C5a Receptor Expression in Severe Sepsis and Septic Shock

MIA FUREBRING
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Abstract

In patients with sepsis, the activation of the cascade systems, for example the complement system with the generation of C5a, is followed by a state of immunosuppression with impaired bactericidal capacity caused by suppression of the neutrophil granulocytes. To inhibit the C5a-induced systemic inflammatory and the following anti-inflammatory responses, different anti-C5a strategies have been successful in experimental models of sepsis. In animals and in healthy volunteers after injection of lipopolysaccharide (LPS), an up-regulation of the C5a receptor (C5aR) has been reported. Before designing clinical studies, it was of importance to increase the knowledge of C5a and C5aR regulation in humans.

At the time when the diagnosis of severe sepsis or septic shock can be established clinically, granulocyte C5aR expression, analysed by flow cytometer, was shown to be reduced, whereas monocyte C5aR expression was unchanged. There was a correlation between granulocyte C5aR expression and the severity of disease, as measured by the APACHE II score.

Ex vivo incubation of whole blood with LPS resulted in a reduction in granulocyte C5aR expression. Such a reduction was not found in isolated cells, indicating that the effect was mediated via plasma factors, such as C5a, IL-8 and TNF-α which all were shown to reduce C5aR expression ex vivo.

Although there was a trend between chemotaxis, as measured by migration in a modified Boyden chamber, and C5aR expression on granulocytes from patients with severe sepsis or septic shock or from healthy individuals, the correlation failed to reach statistical significance.

It is concluded that granulocyte C5aR expression is affected by several plasma factors and that a reduction is clinically evident at the time of the sepsis diagnosis. Reduced granulocyte C5aR expression is associated with an impaired chemotaxis but does not alone limit the chemotactic response.

Keywords: C5a receptor, granulocyte, severe sepsis, septic shock, chemotaxis, anti-C5a treatment, ex vivo incubation, C5a, interleukin-8, LPS

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List of Papers

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


IV. Furebring, M., Håkansson, L., Venge, P., Sjölin, J. C5a receptor expression and impaired chemotaxis in granulocytes from septic patients and from healthy subjects after exposure to C5a and interleukin-8 ex vivo. (manuscript)

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### Abbreviations

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<tr>
<td>APACHE II</td>
<td>Acute Physiology and Chronic Health Evaluation II</td>
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<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
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<td>CARS</td>
<td>compensatory anti-inflammatory response syndrome</td>
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<tr>
<td>CLP</td>
<td>cecal ligation and puncture</td>
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<tr>
<td>C5aR</td>
<td>C5a receptor</td>
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<tr>
<td>CR1</td>
<td>complement receptor 1</td>
</tr>
<tr>
<td>CR3</td>
<td>complement receptor 3</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>FiO₂</td>
<td>fraction of oxygen in inspired air</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-methionin-leucin-phenylalanin</td>
</tr>
<tr>
<td>HDC</td>
<td>high-dose corticosteroids</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>ICU</td>
<td>intensive care unit</td>
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<tr>
<td>IL-8</td>
<td>interleukin-8</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAB</td>
<td>monoclonal antibody</td>
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<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MBL</td>
<td>mannan-binding lectin</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>arterial partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PaO₂</td>
<td>arterial partial pressure of oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear granulocytes</td>
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<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>TCC</td>
<td>terminal complement complex</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
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</table>
Introduction and background

Severe sepsis and septic shock represent leading causes of mortality in patients in the intensive care unit (ICU). Despite extensive attempts to find new therapies, this high mortality rate has practically gone unchanged during the past 20 years. The septic disease is characterised by a widespread activation of host innate immune response initiated by gram-positive or gram-negative bacteria or more seldom, fungi and in rare cases viruses, parasites and rickettsia [1]. This systemic inflammatory response syndrome (SIRS) can also be caused by other conditions, including burns, trauma, pancreatitis or major surgery. The term sepsis is used when SIRS follows from an infection. SIRS is defined as two or more of the following: increased or decreased temperature, increased or decreased leukocyte count, tachycardia and increased respiratory rate. If the sepsis is accompanied by signs of hypoperfusion, organ dysfunction or hypotension, it is called severe sepsis and if the hypotension persists despite adequate fluid resuscitation, along with any symptom of hypoperfusion or organ dysfunction, septic shock has developed [2].

Pathophysiology of severe sepsis and septic shock

Surface molecules of microorganisms such as endotoxin (lipopolysaccharide, LPS), lipoproteins, peptidoglycan, lipoteichoic acid, as well as exotoxins from gram-positive bacteria are recognised as internal danger signals by the immune system [3]. Of these, endotoxin has been most investigated and it has often been used in animal models for the study of the septic inflammatory response. Exposure to these substances leads to an activation of macrophages and monocytes and initiation of several cascade pathways: the complement, coagulation, fibrinolytic and kallikrein-kinin systems (Fig 1). Activated macrophages release cytokines, initially tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) above all, leading to activation of neutrophil granulocytes, T-lymphocytes and endothelial cells. As a result of this process, activated granulocytes adhere to the endothelium that is followed by increased endothelial permeability and capillary leakage, which are characteristic phenomena in patients with severe sepsis and septic shock [4]. Furthermore, the activated coagulation system and the activated endothelium
with its increased expression of tissue factor, together with the activated platelets, form microthrombosis. In advanced cases, the coagulation activation may lead to bleeding because of the consumption of coagulation factors and platelets [5].

Already during the early phases of immune system activation anti-inflammatory substances are released to modulate the inflammatory response [6]. The IL-1-receptor antagonist, soluble TNF-receptors and prostaglandin E2 are examples of this activity [7]. Furthermore, type 2 T-helper cell secretes cytokines with anti-inflammatory effects (e.g., IL-10 and IL-4) [8] and

*Figure 1.* Intravascular activation of host response mechanisms in septic shock.
increased levels of corticosteroids and catecholamines result in anti-inflammatory activities [7].

Stimulation of the inducible form of nitric oxide (NO) synthase by inflammatory mediators leads to increased release of NO from endothelial cells, vascular smooth muscle cells and macrophages [1]. NO is considered to be a major mediator of vasodilation and hypotension in septic shock [9]. The vasodilation, in conjunction with tissue oedema and microthrombosis, results in hypoperfusion with a reduced oxygen uptake in tissues and organs, which is manifested by raised blood lactate concentration. Oxygen extraction is further impaired by hypovolaemia that is caused by a combination of capillary leakage, vasodilation and dehydration [10].

The initial haemodynamic alterations in septic patients are characterised by tachycardia, high cardiac output and low vascular resistance, resulting in hyperdynamic circulation. Despite the high cardiac output, there is often a myocardial dysfunction that is caused by circulatory myocardial depressants, such as NO, TNF-α and IL-1. If the hypovolaemia is not corrected, a reduced venous return, cardiac output and lowered blood pressure will cause further tissue hypoxia and the eventual development of hypodynamic septic shock [1]. Furthermore, if the inflammatory reaction leads to acute respiratory distress syndrome (ARDS), oxygen delivery to the organs will be additionally impaired [1].

The decreased oxygen delivery and uptake cause an anaerobic metabolism and metabolic acidosis which, together with the inflammatory process, may result in multiple organ dysfunction.

