Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 1

Introduction and Outline of the Thesis
General Introduction

The host response to an infection is the outcome of interplay between innate immunity, adaptive immunity and pathogens and their products. This response is an absolute requirement of a successful host defense, aimed at destroying invading organisms, repairing damaged tissue and restoring normal tissue function. A pivotal role in the orchestration of the inflammatory response is reserved for cytokines, which act as soluble or membrane bound intercellular messengers to relay information between inflammatory cells. Nonetheless, inflammatory reactions may be disproportionate to the magnitude of immune challenge, and can be associated with tissue injury and organ failure. As part of excessive immune activation, such as can occur in sepsis, the coagulation system can be triggered, which during severe infections can give rise to the clinical syndrome of disseminated intravascular coagulation (DIC), characterized by extensive fibrin depositions in multiple organs and microvascular thrombosis.

The research described in this thesis focuses on the role of the host response to (myco)bacterial pathogens and their products. The first part of this research comprises experiments in which we investigated the role of the interferon (IFN)-γ stimulating cytokines interleukin (IL)-12 and IL-18, together with IFN-γ in the host response against bacteria or their products; the second part describes studies in which the immunomodulatory functions of the phospholipid mediator platelet activating factor (PAF) were examined. In the third part of this thesis, the results of multiple studies on the different links between the innate immunity and the coagulation/fibrinolytic system are presented.

The general aim of this thesis was to obtain more insight in the regulation of the host immune response to different pathogens. The more specific objectives of each individual study included in this thesis are delineated in the respective chapters.

In this introductory chapter we will first briefly discuss the infections that are studied in this thesis, and the models used for these diseases. Thereafter we will discuss the host response pathways that are investigated together with the outline of this thesis.

Infectious diseases studied and models used

Sepsis, peritonitis and pneumonia

Sepsis is as a clinical syndrome that results from a systemic response of the host to an infection. The outcome of sepsis is poor, and mortality rates remain as high as 30-40%. Sepsis is associated with activation of multiple inflammatory pathways, including the cytokine network and the coagulation system. Indeed, a large series of animal experiments has established that in severe overwhelming infection, such as induced by the intravenous administration of bacteria, the host can react with massive activation of multiple inflammatory cascades, and that this response may importantly contribute to the morbidity and mortality of experimental sepsis. More recent research has indicated that many patients with sepsis syndrome demonstrate evidence for an anti-inflammatory state, characterized by elevated levels of anti-inflammatory
cytokines and cytokine inhibitors, as well as by a condition of cellular hyporesponsiveness in which immunocompetent cells appear unable to release inflammatory mediators upon stimulation with bacterial antigens. It can be assumed that during clinical sepsis the early host response is characterized by hyperinflammation at the systemic level, which is followed by a phase of hyporesponsiveness and immunodepression (Figure 1). The first inflammatory phase may last for only a brief time span and cause early mortality; the second refractory phase may last considerably longer. In this thesis studies are presented that examined both the initial proinflammatory phase of the host response to a systemic bacterial challenge (i.e. lipopolysaccharide or LPS derived from the outer membrane of Gram-negative bacteria; chapter 12) and the subsequent hyporesponsive phase (chapter 4).

![Excessive systemic inflammation and Immunoparalysis](image)

**Fig. 1**: Pro- and anti-inflammatory events during sepsis. Sepsis likely is associated with an early phase of excessive systemic inflammation mediated by various proinflammatory mediators of the innate immune system (e.g. proinflammatory cytokines). Shortly after this initial phase, which may lead to early mortality, counterregulatory pathways become activated, including anti-inflammatory cytokine release and the development of a refractory state characterized by a decreased capacity of immune cells to produce proinflammatory cytokines. This later phase is accompanied by immunodepression or “immunoparalysis”, and presumably is at least in part responsible for the development of secondary nosocomial infections and late mortality.

