there is a window when glucocorticoids improve recovery and survival. In our mouse models, it seems that glucocorticoid treatment is beneficial in the early phase presumably predominated by hyperinflammation (Figure 1). As deaths in this early phase is usually caused by ‘cytokine storms’ leading to refractory shock and multi-organ failure, it is plausible that the hyperinflammatory phase develops at a rate faster than the balancing glucocorticoids can be synthesised and released. This is particularly relevant considering all circulating immune cells and endothelium is likely to contribute to the immune response against a systemic infection, while the adrenal glands, modest in comparison, are the main source of the anti-inflammatory response. Cortisol is not stored ready for release in the adrenal glands, but synthesised upon demand, with a rate limited by the active transfer of free cholesterol to the outer membrane of the mitochondria (Nussey & Whitehead 2001). Thus, the life-saving action of early glucocorticoid treatment may be to blunt an exaggerated pro-inflammatory response when the endogenous glucocorticoid production and release is not yet adequate and the essential balance is lost (Figure 22).

**Figure 22.** Hypothetical mechanism of beneficial early glucocorticoid treatment. Exogenous glucocorticoid treatment could blunt the exaggerated hyperinflammation in early sepsis, which without treatment may lead to refractory shock and death due to cardiovascular collapse.
The observed increased survival from early dexamethasone treatment in Paper I and II may be caused by something other than anti-inflammatory effects. For instance, a recent study in porcine endotoxemic shock showed that early treatment with hydrocortisone improved mean arterial pressure, systemic vascular resistance and heart rate, and that these hemodynamic effects were not mediated through TNF or IL-6 (Soderberg et al. 2012). Enhanced vasopressor effect of glucocorticoid treatment in sepsis is thought to be caused by improved vascular reactivity to catecholamines (d'Emmanuele di Villa Bianca et al. 2003). Dexamethasone, a synthetic glucocorticoid, has been associated with no benefit or even harm when used in sepsis. It is traditionally considered to have no mineralocorticoid effect, which has been suggested the reason for the benefit of hydrocortisone in sepsis as compared to dexamethasone (Druce et al. 2008). It is not known exactly what mediates the hemodynamic effects of glucocorticoids, but it does not appear to be mediated by MR binding, as dexamethasone has been proven to have hemodynamic effects in spite of being known to lack MR activation potential (d'Emmanuele di Villa Bianca et al. 2003).

An experimental study showed that glucocorticoid treatment was beneficial when administered immediately after a lethal infection with S. aureus, but not after a sub-lethal dose (Hicks et al. 2012). This suggests that in addition to the importance of time for a beneficial effect, severity of illness may be another factor to consider.

However, in cases of prolonged sepsis where the initial hyperinflammatory reaction has been met by endogenous glucocorticoid production, patients may have an ongoing immune response trying to evade a persistent pathogen, with detectable high circulating levels of cortisol. Glucocorticoid treatment in this phase could hypothetically be detrimental, as additional cortisol may disturb the balance, cause apoptosis of immune cells and leave the patient immunosuppressed and defenceless against secondary infection (Figure 23). In addition, GR may be unavailable in inflammatory cells in this late phase, as suggested by Paper I, which would further aggravate the negative effects of supranormal circulating cortisol, as it cannot be cleared by its main target, GR.
Figure 23. Hypothetical risk of late glucocorticoid treatment. Exogenous hydrocortisone at a late timepoint could disturb the immunological balance, tipping the response into immunosuppression, resulting in adverse effects such as secondary infection.

The GC treatment window in patients has been suggested to be within the first 24 hours of sepsis (Annane 2010, Park et al. 2012). As patients have very different immunological preconditions, severities and treatment needs, perhaps a future treatment regimen could be to accurately predict the individual patient’s response to glucocorticoid treatment. The ideal treatment would be balanced; reducing the hyperinflammation-associated pathology at the same time as allowing adequate host defence against infection.

An ex vivo assay discerning whom and when to treat?

Despite the many investigations and meta-analyses, glucocorticoid treatment in sepsis remains controversial, which emphasises the urgent need for identification of appropriate target patients who could benefit from glucocorticoid treatment. Random cortisol measurements are a poor prognostic marker of disease severity and putative beneficial effects of glucocorticoid treatment (Annane et al. 2000). We know that low dose glucocorticoid treatment for septic shock reduces vasopressor dependency and length of stay in the intensive care unit, without increasing the risk of superinfections (Annane et al. 2009, Moran et al. 2010, Sligl et al. 2009). The current guidelines recommend glucocorticoid therapy based on vasopressor dependency (Dellinger et al. 2013). A risk/benefit ratio of glucocorticoid treatment should perhaps be determined in each patient, based on the individual’s need for replacement and cellular GR availability. An ex vivo test determining the endogenous cortisol level as well as the availability and activity of GR with cellular spec-
pecificity may be of interest to the intensive care setting. Perhaps in the future, glucocorticoid treatment could be targeted to specific tissues, and avoid putative adverse effects of systemic glucocorticoid treatment, while permitting treatment at the optimal time and with the adequate dose.
Conclusions

