Leukocyte activation in systemic vasculitis and sepsis
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INTRODUCTION

Activation of leukocytes is the initial step in the multistep process of inflammation. The inflammatory response is characterized by closely intertwined humoral and cellular components. During this process leukocytes, in particular neutrophils and monocytes, are initially triggered by proinflammatory mediators or bacterial products, such as endotoxin, in order to optimally perform their task. Upon activation, circulating leukocytes adhere to and transmigrate through the activated endothelial monolayer. Activation of endothelial cells is a prerequisite for neutrophil adherence to and transmigration through the endothelial monolayer. Guided by a concentration gradient of chemoattractants, these activated neutrophils migrate towards the site of infection. Adhesion molecules play a central role in this process of adherence and transmigration. Once there, these activated neutrophils and monocytes recognize, phagocytose and subsequently kill the invading microorganisms. Neutrophils and monocytes possess a whole arsenal of intracellularly stored enzymes, which serve to kill and degrade phagocytosed material. In addition, these cells are capable of, when activated, producing and secreting reactive oxygen species (ROS) for this same purpose. When, however, released uncontrolled, these products are harmful to surrounding cells. This uncontrolled release of lytic enzymes and oxygen radicals is thought to play an important role in the pathophysiology of inflammatory disorders, such as ANCA associated vasculitides and sepsis. Both inflammatory diseases are characterized by systemic inflammation and leukocyte activation, but differ markedly in clinical manifestation. This thesis focuses on the relation between leukocyte activation and its role in the pathophysiology and clinical manifestations in both ANCA associated vasculitides and sepsis, as outlined in chapter 1.

PART I: LEUKOCYTE ACTIVATION IN ANCA ASSOCIATED VASCULITIDES

Cell biological mechanisms of renal disease: Pathophysiology of ANCA associated vasculitides

In vitro experiments have demonstrated that anti-neutrophil cytoplasmic antibodies (ANCA) are capable of activating neutrophils and monocytes. Little is known, however, of the in vivo relevance of these in vitro observed effects of ANCA and how these effects may finally result in in vivo systemic inflammation, damage of blood vessels and pauci-immune glomerulonephritis. In chapter 2 the international literature, including some of the work described in this thesis, on ANCA, leukocyte activation and its role in the pathophysiology of ANCA associated vasculitides is reviewed. Particular attention is given to the relation between those in vitro findings and the in vivo observations in patients with ANCA associated vasculitis/glomerulonephritis.
Leukocyte membrane expression of proteinase 3 correlates with disease activity in patients with Wegener’s granulomatosis

ANCA in patients with Wegener’s granulomatosis (WG), one of the idiopathic systemic vasculitides, are directed against proteinase 3 (Pr3) in most of the cases. As reviewed in chapter 2, upon neutrophil priming in vitro, ANCA antigens, including Pr3 are expressed on the cell surface of neutrophils and monocytes, thereby becoming available for interaction with ANCA\(^1\)\(^,\)\(^2\). This interaction, subsequently, results in activation of these cells\(^1\)\(^,\)\(^2\). Since ANCA can only interact with leukocytes when the ANCA antigens are present on the cell surface, we investigated in chapter 3 whether Pr3 was expressed on the membranes of circulating granulocytes and monocytes of patients with anti-Pr3 associated WG. In addition, we investigated whether Pr3 expression was related to disease activity, so explaining the systemic nature and severity of the disease.

The expression of Pr3, and other ANCA antigens, that is, myeloperoxidase (MPO) and elastase (HLE), was analyzed on circulating granulocytes and monocytes by flowcytometry, using a non activating whole blood method. Disease activity was quantitated using the Birmingham Vasculitis Activity Score (BVAS). We found that the expression of proteinase 3 on neutrophils was increased in patients with active WG compared to patients with quiescent disease and healthy controls. On monocytes no differences in Pr3 expression were found between those groups. Furthermore, the expression of MPO and HLE did not differ between patient groups and healthy controls. Upon follow up, the expression of Pr3 on neutrophils from patients with active WG decreased when patients went into remission. Pr3 expression on neutrophils correlated with disease activity, as expressed by the BVAS score, suggesting that the availability of Pr3 for interaction with ANCA plays a central role in the disease process.

