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**ON THE ROLE OF HMGB1
AND RESISTIN IN SEVERE
SEPSIS AND SEPTIC SHOCK**

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To Vera, Arvid, Elis and Sara

ABSTRACT

Severe sepsis and septic shock are serious manifestations of infectious diseases. Mortality ranges from over 30 % in severe sepsis up to 60% in septic shock. Early antibiotic treatment and intensive care are mainstays of therapy, but outside of this, new treatments have only marginally improved survival.

The innate immune response is a powerful part of vertebrate defence against infections. It is likely that an over-reactive innate response, rather than infections themselves, causes much of the mortality in severe infections. Subduing that immune response could improve survival. The most important signalling proteins in innate immunity are cytokines. Clinical trials aimed at reducing proinflammatory cytokines in sepsis have been disappointing, probably because the brief, powerful cytokine burst has often passed by the time patients are admitted for treatment. The hunt has therefore been on for pro-inflammatory proteins which are still elevated, and thus susceptible to therapy, when patients are admitted to hospital.

This thesis focuses on two proinflammatory proteins with cytokine-like properties, HMGB1 and resistin. Both exert a wide array of inflammatory effects, and HMGB1 reducing therapy in animal sepsis models considerably reduces mortality. We performed two prospective studies, with a total of 109 patients with severe sepsis or septic shock, primarily treated in the intensive care unit. We showed that both proteins had sustained secretion profiles, and remained elevated up to one week, long after other studied cytokines had returned to low values. For resistin, but not for HMGB1, we could also show significant correlations to disease severity as measured by SOFA and APACHE II – scores, and also to other laboratory markers of sepsis.

We studied putative sources of both proteins and could show that HMGB1 was secreted from endothelial cells and that resistin, previously believed to be secreted only from monocytes or adipocytes, was secreted from neutrophils, systemically and in biopsies from soft tissue infections. This is very interesting since both endothelial cells and neutrophils play critical roles in innate immunity. We found that resistin was secreted at higher concentrations in gram-positive infections compared to gram-negative, *in vitro* as well as in patients. In pathophysiological studies, we showed that HMGB1 induces resistin release from monocytes – which might explain their similar secretion profiles. Resistin in itself induces the upregulation of the cell adhesion protein ICAM-1 on monocytes. Furthermore, we could show that the proinflammatory effects of HMGB1 on monocytes and endothelial cells were dose-dependently inhibited by Dexamethasone, a glucocorticoid, and that CNI-1493, an experimental pharmacological agent, and A-box of HMGB1, inhibited HMGB1 effects on monocytes, but not on endothelium.

In summary, HMGB1 and resistin have pro-inflammatory properties, are secreted by important cells of the innate immune system and have persisting secretion profiles in severe sepsis and septic shock. Some further research is required, but both proteins are interesting potential targets in severe infections. Successful reduction could tame hyperinflammation and improve survival in these life-threatening syndromes.

LIST OF PUBLICATIONS

- I. Activation of human umbilical vein endothelial cells leads to relocation and release of high-mobility group box chromosomal protein 1. G. E. Mullins, J. Sundén-Cullberg, A.-S. Johansson, A. Rouhiainen, H. Erlandsson-Harris, H. Yang, K. J. Tracey, H. Rauvala, J. Palmblad, J. Andersson & C. J. Treutiger *Scand J Immunol.* 2004 Dec;60(6):566-73.
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- III. Pronounced Elevation of Resistin Correlates with Severity of Disease in Severe Sepsis and Septic Shock Jonas Sundén-Cullberg, Thomas Nyström, Martin L. Lee, Gail Mullins, Jan Andersson, Leif Tokics, Anna Norrby-Teglund, Carl Johan Treutiger *Crit Care Med.* 2007 Jun;35(6):1536-42
- IV. Neutrophil-derived resistin: a novel player in severe acute infections caused by Gram-positive bacteria Linda Johansson, Anna Linnér*, Jonas Sundén-Cullberg*, Carl-Johan Treutiger, Anna Norrby-Teglund * Contributed equally Submitted manuscript

CONTENTS

1	Background.....	1
1.1	Definition and epidemiology of severe sepsis and septic shock	1
1.2	Etiology.....	2
1.3	Pathophysiology of severe sepsis and septic shock.....	2
1.3.1	Normal immune response	2
1.3.2	Cytokines.....	4
1.3.3	Development of severe sepsis and septic shock.....	4
1.4	Modern treatment of severe sepsis and septic shock.....	6
1.4.1	New treatments.....	6
1.4.2	Potential new therapies.....	7
1.5	HMGB1	9
1.5.1	HMGB1-the intranuclear protein.....	9
1.5.2	HMGB1 - the extracellular protein.....	10
1.5.3	HMGB1-receptors.....	12
1.5.4	Effects of extracellular HMGB1	13
1.5.5	DAMPs, PAMPs, alarmins and a questionmark	14
1.5.6	HMGB1 in organ disorders and diseases other than sepsis	14
1.5.7	HMGB1 in experimental sepsis.....	16
1.5.8	HMGB1 in human sepsis and infectious diseases.....	17
1.6	Resistin.....	19
1.6.1	Background.....	19
1.6.2	Regulation of resistin release	20
1.6.3	Resistin in inflammation	21
2	General aims	23
3	Methods	24
3.1	Patient studies	24
3.2	Measurement of HMGB1	24
4	Results and Discussion.....	25
4.1	HMGB1 and Resistin are highly and persistently elevated in patients with severe sepsis and septic shock.....	25
4.2	Resistin, IL-6, IL-8, IL-10 and TNF-alpha, but not HMGB1, correlate to severity of disease and other measures of critical illness.....	26
4.2.1	Why no correlations between HMGB1 and disease severity?28	
4.3	Origin of HMGB1 and Resistin	29
4.3.1	HMGB1 is released from endothelium.....	29
4.3.2	Resistin is released from neutrophils, and to a greater extent in gram-positive infections.....	29
4.4	Effects of HMGB1 and Resistin	30
4.4.1	HMGB1 stimulates resistin secretion from monocytes	30
4.4.2	Resistin induces upregulation of ICAM-1 on monocytes... ..	30
4.5	HMGB1 signalling and inhibition.....	31
4.6	Anti-HMGB1 and anti-Resistin treatment.....	31
4.6.1	Differences with cytokines.....	31
4.6.2	Are HMGB1 and resistin cytokines?.....	32
4.6.3	Should we treat hyper-HMGB1-emia and hyper-resistinemia?	32

	4.6.4 Potential side effects of anti-HMGB1 and anti-resistin therapy	34
5	Conclusions	35
6	Populärvetenskaplig sammanfattning.....	36
7	Acknowledgements	38
8	References	40

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
APC	Activated protein C
APACHE II	Acute physiology and chronic health evaluation II
CLP	Cecal ligation and perforation
CpG-DNA	Cytosine, phosphate, guanine – DNA
CRID	Cytokine-release inhibitory drug
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorting
G-CSF	Granulocyte-colony stimulating factor
HMGB1	High Mobility Group Box chromosomal Protein 1
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular Adhesion Molecule 1
ICU	Intensive care unit
Ig	Immunoglobulin
IFN- γ	Interferon gamma
IL	Interleukin
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear factor kappa B
NK	Natural Killer cell
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycation endproducts
SIRS	Systemic inflammatory response syndrome
sRAGE	Soluble RAGE receptor
TLR	Toll like receptor
TNF- α	Tumor Necrosis Factor Alpha
VCAM-1	Vascular cell adhesion molecule-1

1 BACKGROUND

1.1 DEFINITION AND EPIDEMIOLOGY OF SEVERE SEPSIS AND SEPTIC SHOCK

Infections of different types – of viral, bacterial and fungal genesis are exceedingly common and an unavoidable part of life. The clinical symptoms of these infections vary widely – from the barely noticeable to rapid death. Severe sepsis and septic shock are serious manifestations of infectious diseases at the outermost, life-threatening extreme of the infectious disease spectrum. Case mortality is high, varying from 30 % up to over 60% if shock is present. Severe sepsis including septic shock are diagnosed in 750 000 cases and cause approximately 215 000 deaths annually in the United States according to one well cited epidemiological survey [1]. This translates to an approximate 23 000 cases/year in Sweden and an expected mortality of roughly 7000/year.

The terms sepsis, severe sepsis and septic shock have been used for decades, but it was not until 1992 that a consensus meeting agreed on universal definitions of the syndromes [2]. Sepsis was defined as systemic inflammatory response syndrome together with a clinical suspicion of infection; severe sepsis as sepsis in addition to signs of acute reduction of organ perfusion (not related to primary septic focus or underlying chronic disease); and septic shock as severe sepsis in addition to hypotension requiring vasopressor support, or lack of response to adequate fluid resuscitation (see table 1 for definitions).

Syndrome	Criteria
Systemic Inflammatory Response Syndrome (SIRS)	At least two of the following: fever or hypothermia (temperature > 38.0, or < 36.0°C, respectively), tachycardia (heart rate >90 beats/min), tachypnea (respiratory rate > 20 breaths/min, or PaCO ₂ <32 torr (4.3 kPa) or the requirement of mechanical ventilation), and white blood cell count > 12 or < 4 x 10 ⁹ /L (or > 10% immature white blood cells).
Sepsis	SIRS + clinical suspicion of infection
Severe sepsis	<i>Sepsis</i> in addition to signs of acute reduction of organ perfusion (not related to primary septic focus or underlying chronic disease) as manifested by at least one of the following: a) acute deterioration of mental status; b) arterial hypoxemia (PaO ₂ < 75 torr [10 kPa] without evidence of primary lung disease); c) oliguria (urine production < 0.5 mL/kg/hr for ≥ 2 hrs); d) acute deterioration of liver function (S-bilirubin > 43 μmol/L, or S-alanine transaminase more than twice elevated above reference value; e) metabolic acidosis (plasma lactate elevated above normal levels or negative base excess ≥ 5 mEq/L); or f) recent coagulation abnormality (prothrombin time or activated partial thromboplastin time ≥ 1.2 times the upper limit plus D-dimer ≥ 0.5 mg/L or platelets ≤ 75 x 10 ⁹ /L or a 50% reduction in 24 hrs).
Septic shock	<i>Severe sepsis</i> in addition to hypotension requiring vasopressor support, or mean arterial pressure < 70 mm Hg for ≥ 30 mins despite adequate fluid resuscitation.

Table 1. Study definitions of Systemic Inflammatory Response Syndrome (SIRS), sepsis, severe sepsis and septic shock.

1.2 ETIOLOGY

Severe sepsis and septic shock are in the majority of cases caused by bacteria. Gram-positive and gram-negative infections each constitute about half of verified bacterial infections [3-5]. Fungi are emerging as important pathogens in subgroups of patients and viruses underlie sporadic cases. Gram-positive and gram-negative bacterial infections mostly have clinically distinct presentations, as do many of the bacteria within each group. In more serious infections, however, the pathological events converge and critically ill patients tend to develop similar symptoms. This may be because, in the most severe infections, symptoms are caused not primarily by the bacteria, viruses or fungi in themselves, but by how the immune system responds to the infectious challenge.

1.3 PATHOPHYSIOLOGY OF SEVERE SEPSIS AND SEPTIC SHOCK

1.3.1 Normal immune response

In the vast majority of infections, the human immune system performs its job flawlessly. The diagnoses severe sepsis and septic shock are exceedingly rare compared to the myriad daily attacks deflected by this environmental armour. What then causes this normally fine-tuned mechanism to go out of control?

In order to understand what happens in the severely ill, we must first understand the central components of the human defence against microbial assault. The initial obstacles for any pathogen to overcome are the surface barriers constituted by the skin or, in body cavities, mucous membranes. If they break through that defence, invaders are prey to a broad, powerful, and immensely sophisticated immune response. Traditionally, that response is divided into two parts, innate and adaptive immunity [6]. The innate immune response is an evolutionarily well conserved system present in all multicellular organisms. It can be quite powerful and is often sufficient to eradicate infections on its own. It is characterized by rapid onset but short action, and has no memory function, *i e* will repeatedly react the same way to the same stimuli. The adaptive immune system, by contrast, is an evolutionarily more recent addition present in vertebrate organisms. It is a slower, second line of defence, but has a memory function and will mount faster, more powerful responses after each new exposure to a particular pathogen.

The innate and adaptive immune responses both have cellular and humoral (*i e* soluble in body fluids) components. The cells of the innate immune response are the monocytes/macrophages, neutrophilic granulocytes, natural killer (NK), and mast cells. Humoral components are the complement-, coagulation- and fibrinolytic systems, cytokines, antimicrobial peptides and acute phase proteins. The adaptive immune system is built up of the B- and T-lymphocytes, antigen presenting cells (APC's) and humoral components such as cytokines and antibodies. Although most research has focused on how innate and adaptive immunity work each on their own, there is a growing understanding of the interaction between the two [6].