The complement system

The complement system represents an activation cascade consisting of soluble and membrane-bound proteins (Fig 2). It is under strict homologous control by positive and negative regulators. The cascade can be activated via three pathways: (1) the classical antibody-dependent pathway, which starts with the binding of complement factor C1 to an antigen-antibody complex, (2) the lectin pathway, which is triggered by the binding of mannan-binding lectin (MBL) or ficolin to microorganism carbohydrate structures, resulting in activation of serine proteases (MASPs) and (3) the alternative pathway that is initiated by low doses of C3b formed by inflammatory proteases, or by C3b formed during the complement activation process. Consequently, this latter activation pathway is also called the complement feedback loop [11] (Fig 2).
The complement system is an important component of the innate immunity in the defence against invading microorganisms [12]. This system has several functions, including: 1) clearance of immune complexes from the blood; 2) opsonisation of pathogens by targeting them for phagocytosis by granulocytes, macrophages and NK cells; 3) direct lysis of invading pathogens through formation of the membrane attack complex (MAC; terminal complement complex, TCC; C5b-C9); 4) generation of the pro-inflammatory peptides C5a and C3a; 5) activation of immune cells via cellular complement receptors and 6) cell adhesion via complement receptor type 3 (CR3) [11].
C5a which is produced during complement activation (Fig 2), is a strong chemoattractant for all myeloid cells, including granulocytes, monocytes and macrophages, as well as for mast cells [11]. C5a activates neutrophil granulocytes by induction of the oxidative burst and enhances phagocytosis and release of neutrophil granula enzymes [11, 13]. C5a has also been demonstrated to be a vasodilator [14]. Moreover, it has been shown that C5a is involved in modulation of cytokine expression [15-17] and in activation of the coagulation system and the generation of tissue factor [18-20]. Finally, C5a has been presented to reduce neutrophil apoptosis but increase thymocyte apoptosis [21, 22] and to induce up-regulation of neutrophil adhesion molecules (CD11b/CD18) [23]. In addition, C5a has a direct effect on the endothelial cells, an activity that results in expression of adhesion molecules (P-selectin) and release of the clotting protein von Willebrand factor [24].

The C5a receptor (C5aR; CD88) belongs to the rhodopsin family of G-protein-coupled receptors with seven transmembrane segments [25]. After binding of C5a, the C5a-C5aR complex is rapidly phosphorylated and internalised and thus cleared from plasma within minutes [26, 27]. The neutrophil C5aR is in an in vitro model recycled to about 75% in 90 min [28].

Anti-inflammatory therapies

The first attempts to interfere with the inflammatory response were done in the 1970s, at that time with non-selective drugs in the form of high-dose corticosteroids (HDC) [29]. Although positive results have been reported in experimental models, treatment with HDC significantly reduced mortality only in a well-defined group of patients with typhoid fever [30]. Corticosteroids in these very high doses were demonstrated to increase the risk of secondary infections and consequently corticosteroids were abandoned in the treatment of septic patients [31]. Later, lower doses of corticosteroids have been presented to have several beneficial effects in sepsis, where they have also been shown to reduce the mortality in septic shock [32].

Non-steroidal anti-inflammatory drugs have also been used in trials, as well as several attempts to target LPS; however, both strategies have not been found to improve survival [33-36]. A number of substances blocking one factor in the inflammatory cascade have also failed to reduce mortality. Anti-TNF antibodies, soluble TNF receptors, IL-1-receptor antagonist and an NO inhibitor represent examples of this [37].

Furthermore, polyvalent intravenous immunoglobulin, interferon-γ and granulocyte colony stimulating factor have all been shown to correct a num-
ber of immune function variables without any impact on the outcome [37]. Moreover, different approaches to replace inhibitors of the coagulation cascade have been used in trials, but only recombinant human activated protein C has been demonstrated to reduce the high mortality rate in patients with severe sepsis [37, 38].

At present, there are a considerable number of strategies to interfere with the immune response in experimental phases. One such target is complement factor C5a [18]. During the past decades, different strategies to neutralise C5a have been studied in experimental models. Blockade of C5a by different IgG antibodies to primates and rats has been demonstrated to improve outcome in sepsis [39-42]. C5a receptor antagonists [43, 44] and antibodies to the C5a receptor [16, 45] have had favourable effects in septic rats and mice, respectively. Moreover, soluble complement receptor type-1 has been shown to protect rats from lethal septic shock following LPS injection [46]. Except for reducing mortality, anti-C5a treatment has, been presented to retain the *in vitro* chemotactic response to C5a and fMLP [39, 47] and to reverse the acquired defect in hydrogen (H₂O₂) production of C5a-exposed neutrophils [17]. Moreover, the production of such inflammatory cytokines as IL-6 and TNF-α has been shown to be reduced [16, 17] and coagulation/fibrinolytic protein changes to be ameliorated by anti-C5a treatment [20].

Obtaining the true state of affairs in clinical trials that have been designed to study the effect of different immunomodulating substances for the treatment of severe sepsis or septic shock has long been considered extremely difficult. More than 70 well-designed randomised clinical trials have been performed. Despite dramatic effects in animal models, only recombinant activated protein C and the low-dose corticosteroids have been found to reduce mortality in patients with septic shock [37, 48]. It was speculated some years ago that it might even be impossible to perform such studies with a reasonable magnitude of the statistical β-error, that describes the risk of failing to find an effect that in fact does exist [49]. Besides a true lack of effect, other reasons have been discussed, such as inappropriate dose, duration and timing of therapy [37], as well as importance of the port of entry of the infection [50]. Moreover, the target population is very heterogeneous and patients with too mild forms of sepsis may have been included [50]. Furthermore, in the post-hoc analysis several of the included patients were found not to be in the intended target group, for example in the IL-1 receptor antagonist trial in which the most common organisms isolated were *Candida spp*, coagulase-negative staphylococci and enterococci, pathogens associated with immunoparalysis and not with the hyper-inflammatory phase [50, 51]. Another reason why agents have failed to demonstrate clinical efficacy, despite promising preclinical evidence, is that many of the preclinical trials were con-
ducted on models of sepsis that did not adequately reflect clinical realities [52].

In summary, the largest problem has probably been to select the optimal patient population for a beneficial effect of the treatment under evaluation. It cannot be excluded that in the previous studies such an effect in one sub-population may have been abolished by the absence of an effect or an adverse effect in another [10, 37, 48].
Aims of the study

Different anti-C5a therapies in several models have thus been successful in improving outcome in experimental septic shock. In order to possibly find markers for an optimal selection of patients into clinical anti-C5a trials, it was of importance to study the C5a receptor (C5aR) expression and its regulation and relation to neutrophil function.

The specific aims of the present investigation were:

- To study the expression of the C5a receptor on granulocytes and monocytes in patients with severe sepsis and septic shock.

- To analyse the effect on C5aR expression in whole blood and on isolated cells of an important bacterial toxin by the exposure of granulocytes and monocytes from healthy donors to lipopolysaccharide (LPS) ex vivo.

- To investigate the effect on C5aR expression in whole blood and on isolated cells of complement factor C5a, tumor necrosis factor (TNF-α) and interleukin-8 (IL-8) by the exposure of granulocytes and monocytes from healthy donors to these activators ex vivo.

- To study whether the C5aR expression was correlated to the granulocyte function as measured by the chemotactic response to C5a and IL-8 in patients with severe sepsis or septic shock and in granulocytes from healthy donors after incubation of whole blood with C5a and IL-8 ex vivo.

- To examine the expressions of complement receptors 1 and 3 on granulocytes and monocytes in patients with severe sepsis and septic shock and after exposure of whole blood and isolated cells from healthy donors to LPS, C5a, IL-8 and TNF-α ex vivo and to relate these changes to that of the C5aR.
Material and methods

Blood donors in the *ex vivo* experiments and healthy controls to the patients with sepsis (I, II, III, IV)

Study I: Twenty healthy individuals with a median age of 46 years (range: 22-60) served as controls.

Study II: Nine healthy persons with a median age of 50 years (range: 38-54) participated in this study.

Study III: Twenty-three healthy individuals with a median age of 49 years (range: 23-61) served in study III.

Study IV: Eight healthy individuals with a median age of 52 years (range: 40-61) took part in the *ex vivo* experiments and at each patient analysis, leukocytes from healthy individuals with a median age of 44 years (range, 21–60) served as controls.

Venous blood was collected in heparinised tubes (Venoject, Terumo Corporation, Belgium) and processed within 2 h.

Patients in the clinical studies (I, IV)

Patients who fulfilled clinical criteria supporting a presumptive diagnosis of severe sepsis or septic shock were prospectively recruited. The criteria for severe sepsis and septic shock were a modification of those defined by Bone et al [2].