Are circulating neutrophils intravascularly activated in patients with ANCA associated vasculitides?

Vascular injury in vasculitis may be due to activation of circulating neutrophils resulting in their increased adhesiveness to locally activated endothelium (Shwartzman phenomenon). Since upregulation of endothelial ICAM-1 and VCAM-1, as a marker for endothelial cell activation, was already demonstrated in biopsies from patients with ANCA associated vasculitis\(^3\), thus enabling the interaction with cells expressing the relevant adhesion molecules, we investigated in chapter 4 the expression of adhesion molecules (CD11b, ICAM-1, VLA-4, L-selectin) and activation markers (CD66b, CD64, CD63) on circulating neutrophils from patients with ANCA associated vasculitis in comparison to their expression on cells from healthy volunteers and patients with sepsis, a condition in which intravascular activation of neutrophils is known to take place. In this study we found that the expression of activation markers, but not the expression of adhesion molecules was increased on neutrophils from patients with active vasculitis. The expression of CD63 and CD66b on neutrophils correlated with disease activity as determined by the Birmingham Vasculitis Activity Score.
Score (BVAS). In contrast to patients with active vasculitis, patients with sepsis showed upregulation of all markers, including adhesion molecules, suggesting that circulating neutrophils are fully activated in sepsis. We concluded that in ANCA associated vasculitis, circulating neutrophils are not fully activated, but primed, since they do not express increased levels of adhesion molecules like in sepsis or in the Shwartzman reaction. These findings are compatible with the concept that in vivo vascular damage in ANCA associated vasculitides does not occur due to a Shwartzman-like reaction but only after ANCA induced neutrophil activation at the endothelial cell surface. It appears that β2 integrin mediated outside-in signaling seems to be instrumental in ANCA induced neutrophil activation.

**Monocyte activation in patients with Wegener’s granulomatosis**

Most studies have focused on the capacity of ANCA to induce neutrophil activation. Apart from neutrophils, however, also monocytes/macrophages play a pivotal role in lesion development in WG. An important histopathological feature in patients with WG is granulomatous inflammation. Granuloma formation is thought to result from activated monocytes/macrophages which are locally present and which participate in granuloma formation by synthesizing and secreting a variety of chemoattractants, growth factors and cytokines. Furthermore, monocytes/macrophages may induce vascular damage by the production and secretion of reactive oxygen radicals and lytic proteases. Monocytes also contain the ANCA antigens Pr3 and MPO in their granules. Like neutrophils, they express ANCA antigens upon priming, which subsequently may initiate a process of cell activation similar as has been described for neutrophils after interaction with ANCA. In view of these findings, we evaluated in chapter 5 in vivo monocyte activation in patients with active or quiescent WG. Patients with sepsis served as positive controls for intravascular monocyte activation. Monocyte activation was analyzed by measuring plasma levels of soluble products of monocyte activation, that is neopterin and interleukin-6, which reflect the state of activation of circulating, adherent and transmigrated monocytes, and by quantitating the surface expression of activation markers on circulating monocytes by flowcytometry. We found evidence that monocytes, circulating or otherwise, are activated in patients with vasculitis, although compared to patients with sepsis, all markers of monocyte activation were lower. We concluded that disease activity in WG correlates with the extent of activation of monocytes, which is compatible with their role in the pathophysiology of this disease.

**In vitro neutrophil activation by antibodies to Pr3 and MPO from patients with crescentic glomerulonephritis**

Patients with necrotizing crescentic glomerulonephritis (NCGN) and ANCA directed against proteinase 3 (anti-Pr3) have been shown to faster deteriorate their renal function and to have more active renal vasculitic lesions than patients with antimiylperoxydase (anti-MPO) antibodies. Since ANCA induced neutrophil activation is thought to play an important role in the pathophysiology of this form of glomerulonephritis.
Summary and General Discussion

In sepsis, the role in activation, since granule constituents upon stimulation with ANCA positive IgG preparations. We found that IgG fractions from anti-Pr3 positive patients with NCGN were more potent activators of primed neutrophils than IgG fractions from anti-MPO positive patients. This is in agreement with our hypothesis that the faster deterioration of renal function and the more active renal vasculitic lesions observed in anti-Pr3 positive patients, in comparison to anti-MPO positive patients, is associated with a higher neutrophil activating capacity of anti-Pr3 IgG of the former patients.

