On Toll-like receptors and the innate immune response in sepsis caused by Burkholderia pseudomallei (melioidosis)

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On Toll-like receptors and the innate immune response in sepsis caused by *Burkholderia pseudomallei* (melioidosis)

Willem Joost Wiersinga
On Toll-like receptors and the innate immune response in sepsis caused by *Burkholderia pseudomallei* (melioidosis).

Dissertation, University of Amsterdam, the Netherlands

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Cover figures. On front cover: rice farmers in south-east Asia are at increased risk of contracting melioidosis; hospital ward in Sappasitthiprasong Hospital, Ubon Radchathani, Thailand; clinical isolates of *Burkholderia pseudomallei* (picture courtesy of Vanaporng Wuthiekanun and Narisara Chantratita); pulmonary infiltrates in a mouse infected with *B. pseudomallei* (picture courtesy of Sandrine Florquin). On the back cover: encounter between *B. pseudomallei* (arrows) and a mouse monocyte (scanning electron microscope); schematic representation of host-pathogen interaction in melioidosis.

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On Toll-like receptors and the innate immune response in sepsis caused by *Burkholderia pseudomallei* (melioidosis)

Academisch proefschrift

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op vrijdag 7 november 2008, te 14.00 uur

door

Willem Joost Wiersinga

geboren te Amsterdam
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Voor mijn ouders en Ilja
# Table of Contents

List of contributors 8

Common abbreviations 11

1. Setting the scene: introduction and outline of the thesis. 13

## Part I: Melioidosis, insights into the pathogenicity of *B. pseudomallei*


3. Inflammation patterns induced by different *Burkholderia* species in mice. *Cellular Microbiology*, 2008 45


## Part II: Toll-like receptors and the innate immune response in melioidosis


9. MyD88 dependent signaling contributes to protective host defense against *Burkholderia pseudomallei*. Submitted 145
10 CD14 impairs host defense against gram-negative sepsis caused by *Burkholderia pseudomallei* in mice. *Journal of Infectious Diseases, 2008*

11 Immunosuppression associated with IRAK-M upregulation predicts mortality in gram-negative sepsis (melioidosis). *Critical Care Medicine, 2008*

12 Expression profile and function of triggering receptor expressed on myeloid cells (TREM)-1 in melioidosis. *Journal of Infectious Diseases, 2007*

13 Expression and function of macrophage migration inhibitory factor (MIF) in melioidosis. *Submitted*

**Part III: Coagulation and fibrinolysis in melioidosis**

14 Coagulation in sepsis. *Update in Intensive Care and Emergency Medicine, 2007*

15 Activation of coagulation with concurrent impairment of anti-coagulant mechanisms correlates with a poor outcome in severe melioidosis. *Journal of Thrombosis and Haemostasis, 2008*

16 Urokinase receptor is necessary for bacterial defense against pneumonia-derived septic melioidosis by facilitating phagocytosis. *Submitted*

17 Summary, general discussion and conclusion.

Samenvatting voor niet-ingewijden

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### COMMON ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALAT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ASAT</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BAL(F)</td>
<td>Bronchoalveolar lavage (fluid)</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit(s)</td>
</tr>
<tr>
<td>HMGB-1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>IRAK-M</td>
<td>IL-1R-associated-kinase-M</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MLPA</td>
<td>Multiplex ligation-dependent probe amplification</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary-response gene 88</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor type I</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PAPc</td>
<td>Plasmin-α2-antiplasmin complexes</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin-time</td>
</tr>
<tr>
<td>PTT</td>
<td>Activated-partial-thromboplastin-time</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHIP-1</td>
<td>Src-homology-2-domain-containing inositol-5-phosphatase-1</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>Single-immunoglobulin-interleukin-1R-related-molecule</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor-of-cytokine signalling</td>
</tr>
<tr>
<td>TATc</td>
<td>Thrombin-antithrombin complexes</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>TREM-1</td>
<td>Triggering receptor expressed on myeloid cells</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor protein-inducing interferon-β</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase receptor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
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Chapter 1

Setting the scene:
General introduction and outline of the thesis

W. Joost Wiersinga

Center for Infection and Immunity Amsterdam (CINIMA), Center for Experimental and Molecular Medicine, University of Amsterdam, Academic Medical Center, Amsterdam, the Netherlands
Melioidosis and *Burkholderia pseudomallei*

*Burkholderia pseudomallei*, the aerobic gram-negative bacterium causing melioidosis, is an environmental saprophyte that can be isolated from wet soils and rice paddies in endemic areas. The disease is acquired through soil or water contamination of skin abrasions (rice farmers), or by inhalation. The majority of infections occurs in south-east Asia and northern Australia, predominantly during the rainy season. In north-eastern Thailand melioidosis accounts for 20% of all community acquired septicemias, with a high mortality rate of almost 40%. The clinical spectrum of melioidosis and Australia is similar, with a high proportion (50%) of cases presenting as pneumonia. The clinical presentation of melioidosis and its severity are highly variable, however, and besides sepsis more chronic localized infections or even subclinical infections occur. Indeed, the seroprevalence of *B. pseudomallei* can be as high as 80% in endemic areas. Clinical trial evidence supports the use of ceftazidime or a carbapenem antibiotic for initial parenteral therapy. This is followed by a prolonged course of oral antimicrobial therapy with trimethoprim-sulfamethoxazole (TMP-SMX) with or without doxycycline to prevent relapses.

Interest in the pathogenesis of *B. pseudomallei* has increased following their recent classification as category B agents by the US Centers for Disease Control and Prevention. It now well established that the pro-inflammatory cytokine interferon (IFN)-γ plays an important role in early resistance against *B. pseudomallei* infection. Inhibition of IFN-γ expression in mice lowered the LD50 from $>5 \times 10^5$ to $\sim$2 colony-forming units (CFUs) and was associated with an 8,500- and 4,400-fold increase in bacterial loads in liver and spleen, respectively. Inhibition of interleukin (IL)-12 or IL-18, the predominant endogenous inducers of IFN-γ production, resulted in increased mortality in the same model. The pro-inflammatory cytokine tumor-necrosis factor (TNF)-α is also likely to be an important element of the early immune response, as passive immunization against this mainly macrophage derived cytokine increased mortality in experimental murine melioidosis. Serum IFN-γ, IL-12 and TNF-α concentrations are elevated in melioidosis patients; the involvement of TNF-α in human disease is further suggested by a report that the $-308$ TNF-α promoter polymorphism, which is related to severity of disease for several other infectious diseases, was associated with both the occurrence and severity of melioidosis.