After pathogens breach the outer barrier of the skin or mucosa, the next line of defence will be the foot-soldiers of the innate immune system – the monocytes/macrophages and the neutrophils - all phagocytic cell types. These react to a wide array of pathogens, mainly by recognizing evolutionarily conserved patterns on the surface of microbes, so called PAMP's – pathogen-associated molecular patterns, which are essential for microbe survival. Important examples of PAMPs are: Lipopolysaccharide (LPS) from gram-negative bacteria, flagellin, lipoteichoic acid from Gram positive bacteria, peptidoglycan from the bacterial cell wall, various structures, such as zymosan, from fungi, and nucleic acid associated with viruses, like double-stranded RNA (dsRNA) or unmethylated CpG motifs [7]. PAMPs bind to pattern recognition receptors (PRR) on or within innate immune cells. Three PRR's are: 1) the transmembrane toll like receptors (TLR's); 2) the intracellular nucleotide-binding oligomerization domain (NOD) leucine-rich repeat proteins; and 3) the retinoic-acid-inducible gene I (RIG-I)-like helicases [8]. Binding will initiate various processes including phagocytosis of intruders, and production and release of inflammatory mediators, including cytokines.

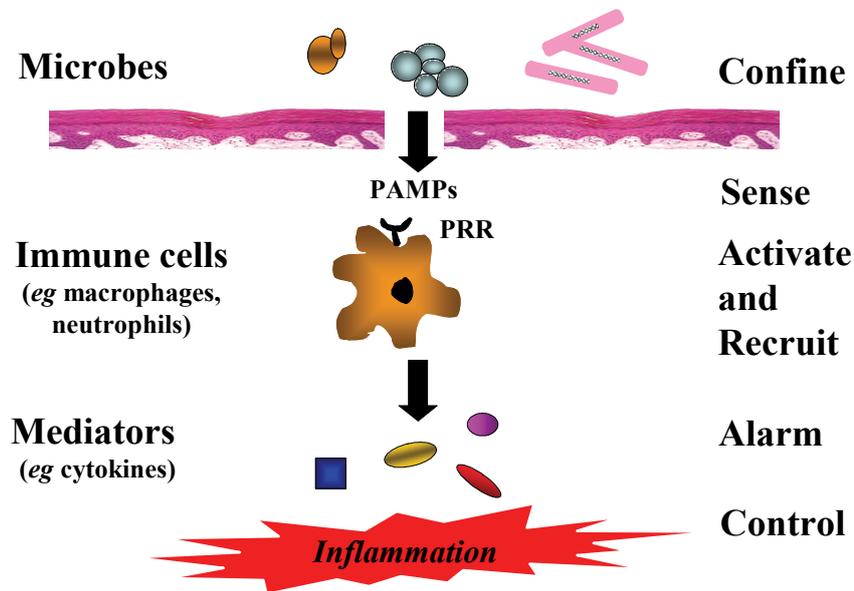


Fig 1. The primary purposes of the innate immune system is to confine pathogens, sense barrier penetration, activate and recruit immune system components, alarm, and ultimately control infections. PAMP (Pathogen associated molecular pattern). PRR (Pattern recognition receptor).

The local release of inflammatory mediators activates endothelial cells along the walls of blood vessels, which then express a number of adhesion molecules such as the immunoglobulin superfamily, integrins, selectins and a number of carbohydrate ligands, the latter of which are also expressed on leukocytes, lymphocytes and platelets. Activated leukocytes will thanks to these adhesion molecules first roll along the walls of blood vessels, then attach themselves to, and finally migrate through the vessel walls, following a chemotactic gradient to the site of infection. The increased capillary permeability that results from this process causes many of the symptoms in localized inflammatory processes – heat, pain, redness, and swelling (calor, dolor, rubor, and tumor), the four classical signs of inflammation, and also contributes to the systemic

symptoms of severe sepsis and septic shock - hypotension, hypoperfusion and organ dysfunction. An important part of severe sepsis pathogenesis is also an over-activation of the coagulation system which can lead to microcirculation thrombosis and disseminated intravascular coagulation (DIC) [8].

1.3.2 Cytokines

A central role in the inflammatory process is played by the cytokines. These are soluble proteins and peptides which act as messengers within the immune system, and also interact with a number of other organ systems. They are produced by many cell types in response to a variety of stimuli. They regulate proliferation, differentiation and functional activity of individual cells and orchestrate general immune responses. They also carry information to other organ systems and are involved in hematopoiesis, tumor surveillance and tissue repair. Cytokines can have pro- as well as anti-inflammatory effects. They are essential components of the innate immune system, but also influence the adaptive immune response. Cytokines have variously been called interleukins, chemokines and lymphokines based on their presumed origin, effects or targets.

A large number of cytokines have been described with various functions. The following are the ones most often mentioned in the context of sepsis. *TNF- α* is mainly secreted by macrophages, but also by a range of other cell types. It is rapidly released in animals and humans after bacterial infection or inoculation. It has strong proinflammatory properties, and is probably the major driving force in the early cytokine storm of severe infections. *IL-1 β* , secreted by monocytes and macrophages, appears shortly after *TNF- α* and has similar proinflammatory properties. Endothelial activation with vasodilation and increased capillary permeability is one example of IL-1 and TNF effects. *IL-6* is secreted by T-cells and macrophages. It is one of the most important mediators of the acute phase response. *IL-8* is secreted by innate immune cells in response to TLR-signalling and attracts other immune cells through chemotaxis. *IL-10* is produced mainly by monocytes and to some extent by lymphocytes. It has anti-inflammatory effects, partly by suppressing the synthesis of other pro-inflammatory cytokines. IL-12 and IL-18 are also important cytokines in sepsis. They are released from monocytes/macrophages and stimulate NK cells and T-cells to produce IFN- γ and various cytokines. They also promote the development of cell mediated immunity, a so called TH 1 response.

1.3.3 Development of severe sepsis and septic shock

1.3.3.1 Proinflammatory phase

After this brief summary of normal immune responses, let's return to the mechanisms that precipitate severe sepsis and septic shock. Currently, it's believed that the main culprits are events in the innate immune response, and that adaptive immunity has a limited part in this process. As mentioned, the response to pathogen associated molecular patterns (PAMPs) is immediate and potentially very powerful. Normally, the innate response will result in an inflammatory reaction at the pathogens point of entry. In severe sepsis, the inflammatory reaction moves far beyond this breach, spreading

like wildfire throughout the bloodstream while igniting multiple inflammatory cascade systems, thus creating an inferno of proinflammatory signalling. Cytokines are central actors in this process and the early phase of a severe infection is often referred to as a “cytokine storm”. This storm leads not only to systemic activation of, but sometimes also damage to endothelial cells, which in turn causes universal capillary leakage. Furthermore, a great number of inflammatory molecules like TNF- α , Platelet activating factor, bradykinins, histamine and prostaglandins, often mediated by nitric oxide (NO), cause arterial as well as venous vasodilation. At the same time other areas of circulation may be closed off due to vasoconstriction, causing hypoperfusion, anaerobic metabolism and production of lactate. Myocardial contractility is also compromised, leading to reduced cardiac output, mediated, at least in part, by IL-6 signalling and through NF κ B activation in cardiomyocytes [9, 10]. These pathophysiological events at the cellular level leads to clinical symptoms like fever, blood pressure fall, hypoperfusion and often organ failure.

It is important to underline that the intense reaction described above is very uncommon, even in patients hospitalized because of infections. Why some patients overreact to certain infectious stimuli, while others have much milder reactions, is incompletely understood. Some of the differences are probably explained by varying exposure dose, and some by dissimilar genetic makeup in patients, which result in divergent inflammatory responses in general, and also different responses to individual pathogens.

1.3.3.2 Anti-inflammatory phase

After the intense initial response follows a period which is described by many authors as a time when anti-inflammatory components of the innate immune system, including anti-inflammatory cytokines, reassert themselves. Central components of the immune response are down-regulated, including monocytes, macrophages, and T-cells. Lymphocytes (but not neutrophils) undergo accelerated apoptosis [11]. During this phase there is a rapid drop in proinflammatory cytokines. Clinically, the patients enter a fragile period during which normal immune functions are impaired, and they are highly susceptible to secondary infections. Many normally immunocompetent patients, having survived an initial severe infection, will contract some opportunistic bacterial or fungal infection that delay their recovery, or may even be fatal. The symptoms of these secondary infections are typically milder, but more protracted. Sometimes this phase is described as “tertiary sepsis”, see next section.

Although there is a substantial amount of evidence indicating that an anti-inflammatory phase indeed exists, it should also be pointed out that during this period there are still bountiful clinical signs of proinflammatory activity. And even though lymphocytes may be downregulated and go into apoptosis during sepsis; neutrophils, of special interest in this thesis, are present in higher concentrations, have an extended lifespan and increased functional activity [12]. As to the assertion that the anti-inflammatory cytokines “take over” and dominate over proinflammatory cytokines after a certain time point, a recent longitudinal study of patients hospitalized with pneumonia found no such immunological dissonance [13].

1.3.3.3 Primary, secondary and tertiary sepsis

The terms primary, secondary and tertiary sepsis are sometimes used to describe typical inflammatory patterns in sepsis. Different patient groups typically belong to one of the three categories. Primary sepsis denotes a rapid and powerful inflammatory reaction in response to virulent bacteria, like *pneumococci*, *meningococci* or *e. coli*. The typical patient is previously healthy. Secondary sepsis describes an infection after some previous insult such as an operation, other trauma or other infection. The disease trajectory is often less intense but more protracted than in primary sepsis. Tertiary sepsis describes intensive care patients who have entered the anti-inflammatory phase after an initially intense period of severe sepsis or septic shock, as described above.

1.4 MODERN TREATMENT OF SEVERE SEPSIS AND SEPTIC SHOCK

The most important treatment of severe infections is adequate [14, 15] and timely [16] administration of antibiotics which eliminates the main root of evil. Often, patients will be hypovolemic because of dehydration or capillary leakage and will need intravenous fluids and oxygen to maintain adequate tissue perfusion and oxygenation. Treatment is intensified if blood pressure does not respond accordingly. At this point many patients are transferred to the intensive care unit (ICU) for treatment with inotropic drugs and intensive surveillance. In the ICU, many end up with mechanical ventilation because of inadequate respiration due to affection of the lungs or general exhaustion. When patients are in the ICU for longer than a few hours, parenteral nutrition is initiated.

1.4.1 New treatments

For many decades, the interventions summarized above were the mainstay of treatment for severe infections. Based on studies published in 2001 and 2002, four new treatment modalities were introduced in ICU's all over the world. Recently, however, results and conclusions in three of the original papers have been criticised or modified by new studies.

Drotrecogin alpha, activated protein C (APC) is an endogenous protein that promotes fibrinolysis and inhibits thrombosis and inflammation. In 2001, a clinical trial showed a mortality reduction in patients with severe sepsis from 30.8% in the placebo treated group to 24.7% in those treated with Drotrecogin alpha [4]. However: two subsequent trials - one in patients with severe sepsis but with a low risk of death [17], and one in pediatric sepsis patients - have been terminated due to futility, and questions have been raised concerning the safety profile of the drug. Calls are now heard for very restrictive use of APC pending new studies [18, 19].

Intensive Insulin treatment. Many critically ill patients develop insulin resistance and hyperglycemia, regardless of whether they have diabetes before or not. A third study in 2001 [20] tested the effects of intensive insulin treatment in a population of surgical intensive care patients on mechanical ventilation. The investigators found a 42% reduction in 12 month mortality in treated compared to conventional treatment. The greatest effect was seen in a reduction of deaths due to proven infections. However, a

follow up study in *medical* ICU patients [21] failed to show a mortality reduction, though it did find evidence of reduced morbidity in the intensive treatment group. Some argue that this lack of mortality reduction should limit the use of intensive insulin therapy to post-operative patients, especially considering the potential side effects of severe hypoglycemia.

Low-dose cortisone treatment in septic shock. The most obvious candidate substances for reducing hyperinflammation are glucocorticoids which are widely used as anti-inflammatory agents in medical practice. However, several trials from the 60's and onwards failed to show any beneficial effect of *high dose*, hyperphysiologic steroids in the treatment of severe infections [22]. A study published in 2002 [5] tested the effect of treatment with physiologic *low-dose* hydrocortisone and fludrocortisone or placebo on 300 patients with septic shock. It found a relative reduction in the risk of death of 16% at day 28 which was attributed to hemodynamic stabilisation thanks to the steroids. Administration of low-dose hydrocortisone to septic shock patients was widely implemented in ICU's, but then a subsequent larger study of cortisol with the same end points was aborted due to futility [23]. Although shock resolved earlier in the hydrocortisone treated group, side effects included nosocomial infection, new sepsis, new septic shock, and hyperglycemia and in sum, mortality did not differ significantly between the groups. The principal investigator of that study recommends restrictive use of glucocorticoids in ICU's to only those patients who have not responded to fluid and vasopressor therapy within one hour [22].