The patients had to fulfil all of the following: (1) the acute disease was thought to be caused by an infection (positive blood culture not required); (2) presence of a SIRS defined as ≥ 2 of the following criteria: (a) temperature of ≤ 35.6°C or ≥ 38.3°C, (b) heart rate of ≥ 90 beats in the absence of a pacemaker, (c) respiratory rate of ≥ 20 breaths per minute or PaCO2 of ≤ 4.3 kPa (32 mm Hg) and (d) white blood cell count of ≥ 12 x 10⁹/l, ≤ 4 x 10⁹/l,
or > 10% immature band forms; (3) presence of one of the following parameters indicating organ dysfunction: (a) acute alteration of mental status defined as Glasgow Coma Scale of < 15, not confounded by sedative, hypnotic or other agents with central nervous system depressive effects, (b) metabolic acidosis with pH < 7.30 or a base deficit of ≥ -5 mEq/l, (c) hypoxia, in the absence of a pneumonia, defined as PaO₂ of < 9.3 kPa (70 mm Hg) on air ventilation, acute reduction of PaO₂ of > 2 kPa (15 mm Hg) on air ventilation, PaO₂/FiO₂ of < 37.3 kPa (280 mm Hg) or hypoxia requiring mechanical ventilation, (d) coagulation abnormalities defined as a platelet count of < 100 x 10⁹/l or < 50% of a value measured within the previous 24 h, increase in INR or partial thromboplastin time of > 50% above the normal value or a D-dimer concentration of > 0.5 mg/l, (e) oliguria with urine output of < 30 ml/hour or < 0.5 ml/kg/h for ≥ 1 h and (f) hypotension defined as persistent systolic blood pressure of ≤ 90 mm Hg or reduction of > 40 mm Hg from a value measured within the previous 24 h despite adequate fluid resuscitation; (4) an informed consent or a presumed consent if the patient was not capable of making decisions because of altered mental health or sedation.

Exclusion criteria were age < 18 years, rapidly progressing underlying disease, HIV/AIDS, cardiogenic shock as the primary underlying disease, haematological underlying disease or cytotoxic therapy given within the previous week expecting neutropenia.

The studies were performed with permission from the Ethics Committee, Faculty of Medicine, Uppsala University.

The APACHE II score was calculated according to Knaus et al [53]. Venous blood was collected in heparinised tubes (Venoject, Terumo Corporation, Belgium) and processed within 2 h.

In study I, this resulted in 12 patients admitted to the ICU. The patients median age was 58 years (range 21 - 85). Three patients were admitted to the ICU with a severe infection as the principal cause. Six patients had probable post-operative infections, one patient post-burn infection, one patient pancreatic abscess and one patient developed pneumonia after having been admitted to the ICU because of severe hypercalcemia and malnutrition. Infection was verified in all but one patient. Although an infection could not be excluded in this patient, a retroperitoneal haematoma might have explained the post-operative deterioration at the time of enrolment, but as this patient fulfilled the prospectively designed inclusion criteria for severe sepsis and would have been recruited in a clinical trial using these criteria, this patient was included in further analyses.
Median APACHE II score at onset of severe sepsis was 17 (range 12–28). ICU, 28-day and hospital mortality were 8%, 17% and 33%, respectively.

In eight patients, the first blood samples were obtained within 24 h after having fulfilled the criteria for severe sepsis and in another four patients within 36 h.

In study IV, 17 patients (all were admitted to the ICU) with a median age of 63 years (range 41 – 86) fulfilled the criteria and were therefore entered into the study. Nine patients were admitted to the ICU with a severe infection as the principal cause. Four patients had probable post-operative infections, two had pancreatic abscess and four had gastrointestinal perforation. Infection was verified in 11 patients. In five patients, no cultures were taken before treatment with broad-spectrum antibiotics. Of these five patients, three had a perforation of the gastrointestinal tract and one had pneumonia; in the fifth patient, the systemic inflammatory response was most probably due to pancreatitis and the presence of an additional infection could not be verified. However, as the patient fulfilled the prospectively defined inclusion criteria for severe sepsis and would have been enrolled in a clinical trial using these criteria, this patient was included in further analyses. The infectious cause of the suspected septic shock could not be confirmed in one patient by culture findings, but there were clinical signs of septic shock with hypotension, tachycardia, acidosis and leukocytosis without other explanations to account for the deterioration.

The APACHE II score at inclusion was 26 (range, 17-38). The 28- and 90-day mortality rates were 35% and 53%, respectively.

Stimulation of whole blood from healthy donors (II, III, IV)

In study II, blood was incubated in tubes with LPS from Escherichia coli O26:B6 (Sigma, St. Louis, MO, USA) 5 EU/ng (Limulus lysate assay) at final concentrations of 0.10, 1.0 and 10 ng/ml for 45 min at room temperature. The tubes were pre-treated with human serum albumin (HSA) (0.2% w/v) in order to avoid adhering of the leucocytes. Blood incubated with dilution buffer consisting of phosphate-buffered saline (PBS) with sodium citrate (0.013 mol/l) and HSA (0.1% w/v) (PBS/citrate/HSA) was served as control.
In the studies III and IV, human recombinant C5a (Sigma, St. Louis, MO, USA) at the final concentrations of $1 \times 10^{-9}$, $1 \times 10^{-8}$ and $1 \times 10^{-7}$ mol/l, IL-8 (R&D Systems Europe Ltd, Abingdon, UK) at 1.0, 10 and 100 ng/ml and TNF-α (R&D Systems Europe Ltd, Abingdon, UK) (only study III) at 1.0, 10 and 100 ng/ml were used. These concentration levels were selected on the basis of previous experience [23, 54, 55].

**Isolation of cells from whole blood and stimulation of isolated leukocytes (II, III)**

The leukocytes were isolated by means of dextran sedimentation [56]. Briefly, 5 ml blood was smoothly mixed with 5 ml 2% dextran (Pharmacia, Uppsala, Sweden) and then kept at room temperature for 30 min to allow sedimentation of the erythrocytes. The leukocyte-rich plasma was collected and centrifuged for 5 min at 160 x g. The cell pellet was suspended in 5 ml Gey’s buffer [57] and centrifuged at 160 g for 5 min. The remaining erythrocytes were removed by hypotonic lysis and the cell suspension was washed once again before the cells were diluted to a concentration of $3 \times 10^9$/l with Gey’s buffer. Finally, the cells were incubated with C5a, IL-8 and TNF-α at the same concentrations as whole blood, or in the case of LPS, at the concentrations of 10, 100 or 1000 ng/ml or with PBS/HSA as control for 45 min.

**Preparation of leukocytes for receptor analyses from whole blood after stimulation (II, III, IV) and from blood from septic patients (I, IV)**

Leucocytes were prepared according to a method described by Hamblin et al [58]. Briefly, 1 ml blood was mixed with 1 ml 0.4% (w/v) paraformaldehyde in PBS. The mixture was then incubated for 4 min at 37°C. Next, 40 ml 0.83% (w/v) NH₄Cl in 0.01 mol/l Tris-HCl-buffer [Tris(hydroxymethyl)-aminomethane 0.01 mol/l, pH 7.4] was added and the mixture was incubated for another 18 min at 37°C. The cells were centrifuged for 5 min at 350 x g at room temperature after which the supernatant and the red blood cell debris were removed. The remaining leucocytes were washed twice with PBS/citrate/HSA. Finally, the cells were diluted to a concentration of $3 \times 10^9$ neutrophils/l with PBS/citrate/HSA.
Labelling of leukocytes with antibodies to cell surface antigens (I, II, III, IV)

To each tube, 100 μl cell suspension, optimally titrated fluorescein isothiocyanate (FITC)-labelled anti-CD35, anti-CD11b and anti-CD18 (DAKO A/S, Glostrup, Denmark), or anti-CD88 (Serotec, Raleigh, NC, USA), or FITC-labelled isotype control antibodies (DAKO A/S) and phycoerythrin-labelled anti-CD14 (DAKO A/S) were added. The samples were then incubated on ice for 30 min. The cells were subsequently washed twice with ice-cold PBS/citrate. The anti-CD88 labelled cells were further incubated with FITC-conjugated goat anti-mouse antibodies for another 30 min and were washed twice with ice-cold PBS/citrate. Afterwards, the leukocytes were diluted with PBS/citrate/HSA and kept on ice.