Host defense against invading pathogens

The immune response to microbial pathogens relies on both innate and adaptive components. Adaptive immunity is mediated by clonally distributed T and B lymphocytes that provide immunological specificity and memory. In contrast, the innate immune response traditionally has been considered non-specific. In the last decade, it has become clear that the innate immune system can specifically recognize groups of microorganisms, and that it not only provides a first line of antimicrobial defense, but also has a profound impact on the establishment of adaptive immune responses. The innate immune system discriminates potential pathogens from self with the use of a series of receptors that recognize conserved motifs on pathogens that are not found
Toll-like receptors (TLR) and pathogen recognition. The TLR family discriminates between specific patterns of microbial components. TLR2, which can associate with TLR1 and TLR6, is essential for the recognition of microbial lipopeptides. TLR4 recognizes lipopolysaccharide (LPS). LPS first binds to LPS-binding protein (LBP), which transfers LPS to CD14. Binding of LPS to CD14 leads to the association of CD14 with MD2 and TLR4. TLR5 is a receptor for flagellin. TLR9 is the CpG DNA receptor, whereas TLR3 and TLR7 are implicated in the recognition of viral double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA), respectively. After TLR stimulation, the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88) is recruited. This will lead to the release of nuclear factor (NF)-κB, which will result in the transcription of a whole range of inflammatory genes. Next to MyD88, the adaptor molecules Toll/interleukin receptor (TIR) domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor protein-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) have been identified. TIRAP is essential for MyD88-dependent signaling through TLR2 and TLR4. TRIF is essential for the TLR3- and TLR4-mediated activation of the MyD88-independent pathway. TRAM is involved in TLR4-mediated MyD88-independent/TRIFdependent signaling pathways. Figure adapted from: Wiersinga WJ and van der Poll T, Is the septic response good or bad, Current Infectious Disease Reports 2007, 9:366–37317.
in higher eukaryotes. These motifs have been termed “pathogen-associated molecular patterns” or PAMPs, whereas their cognate binding partners on host cells involved in the innate immune response have been named “pattern-recognition receptors” or PRRs\(^\text{10}\). Examples of PAMPs include lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria, peptidoglycan (present in most bacteria), flagellin, and mannans in the yeast cell wall (see Figure).

**Toll-like receptors**

The family of Toll-like receptors (TLRs) has been implicated to play a central role as PRRs in the initiation of cellular innate immune responses\(^\text{10,11}\). TLRs are distinguished from other PRRs by their ability to recognize, and more significantly, discriminate between different classes of pathogens. TLR4 is considered to be the principal (signaling component of) the LPS receptor. Activation of TLRs is generally considered important for mounting an effective innate immune response. For gram-negative infection and TLR4 this notion is primarily derived from early studies with C3H/HeJ mice, which have a mutant, nonfunctional TLR4 gene\(^\text{12}\) and display a diminished capacity to clear intraperitoneally administered *Salmonella typhimurium*\(^\text{13}\). Our laboratory extended this observation to pneumonia caused by the gram-negative bacterium *Klebsiella pneumoniae*, *i.e.* TLR4 mutant mice demonstrated an enhanced bacterial outgrowth in their lungs associated with an increased lethality\(^\text{14}\). However, TLR4 mutant mice displayed a normal resistance against *Legionella pneumophila*\(^\text{15}\), like *B. pseudomallei* an intracellular gram-negative bacterium, indicating that TLR4 might not be essential for an adequate host defense against all gram-negative (LPS containing) microorganisms.

**Inflammation and coagulation**

Activation of inflammatory and coagulation pathways is an important event in the pathogenesis of sepsis\(^\text{16}\). In sepsis, which can be defined as the disadvantageous systemic host response to infection, the blood coagulation system is triggered. Activation of coagulation and deposition of fibrin as a consequence of inflammation can be considered instrumental in containing inflammatory activity to the site of infection. However, inflammation-induced coagulation may be detrimental in those circumstances when the triggered blood coagulation system is insufficiently controlled, which can lead to the clinical syndrome of disseminated intravascular coagulation and microvascular trombosis. In recent years, the roles of several elements of the hemostatic mechanism during sepsis have partly been unraveled, including those of tissue factor, thrombin, protease-activated cell receptors, urokinase receptor (uPAR) and activated protein C. Knowledge of the role of the coagulation and fibrinolysis system in the pathogenesis of melioidosis and its interplay with inflammation is highly limited.
Chapter 1

Aim and outline of this thesis

The overall aim of this thesis is to increase our insight into the pathogenesis of melioidosis in order to identify possible new treatment targets for this debilitating disease. More specifically we focused on host-pathogen interactions in melioidosis. Our key objective were (I) to further characterize the innate immune response during melioidosis, (II) to determine the potential role of TLRs in the innate recognition of \textit{B. pseudomallei} and (III) to obtain more insight into both coagulation and fibrinolysis in melioidosis. To evaluate these three key objectives the thesis is divided into three parts.

Part I starts with \textbf{chapter 2} which serves as a general introduction into the world of melioidosis, \textbf{chapter 3} describes our mouse model of melioidosis and typifies the inflammatory patterns elicited by different \textit{Burkholderia} species in mice, while \textbf{chapter 4 and 5} characterize the inflammatory gene-expression profile in humans and mice with severe melioidosis. More specifically, \textbf{chapter 6} investigates the role of IL-18 in disease pathogenesis.

Part II focuses on the role of TLRs in melioidosis and sets off with an overview of current knowledge on TLRs in sepsis (\textbf{chapter 7}). \textbf{Chapter 8} characterizes the TLR expression profile in melioidosis and studies the role of TLR2 and TLR4 as recognition receptors in this gram-negative disease. Additionally, in \textbf{chapter 9} the function of myeloid differentiation primary-response gene 88 (MyD88), the key TLR signaling molecule, is investigated during experimentally induced melioidosis, whereas \textbf{chapter 10} addresses the role of CD14 in host defense against gram-negative sepsis caused by \textit{B. pseudomallei}. The last chapters of Part II describe studies on the regulation of TLR signaling during melioidosis: \textbf{chapter 11} is on immunosuppression and the negative regulators of the TLR-cascade, \textbf{chapter 12} centers on triggering receptor expressed on myeloid cells (TREM)-1, which has been identified as an amplifier of TLR signaling. Finally, \textbf{chapter 13} is on macrophage migration inhibitory factor (MIF) which has been described to be capable of TLR4 upregulation.