Early Goal Directed therapy: In 2001 another study [24] tested the effect of a program of active surveillance of high risk septic patients with invasive monitoring of central venous oxygenation and aggressive treatment with fluids, inotropic drugs and sometimes blood transfusions. The point of these interventions was to adjust cardiac preload, afterload and contractility, and ultimately to balance oxygen delivery with oxygen demand. Early Goal Directed therapy significantly reduced mortality in patients with severe sepsis or septic shock from 46.5% in the standard therapy group (n=133) to 30.5% in the treatment group (n=130). This fourth study has generated much debate, but no really damning criticism. Subsequent reports have lent further credence to results in the original study [25]. The main thrust of the program, *i e* early identification and aggressive treatment of septic shock, a high fatality condition, mirrors the historical care improvements in many other medical conditions such as myocardial infarction, stroke and trauma.

1.4.2 Potential new therapies

1.4.2.1 Many failures

The four new treatments described above are the very few that have made it through the needles eye of clinical trials, but three may nevertheless end up with other aborted sepsis treatments in what has been described as “the graveyard of the pharmaceutical industry”. Other approaches to reduce inflammation and improve survival in sepsis that have been attempted in the last few decades [26], include trials with antagonists against bradykinin, platelet-activating factor, phospholipase A₂, nitric oxide (NO) and prostaglandins and multiple trials aimed at blocking lipopolysaccharide (endotoxin), the

most toxic component of the Gram-negative bacterial cell wall. Bids have also included the use of polyvalent immunoglobulins to neutralise a multitude of potential antigens. None of the described anti-inflammatory strategies have been conclusively shown to reduce mortality in human sepsis. Nor have trials aimed at reversing immune suppression function in the anti-inflammatory phase of sepsis, through the use of interferon and granulocyte colony stimulating factor (G-CSF) been clearly beneficial.

The main thrust of this research project has been to evaluate the pathogenic role in severe sepsis of HMGB1 and Resistin, two proteins involved in the proinflammatory signalling in infections. There is still some uncertainty whether these proteins should be characterized as cytokines, but at the very least they share many characteristics with that class of molecules. Let us therefore take a look at what has been attempted in the area of cytokine therapy in infections.

1.4.2.2 Animal studies

As described earlier, cytokines play a central role in both the pro- and anti-inflammatory stages of an infection. Two prominent proinflammatory cytokines are TNF- α and IL-1 β .

Therapeutic animal experiments showed very promising results with substances aimed at reducing levels of these substances. Pretreatment with antiserum to TNF- α protected mice against lethal doses of intravenous lipopolysaccharide (LPS). A monoclonal antibody against TNF- α protected baboons against Gram-negative bacteraemia and many other IL-1 and TNF-blocking strategies proved successful in reducing mortality in experimental animal sepsis [27].

1.4.2.3 Clinical trials

Given the success in animal trials, much hope was pinned onto therapies aimed at blocking the effects of TNF- α and IL-1 in human sepsis. Several trials used different monoclonal TNF- α antibodies and three used soluble TNF- α receptors. All but one of the individual antibody studies failed to show significant improvements in day 28 survival, although a metaanalysis of these studies did indeed find a modest overall increase in survival of 3,5 % in those treated with study drug vs placebo. There was no such trend to effect in any of the TNF-receptor trials, of which one study in fact showed higher mortality in the treatment group. Three trials evaluated anti-IL-1-therapy in a similar way, and though two of these found positive effects, a third, larger study was terminated for lack of efficacy. These articles are reviewed comprehensively by Marshall [28].

1.4.2.4 Why does anti-cytokine therapy work poorly?

The results of the anti-TNF and -IL1 clinical trials have resulted in much speculation as to why experimental studies on animals were so promising, but human trials so ultimately disappointing. Various explanations for these differences have been proposed.

Animal studies: 1) The animal models of sepsis are probably poor proxies for human sepsis. Bacteria or bacterial products are directly inoculated, resulting in a systemic, sudden and very intensive infection/inflammation, which is dissimilar to infections in patients which tend to develop in a localised area, from which they gradually spread. 2) Treatment in most animal studies is started before or simultaneously with endotoxin or microbial challenge, compared with the often considerable patient delay seen in the clinical setting.

Clinical trials: 1) The consensus criteria for diagnoses of sepsis, severe sepsis or septic shock were developed to include patients into studies early after admittance to hospital, before any laboratory confirmation of infection. These inclusion criteria yield a very wide and heterogeneous patient population with respect to clinical background, microbiological aetiology and circulating proinflammatory mediators. 2) The most commonly used outcome measure, 28-day all-cause mortality is admittedly the most important. It is, however, insensitive to smaller, but nevertheless interesting, changes in clinical status. 3) The immune response in serious infections is massive and a multitude of redundant proinflammatory proteins is released. Targeting just one of these for reduction may not make much of a difference as its part will be taken up by comrades in arms.

The criticisms listed above apply to most of the clinical trials discussed earlier. Regarding the anti-cytokine therapies, a further explanation was proposed, namely that the wrong proteins were chosen. Both IL-1 and TNF- α are among the first cytokines to be produced, and their peak production has often passed by the time of admission. Could targeting cytokines that exert their effect at a later time point be more effective as an anti-inflammatory strategy? That question points forward to the next part of this background story, which tell the tales of two proinflammatory proteins, HMGB1 and resistin.

1.5 HMGB1

In 1999, in an experiment designed to identify possible late mediators of sepsis, Wang et al found that macrophages stimulated with Lipopolysaccharide (LPS) secreted a protein beginning at 16 h – long after the peaks of previously studied proinflammatory cytokines [29]. The protein was identified as the nuclear protein High Mobility Group-1 Box chromosomal protein (HMGB1) and it was further demonstrated that, by administering anti-HMGB1 antibodies, in an experimental model of murine endotoxemia, lethality could be reduced from 100 to 30%. These promising findings dramatically increased the interest in HMGB1 as a proinflammatory cytokine or cytokine-like molecule.

1.5.1 HMGB1-the intranuclear protein

HMGB1 is a 215 amino acid protein, encoded on chromosome 13q12, which is highly conserved between species. There is 99 % species homology between rodents and humans, the proteins differing by only two amino acids. It has three domains: two internal repeats of positively charged residues, the A- and B-box; and a negatively charged COOH terminus (fig2). The two boxes bind to the minor groove of chromatin,

thus modifying DNA architecture. This facilitates the binding of regulatory proteins, including various gene transcription factors, to form stable complexes with the DNA. It likely plays a role in DNA repair and replication [30]. Interestingly, HMGB1 is not essential for fetal development as HMGB1 $-/-$ mice are born full-term. However, they die shortly after birth due to hypoglycemia [31].

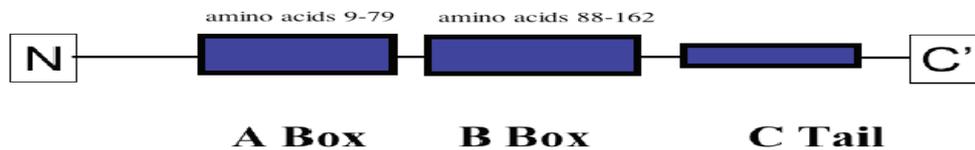


Fig 2. Schematic structure of HMGB1

1.5.2 HMGB1 - the extracellular protein

1.5.2.1 Release of HMGB1

Release of HMGB1 is stimulated by Toll signaling. Thus most bacterial antigens, for example lipopolysaccharide (LPS), can trigger HMGB1 release. Release is also triggered by endogenous proinflammatory signals such as the cytokines TNF- α , interleukin (IL)-1 β [29] or interferon (IFN)- γ [32]. Cells that actively release HMGB1 include monocytes [29], tissue macrophages [33], endothelial cells (paper I), enterocytes [34], pituicytes [35], mature myeloid dendritic cells (DCs) and activated natural killer (NK) cells [36]. The mechanisms by which HMGB1 is exported from the nucleus to the extracellular environment are only partly understood. HMGB1 lacks a classic leader sequence and, like the IL-1 family, it is released through an endolysosomal vesicle-mediated pathway [37]. When actively released after stimulation HMGB1 is heavily acetylated, an acetylation that occurs in the nucleus, and that prevents re-entry once it has reached the cytosol [33]. Acetylated, cytosolic HMGB1 migrates to cytoplasmic secretory vesicles, where it awaits extracellular release. Phosphorylation of the protein has a similar effect in promoting secretion from the nucleus to the cytosol and thence extracellularly [38]. Another source of extracellular HMGB1 is cells undergoing necrosis. HMGB1 is only loosely bound to chromatin and is easily set free during the necrotic process, triggering further inflammation.

In cells undergoing apoptosis, by contrast, binding to chromatin is much stronger, possibly due to a generalized underacetylation of histone. Programmed cell death therefore, it has been proposed, causes no extra-cellular spill of HMGB1 [39]. More

recent reports, however, indicate that HMGB1 is indeed released from apoptotic cells under the right circumstances [40, 41].

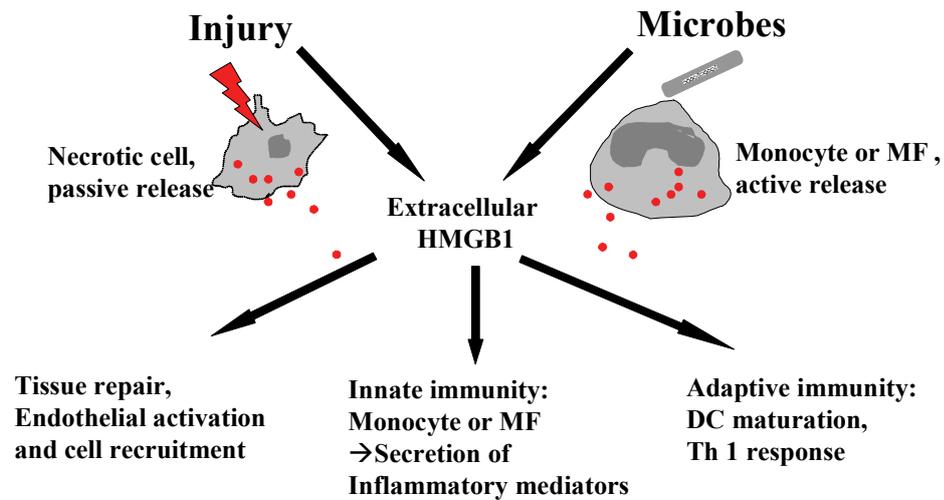


Fig 3. HMGB1 is passively set free during cell necrosis or may be actively released from many cell types after stimulation. Extracellular HMGB1 has many different functions depending on the setting. It can participate in either wound healing or various inflammatory processes. MF (macrophage).

1.5.2.2 Inhibition of release

There are three strategies for inhibiting the actions of HMGB1.

1. Preventing the release of HMGB1 from activated cells by using cytokine-release inhibitory drugs (CRIDs). This class includes ethyl pyruvate [42], cholinergic agonists nicotine and acetylcholine [43], stearyl lysophosphatidylcholine [44], and steroid-like pigment tanshinone IIA [45]. These small-molecule chemical compounds interfere specifically with HMGB1 release from the nucleus into the extracellular space, without affecting its mRNA or protein levels. In contrast, many other steroidal drugs (such as dexamethasone and cortisone) and nonsteroidal anti-inflammatory drugs (such as aspirin, ibuprofen, and indomethacin) fail to inhibit the extracellular release of HMGB1 significantly.
2. Direct inhibition of released, extracellular HMGB1 by anti-HMGB1 antibodies or other compounds. One small-molecule chemical compound, glycyrrhizin, which is a natural anti-inflammatory and antiviral triterpene in clinical use, also binds to and partially inhibits extracellular HMGB1 [46]. Soluble RAGE receptor, (s-RAGE), acts as a competitive inhibitor to membrane bound RAGE, binding and inhibiting HMGB1 [47]. We've found that, in healthy volunteers exposed to endotoxin, released soluble RAGE (s-RAGE) inversely relates to HMGB1 levels, and also that kinetics of HMGB1 and s-RAGE in patients with severe sepsis exhibit a mirror-like pattern (unpublished results). Strategy two has the advantage that it blocks *all* extracellular HMGB1, regardless of whether it comes from actively secreting cells (the targets of strategy one) or from passive release from necrotic (and possibly also apoptotic) cells.