Flow cytometry (I, II, III, IV)

The samples were analysed by the use of the EPICS-PROFILE II or the EPICS XL-MCL flow cytometers (Coulter Company Inc., Hialeah, FL, USA). Daily maintenance controls were performed according to the manufacturer’s recommendation. The samples were analysed using a fixed protocol with the same settings maintained for forward and side scatter throughout the study. The neutrophils and monocytes were identified on the basis of their forward and side scatter patterns; staining with anti-CD14 was subsequently used as an additional identification of the monocytes. Gates were set around the granulocyte and monocyte populations and the FITC fluorescence within the gates was measured. A minimum of 2000 events in the monocyte gate was counted. The cell surface expression of CD11b, CD18, CD35 and CD88 were measured as specific mean fluorescence intensity (MFI) of the whole populations of granulocytes and monocytes. The specific MFI of the granulocyte and monocyte expression of each receptor was calculated by subtracting the background MFI obtained with the negative isotype control monoclonal antibody from the value obtained with each of the anti-receptor monoclonal antibodies.

The maximum inter-assay variation was 9.7% for granulocyte receptors and 7.2% for monocyte receptors.
Measurement of granulocyte migration (IV)

Granulocyte (PMN) migration was measured by means of the leading front technique, using a modified Boyden chamber [59] with a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD, USA). A nitrocellulose filter (Millipore Corporation, Bedford, MA, USA) with 3 µm pore size was placed between the upper and lower compartments. The routines used to study chemokinesis (migration in the presence of albumin) and chemotaxis have been described previously [60, 61]. Briefly, the chemokinesis and the chemotactic response were assayed using PMN 1.5x10^9/l in Gey’s buffer with HSA (2g/l) above the filter. Below the filter, Gey’s buffer was located for the determination of chemokinesis, and for the analysis of chemotaxis C5a (1x10^-9mol/l and 1x10^-8mol/l) in Gey’s buffer or IL-8 (1x10^-10 and 1x10^-9 mol/l) in Gey’s buffer with HSA (1 g/l) was added. All tests were performed in duplicates. The incubation time was 60 min and the temperature 37°C. The results were expressed as µm/h. The chemotactic response to C5a or IL-8 was expressed as the difference between chemotaxis toward C5a or IL-8, respectively, and the migration toward buffer (chemokinesis). The maximum inter-assay variation was 7%.

Analyses of complement components (I)

The blood for complement analyses was collected in EDTA tubes (Becton Dickinson, Plymouth, UK) and kept on ice until centrifugation. The plasma was collected and stored at -70°C.

The concentration of C3a was analysed by an enzyme immunoassay (EIA), which is a sandwich EIA that employs the monoclonal antibody 4SD17.3 as the capture antibody. EDTA plasma was diluted 1/500 and analysed as described previously [62]. Bound C3a was detected with biotinylated rabbit anti-C3a followed by horseradish peroxidase (HRP)-conjugated streptavidin (Amersham, UK). Zymosan-activated serum [63], calibrated against a solution of purified C3a [64], served as standard. The values are given in ng/ml.

Soluble C5b-9 was analysed by a modified EIA, as described by Mollnes et al [65]. Plasma samples, diluted 1/5, were added to microtitre plates coated with anti-neoC9 mAb MCaE11. Soluble C5b-9 was detected by polyclonal anti-C5 antibodies diluted 1/500, followed by HRP-conjugated anti-rabbit immunoglobulin diluted 1/500 (both from Dako A/S, Denmark). Zymosan-activated serum, defined as containing 40 000 arbitrary units (AU) per ml,
served as the standard. Maximum inter-assay variation of the C3a and the C5b-9 analyses was 10%.

Leukocyte count (I)

Leukocyte counts were made on Coulter STKS (Beckman Coulter Inc, FL, US).

Statistics

Cells from healthy individuals (II, III, IV)

Receptor expression and chemotaxis in the healthy individuals approximated to normal distribution. Therefore, a repeated measures ANOVA was performed to test the relationships between concentration of the stimuli and receptor expression as well as chemotaxis. Incubation concentration was set as the repeated increasing variable and receptor expression and chemotaxis as the dependent variables. Pearson’s correlation coefficient was computed for correlation analyses.

Cells from the patients (I, IV)

In the patient group, deviation in chemotaxis and receptor expression was greater and approximation to normal distribution was more difficult to ascertain. Therefore, statistical analysis was performed in a nonparametric manner using the Mann-Whitney U-test for comparison of data between patients and controls and the Spearman rank test for correlation analyses. One healthy control was used at each analysis.

For comparison of individual data within the patient group in paper I the Wilcoxon matched pairs test was employed.

I, II, III, IV

Unless otherwise stated, values are expressed as mean±SE in the healthy controls and median and range in the patients. A difference was considered significant when the p-value was <0.05. The software STATISTICA (StatSoft Inc.Tulsa, OK, USA) was used in the statistical analyses.
Results and discussion

Expression of the C5a receptor (CD88) in patients with severe sepsis and septic shock (I)

The granulocyte median CD88 expression in the septic patients on day 1 was significantly lower as compared with the granulocytes in the healthy control group (36 vs. 63, p<0.001) (Fig 3). In comparison with the value obtained from the healthy individuals analysed on the same occasion, CD88 expression was lower in all patients except in the patient without a verified infection.

![Figure 3. Granulocyte and monocyte expression of CD88 in patients with severe sepsis on days 1, 3 and 15. Values are expressed as median and 25th and 75th percentiles.](image)

On day 3, there was some increase in granulocytes (from 36 to 37) and after 2 weeks, the increase was significant in comparison with day 1 (36 vs. 51, p<0.05). However, the granulocyte CD88 expression was still significantly reduced on both day 3 and day 15 in comparison with that of the healthy controls (p<0.05). The recovery followed an individual course, as the granulocyte CD88 values on day 15 were highly correlated with those on day 1.
(r=0.80, p<0.01). On day 1, the median granulocyte CD88 expression in patients who remained at the ICU on day 15 was 35 (range 2.5-59), which can be compared with 44 (range 26-57) in those who were discharged from the ICU on day 15 (p<0.05).

Monocyte CD88 expression was considerably lower than granulocyte CD88 expression, both in the healthy controls (p<0.01) and in the patients (p<0.05). The monocyte CD88 expression went completely unchanged over the time of observation and there was no difference between the patients and the controls (Fig 3).

No correlation was noted between granulocyte count and CD88 expression on day 1 (r=0.22) or day 3 (r=0.03), indicating that the reduction in C5a receptor expression during acute infection cannot be explained by a variation in the number of leukocytes.

The granulocyte expression of CD88 on day 1 correlated negatively to the severity of disease as measured by the APACHE-II score at inclusion (r=-0.59, p<0.05) (Fig 4).

*Figure 4. Correlation between the granulocyte CD88 expression and the APACHE II score on day 1. Upward directed arrows indicate an increase from onset of the severe sepsis in APACHE II score of > 2 and downward directed arrows a decrease of > 2. Double-directed arrows represent changes < 2. ○ Represents a patient with a duration of sepsis of 12 h, ◰ a duration of 13 – 24 h and ◻ a duration of > 24 h. Patients who died within 28 days are marked with filled symbols. A regression line has been calculated using the method of least squares.*
The median C5b-9 concentration was 147 AU/ml (range 64-562), which means it was increased in all patients (normal range 12-56 AU/ml) and the C3a concentration was increased in all but one patient on day 1 (median 1106 ng/ml, range 259-1529) as compared with the normal range (92-268 ng/ml). There were no correlation between C5b-9 or C3a levels and the CD88 expressions; nor were there any correlations to the granulocytes or to the monocytes.