The last part, \textbf{part III}, describes our studies on coagulation and fibrinolysis in melioidosis. \textbf{Chapter 14} represents a review of the interplay between inflammation and coagulation in sepsis. \textbf{Chapter 15} reports on activation of coagulation and fibrinolysis in patients with septic melioidosis. Lastly, \textbf{chapter 16} describes the role of the fibrinolytic actor uPAR in the host defense against melioidosis. The results and potential implications of our studies are summarized and discussed in \textbf{chapter 17}. We sincerely hope that the increased knowledge of the innate immune response to \textit{B. pseudomallei} gained by the work presented here can make a contribution towards the development of new therapeutic strategies in patients with melioidosis.
REFERENCES

Part I

Melioidosis, insights into the pathogenicity of *B. pseudomallei*
Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*


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Chapter 2

**ABSTRACT**

Melioidosis is caused by the aerobic Gram-negative soil-dwelling bacillus *Burkholderia pseudomallei* and is an important cause of severe sepsis in Southeast Asia and Northern Australia. The high associated mortality rate, wide availability from the environment in endemic areas, intrinsic resistance to many antibiotics and the potential for aerosol spread has made this organism a potential bioterror agent.

The genome of *B. pseudomallei* consists of a large chromosome (4.07 Mb) carrying genes mainly associated with cell growth and metabolism, and a smaller chromosome (3.17 Mb) which has a greater proportion of genes encoding accessory functions such as adaptation and survival in different environments. Approximately 6% of the genome is made up of genomic islands that have probably been acquired via horizontal gene transfer.

Factors associated with disease acquisition in endemic regions include adverse weather conditions, route and size of inoculum and integrity of the host immune system. The geographical incidence of *B. pseudomallei* and typical clinical features of the disease are reviewed here. No single *B. pseudomallei* determinant has been shown to have a role in virulence during human disease, persistence or latency. However, putative virulence factors include quorum sensing, type III secretion system, capsular polysaccharide and, with less conclusive evidence, lipopolysaccharide and flagella.

*B. pseudomallei* is an intracellular pathogen that multiplies within macrophages. Recent data have shed light on the mechanisms that this bacterium uses to adapt to and exploit the intracellular environment. IFNγ and TNFα play an important role in early resistance against *B. pseudomallei* infection. Although more is becoming known about the pathogenesis of this bacterium in disease, host-pathogen interactions are still ill defined.

Potential new therapies which warrant further clinical evaluation include granulocyte colony-stimulating factor, CpG (bacterial DNA), low dose hydrocortisone and activated protein C. Development of an effective human melioidosis vaccine is a research priority.
Melioidosis

**PREFACE**

*Burkholderia pseudomallei* is a recognized bioterror agent and the cause of melioidosis, a severe disease endemic to areas of Southeast Asia and Northern Australia. Infection is often associated with bacterial dissemination to distant sites; there are many possible disease manifestations, melioidosis septic shock being the most severe. Eradication of the organism following human infection is difficult, with slow fever clearance time, the need for prolonged antibiotic therapy and a high rate of relapse if therapy is not completed. Mortality in melioidosis septic shock remains high despite appropriate antimicrobial therapy. Prevention of disease, and a reduction in mortality and the rate of relapse are priority areas for future research efforts. Studying how the disease is acquired together with the host-pathogen interactions involved underpins such efforts; this review presents an overview of current knowledge in this area, highlighting key topics for evaluation.

**INTRODUCTION**

Melioidosis is a serious disease caused by the aerobic Gram-negative soil-dwelling bacillus *Burkholderia pseudomallei* and is most common in Southeast Asia and Northern Australia — for example, melioidosis is responsible for 20% of all community-acquired septicemias and 40% of sepsis-related mortality in northeast Thailand. Reported cases are likely to represent ‘the tip of the iceberg’ as confirmation of disease depends on bacterial isolation, a technique that is not available in many of the affected areas. Melioidosis often affects individuals with one or more pre-existing conditions associated with an altered immune response, most common of which is diabetes mellitus (50% of cases). The most feared clinical picture is melioidosis septic shock, often associated with pneumonia and bacterial dissemination to distant sites (Fig. 1). Melioidosis has been called the great mimicker and can present with a wide array of signs and symptoms. There can be a prolonged period between exposure and the clinical manifestations of infection (the longest recorded incubation period documented is 62 years). Furthermore, recurrence of infection is common despite adequate antimicrobial therapy. *B. pseudomallei* is intrinsically resistant to many antibiotics (including penicillin, first- and second-generation cephalosporins, macrolides, rifamycins, colistin and aminoglycosides), but is usually susceptible to amoxicillin-clavulanate, chloramphenicol, doxycycline, trimethoprim-sulfamethoxazole, ureidopenicillins, ceftazidime and carbapenems. Treatment is required for 20 weeks and is divided into intravenous and oral phases. Initial intravenous therapy is given for 10-14 days; ceftazidime or a carbapenem are the drugs of choice. Overall mortality for primary disease is 50% in northeast Thailand (35% in children), and ~20% overall in the higher technology setting of Northern Australia. Interest in the pathogenesis of *B. pseudomallei* and the related bacterium *Burkholderia mallei* has increased following their classification as category B agents by the US Centers for Disease Control and Prevention. This review presents an overview of current knowledge regarding disease acquisition and host-pathogen interactions.
Figure 1. Selected clinical features of melioidosis, the ‘great mimicker’
Knowledge of common manifestations can aid the recognition of melioidosis. The most feared clinical picture is melioidosis septic shock, often associated with bacterial dissemination to distant sites such as lung, liver and spleen. Lung is the most commonly affected organ in adults, and may manifest as localized or disseminated pulmonary infection, abscess formation or empyema. Chronic lung disease may occur and can be difficult to distinguish from pulmonary tuberculosis. Clinical features of disease in Thailand and Northern Australia (where most cases are reported) are largely shared, but there are some striking differences. Acute suppurative parotitis is the presenting feature in one third of Thai pediatric cases but is uncommon in Australia; conversely, prostatic abscesses and brainstem encephalitis are more frequent in Australia\(^2\), \(^4\). Pictures courtesy of Dr Wirongrong Chierakul.