The varying effects of glucocorticoid treatment in sepsis are poorly understood, with consequences for the clinical guidelines for treatment. In an experimental model of sepsis we showed that GR expression is progressively downregulated in late phase sepsis and that leukocytes have a decreased translocation of exogenous glucocorticoids. In the acute phase of endotoxic shock, our data demonstrate that neutrophils, the predominant leukocyte in sepsis, have a decreased ability to bind exogenous glucocorticoids in spite of increased GR expression. In an observational explorative study, patients with septic shock were studied at five timepoints during sepsis and after recovery, and compared to healthy subjects. While GR expression was found increased in most subsets of circulating leukocytes, GR binding capacity was found decreased in several cell types, most significantly in neutrophils. In a prospective observational study of patients with burn injury, GR expression and binding capacity was found to be increased in most leukocyte subsets. In addition, while lymphocytes underwent apoptosis in sepsis and endotoxic shock as well as after burn injury, neutrophils remained and even expanded in number.

Taken together, these studies suggest that infection may cause different GR regulations compared to severe inflammation without bacterial components. In both experimental models, glucocorticoid treatment was only beneficial when administered early. The blunted response to late glucocorticoid treatment may have been caused by the observed decreased GR expression or the decreased GR binding capacity in the predominant neutrophils. Therefore, perhaps glucocorticoid treatment in sepsis and septic shock should not be abandoned, but given only when early administration is possible. The underlying mechanisms behind insufficient response to glucocorticoid therapy in sepsis may be decreased GR expression, glucocorticoid binding and receptor translocation or even impaired binding to DNA. Furthermore, patients are admitted to the intensive care unit with varying ages, aetiologies, co-morbidities and most importantly, often late after the time of infection. Hopefully, further research in this area will lead to a more individualized
approach to glucocorticoid treatment in sepsis and septic shock, taking into account both the GR expression and availability, as well as the balance of the immune response to target the optimal time to treat.


Sepsis (blodförgiftning) är en allvarlig infektion med bakterier i blodet, och en av de vanligaste orsakerna till dödlighet inom intensivvården, trots antibiotikabehandling. Immunförsvarets reaktion på infektionen är komplicerad och innebär bland annat ökad frisättning av det kroppsegna stresshormonet kortisol som bildas av binjurarna. Dödlighet i sepsis beror oftast på organsvikt till följd av en överdriven immunreaktion som svar på den omfattande infektionen. För att hämna en okontrollerad immunreaktion under svår inflammation och sepsis kan man behandla patienter med kortison, som är kortisol framställt på konstgjord väg. Kliniska studier av kortisonbehandling under sepsis har visat varierande resultat, från förbättrad överlevnad med behandling till oförändrad dödlighet och ökad risk för sekundära infektioner. Det är idag okänt varför kortisonbehandling har så varierande effekt hos olika patienter.

Kortison är starka anti-inflammatoriska läkemedel som har effekt genom att binda till steroidreceptorn (mottagaren) i cellerna. Djupare förståelse av steroidreceptorns signalering under sepsis skulle kunna förbättra kortisonbehandling i framtiden. Syftet med den här avhandlingen var att få ytterligare information om steroidreceptorn och dess mekanismer under experimentell och human sepsis och svår inflammation. Vi har analyserat steroidreceptorns förekomst och förmåga att binda kortison i vita blodkroppar från möss med experimentell sepsis och bakterietoxinshock (arbete I och II) och patienter med sepsis och bränskada (arbete III och IV).

I delarbete I fann vi i en musmodell av sepsis att steroidreceptorn förekom i lägre utsträckning under sen fas av sjukdomen. Kortisonbehandling hade god effekt endast då behandling gavs tidigt under sjukdomsförloppet. Det verkar därför som att steroidreceptorn måste finnas vid en tillräckligt hög nivå för att kortisonbehandling ska kunna ha effekt, och att det har stor betydelse när behandlingen påbörjas.

Populärvetenskaplig sammanfattning på svenska


För att kunna utforska steroidreceptorn under inflammation utan förekomst av bakterieinfektion, följde vi under patienter som intensivvårdades för brännskador genom upprepade provtagnings (delarbete IV). Blodprover togs dels vid patienternas ankomst till brännskadecentrum, mest troligt innan någon infektion tillstöt, och sju dagar senare, då flera av patienterna hade fått diagnosen sepsis. Steroidreceptorns förekomst och bindningsförmåga var högre hos patienter vid ankomst jämfört med hos friska personer. De patienter som utvecklade sepsis på dag sju hade ytterligare ökad förekomst av steroidreceptorer hos lymfocyter. Studien visar att steroidreceptorns förekomst och bindningsförmåga ökar i vita blodkroppar under svår inflammation utan pågående infektion. Steroidreceptorns bindningsförmåga hos patienterna minskade ju större brännskadan var, 14 dagar efter att skadan inträffat.

