Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Chapter 1

General introduction
Setting the scene...

The global burden of severe bacterial infections

Severe bacterial illnesses remain a leading cause of death. Tuberculosis (TB) is one of the most common causes of bacterial infection worldwide\(^1,2\). Melioidosis, caused by the Gram-negative bacterium *Burkholderia (B.) pseudomallei*, is a common cause of community-acquired sepsis in Northeast Thailand and Northern Australia\(^3\). The clinical presentation of melioidosis may mimic tuberculosis as both cause, for example, chronic suppurative lesions unresponsive to conventional antibiotics and both commonly affect the lungs. The two diseases have overlapping risk profiles (e.g., diabetes, corticosteroid use) and both *B. pseudomallei* and *Mycobacterium (M.) tuberculosis* are intracellular pathogens. There are however important differences: the majority of melioidosis cases are acute, not chronic, and present with severe sepsis and a mortality rate that approaches 50% despite appropriate antimicrobial therapy\(^3\). By contrast, TB characteristically is a chronic illness with a mortality <2% with appropriate antimicrobial chemotherapy yet with an enormous morbidity\(^1,2\). Not surprisingly, numerous groups work on the unravelling of the pathogenesis of both TB and melioidosis. The coagulation system plays a central role in the immunological derangements which occur during severe bacterial infections. In its most extreme form the coagulopathy of severe sepsis manifests as disseminated intravascular coagulation, which has been shown to be an independent predictor of organ failure and mortality. The role of the coagulation system during TB and melioidosis however remains ill defined.

Tuberculosis

**Epidemiology**

TB is one of the most devastating infectious diseases\(^1,2,4\). Overall, about one-third of the world population, which involves $2 \times 10^9$ people, is currently infected with *M. tuberculosis*. However, 10% of these infected people will develop the active disease, while in 90% the pathogen is contained as asymptomatic latent infection\(^2,4\). In 2012, 8.7 million people worldwide were registered by the World Health Organisation with active TB and 1.4 million people died from this disease that year\(^1\). HIV-infection and other conditions during which T-cell immunity is suppressed are associated with higher incidences of (reactivation of) TB\(^1,2,4\). TB typically is a disease of developing countries as the incidence in Western Europe and Northern America is low. The majority of cases in these regions occur in foreign-born residents and recent immigrants from countries in which TB is endemic. High incidences are especially found in countries in Southeast Asia, such as India, China, Cambodia, Myanmar and Bangladesh and in sub-Saharan Africa, including South-Africa, Mozambique and Zimbabwe (for example, up to 993 new cases per 100.000 in South Africa in 2012)\(^3\). In comparison, in the Netherlands the incidence in 2011 was 6 per 100.000 and this number is still decreasing over time\(^3\).
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Clinical presentation
TB is acquired via mycobacteria-containing aerosols, spread by patients suffering from pulmonary TB who may expel bacteria after severe coughing. Infection with *M. tuberculosis* typically causes pulmonary TB, with symptoms of chronic cough, sputum production, loss of appetite, weight loss, fever, night sweats and hemoptysis. However, also extrapulmonary disease manifestations occur upon either lymphogenous or hematogenous dissemination of bacteria, which are present in 20%-40% of all TB patients. Extrapulmonary TB can affect any organ such as the lymph nodes, pleural cavity, the musculoskeletal system or the central nervous system. Musculoskeletal TB in particular, if left untreated, can cause severe complications such as paraspinal abscesses, spinal cord compression, spine deformations and neurological deficits, including radicular pain and even severe paraplegia. Without treatment, mortality rates due to TB are high. In studies of the natural history of the disease among sputum smear-positive and HIV-negative cases of pulmonary TB, around 70% died within 10 years. Additionally, multi-drug resistant TB has become a serious threat.

Pathogenesis
High mortality rates and difficulties in treatment of multi-resistant TB emphasize the importance of understanding antimycobacterial host defense mechanisms. Once inhaled by the host, *M. tuberculosis*-containing aerosols are phagocytosed by alveolar macrophages in the lungs. This induces a localized pro-inflammatory response, characterized by recruitment of mononuclear cells from the neighbouring blood vessels and upregulation of several pro-inflammatory cytokines and chemokines. Tumour necrosis factor (TNF)-α, produced by infected macrophages, plays a dominant role. Activation of TNF-α, together with activation of various chemokines induces recruitment of lymphocytes including natural killer cells, CD4+ and CD8+ T-cells and B-cells. Macrophages and lymphocytes are the main component of granuloma or tubercles, which are typical for TB. Granuloma consist of a kernel of infected macrophages, surrounded by foamy macrophages and other mononuclear phagocytes, with a mantle of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components. Tubercle bacilli are ‘walled-off’ inside granuloma resulting in a chronic pulmonary pro-inflammatory state during which mycobacteria are viable but not able to replicate. A proper T helper-1 (TH1) response is essential for maintaining this chronic, balanced state and mainly requires production of interferon (IFN)-γ and interleukin (IL)-12. IFN-γ, produced by CD4+ T-cells, activates phagocytes to contain mycobacteria by stimulation of intracellular killing. IL-12 promotes differentiation of pluripotent T-cells into CD4+ TH1-cells. The importance of IFN-γ was nicely demonstrated by IFN-γ deficient mice, that were unable to turn-off the pro-inflammatory response after infection with *M. tuberculosis* resulting in fatal pathology in their lungs. Moreover, patients with genetic deficiencies in their IFN-γ of IL-12 signalling pathways have an increased risk for TB. When the immune status of the host changes, for example at old age, malnutrition or HIV co-infection, granuloma may become necrotic, rupture and thousands of viable infectious mycobacteria might be spread from the airways via aerosols facilitated by a productive cough.
Introduction