The most frequent cause of sepsis is pneumonia, accounting for approximately 50% of cases in recent clinical sepsis trials. The second most frequent cause of sepsis is peritonitis. In this thesis we used models of pneumonia and peritonitis to investigate the role of certain inflammatory responses and the procoagulant/anticoagulant balance in the outcome of these diseases. Specifically, we used *Streptococcus (S.) pneumoniae* to induce respiratory tract infection in mice (chapters 7, 9 and 11). *S. pneumoniae*, the pneumococcus, is a Gram-positive pathogen that colonizes the upper respiratory tract and causes life-threatening diseases, including pneumonia. In adults, *S.*
pneumoniae is the most frequently isolated pathogen in community-acquired pneumonia. In the United States alone, more than half a million cases of pneumococcal pneumonia are reported each year, with a fatality rate of 5-7\%. Bacteremia with S. pneumoniae is in almost 90\% of cases the consequence of pneumococcal pneumonia. In addition, in recent sepsis trials, S. pneumoniae was an important causative pathogen especially in the context of pneumonia. The mortality rate of 40,000 per year caused by S. pneumoniae in the United States is larger than the mortality rate caused by any other bacterial pathogen. Infections caused by S. pneumoniae are increasingly difficult to treat due to the emergence of antibiotic resistant strains. In this thesis we used Escherichia coli to induce abdominal sepsis in mice (chapters 2, 3, and 8). Acute bacterial peritonitis is a life-threatening condition characterized by the presence of bacteria in the otherwise germ-free peritoneal cavity, almost invariably caused by perforation of intestines. Consequently, the most commonly encountered pathogens are enteric Gram-negative bacteria, among which Escherichia (E.) coli can be found in up to 60\% of cases. Despite advances in surgery and antimicrobial therapy the mortality rate of peritonitis ranges between 30 and 50\%. Above all, in sepsis that originates from peritonitis mortality rates can be as high as 80\%. Altogether it is clear that respiratory tract infection by S. pneumoniae and abdominal infection by E. coli importantly contribute to sepsis mortality and represent major health care problems.

Another important cause of community-acquired pneumonia is Legionella (L.) pneumophila. In addition to the mouse studies described above, we investigated the responsiveness to IFN-\(\gamma\) and L. pneumophila in patients recovered from pneumonia caused by this bacterium (chapter 5).

**Tuberculosis**

*Mycobacterium (M.) tuberculosis* is one of the most effective human pathogens, with an estimated one-third of the world’s population being infected, resulting in 8 million new cases of disease and over 2 million deaths per year. Of persons infected with *M. tuberculosis*, 5-10\% develop tuberculous disease; most people become healthy tuberculin reactors. Hence, the host response to infection with *M. tuberculosis* in general is successful in containing, although not eliminating, the pathogen. Cell mediated immunity is considered instrumental for a protective immune response to *M. tuberculosis*, primarily because mycobacteria are intracellular organisms residing in macrophages, and thus T cell effector mechanisms are required to control the infection. Indeed, efficient eradication of *M. tuberculosis* in macrophages is mediated by a T helper 1 immune response, characterized by the production of IFN-\(\gamma\). In this thesis we used a mouse model of lung tuberculosis, in which live virulent *M. tuberculosis* is instilled intranasally, to study certain immune mechanisms in host defense against this disease (chapters 6 and 10).
Host response pathways studied and outline of the thesis

**Proinflammatory cytokines**

Cytokines are a family of small proteins that play an essential role in the regulation of the immune response. Cytokines can be produced by many different cell types, including monocytes/macrophages, neutrophils, endothelial cells, epithelial cells, and fibroblasts. They function in a complex network in which they can influence each other's production and activity. Often, cytokines are divided into 3 groups, i.e. proinflammatory cytokines, anti-inflammatory cytokines and soluble inhibitors of proinflammatory cytokines. Several studies in experimental models of infectious diseases have demonstrated the importance of locally produced proinflammatory cytokines for an adequate host response against bacteria. The first part of this thesis contains the results of work done to elucidate the role of the proinflammatory cytokines IL-12 (chapter 2), IL-18 (chapter 3) and IFN-γ (chapters 4 and 5).

IFN-γ, IL-12 and IL-18 at least in part function as a “cytokine axis” in which IL-12 and IL-18 potently enhance the production of IFN-γ. The main producers of IFN-γ are activated natural killer cells, T helper 1 cells and cytotoxic T cells. Biologically active IFN-γ exists as a noncovalent homodimer. IFN-γ actions related to inflammation are induction of class II major histocompatibility complex antigen expression on different cell types and macrophage activation. Furthermore, IFN-γ likely plays an important role in the production of IgG against bacterial polysaccharides. IL-12 is predominantly synthesized by monocytes and macrophages. The major cellular targets of IL-12 are T and natural killer cells, inducing the production of IFN-γ, stimulating proliferation and enhancing cytotoxic activity. Biologically active IL-12 is a heterodimer consisting of a p35 and a p40 subunit, each encoded by a separate gene. The p40 subunit mediates binding to the IL-12 receptor (but does not induce signal transduction), while the p35 subunit is critical for signal transduction. The p40 subunit is able to form homodimers, which bind to the IL-12 receptor with affinities similar to the IL-12 heterodimer without eliciting a cellular effect. Therefore p40 homodimers act as inhibitors of IL-12 activity by blocking IL-12 receptor binding sites, although they may also have some proinflammatory activities. The production of p35 and p40 is differentially regulated, and to a given stimulus cells secrete a 10- to 100-fold excess of free p40 over the biologically active p35-p40 heterodimer. IL-18 was first described as IFN-γ-inducing factor (IGIF) and with pro-IL-1β, the IL-18 precursor (pro-IL-18) does not contain a signal peptide required for removal of the precursor amino acids and subsequent secretion. Like pro-IL-1β, pro-IL-18 is cleaved by caspase 1, although recent evidence indicates that processing of pro-IL-18 can also take place via an caspase 1 independent mechanism. Mature IL-18 shows a high degree of three-dimensional structural similarity to mature IL-1β. Although IL-18 is an inducer of IFN-γ (in particular in the presence of IL-12), IL-18 is a cytokine with proinflammatory properties not directly linked to IL-12 and/or IFN-γ.