Despite increased levels of C3a and C5b-C9, which indicates ongoing complement activation in agreement with the animal experiments and a previous clinical study [66-68], the C5aR expression on the granulocytes was markedly reduced in contrast to the findings in the animal experiments. A transient increase in C5a binding to granulocytes in humans has been demonstrated at 3 h after low-dose endotoxin administration [67]. After that, there was a successive decrease and after 24 h the mean value was below that at baseline. Recently, it has been shown that C5aR on rat neutrophil granulocytes decreased sharply during the first 24 h and recovered only partially after 48 h in experimental sepsis [45]. It has also been shown that prolonged down-regulation of C5aR of neutrophils is caused by receptor degradation [69].

In contrast to the changes in the granulocyte C5aR expression, there were no changes in the monocyte expression. The reason for this discrepancy is not known but may be due to a differential effect on granulocytes and monocytes of pro- or anti-inflammatory substances, differences in the affinity to C5a or bone marrow effect in combination with varying half-lives.

In summary, a reduction in granulocyte C5a receptor expression was demonstrated in patients at the time when the diagnosis of severe sepsis or septic shock can be clinically established.

Expression of complement receptors on granulocytes and monocytes after incubation with LPS, C5a, IL-8 and TNF-α (II, III)

Granulocyte expression of CD88 after LPS incubation of whole blood showed a significant reduction, mainly at the highest LPS concentration (p<0.05) (Fig 5a). C5a significantly reduced granulocyte expression of CD88 after stimulation in whole blood and of isolated cells (p<0.001 for both) (Figs 5a and 5b). The decrease in granulocyte CD88 expression on isolated cells was more pronounced than in whole blood at the higher concentrations.
(p<0.05). Incubation of whole blood, both with IL-8 and TNF-α, decreased granulocyte CD88 expression (p<0.001 for both) and similar results were noted on isolated cells (IL-8, p<0.05 and TNF-α, p<0.001).

Figure 5a and 5b. Granulocyte CD88 expression after stimulation of whole blood (a) and isolated cells (b), with increasing concentrations of LPS, C5a, IL-8 and TNF-α. Values are presented as the ratio to that of the control and expressed as mean±SE.

Monocyte CD88 expression is presented in Table 1. Monocyte CD88 expression was also significantly reduced by C5a in whole blood (p<0.001) and on isolated cells (p<0.05), whereas LPS and IL-8 did not have any effect on the monocytes. TNF-α slightly increased the CD88 expression on monocytes in whole blood, whereas no effect was seen on isolated monocytes.
Table 1. Monocyte expression of C5a receptor (CD88) after incubation of whole blood with LPS, C5a, IL-8 and TNF-α expressed as percent of the control (mean±SE).

<table>
<thead>
<tr>
<th>Incubation concentration</th>
<th>LPS</th>
<th>C5a</th>
<th>IL-8</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>103±6.3</td>
<td>97±4.2</td>
<td>95±1.7</td>
<td>112±16.9</td>
</tr>
<tr>
<td>Medium</td>
<td>102±6.3</td>
<td>91±5.2</td>
<td>93±2.2</td>
<td>102±3.8</td>
</tr>
<tr>
<td>High</td>
<td>99±6.5</td>
<td>90±3.6</td>
<td>79±1.6</td>
<td>96±1.2</td>
</tr>
<tr>
<td>Isolated monocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>107±0.7</td>
<td>102±2.4</td>
<td>100±2.2</td>
<td>107±0.7</td>
</tr>
<tr>
<td>Medium</td>
<td>111±1.4</td>
<td>96±4.0</td>
<td>111±1.4</td>
<td>92±3.6</td>
</tr>
<tr>
<td>High</td>
<td>109±1.4</td>
<td>101±3.6</td>
<td>109±1.4</td>
<td>94±5.1</td>
</tr>
<tr>
<td>p-value</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
The results of stimulation with C5a, IL-8 and TNF-α on the granulocyte expression of CD35 are depicted in Figs 6a and 6b. The granulocyte expression of CD35 was significantly increased by C5a and TNF-α incubation of whole blood as well as of isolated cells (p<0.001), but the magnitude of the effect on whole blood was substantially higher than on isolated granulocytes (p<0.05). Furthermore, LPS and IL-8 incubation of whole blood increased granulocyte CD35 expression (p<0.001), whereas no such effect was noted on isolated cells.

Figure 6a and 6b. Granulocyte CD35 expression after stimulation of whole blood (a) and isolated cells (b), with increasing concentrations of LPS, C5a, IL-8 and TNF-α. Values are presented as the ratio to that of the control and expressed as mean±SE.
Table 2. Granulocyte expression of CD11 after incubation of whole blood with LPS, C5a, IL-8 and TNF-α expressed as percent of the control (mean±SE).

<table>
<thead>
<tr>
<th>Incubation concentration</th>
<th>LPS</th>
<th>C5a</th>
<th>IL-8</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole blood</td>
<td>Isolated granulocytes</td>
<td>Whole blood</td>
<td>Isolated granulocytes</td>
</tr>
<tr>
<td>Low</td>
<td>112±13</td>
<td>101±4.2</td>
<td>159±21</td>
<td>112±5</td>
</tr>
<tr>
<td>Medium</td>
<td>116±17</td>
<td>101±6.4</td>
<td>292±41</td>
<td>128±6</td>
</tr>
<tr>
<td>High</td>
<td>135±18</td>
<td>103±4.2</td>
<td>383±77</td>
<td>141±12</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
Table 3. Granulocyte expression of CD18 after incubation of whole blood with LPS, C5a, IL-8 and TNF-α expressed as percent of the control (mean±SE).

<table>
<thead>
<tr>
<th>Incubation concentration</th>
<th>LPS</th>
<th>C5a</th>
<th>IL-8</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole blood</td>
<td>Isolated granulocytes</td>
<td>Whole blood</td>
<td>Isolated granulocytes</td>
</tr>
<tr>
<td>Low</td>
<td>98±2.6</td>
<td>104±5.3</td>
<td>112±5</td>
<td>111±6</td>
</tr>
<tr>
<td>Medium</td>
<td>99±3.9</td>
<td>99±1.6</td>
<td>132±6</td>
<td>138±5</td>
</tr>
<tr>
<td>High</td>
<td>106±4.5</td>
<td>97±6.0</td>
<td>135±8</td>
<td>137±8</td>
</tr>
<tr>
<td>p-value</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
The effects on the granulocyte CR3 are summarised in Tables 2 and 3. CD11b was significantly increased, being most pronounced at the highest concentration of LPS on whole blood (p<0.05), whereas C5a, by increasing concentrations, gradually increased CD11b expression in whole blood and on isolated cells as well (p<0.001). IL-8 and TNF-α stimulation resulted in a similar response. The effect on the CD18 receptor followed that on the CD11b receptor with a response that was of similar magnitude or less, except for LPS which did not affect granulocyte CD18 expression.

Correlations between the individual changes of granulocyte receptor expression in response to C5a in whole blood at different concentration levels are shown in Table 4. A significant correlation was noted between CD11b and CD35 at the two lower concentrations. At the highest concentration, CD11b was further increased, whereas CD35 was not. Concerning CD11b and CD88, a significant negative correlation was demonstrated at the highest concentration (p<0.001).

Table 4. Correlation (r-values) between individual changes in granulocyte receptors after C5a stimulation.