Figure 2. Images of clinical isolates of \textit{B. pseudomallei} (A) Typical colony morphology of \textit{B. pseudomallei} on Ashdown’s agar after incubation at 37°C in air for 3 days. (B), (C) Colony variation is commonly seen during culture of clinical isolates on Ashdown’s agar. Figure C demonstrates variable colony morphology from a single sample; genotyping demonstrates one clonal type. Colony variation can be seen within a single colony, as shown in figure B in which the parental colony (pink) has given rise to a second morphotype (red). Pictures courtesy of Mrs Vanaporn Wuthiekanun and Mrs Narisara Chantratita.
Taxonomy and genomics

*Burkholderia pseudomallei* is an aerobic, motile, non-spore-forming bacillus (Fig. 2). The genus *Burkholderia* contains over 30 species, the most pathogenic members of which are *B. pseudomallei, B. mallei*, and in certain clinical conditions such as cystic fibrosis *B. cepacia* (Fig. 3). The genus includes *Burkholderia thailandensis*, which co-exists with *B. pseudomallei* in the soil in Thailand but rarely

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**Figure 3. Phylogenetic tree of Burkholderiaceae** Phylogenetic tree based on 16S RNA gene sequence. Most *Burkholderia* species are plant pathogens, but two groups may cause disease in man. *Burkholderia cepacia* complex (Bcc) are important opportunistic pathogens for individuals with cystic fibrosis (CF). *B. cenocepacia* causes 70% of cases of Bcc infection. *B. pseudomallei* and *B. mallei* have the potential to cause human disease, but the related *B. thailandensis* is rarely pathogenic. Modified with permission from REF.101.
causes disease and is >10^5-fold less virulent than \textit{B. pseudomallei} in Syrian hamsters or mice\textsuperscript{6}. \textit{B. mallei} causes glanders in horses and is potentially highly virulent in man, but natural disease in any host is now extremely rare. The genome of \textit{B. pseudomallei} (strain K96243 from Thailand) has been sequenced and comprises two chromosomes of 4.07 Mb and 3.17 Mb\textsuperscript{7}. The large chromosome carries many genes associated with core functions such as cell growth and metabolism, while the smaller chromosome carries more genes encoding accessory functions that could be associated with adaptation and survival in different environments. Around 6\% of the genome is made up of putative genomic islands that have probably been acquired via horizontal gene transfer. These are mostly absent from the \textit{B. thailandensis} genome (and are absent from the \textit{B. mallei} genome); it is unclear whether these regions have a role in disease pathogenesis. The Institute of Genomic Research (Rockville, MD, USA) is currently sequencing nine further \textit{B. pseudomallei} isolates and 25 \textit{B. pseudomallei} bacteriophage genomes originating from various sources\textsuperscript{8}. In addition, shotgun sequencing of the \textit{B. thailandensis} (strain E264) genome is now complete (www.tigr.org). The molecular epidemiology of \textit{B. pseudomallei} has been investigated using multilocus sequence typing, findings from which suggest a high rate of genetic recombination\textsuperscript{9}. Whole-genome comparison between \textit{B. pseudomallei} and \textit{B. mallei} suggests that \textit{B. mallei} has evolved through ‘genomic down-sizing’ from a single clone of \textit{B. pseudomallei} (this is consistent with a previous conclusion drawn from multilocus sequence typing\textsuperscript{9}). A DNA microarray based on the whole genome sequence of \textit{B. pseudomallei} K96243 has been used to compare isolates of \textit{B. pseudomallei}, \textit{B. mallei} and \textit{B. thailandensis}\textsuperscript{10}. Deleted regions in \textit{B. mallei} had significant genomic clustering compared with those in \textit{B. thailandensis}, which were more uniformly dispersed. This suggests that the evolutionary processes that result in divergence of the three species might have distinct mechanisms. Subtractive hybridization between \textit{B. pseudomallei} and \textit{B. thailandensis} has revealed several loci that are unique to the former\textsuperscript{11, 12}; publication of whole-genome comparisons between the two species is currently awaited.

\section*{FACTORS ASSOCIATED WITH DISEASE ACQUISITION}

Melioidosis is limited to individuals exposed to environments containing \textit{B. pseudomallei}; infection is acquired through inoculation, inhalation and aspiration. Factors associated with disease acquisition in endemic regions include environmental and host factors. There is no evidence that some isolates are intrinsically more infectious than others.

\section*{Exposure to \textit{B. pseudomallei} in the environment}

There is a positive association between disease incidence and extent of environmental contamination with \textit{B. pseudomallei}\textsuperscript{13-15}. However, few environmental sampling studies have been published, and a more complete picture of the geographical distribution of \textit{B. pseudomallei} can be derived from reported cases of melioidosis. This topic has recently been reviewed by Cheng and Currie\textsuperscript{4}. The disease is endemic in parts of Thailand, Northern Australia, Malaysia, Singapore, Vietnam and Burma. Possible endemic areas include Southern India, Southern China and Hong
Melioidosis

Kong, Taiwan, Brunei, Laos, and Cambodia. Sporadic cases and occasional clusters have been reported from large areas of Asia, the Americas (notably Brazil) and Caribbean, the Pacific, Africa and the Middle-East.

Weather conditions, route of acquisition and inoculum

Melioidosis is seasonal in the tropics where most cases occur during the rainy season. This can be explained by increased contact with the organism. Rice farmers plant at the start of the monsoon and work in flooded rice paddies until harvest. Thai farmers rarely wear protective footwear and their feet often show signs of repeated trauma and injuries. Extreme weather may be associated with a shift in the mode of acquisition of infection. Aerosols are created during heavy rain, and this may result in repeated inhalation of the organism. Heavy rainfall and winds consistently result in a shift towards more pneumonia in patients presenting with melioidosis in Northern Australia\(^\text{16}\). Severe or penetrating injury and near-drowning are known risk factors for melioidosis, as highlighted by a study of a cluster of melioidosis cases in Southern Thailand following the 2004 tsunami\(^\text{17}\).

Integrity of host immunity

In northeast Thailand, 80% of the population belongs to rice-farming families. Children have extensive contact with the organism, yet only one-fifth of all melioidosis cases here occur in children <14 years of age, with disease incidence peaking later between the 4\(^{\text{th}}\) and 6\(^{\text{th}}\) decade. Most affected adults (>80%) have one or more underlying diseases (most commonly diabetes mellitus or renal failure). By contrast, children have an identifiable risk factor in fewer than 30% of cases (most commonly trauma). It is unclear whether affected children have a greater genetic susceptibility for disease. It is also possible that disease in childhood is caused by a subset of the bacterial population with increased pathogenic potential.

PUTATIVE BACTERIAL VIRULENCE FACTORS

There is a paucity of knowledge in this area compared with other Gram-negative bacteria. The factors described here are included on the basis of a known role in virulence for other pathogens, or virulence in experimental models, and have been grouped according to the strength of existing evidence.