Can endothelial cells be activated by ANCA?

Activation of endothelial cells is a prerequisite for neutrophil adherence to and transmigration through the endothelial monolayer. Furthermore, as discussed earlier, $\beta_2$ interaction is a prerequisite for ANCA induced neutrophil activation. In vivo, endothelial cell activation in patients with ANCA associated vasculitides has been demonstrated by several investigators. Endothelial cells may become activated upon the release of pro-inflammatory mediators, such as Tumor Necrosis Factor $\alpha$ or Interleukin 1. In addition, anti-neutrophil cytoplasmic antibody (ANCA) have been demonstrated to activate endothelial cells in vitro. The precise mechanisms by which ANCA might activate endothelial cells is unclear. ANCA antigens, like proteinase 3 (Pr3) and myeloperoxidase (MPO), may bind to endothelial cells or alternatively be expressed on the surface of endothelial cells, as was previously demonstrated for proteinase 3, but not for MPO. Subsequently, these antigens may be recognized by ANCA and this interaction may result in endothelial cell activation. Otherwise, anti-endothelial cell antibodies (AECA) are directed against endothelial antigens and are often present in patients with ANCA associated vasculitides. These AECA may also be responsible for the previously described capacity of ANCA positive IgG preparations to induce endothelial cell activation in vitro. We related endothelial cell activation to the presence of both ANCA and AECA in these IgG preparations. The extent of endothelial cell activation was assessed by measuring E-selectin upregulation and the production of interleukin 6. We found that 39 % of the ANCA positive IgG samples were capable of activating endothelial cells (either by inducing IL-6 production and/or E-selectin expression). Sixty four percent of these endothelial cell activating ANCA positive IgG samples contained AECA. In addition, AECA positive samples which induced endothelial cell activation had higher AECA titres than samples that did not induce endothelial cell activation. Endothelial cell activation by ANCA alone could only be attributed to 14 % of the ANCA positive samples, all these samples contained anti-Pr3 antibodies. We concluded that only a minority of

glomerulonephritis, we investigated in chapter 6 whether anti-Pr3 antibodies were capable of inducing a more pronounced activation of neutrophils in vitro than anti-MPO antibodies. Neutrophil activation was determined by measuring the oxygen radical formation and the release of granule constituents upon stimulation with ANCA positive IgG preparations. We found that IgG fractions from anti-Pr3 positive patients with NCGN were more potent activators of primed neutrophils than IgG fractions from anti-MPO positive patients. This is in agreement with our hypothesis that the faster deterioration of renal function and the more active renal vasculitic lesions observed in anti-Pr3 positive patients, in comparison to anti-MPO positive patients, is associated with a higher neutrophil activating capacity of anti-Pr3 IgG of the former patients.
anti-Pr3 ANCA positive samples induces endothelial cell activation by itself, whereas, samples which also contain AECA are more likely to induce endothelial cell activation.

PART II: LEUKOCYTE ACTIVATION IN SEPSIS

**HLA-DR expression on monocytes as a prognostic marker and a guide for immunotherapy in septic patients**

Sepsis is not a homogeneous entity, but can be regarded as a spectrum of pro-and anti-inflammatory responses. The immune response in sepsis shows a bimodal pattern consisting of an early, frequently exaggerated inflammatory response, followed by a state of hypo-responsiveness often referred to as the compensatory anti-inflammatory response syndrome (CARS) 25. Leukocyte characteristics during these phases range from an exaggerated cell activation towards cell anergy. In chapter 8 the international literature concerning the different immune states in patients with sepsis was reviewed, and new therapies guided by these immunomonitoring techniques are discussed. It was concluded that insight into the disease state could be a promising new approach to the therapy of patients with sepsis.