Melioidosis

Epidemiology

Melioidosis is a serious disease common in South-East Asia and Northern Australia. It is caused by the aerobic soil-dwelling intracellular Gram-negative bacterium *B. pseudomallei*. In the most endemic regions, annual incidence is up to 50 new cases per 100,000. Melioidosis is the third most common cause of death from infectious diseases in Northeast Thailand, exceeded only by HIV-infection and TB. Melioidosis also occurs in Malaysia, Singapore, Vietnam, Cambodia, and Laos. Occasionally, cases of melioidosis are seen in Western Europe, especially in travelers returning from regions in which melioidosis has a high incidence. In endemic areas, *B. pseudomallei* can be isolated from wet soils and rice paddies. Patients usually become infected after percutaneous inoculation, via injured skin (in rice farmers) or via inhalation. After the Asian tsunami in 2004, for example, many people acquired melioidosis after inhalation, ingestion, or aspiration.

Figure 1. Tuberculosis. Mycobacterial cultures on a Middlebrook 7H11-plate (A); Ziehl-Neelsen staining of acid-fast *Mycobacterium tuberculosis* bacteria (purple rods) in human lung tissue (B, magnification 1000x); Granuloma in a human tuberculosis-positive lung tissue section (C, magnification 40x); Macrophage (purple) opsonizing mycobacteria (blue) (D, *printed with permission of S. H. E. Kaufmann*).

Melioidosis

Epidemiology

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of contaminated water, mud and soil\textsuperscript{20, 21}. Since up to 80\% of patients with melioidosis have one or more risk factors for the disease, it has been suggested that melioidosis should be considered an opportunistic infection that is unlikely to be fatal in previously healthy persons, provided that the infection is diagnosed early and appropriate antibiotics and intensive care facilities are available\textsuperscript{16}. Risk factors for melioidosis are diabetes, heavy alcohol use, chronic pulmonary and renal disease, thalassemia and, to a lesser extent glucocorticoid therapy and cancer\textsuperscript{16, 18}.

**Clinical presentation**

Once invaded the host, *B. pseudomallei* spreads rapidly throughout the body. The most common clinical presentation is pneumonia, seen in about 50\% of all infected patients, but also other disease manifestations occur such as genitourinary infections, skin infections, septic arthritis or osteomyelitis or parotitis\textsuperscript{3, 16, 22}. Melioidosis might also rarely present as a chronic disease with subclinical symptoms for over 2 months\textsuperscript{3, 22}. As in these chronic conditions symptoms resemble other diseases such as TB, melioidosis is often called ‘The Great Mimicker’\textsuperscript{23}. The most dramatic presentation of melioidosis is sepsis and septic shock, occurring in about 20\% of all infected patients in Southeast Asia. The mortality of the primary disease is high and varies from 20 to 50\% despite proper antibiotics\textsuperscript{15, 16}. Treatment of patients with melioidosis is often difficult with slow fever clearance-times, a need for prolonged antibiotic therapy and a high relapse rate if therapy is not fully completed and only a limited number of antibiotics are suitable to treat active melioidosis\textsuperscript{3}. Due to these characteristics, *B. pseudomallei* is recently classified as a ‘Tier 1’ disease agent considered to be an exceptional threat to security\textsuperscript{24}.

**Pathogenesis**

*B. pseudomallei* can invade, survive and replicate in a range of phagocytic and nonphagocytic cells and this intracellular behavior is thought to be crucial for disease pathogenesis\textsuperscript{3, 17}. Pattern recog-
Introduction

Pattern recognition receptors, including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) expressed on host cells become activated and initiate the host immune response against invading *B. pseudomallei*. This leads to activation of nuclear factor-κB via the release of pro-inflammatory cytokines of which TNF-α, IL-1β, IL-6, IFN-γ and IL-12 are most important17, 25, 26. Via activated cytokines neutrophils are recruited towards the site of infection and, additionally, the complement and coagulation cascade become activated, all in order to fight the invading bacteria17, 27. An overwhelming pro-inflammatory response, however, can cause collateral damage and might drive severe tissue injury and organ damage, that may further aggravate the clinical condition of the host. Interestingly, gene-expression profiles revealed partially similar host responses to melioidosis and TB as both were dominated by IFN-γ signaling pathways28.