Strong putative candidates

Quorum sensing. Quorum sensing is a cell-density-dependent communication system utilized by Gram-negative bacteria that incorporates N-acyl-homoserine lactones (AHLs) for the coordination of gene expression\(^\text{18, 19}\). LuxI proteins are responsible for AHL biosynthesis while LuxR transcriptional regulators, following association with their cognate AHL(s), mediate gene repression or expression\(^\text{18, 19}\). The \textit{B. pseudomallei} genome is reported to contain three LuxI and five LuxR quorum-sensing homologs\(^\text{19}\). Mass spectrometry analysis of \textit{B. pseudomallei} culture
supernatants has demonstrated the presence of numerous signaling molecules, including N-decanoyl-homoserine-lactone and N-(3-oxotetradecanoyl)-L-homoserine-lactone\(^{19}\). Disruption of the 8 genes encoding LuxIR quorum sensing homologs all lead to a significant increase in LD\(_{50}\) in Syrian hamsters after intraperitoneal challenge, and increased the time to death and reduced organ colonization in aerosolized BALB/c mice\(^{19}\). A LuxI-LuxR homolog termed PmlII-PmlR, which directs the synthesis of N-decanoyl-homoserine-lactone and is involved in regulation of a metalloprotease, is essential for full virulence in a mouse model\(^{20}\). A homolog termed BpsI-BpsR is also required for optimal expression of virulence and secretion of exoproducts\(^{21}\). Quorum-sensing-controlled virulence factors such as siderophore and phospholipase C and biofilm formation are probably partially depended on BpeAB-OprB, a multidrug efflux pump of \(B.\) pseudomallei also known to be responsible for conferring antimicrobial resistance to aminoglycosides and macrolides\(^{22}\). BpeAB mutants showed attenuated cell invasion and cytotoxicity towards human lung epithelial (A549) and human macrophage (THP-1) cells\(^{22}\). This suggests the possibility of attenuating \(B.\) pseudomallei virulence through the use of inhibitors of the BpeAB-OprB efflux pump.

**Type III secretion system (TTSS).** \(B.\) pseudomallei contains three TTSS gene clusters\(^7,23\). One of these (TTSS3) shares homology to the inv/spa/prg TTSS of \(Salmonella\) typhimurium and the ipa/mxi/spa TTSS of \(Shigella\) flexneri\(^{23-25}\). This cluster encodes a secretion apparatus that functions like a molecular syringe; secreted proteins (‘translocators’) interact with the eukaryotic cell membrane and inoculate ‘effector’ proteins into the target cell cytosol where they subvert host cell processes (reviewed by Cornelis and Van Gijsegem\(^{26}\)). Mutations that disrupt the \(Salmonella\) Inv/Spa/Prg apparatus inhibit bacterial invasion and enteropathogenesis. The gene cluster in \(B.\) pseudomallei (designated bsa, \(Burkholderia\) secretion apparatus) encodes proteins that are very similar to the \(Salmonella\) and \(Shigella\) Inv/Mxi-Spa secreted proteins required for invasion, escape from endocytic vacuoles, intercellular spread and pathogenesis\(^{25}\).

Following internalization, \(B.\) pseudomallei escapes from endocytic vacuoles into the cytoplasm of infected cells (Fig. 6). Induction of actin polymerization at one pole leads to the formation of membrane protrusions and cell-to-cell spread. \(B.\) pseudomallei mutants lacking components of the Bsa secretion and translocation apparatus have reduced replication in murine macrophage-like cells, an inability to escape from endocytic vacuoles and cannot form membrane protrusions and actin tails\(^{25}\). Inactivation of BopE, a TTSS protein encoded adjacent to the \(B.\) pseudomallei bsa locus that is homologous to \(Salmonella\) enterica SopE/SopE2, a guanine nucleotide-exchange factor, leads to impaired bacterial entry into HeLa cells, indicating that BopE facilitates invasion\(^{27}\). \(B.\) pseudomallei bipD mutants lacking a component of the translocation apparatus are attenuated following intraperitoneal or intranasal challenge of BALB/c mice, and have impaired bacterial replication in liver and spleen\(^{28}\). \(B.\) pseudomallei BipB has been shown to mediate multinucleated giant cell formation, cell-to-cell spreading of bacteria and apoptosis of infected host cells\(^{29}\). bipB mutants are also associated with attenuated following intranasal challenge of BALB/c mice\(^{29}\).

The TTSS1 gene cluster was first described in 1999\(^{30}\). This is homologous to a TTSS of the plant pathogen \(Ralstonia\) solanacearum but is absent from \(B.\) mallei and \(B.\) thailandensis\(^7,23,31\). TTSS2 is present in \(Ralstonia\) solanacearum, \(B.\) pseudomallei, \(B.\) mallei and \(B.\) thailandensis. The role of these
two systems in pathogenesis is not known. TTSS3 has been shown to be required for full virulence in a hamster model of infection32.

**Capsular polysaccharide.** *B. pseudomallei* produces an extracellular capsular polysaccharide with the structure \(-3\)-2-O-acetyl-6-deoxy-\(\beta\)-D-manno-heptopyranose-(1-1,3). Previously characterized as type I O-polysaccharide of *B. pseudomallei*, this has more recently been considered to be a capsular polysaccharide based on its high molecular mass and genetic homology with group 3 capsular polysaccharides of other organisms. Capsular polysaccharide is required for *B. pseudomallei* virulence in experimental animal models11,34. Capsule expression is induced in the presence of serum, and the addition of purified *B. pseudomallei* capsule to serum bactericidal assays increases the survival of *B. pseudomallei* SLR5, a serum sensitive strain, by 1,000-fold35. Phagocytosis is greater for a capsule-deficient mutant compared to wild-type in the presence of normal human serum35. Both observations can be explained by the finding that deposition of complement factor C3b on the bacterial cell surface is lower in the presence of capsule35. The capsule may act as a barrier, blocking access of the complement receptor-1 (CR1) on phagocytes to the C3b deposited on the bacterial surface35.

**Other putative candidates**

**Lipopolysaccharide (LPS).** *B. pseudomallei* LPS (formerly termed type II O-antigenic polysaccharide) appears to differ in several respects from the LPS of other Gram-negative organisms. *B. pseudomallei* LPS exhibits weaker pyrogenic activity in rodents compared with enterobacterial LPS, but stronger mitogenic activity in murine splenocytes36. LPS-mediated activation of a mouse macrophage cell line in vitro is slower for LPS from *B. pseudomallei* compared with LPS from *E. coli*37. Recognition of LPS by the host is of eminent importance for the initiation of a swift innate immune response to Gram-negative bacteria primarily through activation of the pattern recognition receptor Toll-like receptor (TLR) 4 (see also further)38. Thus, the fact that *B. pseudomallei* LPS apparently is less capable of activating immune cells may at least in part explain why TLR4 does not play a role in host defense against experimentally induced melioidosis in mice, whereas mice deficient for this receptor are highly susceptible to other Gram-negative infections38 (and our own observations).