In chapter 2 the role of endogenous IL-12 is analyzed in the host response to septic peritonitis induced by *E. coli* by making use of p35 gene deficient mice, animals in
which IL-12 is the only cytokine that cannot be produced. In chapter 3, the role of IL-18 in the local and systemic host response to abdominal sepsis caused by *E.coli* is determined using IL-18 gene deficient mice; moreover, this chapter describes the effects of recombinant IL-18 during peritonitis. In chapter 4, the production and effect of IFN-γ during immunoparalysis, induced by intravenous injection of LPS into healthy humans, are examined. In chapter 5, the responsiveness to IFN-γ in patients recovered from *L. pneumophila* pneumonia is investigated.

**Platelet activating factor**

Platelet-activating factor (PAF) is a potent phospholipid mediator that plays an important role in inflammatory and immune responses 18. PAF is produced by a large number of cells, including platelets, endothelial cells, stromal cells, lymphoid tissue and neutrophils. The biological activity of PAF is mediated through a specific G-protein-coupled receptor (PAFR) on the membrane of responsive cells, which has been identified on many haemopoietic cells, including neutrophils, dendritic cells, macrophages and monocytes. PAF and the PAFR may influence host defense mechanisms during infection in different ways. First, PAF itself may play a role in the innate immune response to infection as a pluripotent mediator of inflammation. Second, the PAFR may influence host defense against certain pathogens independent of its interaction with PAF by virtue of its capacity to bind phosphorylcholine, the biologically active component of PAF, but that is also a component of the cell wall of certain pathogens including *S. pneumoniae* 19. Furthermore, recent studies have suggested that endogenous PAF may play an important role in an adequate immune response to intracellular microorganisms, such as *Leishmania amazonensis* and *Trypanosoma cruzi* 20,21. In chapter 6, the role of PAF is evaluated in the immunopathology of the intracellular infection with *M. tuberculosis*. In chapter 7 we sought to obtain more insight into the role of the PAFR in the pathogenesis of pneumococcal pneumonia.

**Coagulation and anticoagulation**

Severe infections can be associated with activation of the coagulation system 2,22. Ample evidence exists that tissue factor (TF) is the central protein that mediates the activation of coagulation during sepsis and endotoxemia. TF, expressed at the surface of endothelial and mononuclear cells, can bind and activate clotting factor VII, and the factor VIIa-TF complex is able to activate factor X and factor IX. The activity of the factor VIIa-TF complex can be inhibited by tissue factor pathway inhibitor (TFPI), which can bind factor Xa, and thereafter inhibit factor VIIa-TF activity by forming a quaternary Xa-TFPI-VIIa complex. TF cannot be detected on the surface of resting vascular endothelium, and only in very low quantities on circulating blood cells, conceivably because this would lead to undesired procoagulant activity. During infection and after stimulation with LPS or tumor necrosis factor (TNF), TF is expressed on blood mononuclear cells and on vascular endothelium. The evidence that TF is pivotal for the activation of coagulation is derived from observations that a
number of different strategies that prevent the activation of the VIIa-TF pathway in endotoxemic humans and chimpanzees, and in bacteremic baboons abrogate the activation of the common pathway of coagulation. In healthy humans injected with LPS, intravenous infusion of recombinant TFPI at two different doses caused a dose-dependent inhibition of coagulation activation. It should be noted that interventions inhibiting the TF pathway in lethal E. coli sepsis in baboons not only prevented DIC, but also resulted in an increased survival. These findings contrast with interventions that block the coagulation system more downstream, i.e., administration of factor Xa blocked in its active center (DEGR-Xa), failed to influence lethality of bacteremic baboons, while completely inhibiting the development of DIC. This has led to the hypothesis that elimination of the VIIa-TF pathway protects against death not merely by an effect on the coagulation system, and that TF may have effects on inflammatory responses different from the procoagulant response. In accordance with this hypothesis, bacteremic baboons treated with DEGR-VIIa demonstrated significantly lower IL-6 and IL-8 levels than baboons not infused with this compound.