**Leukocyte activation in sepsis; correlations with disease state and mortality**

In chapter 9 we investigated the hypothesis that poor outcome in patients with sepsis is related to the severity of CARS, as reflected in the degree of leukocyte activation. We determined the degree of leukocyte activation by analyzing the surface expression of several activation markers, including HLA-DR, CD11b, ICAM-1, CD66b, CD63 and CD64 on neutrophils and monocytes and by determining plasma concentrations of lactoferrin, interleukin 6, and neopterin at the time of patient inclusion. We related these data to patient survival. Compared to healthy controls, we found increased expression of all markers, except for HLA-DR, which was decreased, on both PMNs and monocytes. In addition, the expression of CD11b on PMNs and ICAM-1 on monocytes was lower in patients who died compared to patients who survived. Furthermore, the combined analysis of all activation markers appeared to be predictive for survival in patients with sepsis. Therefore, we concluded that in sepsis, both PMNs and monocytes are activated compared to healthy controls. However, poor prognosis was associated with a lower expression of activation markers on monocytes and PMNs, suggesting that poor outcome in these patients might have been due to the compensatory anti-inflammatory response. Furthermore, the combined analysis of parameters of neutrophil and monocyte activation appeared to be a promising new tool to investigate the presence of CARS and/or to predict outcome in patients with sepsis.
Interleukin 10 and monocyte HLA-DR expression in patients with sepsis
As discussed in the previous chapters an overwhelming systemic pro-inflammatory reaction is frequently followed by an overactive compensatory anti-inflammatory response (CARS) in which leukocyte function is impaired. The precise mechanisms that are involved in the development of CARS are not well known. In patients with sepsis a decreased monocyte HLA-DR surface expression can be demonstrated during CARS. Furthermore, ongoing decreased expression of HLA-DR on monocytes from patients with sepsis has predictive value for a fatal outcome. Since the expression of HLA-DR on monocytes is downregulated by the anti-inflammatory cytokine interleukin 10, we examined in chapter 10 the hypothesis that IL-10 is a potential candidate for the induction of CARS.

We investigated the expression of HLA-DR on monocytes in 2 patients with sepsis and related our findings to plasma concentrations of interleukin-10 during follow up. We demonstrated in one patient an episode of CARS, as defined as an HLA-DR expression of less than 30% compared to healthy controls. This coincided with an increase of IL-10 plasma concentration and a protracted clinical recovery. HLA-DR expression of the second patient did not decrease significantly and IL-10 levels remained low. We concluded that the anti-inflammatory cytokine IL-10 when measured in combination with HLA-DR expression may be a good way to determine the patient’s individual immune status. The use of IL-10 measurements alone in determining the patient’s individual immune status, should however still be investigated. We believe that this finding may have therapeutic consequences, since patients with sepsis have to be treated according to their individual immune status.

Levels of soluble FcγRIII correlate with disease severity in sepsis
As discussed in the previous chapters, neutrophil activation is thought to play a crucial role in the pathogenesis of sepsis. During activation, neutrophils adhere to and migrate through the endothelium. Therefore, the amount of circulating neutrophils does not adequately reflect the total amount of neutrophils that are involved in the pathophysiological process of this condition. Therefore, in chapter 11 we tested the hypothesis that the severity of sepsis is associated with the total body mass of neutrophils as reflected in the plasma concentration of soluble Fcγ receptor type III (sFcγRIII). We measured soluble FcγRIII plasma concentrations in nineteen patients with sepsis and related these data to clinical and laboratory parameters of disease severity of these patients and to the extent of neutrophil activation. We found that soluble FcγRIII levels were elevated compared to healthy controls and correlated with disease severity as measured by the APACHE II score in sepsis patients. Markers of cell activation were significantly increased in sepsis patients, but did not correlate with the APACHE II score.

In conclusion, this study demonstrates that soluble FcγRIII, but not neutrophil activation, is a useful marker to assess disease severity in patients with sepsis.
GENERAL CONCLUSIONS

The work described in this thesis is focussed on the relation between leukocyte activation and its role in the pathophysiology and clinical manifestations in both ANCA associated vasculitides and sepsis. Both inflammatory diseases are characterized by systemic inflammation and leukocyte activation, but differ markedly in clinical manifestation.