<table>
<thead>
<tr>
<th>Concentration (mol/l)</th>
<th>CD35</th>
<th>CD11b</th>
<th>CD18</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD88</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10⁹</td>
<td>-0.26</td>
<td>-0.35</td>
<td>-0.27</td>
</tr>
<tr>
<td>1x10⁻⁸</td>
<td>-0.15</td>
<td>-0.46</td>
<td>-0.05</td>
</tr>
<tr>
<td>1x10⁻⁷</td>
<td>-0.55</td>
<td>-0.99**</td>
<td>-0.25</td>
</tr>
<tr>
<td><strong>CD35</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10⁻⁹</td>
<td>0.92*</td>
<td>0.90*</td>
<td></td>
</tr>
<tr>
<td>1x10⁻⁸</td>
<td>0.89*</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>1x10⁻⁷</td>
<td>0.61</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td><strong>CD11b</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10⁻⁹</td>
<td></td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>1x10⁻⁸</td>
<td></td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>1x10⁻⁷</td>
<td></td>
<td>0.26</td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05
** p<0.001
LPS incubation of granulocytes *ex vivo* resulted in reduced granulocyte expression of the C5aR, which in part is discrepant with previous results [16, 67]. In the study by Granowitz et al LPS was administered to healthy individuals and an increase in C5a binding to neutrophil granulocytes was observed at the first measurement 3 h after injection of LPS. Riedemann et al used the cecal ligation and puncture model in mice and reported similar findings in lung, liver and kidney after 3 h. In our experiments, the incubation time was shorter than 3 h. However, the cecal ligation and puncture model are known to have a slower induction of the inflammatory reaction compared with intravenous administration of LPS, suggesting that the increase in C5aR expression should be an early event, if present. Furthermore, in agreement with our results, a recently published study by Guo et al demonstrated a reduction in blood neutrophil C5aR expression in experimental sepsis in the rat [45]. The decrease was gradual and without any biphasic course during the first 24 h. The result obtained in our study is also in concordance with the reduced C5aR expression on neutrophils that was observed in patients with severe sepsis (study I). As indicated in the study by Riedemann et al, C5aR expression on myeloid blood cells may be differently regulated from that in such organs as lung, liver, heart and kidney [16]. In the article by Riedemann et al, this was discussed on the basis of unpublished neutrophil granulocyte expression data that were in accord with our results. It is possible that these results represent a phenomenon similar to that observed during other inflammatory disease processes, i.e. a pro-inflammatory state is present at the organ level while a more anti-inflammatory one is predominant in blood [70]. The clinical significance of this is unknown at present.

It must be pointed out that the results from this study do not exclude a very early up-regulation of the neutrophil C5aR and that our *ex vivo* model does not include, for example, endothelial cells that are known to play an important role in inflammatory reactions.

The reduction in granulocyte C5aR expression induced by C5a in this study was of a magnitude similar to that observed in the patients with sepsis (study I). This finding is consistent with a study showing that after binding of C5a to the C5aR, the C5aR complex is internalised [71]. However, to achieve a reduction of the magnitude seen in severe sepsis in the whole blood model high doses of C5a were required. At these high C5a levels, monocyte C5aR expression was also significantly reduced, which is in contrast to the clinical situation in which C5aR expression on mono-
...cytes is unchanged (study I). This finding indicates that most probably other factors than C5a contribute to the reduction in granulocyte C5aR expression in severe sepsis.

The finding that IL-8 decreased C5aR expression on isolated granulocytes is in good correspondence with recently published data [72]. In the present study, it was in addition demonstrated that TNF-α had a similar effect, and at maximal concentrations of the two, the reductions were of similar magnitude. The IL-8 and TNF-α concentrations used in these experiments were in the magnitude of those observed in severe clinical sepsis or higher [73], but even with these doses, no reduction in monocyte C5aR expression was found. Thus, the effects of these two cytokines resemble the findings in patients and therefore our results suggest that IL-8 and TNF-α may also be operative in the reduction in granulocyte C5aR expression in patients with severe sepsis and septic shock.

In contrast to the C5aR, the expressions of complement receptors 1 (CR1; CD35) and 3 (CR3; CD11b/CD18) were increased. Increase in CR3 expression has previously been shown to occur, both after LPS incubation of isolated granulocytes [74] and in patients with sepsis [75-77]. Changes in CR1 receptor expression have been less investigated, but in two studies an increase in CR1 expression was found in patients with sepsis and especially in those with multiple organ failure [75, 77]. In addition to facilitating phagocytosis, CR1 has been shown to be a negative regulator of the complement system by interfering with the formation of C3 and C5 convertases [11] (Fig 2). The protective effect was also demonstrated in a recently published study in which soluble CR1 inhibited complement activation in an endotoxin model [46]. Granulocyte expression of CR1 was up-regulated after exposition of whole blood to LPS, C5a, TNF-α and IL-8. As would have been expected from the discussion above, there was no negative correlation between CR1 and C5aR expression in our study with LPS. In contrast, the two individuals with the highest increases in CR1 expression demonstrated the lowest reduction in C5aR expression. This observation was supported by the findings in study III in which it was demonstrated that the C5a-induced changes of CD11b and C5aR were significantly correlated only at the highest concentration of C5a when the expression of CR1 had reached a plateau. The fact that the correlation between CD11b and C5aR was not significant at the lower concentrations when CR1 was on the rise could suggest that the decrease in C5aR expression might be reduced by the concomitant CR1 increase. In accordance with this position, the correlation between CD11b and CR1
expressions was only significant at the two lower concentrations. A protective role for CR1 expression could not be demonstrated after stimulation with IL-8 or TNF-α. This finding would support to some extent the hypothesis proposed by Goya et al that neutrophil up-regulation of surface inhibitory factors of complement activation, such as CR1, probably allows the neutrophils to survive and function during intense complement activation [77].

In summary, it was shown in study II that LPS stimulation of whole blood, in similarity with the findings in septic patients, resulted in a reduction in the granulocyte expression of C5aR and increased expressions of CR1 and CR3. No such effect was detected after stimulation of isolated cells, indicating an indirect effect mediated by factors in plasma. The data in study III indicate that this indirect effect may be attributed to several factors, including C5a, IL-8 and TNF-α. In patients with severe sepsis or septic shock, as well as after LPS stimulation, the reduction in granulocyte C5aR expression was not followed by a similar one in monocyte expression. This finding indicates that the observed changes are not only a consequence of complement activation but also of inflammatory cytokines, suggesting that these mediators may act in concert.

C5a receptor expression and impaired chemotaxis in granulocytes from septic patients and from healthy controls after exposure to C5a and interleukin-8 *ex vivo* (IV)

**Patient study**

The granulocyte complement receptor expression in the patients is listed in Table 5. The median CD88 expression was significantly lower in the patients as compared with the healthy controls (p<0.001). In relation to the controls the CD88 expression on patient granulocytes was 43% (range, 21-66%). Correspondingly, the granulocyte CD35, CD11b and CD18 expressions in the patients were 540% (range, 203-1096%; p<0.001), 163% (range, 81-239%; p<0.001) and 109% (range 42-237; NS) of those of the controls, respectively.
Table 5. Granulocyte receptor expression.

<table>
<thead>
<tr>
<th>Receptor expression (MFI)</th>
<th>CD88</th>
<th>CD35</th>
<th>CD11b</th>
<th>CD18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>19</td>
<td>5.5</td>
<td>7.3</td>
<td>4.3</td>
</tr>
<tr>
<td>range</td>
<td>9-33</td>
<td>1.9-10.5</td>
<td>5.1-11.0</td>
<td>1.6-7.2</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>42</td>
<td>0.9</td>
<td>4.3</td>
<td>3.8</td>
</tr>
<tr>
<td>range</td>
<td>32-62</td>
<td>0.6-3.2</td>
<td>3.3-10.1</td>
<td>2.6-5.6</td>
</tr>
</tbody>
</table>

The median migration toward C5a and IL-8 in the patients was 81 μm/h (range 44-119) and 82 μm/h (range 52-115), respectively and in the controls 110 μm/h (range 58-136) and 115 μm/h (range 67-138), respectively (p<0.001 for both chemoattractants). Chemokinesis was significantly lower in the patients than in the healthy controls: 50 μm/h (range 40-83) compared with 75 μm/h (range 49-88) (p<0.001). The median chemotactic response to 1x10^{-8} mol/l of C5a was 20 μm/h (range 1-46) in the patients as compared with 32 μm/h (range 9-51) in healthy controls (p<0.01), while the chemotactic response to 1x10^{-9} mol/l of IL-8 in the patients was 29 μm/h (range 4-42) and 44 μm/h (range 18-54) in the controls (p<0.05) (Fig 7).