3. Inhibition of HMGB1-receptors. The known surface receptors for HMGB1 are RAGE, TLR 2 and 4 which are described below. Substances which bind

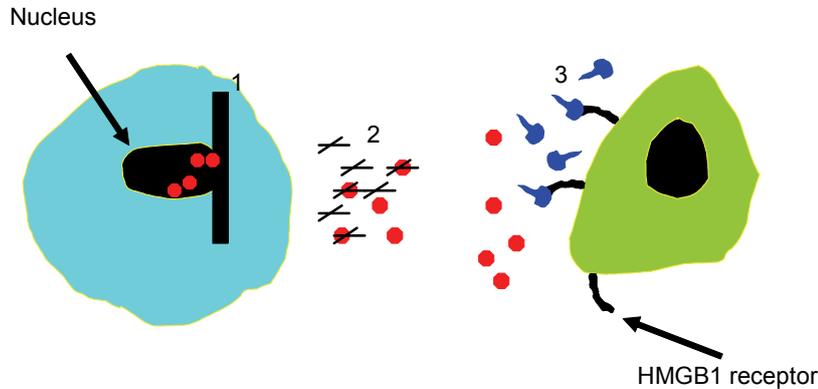


Fig 4. Three mechanisms of HMGB1 inhibition. 1) Cytokine-release inhibitory drugs (CRIDs), eg ethyl pyruvate, nicotine and acetylcholine. 2) Direct inhibition of released, extracellular HMGB1, eg anti-HMGB1 antibodies and s-RAGE. 3) Inhibition of HMGB1-receptors eg A-box of HMGB1.

directly to these without initiating intracellular signalling could block HMGB1 docking. The A-box of the HMGB1 molecule [48] is a partial agonist and competitively inhibits its much more proinflammatory B-box sibling.

1.5.3 HMGB1-receptors

RAGE - the receptor for advanced glycosylated end-products has been identified as a major receptor for HMGB1 [49]. RAGE antibodies, which bind and block membrane bound RAGE and soluble RAGE [47], which binds circulating HMGB1, reduce the proinflammatory activities of HMGB1. Furthermore, RAGE^{-/-} mice show a significantly reduced inflammatory response in tissues compared with wild-type animals when injected intraperitoneally with HMGB1. HMGB1 binding to RAGE leads to activation of the nuclear factor- κ B (NF κ B) pathway and also activation of extracellular signal regulated kinase (ERK) and P38.

TLR-2 and TLR-4 also bind extracellular HMGB1 (fig 5) which leads to a MyD88 (Myeloid Differentiation primary response gene 88)-dependent activation of NF κ B [50-52]. NF κ B moves from the cytosol to the nucleus and binds to transcription sites, activating genes which result in the production of a number of proinflammatory substances. Bacterial unmethylated CpG-DNA or its synthetic analogue CpG-ODN activates the intracellular receptor TLR9, which by way of MyD88 and NF κ B stimulates the production of IL-6, IL-12, TNF- α and iNOS. It turns out that HMGB1 can bind CpG-ODN and augment its interaction with TLR9 [53].

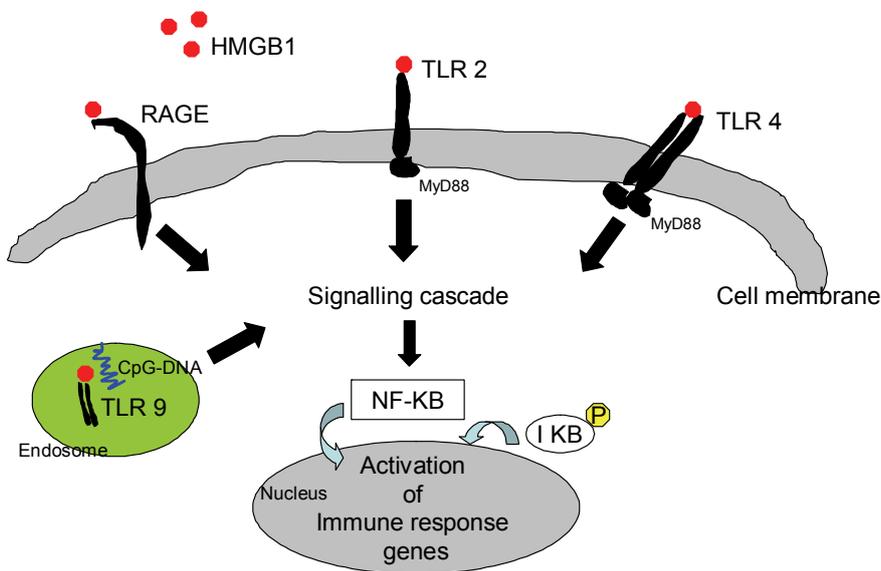


Fig 5. HMGB1 receptors. TLR 2, 4 and RAGE are receptors bound to the cell membrane. TLR9 is an intracellular receptor which pre-associates with HMGB1 and thus augments its binding of CpG-DNA in endosomes.

1.5.4 Effects of extracellular HMGB1

HMGB1 is active in the differentiation of cells, in neurite outgrowth [54] and in the governing of cell motility [49, 55, 56]. After the report by Wang et al which indicated an important role in sepsis, a vast number of investigations have charted the involvement of HMGB1 in various proinflammatory activities. Andersson et al demonstrated that HMGB1 is released from endotoxin stimulated monocytes and that HMGB1 in itself acts as a proinflammatory cytokine, inducing the production of other cytokines and chemokines, including TNF- α , IL-1 α , IL-1 β , IL-1RA, MIP-1 α , MIP-1 β , IL-6 and IL-8 [57]. It activates endothelial cells inducing the release of chemokines and cytokines and the upregulation of adhesion molecules [58], thereby increasing the adhesion of neutrophils and monocytes to stimulated endothelia. An important pathogenic effect is the induction of epithelial-cell barrier leakage in the gut [59] through the downregulation of cell-surface proteins responsible for the adhesion between adjacent epithelial cells.

HMGB1 mediates migration of both monocytes [60] and smooth muscle cells [61]. Interestingly, it stimulates dendritic cell maturation [62] which implicates a role in the switch from the innate to the adaptive immune response. The proinflammatory activity of HMGB1 mainly derives from the B-box while the A-box only has limited proinflammatory activity [63]. HMGB1 was recently shown to promote neutrophil recruitment to an inflammatory site through boosting the functional interplay between the RAGE receptor (see below) and Mac-1-integrin [64].

On an organism level, the summed consequences of HMGB1's effect on different cell types are dose dependent. Low doses on the one hand are, as with other proinflammatory cytokines, usually beneficial and can contribute to confine infections or tissue damage and also to promote wound healing and tissue regeneration [65]. High doses, on the other hand, contribute to fever, inflammatory cell migration, vascular leakage, oedema and eventually hypotension and other symptoms of systemic inflammation.

Nuclear functions	Extracellular functions
DNA stabilization Transcription factor	Aids neurite outgrowth [54] Cell chemotaxis and migration [49, 55, 56] Induces TNF- α , IL-1 α , IL-1 β , IL-1RA, MIP-1 α , MIP-1 β , IL-6 and IL-8 [57]. Activates endothelial cells. Induces cytokines and upregulation of adhesion molecules [58]. Induces epithelial-cell barrier leakage in the gut [59] Mediates migration of monocytes [60] and smooth muscle cells [61]. Stimulates dendritic cell maturation [62] Promotes neutrophil recruitment to inflammatory sites [64] Tumor metastasis [66] Antibacterial activity [67]

Table 2. Intra- and extracellular functions of HMGB1

1.5.5 DAMPs, PAMPs, alarmins and a questionmark

In the section above on the functions of the normal immune system, the role of Pathogen Associated Molecular Patterns (PAMPs), *i.e.* evolutionarily conserved patterns on invading microbes, in triggering the innate immune response was discussed. It has for some time been recognized that serious tissue injury by trauma, burns, irradiation, etc, has consequences similar to severe infections. A new class of molecules, the alarmins, has been proposed. Alarmins are endogenous molecules that signal tissue and cell damage and that, together with the exogenous PAMPs are subgroups of the larger group Damage Associated Molecular Patterns (DAMPs). Characteristics of alarmins are that they: 1) are rapidly released following necrosis but not after apoptosis; 2) may be produced and released from stimulated immune cells even in the absence of necrosis often using specialized secretion systems or the endoplasmic reticulum (ER)-Golgi secretion pathway; 3) recruit and activate receptor-expressing cells of the innate immune system, including dendritic cells, and thus directly or indirectly also promote adaptive immune responses; 4) promote the reconstruction of tissue injured by direct insult or as secondary effects of inflammation. HMGB1 is held forth as a prime example of an alarmin, fulfilling all these criteria. Other alarmins are: Calgranulins, Hepatoma Derived Growth Factor, Heat Shock Proteins, IL-1 α , Uric Acid, Cathelicidins, Defensins, Eosinophil derived neurotoxin, Galactins, Thymosins, Nucleosins and Annexins [68].

The results of a recent study may challenge this and other hypotheses around HMGB1. It suggests that HMGB1 on its own has a rather weak proinflammatory effect, and instead acts as a chaperone protein that binds proinflammatory bacterial substances and further amplifies their effects [69]. This may be HMGB1's main modus operandi and, if confirmed, will necessitate some re-thinking around its pathophysiology.

1.5.6 HMGB1 in organ disorders and diseases other than sepsis

Elevated levels of HMGB1 are seen in several organ disorders and diseases characterized by inflammatory responses:

Lungs: Intra-tracheal administration of HMGB1 in mice induces acute inflammation in the lung with accumulation of neutrophils, edema evolution and the production of proinflammatory cytokines [70]. 21 patients with septic acute lung injury had elevated levels of HMGB1 in plasma and in lung epithelial lining fluid. Although control subjects had no HMGB1 in plasma, concentrations were elevated in lung epithelial lining fluid, suggesting a role of HMGB1 in normal lung function as well as in the pathogenesis of acute lung injury [71]. Nafamostat mesilate, a serine protease inhibitor protected mice against LPS induced lung injury possibly through reduced HMGB1 levels which, the authors postulate, was achieved through the observed phosphorylation of I κ B [72].

Liver: Acute liver damage, following on interrupted blood perfusion or exposure to toxins, results in release of HMGB1 which mediates hepatic injury [73]. In addition HMGB1, along with other RAGE ligands, will limit liver regeneration [74]. Anti-HMGB1 therapy in the form of glycyrrhizin and recombinant A box peptide, by contrast, interfere with HMGB1-induced recruitment of neutrophils and other inflammatory cells in the liver and reduces liver disease in a mouse model of hepatitis B [75]. Glycyrrhizin is commonly used in Japan to treat patients with chronic hepatitis and the effect on HMGB1 may be its main mode of action.

Pancreas: 45 patients with acute severe pancreatitis were assessed for HMGB1 levels which were significantly higher compared to healthy controls, correlated with disease severity score, lactate dehydrogenase, C-reactive protein and total bilirubin. It was also higher in patients with organ dysfunction and infection during the clinical course and higher in non-survivors than survivors [76].

Central nervous system: In human patients with purulent meningitis high levels of HMGB1 are seen in cerebrospinal fluid (Dumpis personal communication) and HMGB1 has proinflammatory activity within the central nervous system, inducing fever, lowered pain thresholds (allodynia) [77], aphagia and taste aversion [78].

Malaria: Patients with cerebral malaria, a disease which is characterized by an intense inflammatory response, have high circulating levels of HMGB1 [79].

HIV: HMGB1 is higher in HIV patients compared to healthy controls, and highest in those with clinical complications [80].

Rheumatoid arthritis: The presence of cytoplasmic and extracellular HMGB1 has been reported in both experimental arthritis models as well as in human RA [81]. Anti-HMGB1 treatment in experimental RA limits disease severity and progression [82].

Churg–Strauss syndrome: In patients with C-S, serum HMGB1 levels were significantly higher than those of asthma patients and healthy volunteers. Levels decreased after steroid therapy, and positively correlated with soluble interleukin-2 receptor, soluble thrombomodulin, and eosinophil cationic protein in sera [83].

Myositis: In muscle biopsies taken from patients with chronic myositis, HMGB1 was detected cytoplasmatically in infiltrating rounded mononuclear cells, vascular endothelial cells and muscle fibers, and also extracellularly, surrounding the inflammatory infiltrates. Systemic corticosteroid treatment led to diminished expression of HMGB1 in this group [84].

Epithelium: HMGB1 induces epithelial-cell barrier leakage in the gut [59]. Exposure of epithelial cells to HMGB1 leads to downregulation of the expression of cell-surface proteins, such as zona occludens, that are responsible for the tight adhesion between adjacent epithelial cells. Epithelial damage is a mechanism that may explain much of the organ dysfunction seen in sepsis.

Cancer: HMGB1 and RAGE upregulation and interaction have been associated with the proliferation, migration and metastasis of many tumor types, including breast, colon, prostate, melanoma, and others [66].