*B. pseudomallei* LPS appears to be largely conserved across this species. LPS profiling of >700 *B. pseudomallei* isolates using proteinase K digestion and SDS-PAGE silver-stained gels, a technique that examines the O-side chain, demonstrated that the majority had a ‘typical’ ladder pattern of extracted LPS, 3% had an ‘atypical’ pattern, and 0.1% do not exhibit a ladder appearance at all39. The different LPS preparations have similar endotoxic activity in the Limulus amebocyte lysate assay. However, there appears to be a difference in the host immune response to these molecules as there is a lack of immunological cross reactivity on Western blot between typical and atypical LPS using patient sera infected with typical and atypical LPS isolates39.

The level of antibody to LPS on admission to hospital is higher in patients with melioidosis who survive compared with those who die, and in patients with non-septicaemic versus septicaemic melioidosis40. These antibodies may act to protect the host against death; alternatively, there may be association between a raised anti-LPS antibody titer and a more efficient host immune response including cell-mediated killing.
Chapter 2

LPS of *B. pseudomallei* (pathogenic) and *B. thailandensis* (non-pathogenic) have been compared. LPS profiling using proteinase K digestion and SDS-PAGE silver stained gel demonstrates identical ladder patterns for the majority of isolates of both species. The two species exhibit similar immunoblot profiles against pooled sera from patients with melioidosis, and with hyperimmune mouse sera\textsuperscript{41}. LPS shedding profiles are also similar between the two species\textsuperscript{42}. This has led to the suggestion that LPS is unlikely to be involved in the virulence and pathogenicity of *B. pseudomallei*\textsuperscript{41}. Other possible explanations are that: (i) LPS from the two species are antigenically very similar but differ in biological activities, or that (ii) LPS from both species have biological activity \textit{in vivo}, but only *B. pseudomallei* has an additional complement of genes that promotes successful invasion and bacterial dissemination within the host. Caution is required when interpreting the significance of LPS in virulence as LPS truncation may impair the insertion, stability and folding of other surface-anchored molecules.

**Flagella.** *B. pseudomallei* is flagellated and motile. The ability of *B. pseudomallei* to invade and replicate in human lung cells \textit{in vitro} is not different between wild-type and an isogenic mutant defective in flagella expression\textsuperscript{43}. In one study, there was no difference between an isogenic mutant and wild-type *B. pseudomallei* in diabetic rat and Syrian hamster infection models\textsuperscript{44}. In a second study, bacterial numbers were markedly reduced in the lung and spleen of BALB/c mice following intranasal infection of an aflagellate mutant compared with wild-type, and the mutant was less virulent following intraperitoneal infection of BALB/c mice as based on LD\textsubscript{50}\textsuperscript{43}.

**Type IV pili mediated adherence.** Adherence is an important virulence mechanism mediated by carbohydrate molecules, pilus, and non-pilus adhesins. Type IV pili are important for virulence in many Gram-negative bacteria. The *B.pseudomallei* K96243 genome contains multiple type IV pilin-associated loci, including one encoding a putative pilus structural protein (pilA)\textsuperscript{45}. A pilA deletion mutant has reduced adherence to human epithelial cells and is less virulent in the nematode model of virulence and the murine model of melioidosis, suggesting a role for type IV pili in *B. pseudomallei* virulence.

**Other candidate virulence factors.** These include a siderophore for iron acquisition\textsuperscript{46} and secreted proteins such as haemolysin, lipases and proteases\textsuperscript{47}. Isolates associated with specific disease manifestations in Australian patients were examined using MLST; no association was found between the type of disease manifestations and bacterial genetic relatedness\textsuperscript{48}. Bacterial colony morphology of clinical *B. pseudomallei* isolates can vary both within a given culture and in cultures from the same patient over time (Fig 2). The relevance of this to human disease is the subject of intensive investigation in our laboratory.

**Down-regulation of virulence.** An arabinose assimilation operon consisting of nine genes is present in *B. thailandensis* and absent from the *B. pseudomallei* and *B. mallei* chromosomes. When this operon is cloned experimentally and re-introduced into a laboratory strain of *B. pseudomallei*, the mutant strain has a reduced ability to cause the death of Syrian hamsters compared with the parent strain\textsuperscript{49}. On microarray analysis, several genes in the TTSS3 cluster are down-regulated
in the mutant when cells are grown in l-arabinose, suggesting a regulatory role for (a metabolite of) l-arabinose. This could be one of many similar examples.

**Immune response in the exposed but healthy population**

Seroprevalence studies in northeast Thailand based on the indirect hemagglutination assay (IHA) show that ~80% of people have antibodies against *B. pseudomallei* by the age of 4 years. Antibodies are maintained throughout adult life in this population. It is not clear whether healthy individuals with high antibody titers are infected and have a quiescent focus (analogous to a quiescent TB infection), or whether repeated environmental exposure in a primed individual maintains high antibody levels. The strong seasonality in disease presentation, with a close association with rainfall, combined with a reported mean incubation period of 9 days, suggests that primary disease occurs as a result of new infection rather than seasonal activation of a persistent focus. It is possible that the development of underlying risk factors causing immune dysfunction is seasonal, and this could be followed by seasonal disease breakthrough as bacteria escape from immune surveillance, but this is less biologically plausible. The lack of seasonality in documented relapse argues against this hypothesis. It is not known whether an antibody response provides any degree of protective immunity.

**Host-pathogen interactions during melioidosis**

*Innate immune response* (Fig. 4). On first encounter with a pathogen, cells of the innate immune system recognize conserved surface motifs termed “pathogen-associated-molecular-patterns” or PAMPs via host cell “pattern recognition receptors”. The family of Toll-like receptors (TLRs) are important members of this surveillance system which initiate the innate immune response, and form a key link between innate and adaptive immunity. This organism expresses several PAMPs for which the corresponding TLR is known. For example, LPS activates the cells of the immune system via a receptor complex that consists of a ligand-binding molecule (CD14) and TLR4 as the signal-transducing element. Other candidate *B. pseudomallei* TLR ligands include peptidoglycan (TLR2), flagellin (TLR5) and bacterial DNA or CpG (TLR9). Although experimental data are limited for melioidosis, it is likely that recognition of TLR ligands expressed by *B. pseudomallei* would activate innate immune defense mechanisms. C3H/HeJ mice that carry a loss-of-function mutation in the *tlr4* gene are resistant to extremely high doses of *B. pseudomallei* LPS (up to 10,000 ng/mouse). However, our own unpublished data indicate that TLR4-deficient mice display an unaltered defense response in a murine model of melioidosis. These differences require further clarification; our laboratory is currently defining the expression and function of a range of TLRs in mice infected with *B. pseudomallei* and in melioidosis patients.