![Chemotactic response to C5a and IL-8](image)

*Figure 7. Chemotactic response to 1x10^{-8} mol/l of C5a and 1x 10^{-9} mol/l of IL-8 in patients with severe sepsis and septic shock and in healthy controls expressed as median with lower and upper quartiles.*
A significant correlation between the chemotactic response to C5a and IL-8 ($r=0.70; p<0.01$) was revealed in the control group; however, no significant correlation could be demonstrated in the septic patients ($r=0.18; p=\text{NS}$).

The correlation analysis between granulocyte CD88 expression and the chemotactic response to C5a is displayed in Fig 8. There was a positive trend between granulocyte CD88 expression and the chemotactic response to C5a. However this positive trend did not hold for two of the three patients with the lowest CD88 expression, resulting in an overall only weak positive trend that did not reach the significance level ($r=0.40; p=0.11$).

*Figure 8. Chemotactic response to $1\times10^{-8}$ mol/l of C5a in relation to granulocyte CD88 expression in patients with severe sepsis and septic shock. Individual values are expressed in percent of the simultaneously analysed healthy control.*
Ex vivo experiments

There was a gradual reduction in CD88 expression at increasing C5a concentrations. Furthermore, similar changes were seen at higher IL-8 concentrations (Table 6). Expressions of CD35 and CD11b increased considerably at higher C5a and IL-8 concentrations, whereas the increase in CD18 expression was more limited.

Table 6. Granulocyte expression of complement receptors after in vitro incubation with C5a and IL-8 (mean±SE).

<table>
<thead>
<tr>
<th>Incubation with</th>
<th>Concentration (mol/l)</th>
<th>CD88 (MFI)</th>
<th>CD35 (MFI)</th>
<th>CD11b (MFI)</th>
<th>CD18 (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5a</td>
<td>Control</td>
<td>39.4±7.9</td>
<td>1.8±1.2</td>
<td>6.9±2.6</td>
<td>4.3±1.7</td>
</tr>
<tr>
<td></td>
<td>1x10^-9</td>
<td>34.4±7.4</td>
<td>2.8±1.6</td>
<td>10.6±4.2</td>
<td>4.9±2.2</td>
</tr>
<tr>
<td></td>
<td>1x10^-8</td>
<td>30.2±9.2</td>
<td>5.6±1.9</td>
<td>18.6±4.9</td>
<td>5.8±2.5</td>
</tr>
<tr>
<td></td>
<td>1x10^-7</td>
<td>22.0±5.9</td>
<td>6.4±1.9</td>
<td>24.0±7.7</td>
<td>5.8±2.2</td>
</tr>
<tr>
<td>IL-8</td>
<td>Control</td>
<td>45.3±3.2</td>
<td>1.3±0.6</td>
<td>5.7±1.2</td>
<td>5.4±1.4</td>
</tr>
<tr>
<td></td>
<td>1x10^-10</td>
<td>44.4±4.1</td>
<td>1.6±0.8</td>
<td>6.8±1.8</td>
<td>5.5±1.5</td>
</tr>
<tr>
<td></td>
<td>1x10^-9</td>
<td>35.5±3.2</td>
<td>3.9±1.0</td>
<td>14.6±3.1</td>
<td>6.4±2.2</td>
</tr>
<tr>
<td></td>
<td>1x10^-8</td>
<td>36.8±2.7</td>
<td>5.1±1.0</td>
<td>20.5±3.3</td>
<td>7.1±2.1</td>
</tr>
</tbody>
</table>

The changes in chemokinesis in relation to that of the non-incubated cells after incubation with the low, middle and high C5a concentrations were -6±3%, -6±3% and +1±5%, respectively. None of these changes was statistically reliable. The chemotaxis toward C5a was significantly reduced, with reductions of 24 to 50% of that of the non-incubated cells (p<0.05) (Fig 9a). The reductions were not significantly dependent on the C5a incubation dose. After stimulation of the C5a incubated cells with IL-8, there was a reduction in the response at the two lower incubation concentrations of 21% and 24%, respectively (p<0.01); at the highest C5a dose, however, the response to IL-8 was not decreased at all.

After IL-8 incubation, there were significant reductions in chemokinesis at the low (14±2%), middle (9±5%) and high (8±5%) incubation concentrations (p<0.01). Similarly, the chemotaxis toward C5a demonstrated reductions of 43%, 51% and 24%, respectively (p<0.05), and toward IL-8
of 36%, 39% and 39%, respectively (p<0.01) (Fig 9b). None of the chemotactants demonstrated any significant dose response.

*Figure 9a and 9b.* Reduction in chemotactic response to $1 \times 10^{-8}$ mol/l of C5a and $1 \times 10^{-9}$ mol/l of IL-8 after incubation with different concentrations of C5a and IL-8.
No correlation between CD88 expression and the chemotactic responses to C5a or IL-8 was found at any of the given incubation concentrations or when all concentration levels were analysed collectively.

We found a reduction in the expression of C5aR on granulocytes obtained from septic patients together with a reduction in the chemotactic response to C5a in study IV. These data would support our hypothesis that the increased generation of C5a during sepsis interferes with the C5a receptor density and thereby affects the functional response to C5a. Patient granulocytes with the lowest C5aR expression were expected to demonstrate the most impaired chemotactic response, as well as a positive over all correlation between individual chemotactic responses and C5aR expressions. However, as demonstrated in Fig 7, there was only a trend toward a positive correlation and two of the three patients with the greatest reduction in C5aR expression demonstrated a relatively well preserved chemotactic response. Analysis of clinical data did not reveal that these patients differed from the others on age, type of infection, duration of infection or severe sepsis, APACHE II score, treatment with steroids, vasopressors, inotropic support or any other treatment. However, their inflammatory response was more on the rise than in the other patients, as depicted by the CRP level on the following day when these patients demonstrated the two highest CRP concentration increases of 132 mg/l or more. There was no difference in CRP concentration at the day of inclusion. Thus, even if there may be some correlation between C5aR expression and the chemotactic response suggesting a diminished response to C5a in patients with low C5aR expression, this is not true for all patients, especially not in those with an increasing inflammatory response.

It was also evident in the septic patients that not only the chemotactic response to various chemotaxins was reduced but also the response to chemokinetic factors (such as albumin), which are necessary facilitators of the chemotactic response to C5a and IL-8.

On the granulocytes from the healthy individuals that were incubated with C5a, there was a gradual decrease in C5aR expression at increasing incubation concentrations. The chemotactic response did not congruently follow this decrease and granulocytes incubated with the highest concentration of IL-8 demonstrated an almost normal chemotactic response to C5a despite a substantial reduction in the C5aR expression. Furthermore, at the individual level, there was no significant correlation between C5aR expression and chemotactic response to C5a. Taken together, these data
indicate that after a short exposure to C5a or IL-8, it is not possible to predict the chemotactic response from the granulocyte expression of C5aR. Accordingly, the results from the ex vivo experiments are in concordance with those from the patients with sepsis.

A close relationship between the two chemoattractants was demonstrated in the ex vivo experiments. Incubation with C5a resulted not only in a decreased response to C5a but also to IL-8, and vice versa. The C5aR and the IL-8 receptors are both members of the G-protein-coupled superfamily of rhodopsin-like receptors [78, 79]. After binding of C5a or IL-8, the ligand-receptor complexes are rapidly internalised [71, 80], a process leading to a rise of the cytosolic free calcium concentration and the activation of protein kinase C. The close relationship between the chemoattractant responses is probably explained by cross-desensitisation of the C5a and IL-8 receptors [81], which has been shown to be able to occur at the level of G protein activation [82].