1.5.7 HMGB1 in experimental sepsis

The kinetics of HMGB1 release and accumulation has been studied in murine models of endotoxaemia and also in ceecal ligation and puncture (CLP), an experimental setup intended to mimic spontaneous peritonitis (the cecum is ligated & punctured, after which peritonitis develops). In Wang's study, animals were injected intraperitoneally with a 50% lethal dose (LD50) dose of endotoxin. After which HMGB1 started to rise in the circulation at 8 hours; increased until 16 h; and thereafter remained stable until 36 h [29]. In a CLP model, HMGB1 started to rise approximately 18 hours after induction of peritonitis, and remained elevated for more than 72 h [48]. The kinetics of HMGB1 release in these animal models is delayed compared to other well-studied proinflammatory cytokines such as TNF- α , IL-1 β and IL-6. More importantly, death of animals paralleled the accumulation of HMGB1 in sera or plasma.

Treatment: In the endotoxemia model, passive immunization with anti-HMGB1 antibodies significantly protected against lethal doses of LPS, even if treatment was delayed until 2 h after exposure [29]. The effect of anti-HMGB1 antibodies was dose-dependent and sustained after the peak of circulating TNF- α had passed. A similar effect was demonstrated in the CLP model [48], in which both anti-HMGB1 and A-box of HMGB1 effectively improved survival even after a delay of 24h. The A-box segment of the protein has only weak proinflammatory activity and is a competitive inhibitor of the much more active B-box. Ethyl pyruvate, a non-toxic food additive and an experimental anti-inflammatory agent [42] that *in vitro* inhibits the release of HMGB1 from macrophages, also decreases lethality when given to mice in these animal models. Treatment with anti-interferon (IFN)- γ reduces mortality in a rat CLP model, an effect that the authors attribute to the reduced levels of HMGB1 achieved with the treatment [85]. The listed and other HMGB1 reducing treatments are summarized in table 3.

Substance	Experimental model	Effect	Ref
Anti-HMGB1 antibodies	Mice. LPS	Significantly and dose-dependently protected against lethal doses of LPS, even if treatment was delayed until 2 h after exposure.	[29]
Anti-HMGB1 and A-box	Mice. CLP	Both improved survival even after 24h delay.	[48]
Ethyl pyruvate.	Mice. LPS and CLP	Decreased lethality in both models.	[42]
Anti-interferon (IFN)- γ	Rat. CLP	Reduced mortality. Reduced levels of HMGB1.	[85]
TSNIIA-SS, a Chinese cardiovascular drug	Mice. LPS	Selectively inhibited HMGB1 and reduced lethality, even after delayed administration	[45]
GTS-21, a Selective $\alpha7$ -nicotinic acetylcholine receptor agonist	Mice. LPS	Improved survival	[86]

Table 3. Substances that inhibit HMGB1. G- (Gram negative). LPS (Lipopolysaccharide). CLP (Cecal ligation puncture)

Thus HMGB1 inhibiting treatment, be it HMGB1 antibodies, the A-box of HMGB1, ethyl pyruvate, anti-IFN- γ , TSNIIA-SS, or GTS-21, all reduce sepsis lethality in mice or rats even if treatment is delayed. Other ways to ameliorate the effects of HMGB1 may be to develop treatments that focus on its receptors. One interesting observation, for example is that RAGE $^{-/-}$ mice exhibit reduced inflammatory responses to injected HMGB1 and reduced lethality in CLP models compared to wild type mice [87].

1.5.8 HMGB1 in human sepsis and infectious diseases

Seven papers directly report levels of HMGB1 in sepsis or serious infections. The first was the seminal work by Wang et al which six years ago started a wave of research on HMGB1 as a late mediator of inflammation. Increased levels of HMGB1 were found in twenty-five critically ill patients with sepsis, and significantly higher levels in those that succumbed to disease compared to those that survived. The kinetics of HMGB1 release was not discussed in that paper. In paper II from 2005, we took a closer look at the kinetics of HMGB1 in patients with severe sepsis or septic shock. We studied 64 patients (whereof 59 with severe sepsis or septic shock) over the first week after admission. For a detailed discussion of this study, please refer to Results and discussion. Five other papers touch on HMGB1 concentrations in infections (table 4). Those that reported kinetics found elevated levels over time, like in our study. Those that used an ELISA technique in their analyses report lower levels of HMGB1 than those which used Western Blot techniques, which is not surprising, since Western Blots yield much higher values than ELISAs, in which plasma or serum components interfere with detection [88]. On the other hand, studies using ELISA's tend to find more consistent correlations between HMGB1 levels and disease severity and other inflammatory markers which could indicate that the technique is better at identifying relevant portions of the protein.

It is also important to establish that no study of HMGB1 in clinical diseases has been able to determine where it comes from – whether it is actively secreted from stimulated cells or passively released from necrotic ones – and, related to this - whether or not it is in acetylated form, and if the protein measured is actually biologically active.

To summarize, the results in these studies reflect some uncertainty concerning what are true levels of biologically active, extracellular HMGB1 in septic patients, but we can safely state that levels are highly elevated in a majority of septic patients, long after they are admitted to the hospital, and in several of the studies, although not in ours, there are important correlations to different markers of severity of disease.

Study type, setting	Inclusion criteria	Kinetics. Method of analysis	Findings	Ref
Retrospective	Critically ill patients with sepsis (n=25).	One sample, unspecified time point. Western Blot.	Increased levels of HMGB1. Higher levels in patients that died.	[35]
Prospective	Acute lung injury and severe sepsis (n=21).	Unspecified sampling scheme over seven days. Western Blot.	Persistent elevation of HMGB1 in patients. Kinetics not reported. No relation to disease severity.	[71]
Prospective study. Infectious disease dept and mixed case ICU	Severe sepsis & septic shock (n=59 + 5 patients with sepsis).	5 time points during 1:st week after inclusion. Western Blot.	HMGB1 highly and persistently elevated in the majority of patients. No correlation to disease severity.	II
Prospective study, Medical ICU	Septic shock (n=42)	Sampling at inclusion and after 3, 7 and 14 days. ELISA.	Correlations at baseline to SOFA-score, Lactate- and PCT-concentrations. No difference at inclusion, but HMGB1 levels diverged from day 3, after which survivors had lower levels than those who died by day 28.	[89]
Prospective	Community acquired pneumonia (n=122)	Sampling daily for 1 week. Western Blot.	Almost all patients had persistently elevated levels of HMGB1. Levels higher in those who died, but also remained elevated in those who fared well.	[90]
Prospective	Suspicion of severe infection (n=154).	One time sampling within 24 hrs of inclusion. ELISA.	HMGB1, LBP, and PCT higher in severe sepsis compared to only sepsis, and in bacteraemic compared to non-bacteraemic pat. Not significantly higher in pat who died compared to survivors. HMGB1 correlated to LBP, IL-6, CRP, WBC and neutrophils.	[91]
Prospective	Suspected DIC (n= 201)	One time sample. ELISA.	HMGB1 correlated to severity of disease. Higher levels of HMGB1 in patients who died.	[92]

Table 4. Clinical studies of HMGB1 in infectious diseases. LBP (LPS Binding Protein), WBC (White blood count). PCT (Procalcitonin). DIC (disseminated intravascular coagulation).

1.6 RESISTIN

1.6.1 Background

Resistin was discovered by three separate groups in 2000-2001 [93-95]. It is a 108-amino acid, 12.5 kDa peptide hormone member of the cysteine-rich secreted protein family also referred to as “resistin -like molecules (RELM)” or “found in inflammatory zone (FIZZ)” molecules. Resistin has mainly been studied in mice in which there is compelling evidence linking the protein to insulin resistance, obesity and type 2 diabetes mellitus (T2DM) [94]. There is only 59% amino acid homology between human and murine resistin [96] and regarding a potential role of resistin in human insulin regulation, obesity and T2DM, findings are as of yet inconclusive [97]. In man, the recent focus of research has come to center instead on the inflammatory effects of resistin.

1.6.1.1 Sources of resistin

In mice, the protein is mainly secreted from adipocytes. There has been controversy concerning the origin of resistin in man. Some evidence has suggested that it is secreted by adipocytes or white adipose tissue [98] but several reports claim that a more important source is monocytes [99-101].

We have found that another, probably quite significant, producer of resistin in human inflammation is the neutrophilic granulocyte (fig 6) which is the main topic of paper IV. Our findings support a recent publication which, in a comprehensive study of the protein content of neutrophilic phagosomes, listed resistin as one of many proteins of undetermined function contained therein [102]. In a review of the literature, we also found that human resistin has been detected in placental tissue [103] and pancreatic islet cells [104].

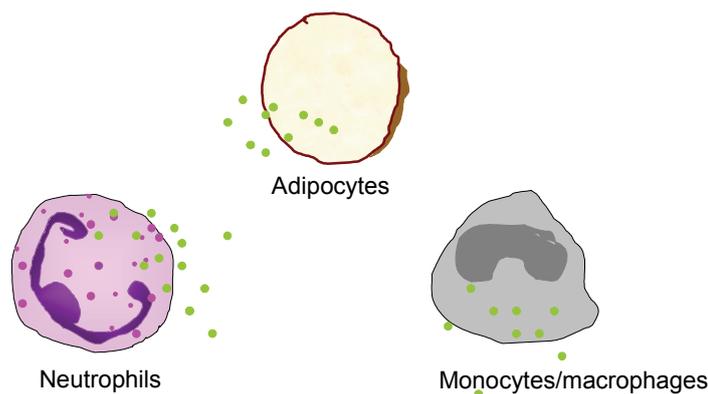


Fig 6. Cellular origin of Resistin in man

1.6.1.2 Resistin isoforms

In mice, resistin circulates in two distinct assembly states; the more abundant high molecular weight (HMW) hexamer, and the low molecular weight (LMW) ‘monomeric’ form. These have different biological actions. The monomer, for example,

is more potent than the hexameric form in impairing hepatic insulin action *in vivo* [105], but the hexamer has a more pronounced effect on cardiac muscle [106]. One study has shown that resistin exists in monomeric and hexameric form in man as well, but the functions of the different forms remain undetermined [107].

1.6.2 Regulation of resistin release

In man: Rosiglitazone (RSG) belongs to a new class of anti-diabetic drugs called thiazolidinediones. These enhance target-tissue sensitivity to insulin *in vivo*. Rosiglitazone treatment lowers resistin expression by human macrophages *in vitro* [99]. *In vivo*, resistin plasma concentrations [108] and also resistin gene expression in adipocytes [109, 110] is suppressed by Rosiglitazone in patients with type II Diabetes. Furthermore, serum resistin levels in overweight women with polycystic ovary syndrome are reduced by Rosiglitazone therapy [111].

Patients with Inflammatory Bowel Disease treated with infliximab, an anti-human TNF- α monoclonal mouse antibody, exhibited significantly reduced levels of resistin after treatment [112].

A short term prospective open randomized trial with the antioxidant Vitamin C (n=40) compared to no treatment (n=40) demonstrated significant reduction of mean resistin levels in the treatment group after only two weeks of an oral intake of 2 g ascorbic acid daily [113].

In rodents: In mouse adipocytes and/or mice/rats, resistin has been reported both to be upregulated and downregulated by Rosiglitazone and insulin; upregulated by glucose, testosterone and growth hormone; and downregulated by adrenalin, isoproterenol (a β_3 -agonist), retinoic acid, thyroid hormones and green tea(-)-epigallocatechin [97] [114].

1.6.2.1 Resistin receptors and signaling pathways

To date, no receptor for resistin has been identified, but intracellularly, it has been shown that human resistin stimulates the synthesis and secretion of the proinflammatory cytokines TNF- α and IL-12 in macrophages via a nuclear factor- κ B (NF- κ B)-dependent pathway [115], a path also used in adipocytes in which the JNK signalling pathway too is implicated [98].

1.6.2.2 Resistin in metabolism

An entirely new field of research exploded with the study by Stepan *et al* which in 2001 identified a new protein that in mice induces insulin resistance, obesity and type 2 diabetes mellitus and which increases in diet-induced and genetic forms of obesity [94]. Further studies have shown that the main target of resistin action in the rodent is the liver, in which it inhibits insulin suppression of hepatic glucose production [116-119] leading to hyperglycaemia. Conversely, resistin -null mice have low fasted blood glucose levels because of reduced hepatic glucose production [118]. Resistin also inhibits glucose uptake [120] and fatty acid uptake and metabolism [121] in rodent skeletal muscle. Adenovirus-mediated hyper resistinemia in mice causes dyslipidaemia.

A possible mechanism of resistin action is through the upregulation of suppressor of cytokine signalling 3 (SOCS-3) which inhibits factors in the insulin signalling cascade [122]. Resistin lives up to its name in mice, but in humans, its metabolic role, if any, is still unclear. Reports conflict on whether it is elevated in obese subjects; increases insulin resistance; or has anything to do with the pathogenesis of type 2 Diabetes Mellitus. For a thorough review, please refer to [97].