The pro-inflammatory cytokine interferon (IFN)-γ plays an important role in early resistance against *B. pseudomallei* infection. Inhibition of IFN-γ in mice lowered the LD_{50} from >5 x 10^5 to ca. 2 CFU and was associated with an 8,500- and 4,400-fold increase in bacterial load in liver and spleen, respectively. Inhibition of interleukin (IL)-12, the predominant endogenous inducer of IFN-γ production, resulted in increased mortality in the same model. IFN-γ and IL-12 play a key role in the type-1 cell-mediated immune response. Recent data indicate that activation of suppressor of cytokine signaling-3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) in *B. pseudomallei*-infected macrophages correlates with a decreased IFN-γ signaling.
Further evidence for the role of a type-1 response in protective immunity against melioidosis comes from studies with inbred mouse strains, in which type-1 response-prone C57BL/6 mice are relatively resistant to *B. pseudomallei* when compared to the type-2 response prone BALB/c mice. The pro-inflammatory cytokine tumour necrosis factor (TNF-α) is also likely to be an important element of the early immune response, as passive immunisation against this mainly macrophage-derived cytokine increased mortality in experimental murine melioidosis. Serum IFN-γ, IL-12 and TNF-α concentrations are elevated in melioidosis patients, the involvement of TNF-α in human disease is further suggested by a report that...
Melioidosis

the -308 TNF-α promoter polymorphism, which is related to severity of disease for several other infectious diseases, was associated with both the occurrence and severity of melioidosis\textsuperscript{59}. Plasma or serum concentrations of several other pro-inflammatory mediators are elevated in patients with melioidosis, including IL-6, IL-15, IL-18, IFN-γ inducible protein (IP)-10, and monokine induced by IFN-γ (Mig)\textsuperscript{57, 60}, as well as the anti-inflammatory cytokine IL-10\textsuperscript{61}. This indicates that multiple inflammatory pathways become activated, including those involving cellular activation. For example, IP-10 and Mig are chemokines primarily induced by IFN-γ which share a common receptor (CXC chemokine receptor 3), expressed by activated T and natural killer (NK) cells. Cytotoxic T-cells and NK cells are further implicated in the immune response to \textit{B. pseudomallei} through the observation that circulating levels of granzymes A and B are elevated. Granzymes are secreted by CT and NK cells and are important for the initiation of apoptosis in the target cell, although their precise role in bacterial infection remains to be established\textsuperscript{62}. NK cells and CD8+ T cells harvested from the spleen of uninfected mice also release IFN-γ on stimulation with \textit{B. pseudomallei} in vitro\textsuperscript{63}.

\textit{B. pseudomallei} rapidly and efficiently activates complement, predominantly via the alternative pathway\textsuperscript{64}. Complement activation results in the deposition of C3 on the bacterial surface and further opsonisation\textsuperscript{64}. However, \textit{B. pseudomallei} is resistant to the lytic action of complement\textsuperscript{64}, a feature shared with several other pathogens including Borrelia, Salmonella, Neisseria and streptococci\textsuperscript{65}. In one study, adherence and phagocytosis of \textit{B. pseudomallei} by granulocytes and macrophages was dependent on the presence of opsonins and mediated by the complement receptors CR1 and CR3 expressed on the macrophage membrane\textsuperscript{64}. The capsular polysaccharide of \textit{B. pseudomallei} contributes to resistance to phagocytosis by reducing deposition of complement factor C3b\textsuperscript{65}.

**Adaptive immune responses.** In patients with melioidosis levels of IgG, IgA and IgM correlate positively with disease severity with higher levels in those with invasive compared with localized disease. Melioidosis is positively associated with specific human leukocyte antigen (HLA) class II molecules in Thai patients; the HLA class II DRB1*1602 allele was positively associated with septicemic melioidosis, whereas DQA1*03 was negatively associated\textsuperscript{66}. Patients who recovered from melioidosis demonstrated evidence for an antigen-specific cell-mediated immune response, as reflected by enhanced lymphocyte proliferation and IFN-γ production in response to \textit{B. pseudomallei} antigens\textsuperscript{67}. In addition, asymptomatic seropositive individuals displayed a stronger cell-mediated adaptive immune response as measured by \textit{Burkholderia}-specific lymphocyte reactions compared with subjects with a history of clinical melioidosis\textsuperscript{68}, suggesting that a strong cell-mediated immune response might protect against disease progression. Conceivably, the production of IFN-γ by CD4+ T cells activates macrophages to become more bactericidal, a notion that is supported by the finding that IFN-γ increases the intracellular killing activity of macrophages \textit{in vitro}\textsuperscript{69}. However, the importance of CD4+ lymphocytes in the control of infection is open to debate since there does not seem to be an association between HIV infection and melioidosis.

The role of both cell-mediated and humoral immune mechanisms in protection against \textit{B. pseudomallei} infection was recently underscored in a vaccine-driven study\textsuperscript{70}. Dendritic cells were utilized as a vaccine delivery vector to induce cell-mediated immune responses to \textit{B. pseudomallei}. Purified dendritic cells were pulsed with heat-killed whole-cell \textit{B. pseudomallei} and used to immunize...
Chapter 2

syngeneic mice. Strong cellular immune responses were elicited, although antibody responses were low. Subsequently, booster immunizations of either a second dose of dendritic cells or heat-killed \textit{B. pseudomallei} were administered to increase the immune response. Immunized animals were challenged with fully virulent \textit{B. pseudomallei}, and protection was demonstrated in those with both a strong humoral and cell-mediated immunity\textsuperscript{70}.

**Intracellular survival of \textit{B. pseudomallei}** (Fig. 5). Data from \textit{in vitro} models indicate that \textit{B. pseudomallei} is equipped for intracellular survival. The organism survives and replicates within neutrophils and monocytes\textsuperscript{71-74}, and employs multiple mechanisms to escape macrophage killing and evade host immunity. These include resistance to human defensins\textsuperscript{71}, and inhibition of DNA and protein synthesis within the host cell\textsuperscript{75}. Ultrastructural studies suggest that \textit{B. pseudomallei}
Melioidosis might reside within membrane-bound compartments\textsuperscript{72}, particularly phagolysosomes\textsuperscript{64}, making use of its ability to survive and grow in acidic environments\textsuperscript{76}. Furthermore, \textit{B. pseudomallei} is able to invade mouse macrophages without activating inducible nitric oxide synthase (iNOS), an enzyme that is required for the generation of reactive nitrogen intermediates and important for intracellular bacterial killing\textsuperscript{77}. \textit{B. pseudomallei} can escape from endocytic vesicles into the cytoplasm by lysing the endosome membranes as early as 15 minutes after internalization by phagocytic cells\textsuperscript{25, 78, 79}. Moreover, intracellular \textit{B. pseudomallei} can be propelled by inducing continuous polymerization of actin at one pole of the bacterial cell\textsuperscript{25, 74, 80}, which results in the formation of membrane protrusions in host cells with the bacteria at the tip end. Such protrusions can project into an adjacent cell, facilitating the spread \textit{B. pseudomallei} from one eukaryotic cell to another\textsuperscript{74}. A \textit{B. pseudomallei} specific protein termed BimA is required for the formation of actin tails. BimA is located at the pole of the bacterial cell at which actin polymerization occurs\textsuperscript{81}. Mutation of the \textit{bimA} gene in \textit{B. pseudomallei} abolished actin-based motility of intracellular bacteria in a macrophage-like cell line. Actin tail formation could be restored by inducible expression of the \textit{bimA} gene on a plasmid, indicating the essential role for this gene in actin tail formation. Of note, mutation of \textit{bimA} does not influence the activity of the Bsa TTSS apparatus or bacterial escape from endosomes.