Sammanfattningsvis tyder våra resultat på att sepsis och svår inflammation påverkar både förekomst och bindningsförmåga hos steroidreceptorer, vilket kan påverka effekten av kortisonbehandling. I experimentell sepsis och svår inflammation är kortisonbehandling effektiv endast då den ges tidigt under sjukdomsförloppet.
Acknowledgements

On the cover of this book, there is only one name - mine. Many people have contributed to the work that resulted in this research, and I could not, and did not, do it without their help. I want to express my sincere gratitude to all of you, especially to

Göran Hedenstierna - A true orbiter of the mind as well as our planet! I have too many things to be grateful to you for than I can list here, but amongst many things for being infinitely kind and unfailingly generous. Thank you for believing in me and for offering me a place in your inspiring group of scientists. Thank you for being a pillar in times of adversity and for sharing your unparalleled scientific excitement, for all the fun, for always listening and for all enthusiasm and encouragement.

Christian Rylander. Thank you for your efforts above and beyond the call of duty. I have learnt a great deal from our discussions and countless drafts and redrafts of every figure and passage of text. Thank you for inviting me into your clinical world and for every now and then lifting my perspective from the microcosmos of nuclear receptors to the macrocosmos – aurora borealis, solar eclipses and the movements of Venus! Also, thank you for always sharing your dessert and for introducing me to

Catharina Lindholm. Thank you for coming to my rescue when I was struggling against the bêtes noires of science and helping me to bring chaos into order. Thank you for being a strong role model and mentor, and for all you have taught me about mouse models, immunology and writing. Thank you for bringing excellent ideas and positive energy!

Cecile Martijn. Thank you for your encouragement and for being generous and unstinting with advice and suggestions. Thank you for teaching me to run qPCR, and for your time and patience in the lab.
Thank you also to all collaborators; Merja, Pernilla, Erik, Morten, Filip, Fredrik and Johanna. All PhD students ought to have such brilliant and astute friends and allies.

All residents at the Hedenstierna Lab; Agneta, Karin, Maria S, Anders N, Monica, Joba, Fernando, Kerstin, Jaime, Staffan, Eva-Maria, Marianne, Bosse and Anna Foyer. Thank you for being helpful, supportive, for giving me scientific input and being there all those times things did not work. A special thank you to Anders L, my step-supervisor of sorts, for suggestions, helpful criticisms and being generous with knowledge and advice. I am also grateful for collaborations, sincere scientific curiosity and devotion provided by transients at the Hedenstierna Lab, especially Kristina, Marco, Gaetano, Manja, Savino and Mariangela.

I am indebted to the extraordinary E2 group; Hans, Ulrika, Alexandra, Angelina, Annica, Louise, Marie, Cissi and Martin. Thank you for all the lab hours, for including me in the group, for invaluable help and good times.

Thank you Kristina and the entire department of Rheumatology, especially Anna S, Hardis, Anna-Carin, Mattias, Jessica, Mikael, Gabriel, Vanja, Esbjörn, Malin, Inger G, Tao, Elisabet, Tove, Nico and Christina. You compose a veritable scientific encyclopaedia and helpdesk I could not have done without.

I am grateful to the very nice and capable staff at the Sahlgrenska ICU, especially Caisa, Anette and Jenny, and at BRIVA in Uppsala, especially Åsa. Thank you for all help with blood sampling and collecting clinical data.

Thank you also to everyone who generously donated blood for experiments.

Far too often, someone’s name is forgotten. If your name is missing because I have forgotten to acknowledge you here, I sincerely apologise and suspect my own pain at your omission is greater than yours. I am deeply grateful to ....................................................... (please fill in your name) for you probably know what.

Thank you for support and understanding, all family and friends who provide me with context, especially in Drottningstorp, Uppsala, Göteborg, Markaryd, Fjällbacka, Hallsberg, Könner and Sydney.
I owe a very warm thank you to my brother Jonas and his lovely wife Lotta, who gave me a microscope when I was much too young. Thank you Jonas for taking me around so many labs, for teaching me to crush hearts in a standardised way, for convincing me to study biology and showing me that research is the greatest thing to do! It should also be noted that this book was his idea in the first place. Please direct complaints accordingly.

Finally, thank you Jörg. For your unfailing encouragement, for your bottomless patience with me, for always listening and for making me laugh every day. I remain deeply grateful to and for you.

Please remember that behind each dry graph or number in this thesis; there are individuals, mice or men, who in most cases were critically ill, and without them this research would not have been possible.

This work was supported by grants from the Swedish Research Council, Regional Research Fund of Västra Götaland, Gothenburg Medical Society, Inga Britt and Arne Lundberg Foundation and Anna-Maria Lundins Scholarships.
Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1016

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)