1.6.3 Resistin in inflammation

A number of recent reports suggest that in man, resistin is likely to be directly involved in inflammation. Resistin itself induces the production and release of Tumor Necrosis Factor (TNF)- α , and IL-6 in human peripheral blood mononuclear cells (PBMC's) [123]; TNF- α and IL-12 in human macrophages [115]; and TNF- α and IL-6 in human adipocytes [98]. In human PBMC's, LPS and the proinflammatory cytokines IL-1, IL-6 and TNF- α [124] increase expression of resistin mRNA. In paper III we showed that HMGB1 stimulated Resistin secretion release from human monocytes and in paper IV, we showed how bacteria stimulate primary neutrophils to produce resistin.

Lipopolysaccharide (LPS) induces resistin gene expression in human adipocytes and monocytes [125] and, interestingly, LPS given to healthy volunteers immediately increases plasma resistin levels [126, 127], an observation we have also confirmed in 16 healthy subjects exposed to endotoxin (Soop et al, submitted).

Previous work has demonstrated that resistin up-regulates vascular cell adhesion molecule (VCAM)-1 on endothelial cells [128]. In study III we showed *in vitro* that ICAM-1 was up-regulated on monocytes in response to resistin.

1.6.3.1 Resistin in organ disorders and diseases in man

Further clues to functions of resistin in man come from publications on in which diseases it is elevated. As evident from table 5, almost all of these are inflammatory conditions.

Disease	Findings	Ref
Severe inflammatory disease and T2DM.	Resistin levels correlated to inflammatory markers, including IL-6, leptin, sTNF-R2 and CRP.	[126, 129]
Rheumatoid arthritis (RA)	Resistin was found in the synovial fluid and plasma of patients with RA, and injection of resistin into mouse joints produced an arthritis-like condition.	[123, 130]
Patients on haemodialysis	Resistin was independently related to hsCRP, IL-6, and residual renal function.	[131]
Alcoholic hepatitis	Resistin correlated with inflammation and fibrosis in the liver of patients with alcoholic hepatitis. Hepatic stellate cells exposed to resistin increased expression of the proinflammatory chemokines MCP-1 and IL-8, through activation of NF-kappaB.	[132]
Coronary atherosclerosis (CA)	In asymptomatic patients, plasma resistin levels were predictive of CA, independently of CRP.	[133]
Heart failure	Serum resistin was related to severity of heart failure (NYHA-score) and was associated with a high risk for adverse cardiac events in patients with heart failure.	[134]

Table 5. Resistin in human disease. sTNF-R2 (soluble Tumor Necrosis Factor - receptor 2), MCP-1 (monocyte chemoattractant protein-1), CRP (C-reactive protein).

2 GENERAL AIMS

The aims of this thesis were to study *in vivo* and *in vitro* characteristics of the proinflammatory proteins HMGB1 and resistin, and specifically their role in the pathogenesis of infections severe enough to require critical care.

Specific objectives

- To determine the kinetics of HMGB1 and resistin in severe sepsis and septic shock, and the relationship of protein levels to etiologic pathogens and to clinical parameters.
- To determine the cellular origin of HMGB1 and resistin.
- To study the effects of HMGB1 and Resistin.
- To study potential inhibitors of HMGB1.

3 METHODS

The methods used in the studies of this thesis are described in the original papers, I-IV. Some overall remarks may help to put things into context.

3.1 PATIENT STUDIES

Included in the papers are data from two studies of patients with severe sepsis and septic shock (for definitions, see Bone et al [2]). The first study included 59 patients with severe sepsis and septic shock, and 5 patients with only a diagnosis of sepsis. In the first study, some patients were never admitted to the intensive care unit. In the second study (paper III, with 50 patients), we restricted inclusion to those who were admitted to the ICU with a diagnosis of severe sepsis or septic shock, or developed the syndromes while in the ICU. The window of inclusion from the point of diagnosis was set to twenty-four hours. In paper IV, we also used data from a few patients in a third study (likely finished by early 2008) with the same inclusion and exclusion criteria as study two. The third study is planned to include 100 patients, plus a control group of thirty severely ill ICU patients without signs of infection at inclusion. Sampling and registration of clinical data were identical in all studies.

3.2 MEASUREMENT OF HMGB1

The serum levels of HMGB1 reported for various clinical conditions, and even for the same condition in different reports, vary widely. On a closer look, it seems that the different results depend on what analytical method was chosen. In all early reports, analysis was based on the Western Blot technique, whereas in more recent reports [76, 83, 89, 91, 92], ELISA was used. That ELISA is preferred comes as no surprise, since it is a much less laborious technique than Western Blot. The ELISA came into production at a late time-point which had to do with difficulties in getting laboratory animals to produce anti-HMGB1 antibodies. This problem follows from the 99% species homology of the protein. The respective analytical methods unfortunately yield different results. The Western Blot typically indicates levels that are up to ten times higher than when measured in similar groups of patients with ELISA. The different results of immunoblotting and ELISA techniques are discussed in a recent paper which shows that serum/plasma components including IgG bind to HMGB1 and interfere with its detection by ELISA systems [88]. We have found that Western Blot techniques, too, can yield different results (paper II). It is possible that the different Blot methods detect different subsets of the protein, or that other serum/plasma components interfere with detection.

4 RESULTS AND DISCUSSION

The original aim of this thesis was to study in depth the proinflammatory protein HMGB1, which at the time had been shown to be a possible late mediator of inflammation. Our approach was two-pronged. Earlier experiments with anti-cytokine therapy had not been successful which probably, at least in part, has to do with how the brief bursts of those cytokines have already passed by the time drug was administered. Our first objective was therefore to study HMGB1 kinetics in critically ill patients. This was to answer the questions whether the protein was still elevated at the time when patients were admitted to the hospital; for how long it persisted at an elevated level; and finally how protein concentrations related to various measures of clinical status. Our second objective was to study *in vitro* cellular mechanisms underlying HMGB1's role in sepsis pathogenesis.

During the course of our studies, we came across resistin, a protein which at the time was generating a lot of interest in the field of diabetes and metabolism, but on which very little had been published concerning potential inflammatory effects. We took an interest based on the hypothesis that the protein might play a role in the development of insulin resistance and hyperglycaemia which are commonly seen in intensive care patients. This we could not show, but we did find that resistin has a prolonged secretion profile very similar to that of HMGB1, and that it correlates well to different measures of disease. These initial findings led on to more in-depth pathogenic studies of its role in inflammation.

4.1 HMGB1 AND RESISTIN ARE HIGHLY AND PERSISTENTLY ELEVATED IN PATIENTS WITH SEVERE SEPSIS AND SEPTIC SHOCK.

The main finding of the two clinical studies was the marked and persistent elevation of HMGB1 and resistin. In all the patients in paper II and half the patients in paper III, four other cytokines (IL-6, IL-8, IL-10 and TNF- α) were analysed at every time point to use as reference for HMGB1 and resistin. Mean and median levels of HMGB1 and resistin on inclusion were higher than those of IL-6 and IL-8, the two standard cytokines which reached the highest levels (fig 7). In contrast to the other cytokines, HMGB1 and Resistin only declined slightly during the studied period. Mean serum concentration for HMGB1 at the final sampling point was 300 times higher than for any of the other studied cytokines. Rates of decline were compared between HMGB1 and the other cytokines, in a comparison of time-dependent differences (interaction) between cytokine levels, and demonstrated that IL-6 and IL-8 declined faster and that there was a trend to a faster decline of IL-10. TNF- α did not decline significantly faster than HMGB1, probably because it had already decayed to near normal levels by the time of study entry, and we only caught the tail of its kinetic curve. We did not analyse these cytokines for all time points in the resistin study, but as evident in fig 7, where results from the two studies are overlaid, resistin kinetics resembled those of HMGB1, demonstrating a pattern of sustained increase.

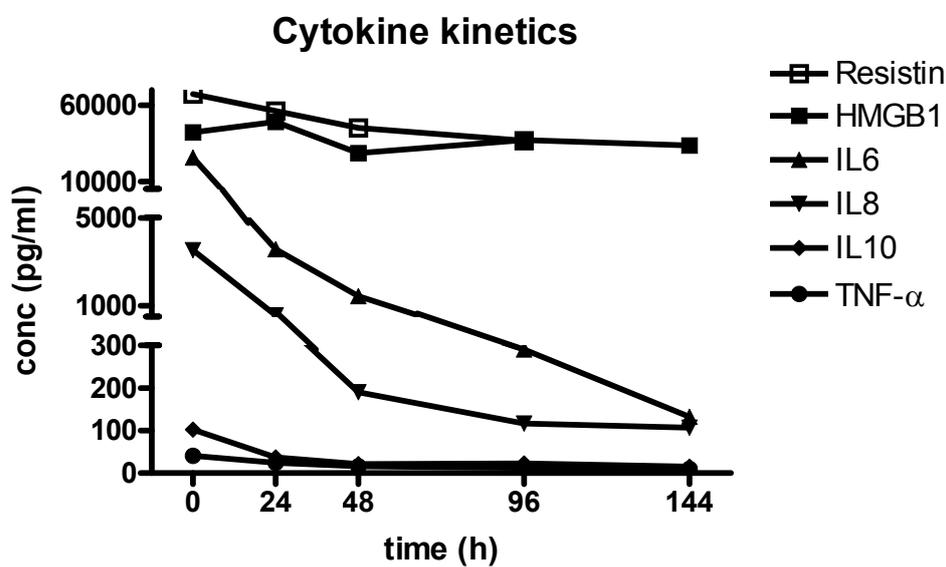


Fig 7. HMGB1, Resistin and cytokine kinetics in pooled patients from papers II and III with severe sepsis or septic shock. The y-axle has three regions. Values are means.

In study III, fifteen patients were sampled on day fourteen, at which point ten still had markedly elevated resistin levels. Overall, the sepsis cohort at this time point had a median value of 24 ng/ml, which is more than three times the median value of healthy controls and indicative of a persisting inflammatory state. All but one of the patients were still hospitalized, and eight were still in the ICU. Resistin levels were higher in the eight ICU patients (median 38 vs. 17 ng/ml) (fig 8D), and despite small groups the difference in levels compared with the seven patients who had been discharged to other wards was almost statistically significant. In paper IV, patients with toxic shock syndrome were sampled on day 28 after admission at which time point they had levels seven times those of healthy controls.

4.2 RESISTIN, IL-6, IL-8, IL-10 AND TNF-ALPHA, BUT NOT HMGB1, CORRELATE TO SEVERITY OF DISEASE AND OTHER MEASURES OF CRITICAL ILLNESS.

Resistin was significantly increased in patients with severe sepsis or septic shock (paper III) with four- to eight-fold higher median levels compared to 14 healthy controls, (fig 8A). In patients with septic shock compared to patients with severe sepsis, serum levels of resistin were significantly elevated at 0h and bordering on significantly elevated at 24h (fig 8B). It was also higher in patients that died compared to survivors (fig 8C), but the difference was not statistically significant.

Comparing by disease scores, we found that resistin and IL-6, IL-8, IL-10 and TNF- α correlated with APACHE II at baseline and daily SOFA-scores (table 2, paper III). Interestingly, APACHE II and SOFA-scores correlated better with resistin values measured 24 or 96 hours later compared with values from the same day, implying that the increase in resistin was delayed in relation to clinical status. An

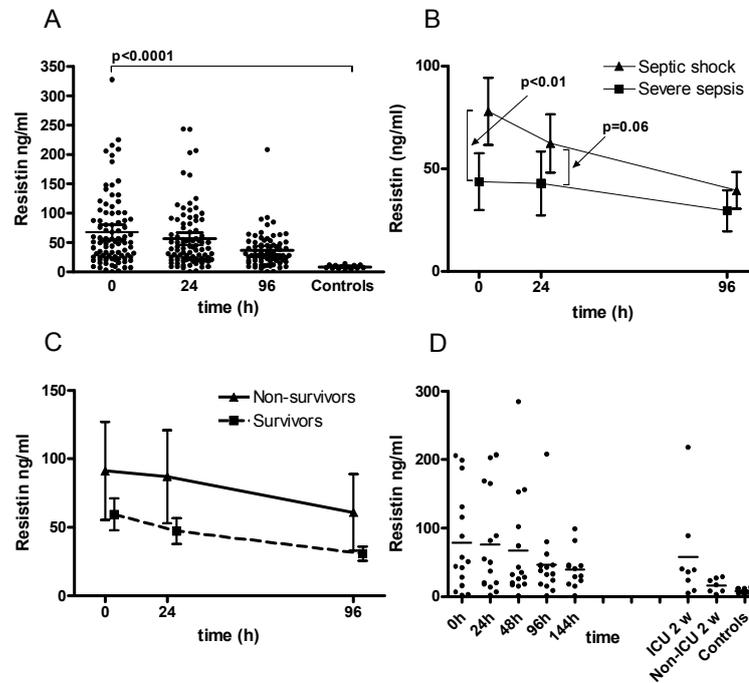


Fig 8. The kinetics of resistin in severe sepsis/septic shock. (A) Resistin levels in sera from patients with septic shock and/or severe sepsis compared to healthy controls. P-value by Kruskal-Wallis nonparametric one-way analysis of variance. (B) Resistin levels in patients with severe sepsis compared to patients with septic shock. P-values by Wilcoxon rank-sum test. (C) Resistin levels in survivors/non-survivors at 28 days. The difference was not statistically significant by the Wilcoxon rank-sum test. (D) Resistin in fifteen patients sampled up to day fourteen after admission. All statistics are given as mean (95% CI).

intact immune system yielded a stronger correlation between disease severity and resistin values; if we excluded twenty-nine patients who were immune-depressed according to APACHE II criteria, the correlation between SOFA-score and resistin in the remaining sixty-six immuno-competent patients rose markedly, from $\rho=0.2$ ($p=0.055$) and 0.3 ($p=0.004$) at 0 and 24 h respectively, to 0.37 ($p=0.002$) and 0.49 ($p<0.0001$). For fifteen patients, we obtained a day fourteen serum sample, which correlated strongly to SOFA-score the same day, $\rho=0.71$ ($p=0.0028$). Of the other studied cytokines only IL-8 correlated to severity of disease as measured by SOFA-score.