We found large differences in the extent of activation of circulating neutrophils and monocytes patients with vasculitis compared to patients with sepsis. Whereas in patients with sepsis circulating neutrophils are fully activated, in patients with vasculitis circulating neutrophils are not fully activated, but ‘primed’. The absence of increased expression of adhesion molecules on circulating neutrophils in patients with vasculitis pointed to this conclusion \(^{122}\). In patients with vasculitis these circulating primed neutrophils express proteinase 3 and myeloperoxidase, the target antigens for ANCA in both patients with vasculitis and patients with sepsis \(^{117}\). We demonstrated that the expression of proteinase 3 on circulating neutrophils from patients with vasculitis correlates with disease activity, as expressed by the BVAS score, suggesting a role for priming and availability of the target antigen proteinase 3 in the disease process \(^{117}\). Once neutrophils and monocytes express proteinase 3 or myeloperoxidase, these cells can interact with ANCA. However, full activation of neutrophils, as a result of stimulation by ANCA, occurs only when cells are bound to a surface, that is, bound to endothelial cells \(^{22}\). Thus, the close contact between endothelial cells and neutrophils, that are locally activated by ANCA, enables these neutrophils to degranulate in the vicinity of endothelial cells, resulting in endothelial cell injury and, eventually, vascular damage \(^{1}\), which does not occur in sepsis where leukocytes are already intravascularly activated.

Most studies have focused on the capacity of ANCA to induce neutrophil activation. Apart from neutrophils, however, also monocytes/macrophages play a pivotal role in lesions developing in Wegener’s granulomatosis. Furthermore, monocytes also contain the ANCA antigens Pr3 and MPO in their granules. Like neutrophils, they express ANCA antigens upon priming \(^{18,117}\), which subsequently may initiate a process of cell activation, similar as has been described for neutrophils after interaction with ANCA. We showed that circulating monocytes in patients with Wegener’s granulomatosis are activated, although less than in patients with sepsis, suggesting that these cells are also only ‘primed’. We further demonstrated that the extent of activation of these cells is correlated with disease activity \(^{125}\), which supports their role in the pathophysiology of Wegener’s granulomatosis, more in particular, in granuloma formation.

In patients with sepsis, however, no clear-cut correlation was found between cell activation and disease severity, as expressed by the APACHE II score \(^{149}\). The immune response in sepsis shows a bimodal pattern consisting of an early, frequently exaggerated inflammatory response, followed by a state of hyporesponsiveness often referred to as the compensatory anti-inflammatory response syndrome (CARS) \(^{21}\). We demonstrated that, whereas circulating cells in patients with sepsis are intravascularly
Summary and General Discussion

fully activated, it appears that in those patients, a relatively diminished cell activation of monocytes, but also neutrophils, is associated with a poor survival. This phenomenon can be attributed to a state of CARS. Furthermore, a state of CARS can be identified by a decreased expression of HLA-DR on monocytes, which coincides with increased plasma concentrations of the anti-inflammatory cytokine IL-10. IL-10 appears to be a good candidate for the induction of CARS, since this cytokine is capable of downregulating monocyte HLA-DR expression in vitro. In view of the lack of success of many experimental, usually anti-inflammatory therapies, insight into the disease state by monitoring the expression of HLA-DR in combination with the analysis of IL-10 plasma concentrations may be helpful in deciding whether to choose pro- or anti-inflammatory therapy in these patients and may determine clinical outcome.

In summary, although systemic vasculitis and sepsis, both being systemic inflammatory disorders, are characterized by systemic activation of circulating leukocytes, these disorders differ in the nature of leukocyte activation, i.e. pro- or anti-inflammatory response, the extent of leukocyte activation, i.e. priming or full activation, and the site of leukocyte activation, i.e. at the endothelial lining or intravascularly. Vessel wall injury in patients with vasculitis may, thus, result from endothelial injury due to leukocyte activation at the endothelial lining, causing endothelial cell injury. In contrast, vascular leakage in patients with sepsis may result from aberrant leukocyte function, such as the inability to transmigrate, which may lead to intravascular leukocyte activation, i.e. release of toxic products as ROS, lytic enzymes and NO in the circulation, but not at the endothelial lining.