Activated protein C (APC) represents an important natural anticoagulant protein. Protein C is activated by the complex of thrombin with the endothelial cell surface protein thrombomodulin; APC is dependent on its cofactor protein S. APC proteolytically inactivates factors Va and VIIIa, thereby rapidly inactivating blood coagulation. The impairment of the protein C system during sepsis is the result of increased consumption of protein S and protein C, and decreased activation of protein C by downregulation of thrombomodulin on endothelial cells. Furthermore, protein S can be bound by the acute phase response protein C4b-binding protein. Treatment of patients with severe sepsis with recombinant APC has resulted in a significantly increased survival. The finding that APC is beneficial in sepsis is supported by a number of preclinical observations. Infusion of APC into septic baboons prevented hypercoagulability and death, while inhibition of activation of endogenous protein C by a monoclonal antibody exacerbated the response to a lethal E. coli infusion, and converted a sublethal model produced by a LD10 dose of E. coli into a severe shock response associated with DIC and death. Furthermore, treatment of baboons with an monoclonal antibody that prevented protein C from binding to the endothelial cell protein C receptor (EPCR), thereby reducing the efficiency by which protein C can be activated by the thrombin-thrombomodulin complex, also was associated with an exacerbation of a sublethal E. coli infection to lethal sepsis with DIC. Hence, like the VIIa-TF mediated pathway, the protein C-protein S system may have other effects on host responses apart from its role in the coagulation system. Indeed, several anti-inflammatory effects have been ascribed to APC, including inhibition of leukocyte activation, inhibition of E-selectin mediated cell adhesion to the vascular endothelium and reduction of tumor necrosis factor (TNF)-α production. Moreover, APC exerts profibrinolytic effects through inhibition of plasminogen activator inhibitor type I (PAI-1), the main inhibitor of plasminogen activation.
Fibrin removal during sepsis is further impaired due to depression of the fibrinolytic system \(^2\,^{22}\). Indeed, experimental models indicate that at the time of maximal activation of coagulation, the fibrinolytic system is largely shut off. Experimental bacteremia and endotoxemia result in a rapidly occurring increase in fibrinolytic activity, most probably due to the release of plasminogen activators from endothelial cells. This pro-fibrinolytic response is almost immediately followed by a suppression of fibrinolytic activity, due to a sustained increase in plasma levels of PAI-1. Interestingly, strategies that are able to completely block the LPS-induced thrombin generation, such as anti-TF antibodies or recombinant hirudin, were without any effect on the activation and subsequent inhibition of fibrinolysis, suggesting an independent regulation of these two processes. Interestingly, mediators and products of fibrinolysis may influence inflammatory responses independent of their proteolytic properties. For example, in vitro, plasmin was demonstrated to stimulate the release of cytokines and other inflammatory mediators by different cell types \(^34\). In addition, the urokinase plasminogen plasminogen activator receptor, that is expressed on many different cells including leukocytes, has been implicated in the recruitment of neutrophils to sites of inflammation \(^35\).

Hence, severe infection frequently is accompanied by a disturbance of the hemostatic balance favoring a procoagulant response. Enhanced expression of the factor VIIa-TF pathway plays a crucial role in the procoagulant response to sepsis, likely in conjunction with concurrent suppression of anticoagulant pathways such as the protein C – protein S – thrombomodulin system, antithrombin and fibrinolysis. Evidence exists that at least some components of the procoagulant and anticoagulant pathways (in particular TF and APC) may influence inflammatory reactions other than the coagulation system. The third part of this thesis investigates the interplay between coagulation and anticoagulation on the one hand, and inflammation on the other hand in more detail. In chapters 8 and 9 the role of the TF/factor VIIa complex was investigated in mice suffering from \(E.\ coli\) peritonitis and pneumococcal pneumonia respectively. For this, the recombinant Nematode Anticoagulant Protein c2 (rNAPc2) was used, a selective inhibitor of the TF/factor VIIa pathway \(^36\). In chapters 10 and 11, the role of the endogenous thrombomodulin/APC system in the pulmonary infection with \(M.\ tuberclosis\) or the bacterial respiratory pathogens \(S.\ pneumoniae\) and \(Klebsiella\ pneumoniae\) is investigated. Finally, in chapter 12 the effect of tranexamic acid (a synthetic anti-fibrinolytic substance) on the activation of the coagulation pathway, granulocytes, endothelial cells and the cytokine network in healthy humans injected with a single dose of LPS was examined.
References