Resistin also correlated with other biomarkers of severe sepsis: creatinine, white blood count and lactate at most time points and to APTT and D-Dimer at baseline. For thirty-nine patients, we had neutrophil counts at baseline (0 h). These correlated with resistin values ($\rho=0.53$, $p=0.0005$). For forty-five patients we studied IL-6, IL-8, TNF- α and IL-10. These correlated at most time points to levels of resistin. In analogy with SOFA and APACHE II scores, inclusion values (0 h) for these four cytokines correlated better with the 24 hour value of resistin compared with the 0 h value, indicating that the release of resistin was delayed in relation to the other cytokines (table 3, paper III).

HMGB1, although highly elevated, did not correlate to any measures of severity of disease or to any other standard laboratory markers of critical illness. Nor were levels higher in patients who died compared to those who survived. In fact, with one of the Western Blot analyses used, levels were significantly higher in those who died

compared to survivors. The other analytical method, however, showed no difference between the groups.

In summary, both HMGB1 and resistin turned out to be highly and persistently elevated in patients with severe sepsis or septic shock. We could show that resistin, but not HMGB1, correlated with many measures of severity of disease.

4.2.1 Why no correlations between HMGB1 and disease severity?

As reviewed in the introduction, recent studies which have used ELISA techniques in analyzing HMGB1 often report positive correlations to various measures of disease severity. We recently ran a set of sepsis sera from paper II on an ELISA, to compare with our earlier Western Blot results (fig 9). We found no correlation between ELISA HMGB1 values and SOFA-score using this method either, but the analysis was based on only 33 pairs of values. We could confirm that in samples where both techniques showed positive results, levels were indeed higher with the Western technique (median 20.0 vs 3.1). On the other hand, out of the 32 samples, 17 had a value of 0 using Western Blot, while only one was also negative using ELISA. It would thus seem that with the Western Blot method, just as with ELISAs [88], there may be factors interfering with detection. Despite the many negative values, there was still an overall correlation between the two methods, $\rho = 0.45$ ($p = 0.01$). We speculate that many false negative values using the Western Blot technique in our study may explain the lack of correlations with clinical and laboratory parameters of sepsis.

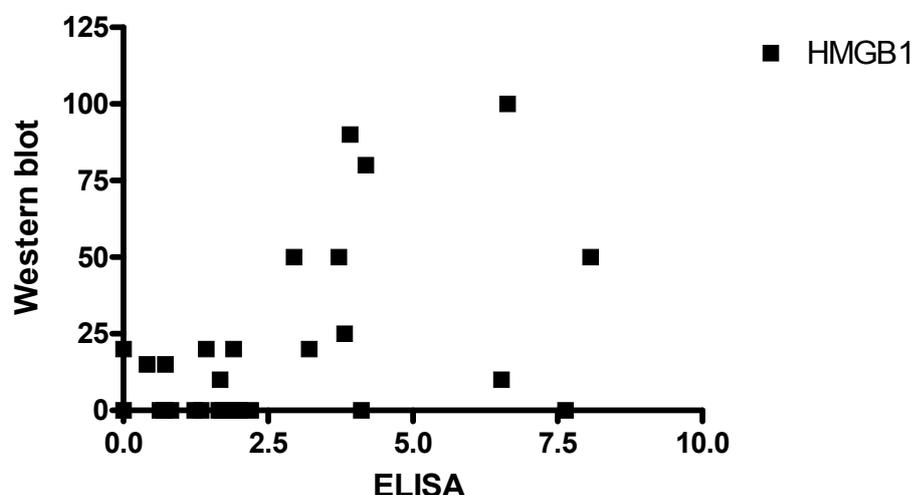


Fig 9. Results of HMGB1 analysis using ELISA vs Western Blot (WB). In samples where both techniques produced positive results, HMGB1-WB-values were typically much higher than HMGB1-ELISA values, but many WB vs only one ELISA analysis showed 0. Despite differences, the tests correlated, $\rho = 0.45$ ($p = 0.01$), Spearman rank correlation.

4.3 ORIGIN OF HMGB1 AND RESISTIN

4.3.1 HMGB1 is released from endothelium

The proinflammatory effects of extracellular HMGB1 were extensively reviewed in the introduction. We contributed the finding that HMGB1 is secreted from human endothelium (human umbilical vein endothelial cells, HUVECs) stimulated with lipopolysaccharide or TNF- α (Paper I). This resulted in a nuclear relocation of HMGB1 to the cytoplasm at 4h and release into extracellular medium as measured at 16 h. Endothelium, which covers the inside of all blood vessels, has a large surface area and could possibly be a major producer of systemic HMGB1 in situations of stress.

4.3.2 Resistin is released from neutrophils, and to a greater extent in gram-positive infections

Currently, resistin is believed to be produced mainly by monocytes/macrophages or adipocytes (please refer to introduction). We have found that a conceivably more significant producer of resistin in human inflammation, and perhaps also in normal situations, is the neutrophilic granulocyte (Paper IV). Our finding started with the observation in paper III that patients with confirmed Gram-positive infections had (non-significantly) higher levels of resistin than patients with confirmed gram-negative infections. In order to explore this finding, we selected 19 patients with confirmed Gram-negative infections and septic shock from papers II and III and compared with 19 patients with toxic shock syndrome caused by the Gram-positive bacteria group A *Streptococcus* and *Staphylococcus aureus*. The two patient cohorts were well matched by sex, age and SOFA-scores. We found that levels of resistin were significantly higher in the Gram-positive cohort (paper IV). In order to study resistin at a local site of infection, we used biopsies from patients with severe group A streptococcal severe soft tissue infections. Here we identified a novel source of resistin, the neutrophilic granulocytes, which turned out to constitute the majority of resistin-expressing cells in the biopsies (median=87%, range 63-89%) compared to macrophages (median=34%, range 12-56%). To see whether neutrophils might be important producers of resistin in circulation too, we checked records from paper III and found an overall correlation of neutrophil counts with resistin levels (n= 39, rho=0.53, p=0.0005). This led on to Flow cytometric analyses of peripheral blood of two patients with septic shock (58 and 63 yy). The vast majority of the resistin-positive cells in circulation were neutrophils (93% and 97%), whereas the corresponding monocyte frequencies were 6% and 1%, respectively. We compared with three healthy volunteers (one man and two women, 36, 38 and 26 yy) who had similar numbers ranging from 72 – 80% and 2 – 9 % of neutrophils and monocytes, respectively. In septic shock, there was also an increase in the absolute number of neutrophils, which likely contributed to the state of hyper-resistinemia found in these patients (36 and 15 ng/ml at baseline in the septic shock patients described above compared to 3-5.5 ng/ml in the volunteers). To be sure, neutrophils were not the only source. Several neutropenic patients also had elevated levels of resistin. To further investigate the noted difference in resistin responses in Gram-positive bacterial sepsis as compared to Gram-negative, and to establish the role of neutrophils as a source of resistin during these infections, we set-up an *in vitro* infection assay. We stimulated primary neutrophils with equivalent concentrations of clinical isolates of *E. coli* and Group A *streptococci* from two septic shock patients.

Both types of bacteria induced resistin production and release, but the *streptococci* infected neutrophils produced significantly more resistin than those infected with *E. coli*. Our findings also support a recent publication which, in a comprehensive study of the protein content of neutrophilic phagosomes, listed resistin as one of many proteins of unspecified function contained therein [102].

These are very interesting findings, since neutrophils play a fundamental role in the innate immune response to bacterial infections. The high expression of resistin in neutrophils, the correlation of resistin levels with neutrophil counts in severe sepsis and the colocalization of resistin with neutrophils in infected tissue all strongly suggest that resistin has an important role in neutrophil function.

Both Gram-positive and Gram-negative infections induce resistin production, but the former appears to be more efficient in this regard, especially if Group A *streptococci* are involved. One mechanism that could be involved is the streptococcal M-protein, which can powerfully activate neutrophils and induce vascular leakage and septic shock [135]. The relationship of M-protein and resistin needs further study.

4.3.2.1 Adipocytes

As for the role of adipocytes in the hyperresistinaemia of sepsis, we could find no correlation between Body Mass Index (BMI) and levels of serum resistin (paper III), in 47 patients for whom we had BMI values recorded. Considering the state of excessive systemic inflammation in this group of patients, one would expect adipose tissue to be involved and activated. Our findings suggest that adiposity plays no significant role in the increase of resistin in response to acute stress.

4.4 EFFECTS OF HMGB1 AND RESISTIN

4.4.1 HMGB1 stimulates resistin secretion from monocytes

Recognizing the similar kinetic profiles of HMGB1 and resistin, we assessed whether HMGB1 contributed to resistin release. *In vitro* incubation of human monocytes with HMGB1 for 16 h led to a dose dependent release of resistin, comparable to that of LPS. This link between the two late mediators of inflammation may be the explanation for their similar dynamic profiles.

4.4.2 Resistin induces upregulation of ICAM-1 on monocytes

Resistin in itself induced upregulation of ICAM-1 expression on monocytes. Monocytes were incubated for 16 h with the recombinant protein in increasing concentrations. Resistin induced a dose-dependent increase of ICAM-1 expression on the surface of the monocytes. ICAM-1 is an important cell adhesion molecule, which promotes the attachment of cells of the immune system to a site of infection. This finding adds to ever increasing evidence in the last few years concerning different pro-inflammatory effects of resistin, please refer to introduction for a detailed account.

4.5 HMGB1 SIGNALLING AND INHIBITION

We investigated the effects of HMGB1 on HUVEC and monocytes and observed that the proinflammatory activity of HMGB1 on both cell types is sensitive to dexamethasone (paper I). Next, we investigated the HMGB1-induced TNF- α release from monocytes and found that it could be inhibited by either the A-box of the protein or the p38 inhibitor CNI-1493. Neither, however, had any inhibitory effects on the HMGB1-dependent upregulation of cell-adhesion molecules on HUVEC, and from these findings we deduce that HMGB1 may use different signalling pathways in monocytes and endothelial cells (fig 10).

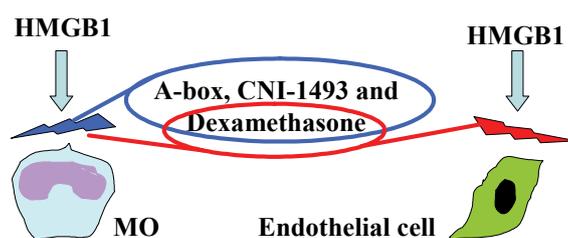


Fig 10. Dexamethasone inhibited the proinflammatory effect of HMGB1 on both endothelium and monocytes but A-box and CNI-1493 inhibited effects only on monocytes.

4.6 ANTI-HMGB1 AND ANTI-RESISTIN TREATMENT

4.6.1 Differences with cytokines

The kinetic profiles of HMGB1 and resistin clearly differ from the other cytokines we studied. First, although many studies, including our own (paper II), report unmeasurable levels of HMGB1 in healthy controls, others have indeed found measurable levels of the protein in many, but rarely all, healthy volunteers tested. We recently did an analysis of plasma HMGB1 and resistin in sixteen healthy volunteers using an ELISA method and found measurable levels of HMGB1 in eleven of them (preliminary results). In the case of resistin, almost all subjects have measurable levels in published studies, as they did in our volunteers. This clearly differs from the other cytokines we have measured in our studies, IL-6, IL-8, IL-10 and TNF- α which all are unmeasurable or at least very low in control subjects. A second difference is the magnitude of response of both HMGB1 and resistin compared to the cytokine group. Although both are released *as quickly* (within hours) as IL-6, IL-10 and TNF- α after endotoxin injection in healthy volunteers, *the magnitude* of the release is much lower than for the other cytokines (Sunden-Cullberg *et al*, manuscript). Both resistin and HMGB1 increased about threefold from baseline levels, whereas IL-6 and TNF- α increased from zero in most cases to median values exceeding 1000 ng/ml and IL-10 from zero to about 50. The third major difference is the prolonged secretion profile of both HMGB1 and resistin, *i e* the main findings of the two clinical studies in this thesis. By the time patients are admitted to hospital, levels of other cytokines are rapidly

falling and reach normal or very low levels within days. Resistin and HMGB1 by contrast, remain high up to two weeks post admission. In the volunteer-endotoxin experiment, which simulates the earliest phases of sepsis, we noted an interesting difference between HMGB1 and resistin in that resistin was still rising at the final, five-hour sampling point in 14 out of 16 volunteers, whereas HMGB1 by that time had started to fall in 11 cases. In a previously published study on resistin secretion after endotoxin injection, mean resistin levels rose until 12 h after injection [126]. In this sense, resistin fits the description of a *late* mediator of inflammation even better than HMGB1.