Coagulation, the protein C system and fibrinolysis

Coagulation

During inflammatory conditions proteins involved in coagulation and fibrinolysis become activated as part of the host immune response. Blood coagulation consists of a cascade of coagulation factors that may activate each other finally leading to formation of cross-linked fibrin, the major component of a solid blood clot29 (Figure 3). The cascade is initiated by binding of coagulation factor VIIa to tissue factor (TF), followed by activation of factor IX and X. Together with factor VIIIa and Va, factor X and II (prothrombin) are activated into active factor Xa and thrombin respectively. Thrombin is able to convert fibrinogen into fibrin, which forms a solid clot, the end product of blood coagulation29. In the physiological situation coagulation is balanced by anti-coagulant factors including TF-pathway inhibitor (TFPI), antithrombin and activated protein C (APC).

The protein C system

APC and the protein C (PC) system are of particular importance during pro-inflammatory conditions, as besides anti-coagulant effects, APC also has distinct cytoprotective, anti-inflammatory and anti-apoptotic properties30. The vitamin K-dependent zymogen PC, discovered in 1976 by Stenflo *et al*31 is activated into APC by the thrombin-thrombomodulin complex (Figure 3). Thrombomodulin is a transmembrane protein expressed on the surface of vascular endothelial and various hematopoietic cells, with distinct domains. Of these the thrombomodulin epidermal growth factor domain mediates PC activation, whereas the lectin-like domain is responsible for several anti-inflammatory functions30, 32. The rate of this conversion of PC into APC is dramatically enhanced when PC binds to the endothelial protein C receptor (EPCR)33. Dissociated from the EPCR, APC exerts its anticoagulant activities when bound to cell membrane surfaces, microparticles and lipoproteins. This results in proteolytical degradation of (activated) coagulation factors Va and VIIIa, with protein S and various lipids acting as cofactors. When APC remains bound to EPCR it may exert cytoprotective effects for which association of protease-activated receptor (PAR)-1 with
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The APC-EPCR complex is required. Cytoprotective activities include APC-mediated alteration of gene expression, anti-inflammatory effects, anti-apoptotic effects and protection of endothelial barrier functions. For example, APC restores vascular barrier disruptions through PAR-1-dependent activation of the sphingosine-1-phosphate receptor-1 (S1P1) pathway. On the other hand, the cytoprotective effects of APC can also be mediated via EPCR-independent, CD11b/CD18-PAR-1 dependent mechanisms, induced via direct binding of APC to activated \( \alpha_{IIb}\beta_{3} \), \( \alpha_{IIb}\beta_{1} \), and \( \alpha_{V}\beta_{3} \) integrins or, as very recent data showed, be mediated via EPCR-PAR-3 dependent pathways. Finally, APC is capable of cleaving histones, major mediators of cell death in sepsis, which also reduces cytotoxicity.

**Fibrinolysis**

Fibrinolysis concerns the process of degradation of fibrin, the end product of coagulation activation (Figure 3). The main enzyme plasmin cuts cross-linked fibrin into smaller fragments that are further
cleared by other proteases. Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are the main activators of the fibrinolytic system by virtue of their capacity to convert the inactive precursor plasminogen into its active form plasmin. The pro-fibrinolytic effects of tPA and uPA are counteracted by plasminogen activator inhibitor type 1 (PAI-1). Furthermore, fibrinolysis can be inhibited directly by alpha-2 antiplasmin (A2AP). Although little is known about other properties of tPA and A2AP that extend beyond its role in activation and inhibition of fibrinolysis respectively, PAI-1 has been implicated in other processes and diseases that are not or only partially related to its capacity to inhibit plasminogen generation, including inflammatory conditions.

Coagulation and fibrinolysis during pro-inflammatory conditions

Coagulation and inflammation
Ample evidence has shown that during severe pulmonary infections and associated sepsis activation of inflammatory pathways is accompanied by hemostatic changes. These changes, often elicited via release of the pro-inflammatory cytokines TNF-α and IL-6, include increased procoagulant activity, decreased expression of anticoagulant factors, of which APC is of major importance, and suppression of the fibrinolytic system, which in most severe cases can result in disseminated intravascular coagulation and microvascular thrombosis. Low levels of PC and APC were measured in the lungs of patients with severe pneumonia and pneumonia-induced sepsis, and these levels appeared to correlate with the occurrence of organ dysfunction and an adverse outcome. As a consequence, interfering in the coagulation system was thought to be a promising strategy in the treatment of severe pneumonia and (pneumo)sepsis and the PC system seemed to be the most attractive target. Indeed, treatment of severe sepsis patients with intravenous recombinant human APC (rhAPC) was reported to strongly reduce mortality in the PROWESS (Protein C Worldwide Evaluation in Severe Sepsis) study published in 2001. However, a recently completed confirmatory trial in septic shock patients (PROWESS-SHOCK) did not show any benefit from APC treatment, which led to the withdrawal of this compound from the market.