This relationship was also demonstrated by the high correlation between the chemotactic responses to IL-8 and C5a in the healthy controls that were analysed concomitantly with the patients. However, this close correlation was no longer observed in the patients with severe sepsis. Furthermore, in the ex vivo experiments, after incubation with the two highest doses of C5a, the chemotactic response to IL-8 was significantly less reduced than that to C5a. These results indicate that the intracellular pathways are not completely the same and that they may be regulated differently. This view concurs with recent findings by Heit et al who demonstrated that there are different intracellular signalling pathways for C5a and IL-8 [83]. However, the reason or mechanism for the improved response to IL-8 at higher C5a incubation doses is not known. It may be speculated that this represents a reserve mechanism by which the granulocytes can function after heavy complement activation. Such speculation might be one explanation to the lost IL-8/C5a chemotaxis correlation in sepsis. Even if there were a trend toward a diminished reduction in the response to C5a at the highest concentration, the decrease in chemotaxis after IL-8 incubation seems less selective. This more unspecific property is further reinforced by the significant IL-8 incubation effect on chemokinesis. Of interest is also that the effects of C5a and IL-8 incubation are seen already at the lowest concentrations, which in this study was demonstrated to be insufficient to elicit a chemotactic response.
Because both IL-8 and C5a are increased in most patients with severe infections [84-86], the clinical relevance of these C5a/IL-8 interactions is uncertain.

In conclusion, the granulocyte C5aR expression did not limit the chemotactic response to C5a, neither of cells from healthy individuals exposed to C5a for a short incubation time nor of cells from septic patients with a most likely longer duration of increased C5a levels in the blood. C5a receptor expression cannot alone be used as an indicator of the chemotactic granulocyte function.
General discussion

Microbial invasion in patients with sepsis leads to an overwhelming inflammatory response with activation of cascade systems and monocytes and macrophages. The following excessive release of pro-inflammatory cytokines and activation of neutrophil granulocytes play an important role in the development of severe sepsis, septic shock and multiple organ dysfunction [48]. This hyper-inflammatory phase has been associated with early mortality in septic shock. The initial pro-inflammatory response may be followed by an anti-inflammatory phase, a phase referred to as the “compensatory anti-inflammatory response syndrome” (CARS) [87] or immunoparalysis [88]. Depression of monocyte antigen-presenting capacity and pro-inflammatory cytokine secretion can often be demonstrated in these patients [89, 90]. The monocyte deactivation is closely associated with a loss of type 1 T-cell response, characterised by a reduction in interferon-γ-producing-T-helper cells and increases in IL-4 and IL-10 levels [91]. Furthermore, sepsis-induced apoptosis of both B and T lymphocytes has been found in some patients [92].

Although neutrophil granulocyte function not has been studied to the same extent, suppressed chemotactic response, depressed enzyme release, impaired bactericidal capacity and decreased production of reactive oxygen radicals have all been demonstrated [77, 93]. This immunodepression state has been associated with late mortality in severe sepsis and septic shock [92].

The complement activation with its production of C5a leads to neutrophil adhesion and activation [13, 23, 24], cytokine release [16, 17], coagulation activation [20] and vasodilation [14]. Therefore, C5a may, together with many other mediators, contribute to the pro-inflammatory initial phase in severe sepsis and septic shock. In support of this proposition, experimental studies of primates have shown that blockade of C5a by antibodies markedly attenuated *Escherichia coli*-induced septic shock and the development of ARDS [42, 94]. However, such a result is not surpris-
ing and has been demonstrated for numerous substances with immunomodulating properties [37]. Because the infections encountered in patients with immunoparalysis are mostly of a bacterial or fungal origin (e.g. enterococci, staphylococci and resistant gram-negative bacteria or invasive Candida infections), whereas infections associated with defect T-cell function (e.g., cytomegalovirus and Pneumocystis Jiroveci infections) are very rare, depression in granulocyte function is probably the most clinically significant defect in this patient population. What makes anti-C5a treatment of special interest for the treatment of severe sepsis and septic shock is that C5a at high and prolonged concentrations seems to have the ability to cause granulocyte dysfunction. There is substantial experimental evidence that the generation of C5a gradually causes a shutdown of crucial neutrophil functions, such as generation of reactive oxygen radicals, release of granular enzymes and phagocytosis [18, 45, 95] and that anti-C5a treatment might prevent this shutdown [17, 39, 47]. In fact, anti-C5a treatment has been demonstrated to attenuate the multi-organ dysfunction syndrome in the rat [39].

Experimental evidence showing a beneficial effect of anti-C5a treatment in the form of animal experiments is rather extensive. In several of the presented studies the cecal ligation and puncture (CLP) model have been used [16, 17, 20, 22, 39, 40, 45, 47, 96]. Advantages with this model include an initial hyperdynamic cardiovascular response and signs of systemic inflammatory response that are similar to those being observed in patients with sepsis [52]. Furthermore, the infection in this model is introduced locally and not directly into the blood.

However, in the animal studies in which anti-C5a strategies were beneficial most of the treatments were given concomitantly or before the endotoxin injection [43, 44] or establishment of the infection [40]. In one study, anti-C5a given 12 h after induction of sepsis with the CLP model in rats still had some effect but not when given after 24 h [47]. Another CLP study in mice demonstrated that antibodies to the C5aR administered after 6 h were without effect [16].

In study I, a reduction in granulocyte C5aR expression in patients was found at the time when the diagnosis of severe sepsis or septic shock could clinically be established. In the light of the time-dependent effect in the animal experiments, it was suggested that this reduction could perhaps implicate a reduced response to C5a and a risk that the clinical response to anti-C5a treatment might be more limited than that seen in
animal experiments. It was further speculated that the C5aR reduction possibly was a consequence of previous complement activation.

In study III, it was demonstrated that not only C5a but also TNF-α and IL-8 affected the granulocyte C5aR expression. In study IV, it was further shown that neither the granulocytes from septic patients nor the C5a incubated granulocytes from healthy individuals demonstrated any significant correlation between the reduced C5aR expression and the impaired chemotaxis. A trend toward a positive correlation was found in the patients, but two of the three patients with the greatest reduction in C5aR expression demonstrated a relatively well-preserved chemotactic response. These two patients had an inflammatory response that was more on the rise than in the other patients. Thus, even if there may be some correlation between C5aR expression and the chemotactic response suggesting a diminished response to C5a in patients with low C5aR expression, this is not true for all patients, especially not in those with an increasing inflammatory response. Theoretically, it was speculated that C5aR expression, as a marker of responsiveness to C5a, could be one of the candidates to predict a result from anti-C5a treatment and therefore to be employed as a method for patient selection in future anti-C5a treatment trials. However, our data demonstrate a complex relationship and if C5aR is to be used as an indicator of effect in a phase II trial, the inflammatory response will also probably have to be taken into account. If such an indicator for selection of patients into a phase III trial could be identified, this might greatly reduce the number needed to recruit with a given statistical power.
Conclusions

- The granulocyte C5a receptor (C5aR) expression is reduced at the time when the diagnosis of severe sepsis or septic shock can clinically be established, whereas the C5aR expression on monocytes in patients is unchanged compared with that of a healthy control.

- Lipopolysaccharide (LPS) incubation of whole blood from healthy donors ex vivo results in reduced granulocyte expression of the C5aR, whereas monocyte C5aR expression is not changed. Despite higher concentrations of LPS, no effect can be demonstrated on C5aR expression of isolated granulocytes or monocytes.

- Incubation of whole blood and isolated cells from healthy donors with C5a, IL-8 and TNF-α ex vivo induces a reduction of the granulocyte C5aR expression; however, only C5a gives rise to reduced monocyte C5aR expression.

- The granulocyte C5aR expression does not limit the chemotactic response to C5a. This holds for cells from healthy individuals exposed to C5a for a short incubation time ex vivo and for cells from septic patients with a most likely longer duration of increased C5a levels in the blood. C5a receptor expression cannot alone be used as an indicator of the chemotactic granulocyte function.

- Expressions of complement receptors 1 and 3 are increased in patients with severe sepsis or septic shock. Similarly, LPS, C5a, IL-8 and TNF-α incubation of whole blood and isolated cells from healthy donors ex vivo increases expressions of the two complement receptors. After C5a incubation of whole blood, a correlation between granulocyte C5aR and CD11b expression can be demonstrated, whereas no correlation is seen between C5aR and the other complement receptors.
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References


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