4.6.2 Are HMGB1 and resistin cytokines?

I've been able to locate no definitive definition of the term "cytokine". The most common definition of a cytokine is broad: "A cytokine is a small protein that has a specific effect on the interactions between cells, on communications between cells, or on the behavior of cells" (MedicineNet.com). Other definitions include structural specifications, so that a cytokine should resemble one of the following: the four-helix bundle family, the IL-1, the IL-17 or the Chemokine family. Yet another classification is by cytokine receptor morphology and includes: the immunoglobulin, the hematopoietic growth factor, the interferon, the Tumor Necrosis Factor and the seven transmembrane helix superfamilies. HMGB1 and resistin fulfill the first definition of a cytokine, but neither fits the second, though RAGE, one of the HMGB1 receptors, belongs to the immunoglobulin superfamily and so fits the third billing. Many authors refer to HMGB1 as a cytokine and to resistin as an adipocytokine (fat cell cytokine), but others prefer wordings like "cytokine-like" or "proinflammatory proteins", which I have used throughout this thesis.

As previously recounted, HMGB1 and resistin secretion profiles have some similarities but also clear differences from the other pro and anti-inflammatory cytokines we have studied in experimental endotoxemia and in severe sepsis and septic shock. Maybe the different profiles have to do with other functions the proteins also have, besides their proinflammatory role - in the case of HMGB1 as both intranuclear transcription factor and extracellular protein with pleiotropic actions (please refer to introduction for detailed review) - and in the case of resistin as a potential player in metabolism. In this discussion, we must also consider new reports which question whether HMGB1 really is very proinflammatory on its own, or if it mainly acts as a "chaperone protein" binding bacterial substances, such as lipids, in order to amplify its own effect [69]. Even if such a mechanism of action is verified, HMGB1 in "amplifier mode" may have cytokine-like properties.

4.6.3 Should we treat hyper-HMGB1-emia and hyper-resistinemia?

Now to the million-dollar question – should we treat high HMGB1 and resistin levels in severe sepsis? The proteins are both involved in promoting inflammation, both are highly and persistently elevated in severe sepsis and both correlate to different measures of severity of disease. So, from this perspective, it seems logical to treat septic patients with high levels of the proteins. There are, however, several important problems and unanswered questions to tackle before any clinical trial can be attempted:

1) Although several studies have found correlations of HMGB1 with severity of disease and different biological markers of sepsis, as we did with resistin in paper III, no study has found consistently higher levels of HMGB1 in septic patients who died compared to survivors of sepsis. We found higher levels of resistin in non-survivors compared to survivors, but the difference was not statistically significant. Here, there is clearly a need to identify subgroups of patients who may benefit from HMGB1 or resistin - lowering therapy.

2) The second question is related to the first and concerns the role of HMGB1 and resistin treatment in the later stages of sepsis progress. As pointed out in the background section, patients with severe infections at some point pass the inflammatory and enter the anti-inflammatory phase of sepsis progression, a state characterized by immune system paralysis. When the pro-anti-line is passed is not well defined, but most would probably agree that it happens some time during the first week after diagnosis of severe sepsis or septic shock. That means that the line is passed at a time when other cytokines have dropped to normal or very low levels, but HMGB1 and resistin remain elevated. If we assume that the duo is still exerting proinflammatory effects (remember – no group has been able to determine the biological activity of the HMGB1 measured in septic patients), one must question whether it is wise to curb those effects at such a time, or if it is more prudent to wait and hope that the proteins have an immunosupportive effect. A counter-argument would be that, even if there is academic consensus on the existence of an anti-inflammatory sepsis phase, many, if not most patients in that phase will, regardless of some laboratory signs of immuno-paralysis, still be showing overt clinical signs of acute systemic inflammation such as fever, hypotension, and organ failure. It is likely that proinflammatory signalling is still going on, and in fact, although lymphocytes undergo apoptosis at this time, neutrophils, central players in innate immunity, exhibit increased numbers, *suppressed* apoptosis [12] and enhanced functional responses. This gives good cause for hoping that severely ill septic patients can benefit from anti-inflammatory, anti-neutrophil and perhaps anti-resistin therapy.

3) Animal experiments: as reviewed in the introduction HMGB1-attenuating therapy has improved survival in several animal sepsis models, but these seldom translate well into clinical practice. There are many objections to the animal models, but in this setting, the most pertinent critique is that they poorly reflect the situation we're looking to treat – late stage sepsis. As for resistin, it is hard to design relevant animal models since rodent and human resistin, not only have low amino acid homology, but also seem to have very different roles, the first probably involved mainly in metabolism, and the latter apparently involved mainly in inflammation. A potential solution would be to identify species other than rodents in whom structural and functional similarity with human resistin is greater.

4) What is the relevance of targeting individual cytokines/proteins in sepsis? We are singling out but one molecule in a cavalcade of others, which all interact in complex antagonistic and synergistic patterns. There is redundancy in these systems with many molecules exerting similar effects. Targeting just one or two of them for therapeutic intervention might seem a futile exercise. But then again, maybe the redundancy is not

so pronounced in late sepsis? Perhaps HMGB1 and resistin really are inflammatory pathway bottlenecks? That would make them perfect targets for intervention.

The listed questions/problems need satisfactory answers before clinical trials can come into question. On the other hand, severe shock is a condition with an unacceptably high mortality, and finding new treatments is imperative. In a comparison of the two proteins, the potential benefit of anti-HMGB1 therapy is more documented than anti-resistin therapy. In the latter case, relevant animal trials are lacking, but our discovery that neutrophils, powerful actors in the innate immune response, are a source of resistin suggests that this protein may well turn out to be an even more important target. Recent evidence implicate neutrophils as central in the pathogenesis of multiple organ failure in sepsis [12].

Treatment may also be considered for other (auto)inflammatory diseases, like Rheumatoid arthritis and Inflammatory bowel disease. Consider the analogy with anti-TNF- α therapy which did not help sepsis patients but revolutionized the treatment of Rheumatoid Arthritis!

4.6.4 Potential side effects of anti-HMGB1 and anti-resistin therapy

What side effects can we expect from therapy directed at reducing HMGB1 and resistin in sepsis? As discussed earlier, it is possible that the proteins have beneficial effects in some or even all sepsis patients, as immuno-supporters, and that reduction of them might consequently be deleterious. As to normal extracellular, non-immunologic functions of the proteins, we know that HMGB1 is active in the differentiation of cells, in neurite outgrowth and in the governing of cell motility (please refer to introduction). Resistin may, apart from its proinflammatory effects, be involved in metabolism, but other functions are not known at present. HMGB1 reducing therapy in rodents has had no obvious side effects, but observation times have been short. Based on the known extra-cellular functions of the proteins, we do not foresee any problems with short-term treatments during sepsis. Considering HMGB1s ubiquitous presence as a nuclear protein, one should make very sure that candidate therapeutics cannot enter the cell and potentially wreak genetic havoc. A final consideration is what HMGB1 or resistin reducing substance is used. As reviewed earlier, there are several aspirant molecules for each protein. These might, irrespective of any effect on the target proteins, have side effects of their own.

5 CONCLUSIONS

This thesis shows that the proinflammatory proteins HMGB1 and resistin are highly and persistently elevated in patients with severe sepsis and septic shock. It also shows that resistin, but not HMGB1, levels correlate with many measures of severity of disease in critically ill patients. We studied putative sources of the proteins and found that, in response to proinflammatory stimulation, neutrophils, central cells in the innate immune response and endothelial cells, respectively release resistin and HMGB1, and that resistin is released at higher concentrations in gram-positive infections compared to gram-negative. Our findings in addition to other current research indicate likely roles for both proteins in maintaining a potentially deleterious inflammatory response in patients with severe sepsis and septic shock. Although crucial research is still lacking, the evidence at hand motivates hope that we can develop anti-resistin or anti-HMGB1 drugs with disease-modifying effects in inflammatory diseases and an anti-inflammatory and life-saving effect in the critically ill.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Svår sepsis (blodförgiftning) och septisk chock är ovanliga, men livshotande, tillstånd som kan uppstå i samband med infektioner. Dödligheten är hög - från 30 % i svår sepsis upp mot 60 % i septisk chock. Tidig antibiotika- och intensivvårdsbehandling har visat sig ha stor betydelse för patienternas överlevnad. Övriga nya behandlingar har dock medfört endast marginell minskning av dödligheten.

Det medfödda immunsvaret (*innate immunity*) är en evolutionärt uråldrig och kraftfull del av ryggradsdjurens försvar mot infektioner. Sannolikt beror en stor del av den höga dödlighet man ser i svåra infektioner inte på bakterier, utan på att det tidiga immunsvaret blir för kraftigt. Att dämpa denna kraftiga *inflammation* skulle kunna öka överlevnaden hos svårt sjuka patienter. Det medfödda immunsvaret använder olika proteiner, av vilka de viktigaste är cytokinerna, för att signalera mellan celler. Ett sätt att dämpa inflammationen vore att slå ut dessa proteiner. Kliniska prövningar av läkemedel som reducerar enskilda cytokiner har dock inte lyckats minska dödligheten. Troligen beror detta på att den kraftfulla, men kortvariga, frisättningen av cytokiner redan är förbi när patienterna kommer till sjukhus. Man har därför letat efter proinflammatoriska proteiner som ingår i det medfödda immunsvaret och som har en fördröjd utsöndringsprofil, sådan att behandling fortfarande är möjlig när patienterna kommer till sjukhus.

Denna avhandling fokuserar på två proteiner med cytokin-lik egenskaper, HMGB1 och resistin. Båda har en mängd proinflammatoriska effekter, och för HMGB1 har man tidigare påvisat en kraftigt sänkt dödlighet i experimentella infektioner hos djur när man givit HMGB1-sänkande behandling.

Vi genomförde två observations-studier med totalt 109 patienter med svår sepsis eller septisk chock. Huvuddelen behandlades på intensivvårdsavdelningen på Huddinge sjukhus. Vi kunde påvisa en förlängd utsöndringsprofil för såväl HMGB1 som resistin. Båda kvarstod kraftigt förhöjda upp till en vecka efter att patienterna kommit med i studierna, långt efter att övriga studerade cytokiner återgått till låga värden. För resistin kunde vi också visa på en samvariation med sjukdomens svårighetsgrad, och med flera klassiska laboriemarkörer för sepsis.

Vi letade efter tänkbara källor till de båda proteinerna och kunde visa att HMGB1 utsöndras från endotel – d v s celler som klär insidan av blodkärlen - och att resistin, som man hittills trott utsöndras endast från monocyter (en vit blodkropp) och fettceller, även kommer från neutrofiler, den vanligaste av de vita blodkropparna. Både endotel och neutrofiler är viktiga beståndsdelar i det medfödda immunsvaret vilket stärker misstanken att proteinerna spelar en viktig roll i det tidiga svaret på infektioner. Vi visade också att resistin utsöndras i högre koncentration vid infektioner med grampositiva bakterier jämfört med gramnegativa.

I vidare studier kunde vi visa att monocyter, som stimulerades med HMGB1, frisatte resistin. Denna koppling skulle kunna förklara proteinernas likartade utsöndringsprofil vid sepsis. Vi kunde visa att resistin orsakar uppreglering av ICAM-1 på monocyter.

ICAM-1 är en cell adhesions molekyl – d v s den hjälper immunceller att binda till inflammerade områden. Slutligen studerade vi tänkbara hämmare av HMGB1 och fann att Dexametason, ett slags kortison, hämmade HMGB1's proinflammatoriska effekter på monocyter och endotel och att CNI-1493, en experimentell läkemedels substans, hämmade dess inverkan på monocyter.

Sammanfattningsvis visar denna avhandling att HMGB1 och resistin har proinflammatoriska effekter; att de utsöndras av celler som är viktiga i det medfödda immunsvaret och att de har en förlängd utsöndringsprofil vid svår sepsis och septisk chock. Detta tyder på att om man kan sänka nivåerna av proteinerna vid svåra infektioner, så skulle det kunna dämpa inflammationen och därmed minska den höga dödligheten i patientgruppen.

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