Coagulation and fibrinolysis in tuberculosis and melioidosis
Previous research has demonstrated that also during TB and melioidosis the coagulation system becomes activated. Pulmonary TB, although a more chronic disease, has demonstrated to be associated with a hypercoagulable state, with elevated plasma fibrinogen levels and increased platelet aggregation, activation of fibrinolysis and downregulation of anticoagulant factors such as antithrombin and PC. Fulminant TB can even result in disseminated intravascular coagulation. Moreover, a link between TB and deep venous thrombosis has been made by several case reports and small series, further pointing to a procoagulant state in these patients. Finally, in vivo studies demonstrated that the protein C system may play an important role during pulmonary TB. Mice with a mutation in the TM-gene resulting in minimal capacity for APC generation...
(TMpro/pro mice) demonstrated uncontrolled lung inflammation and a reduced survival after intra-
nasal infection with *M. tuberculosis*. Little knowledge, however, exists about the possible direct
role of these parameters of coagulation and fibrinolysis during pulmonary TB. Comparative to TB,
melioidosis is also associated with activation of pro-coagulant and fibrinolytic factors and
impairment of anti-coagulant mechanisms as was shown by recent clinical studies in Ubon
Ratchathani, Thailand. These results point to an important role in the pathogenesis of this disease.
However, current data remain descriptive and in-depth analyses of the effects of the separate proteins
involved in coagulation and fibrinolysis are lacking.

**Aim and outline of this thesis**

The overall aim of this thesis is to expand our knowledge on the role of the coagulation system as
part of the host immune response during TB and melioidosis. More specifically, by investigating
the role of a variety of distinct coagulation factors and parameters of anti-coagulation and fibrinolysis
during infection with *M. tuberculosis* or *B. pseudomallei*, we try to gain insight in the contribution
of these parameters to the pathogenesis of these diseases.

Our key objectives were (I) to explore the role of coagulation and fibrinolysis during pulmonary
TB and (II) to investigate the role of coagulation and fibrinolysis during melioidosis. Furthermore,
we performed additional studies concerning lung inflammation and bone TB. To evaluate our key
objectives this thesis is divided into three parts.

After the general introduction, **Part I** describes studies in TB patients and in our experimental
model of murine TB. This part starts with **chapter 2** involving a research project performed in
Chittagong, Bangladesh. We here present results of measurements of a large range of parameters of
inflammation, coagulation, anticoagulation and fibrinolysis in sputum-positive TB patients. Then,
**chapter 3** characterizes the roles of EPCR and APC during murine TB, which is followed by
**chapter 4** focusing on the role of the lectin-like domain of thrombomodulin in the same model of
murine TB. Finally, **chapter 5** characterizes the role of PAI-1 during murine TB.

**Part II** aims to characterize the roles of coagulation and fibrinolysis during murine melioidosis.
The first chapters focus on APC and the protein C system in melioidosis: **chapter 6** describes the
effects of blocking endogenous APC with specific anti-(A)PC antibodies during murine melioidosis,
while **chapter 7** reports on the effects of a thrombomodulin mutation that impairs endogenous
APC generation. **Chapter 8**, on the other hand, explores the outcome during endogenous over-
xpression of APC in murine melioidosis. Next, **chapter 9** focuses on the role of an important recep-
tor for APC, EPCR, by using mice both overexpressing endogenous EPCR and mice deficient for
EPCR. **Chapter 10** describes the effects of deficiency of PAR-1, the receptor responsible for many
of the cytoprotective effects of APC, during murine melioidosis, while finally, chapter 11 reports on the role of the lectin-like domain of thrombomodulin in the same model of murine melioidosis. The last three chapters of Part II concern the topic of fibrinolysis: first, we explore the effects of the pro-fibrinolytic factor tPA during melioidosis in chapter 12, followed by investigations on the role the fibrinolysis inhibitors PAI-1 and A2AP during murine melioidosis in chapters 13 and 14 respectively.

Part III provides additional studies on lung inflammation and TB. Chapter 15 describes a human volunteer study exploring the effects of intrabronchially administrated APC on LPS-induced lung inflammation. Next, chapter 16 describes a new murine model of musculoskeletal TB that we recently developed. The results and potential implications of our investigations are summarized and finally discussed in chapter 17.
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