Multinucleated giant cells are occasionally seen in human tissue infected with \textit{B. pseudomallei}; this may represent giant macrophages\textsuperscript{82}. It is possible that resident macrophages represent a site of intracellular survival, given their relatively long half life and reduced microbicidal capacity in comparison with neutrophils and monocytes\textsuperscript{64}. A recent report indicates that \textit{B. pseudomallei} may evade killing by macrophages through the induction of caspase-1 dependent host cell death; this process requires a functional \textit{bsa} TTSS\textsuperscript{83}. Cell death was accompanied by IL-1\textbeta and IL-18 release\textsuperscript{83}. Survival within microcolonies encapsulated in protective biofilm is an alternative explanation for prolonged quiescent survival within the host. \textit{In vitro}, \textit{B. pseudomallei} can survive for years in distilled water and can also enter and survive within free-living amoebae belonging to the genus \textit{Acanthamoeba}; it is possible that this might enhance bacterial survival in soil and aquatic environments\textsuperscript{84, 85}.

\textbf{Interactions with human epithelial cells in vitro.} \textit{B. pseudomallei} adheres to cultured human epithelial cell lines derived from alveolar, bronchial, laryngeal, oral, conjunctival and cervical tissues\textsuperscript{86}. Adherence of \textit{B. pseudomallei} (but not \textit{B. thailandensis}) to cell lines \textit{in vitro} was enhanced when incubated at a temperature of 30°C compared with 37°C\textsuperscript{86}. \textit{B. pseudomallei} is more efficient in invasion, adherence and induction of cellular damage of respiratory epithelial cells compared to \textit{B. thailandensis}\textsuperscript{87}. A bacterial mutant defective in \textit{pilA} (a putative type IV pilus gene) has reduced adherence to human epithelial cells\textsuperscript{45}. The relevance of these observations for human disease pathogenesis is unknown.
Potential new therapies targeting the immune response

Several conditions including diabetes mellitus, renal failure and alcohol abuse are important risk factors for the development of melioidosis. A common link is that these are associated with impairment of neutrophil function. This had led to interest in the therapeutic role of granulocyte colony-stimulating factor (G-CSF), a cytokine that increases the circulating neutrophil count and stimulates neutrophil function. A retrospective study conducted in Australia reported a fall in mortality of melioidosis patients after the introduction of G-CSF as an adjunctive treatment of those with septic shock\textsuperscript{88}. A mouse model of melioidosis in which outcome was compared between mice given ceftazidime alone or in combination with G-CSF failed to show a benefit from G-CSF\textsuperscript{89}. A randomized clinical trial of G-CSF is currently on-going in Thailand\textsuperscript{90}.

Unmethylated CpG motifs in synthetic oligodeoxynucleotide (CpG ODN) enhance the uptake of bacteria by mouse macrophages in a concentration-dependent manner and induce nitric oxide production by mouse macrophages\textsuperscript{91}. CpG treatment one hour before bacterial inoculation offered protection in a murine model of \textit{B. pseudomallei} infection, associated with the rapid induction of pro-inflammatory cytokines\textsuperscript{92}.

Vaccine development

There is no effective vaccine available that protects against \textit{B. pseudomallei} infection. Current approaches under evaluation include conjugate-, DNA-, attenuated- and heterologous vaccines\textsuperscript{93}. Attenuated mutants that are invasive but have a reduced ability to multiply in phagocytes have been identified using transposon mutagenesis, and these induce a high degree of protective immunity in mice\textsuperscript{94}. A mutant of \textit{B. pseudomallei} that is auxotrophic for branched-chain amino acids induced a protective response against a subsequent challenge with an otherwise lethal dose of wild-type \textit{B. pseudomallei} in an animal model\textsuperscript{95}. Mice inoculated with a \textit{B. pseudomallei bipD} mutant were partially protected against subsequent challenge with wild-type \textit{B. pseudomallei}, although immunization with purified BipD protein was not protective\textsuperscript{28}. However, it seems unlikely that live attenuated vaccination will be feasible for use in humans. Antibodies raised against \textit{B. pseudomallei} flagellin markedly reduced bacterial motility and provided passive protection against experimentally induced \textit{B. pseudomallei} infection\textsuperscript{96}. Evaluation of LPS and capsular polysaccharide as subunit vaccines against experimental melioidosis demonstrated partial protection in a mouse model\textsuperscript{97}. Guinea pig inoculation with \textit{B. thailandensis} provided partial protection from a subsequent challenge with virulent \textit{B. pseudomallei}\textsuperscript{98}. The possible utility of these observations for vaccine development awaits further investigation.
CONCLUSIONS AND PERSPECTIVES

Interest is increasing in *B. pseudomallei* and there has been a significant boost in funding from agencies such as the NIH. The recent advent of molecular genetics and *in vitro* and *in vivo* infection models together with availability of the complete genome sequence of *B. pseudomallei*, *B. mallei* and (in the near future) *B. thailandensis*, has advanced our understanding of virulence mechanisms governing the complex interaction between *B. pseudomallei* and host cells. Preserved mechanisms underlying the intracellular lifestyle of *B. pseudomallei* have been discovered by comparing the *B. pseudomallei* genome with the genomes of other intracellular microbes (e.g. *Salmonella* and *Shigella*)\(^85\). New techniques that allow integrative analyses at genomic, transcriptional and proteomic molecular levels promise to provide further insights into the complex interaction between this pathogen and its environment\(^99\). The genetic diversity within the natural population will be characterized further using techniques such as multi-locus sequence typing and microarray analysis, along with the identification of the *B. pseudomallei* secretome (all secreted proteins secreted) and immunome (identification of immunogenic proteins). Some virulence factors have now been characterized and the study of host-pathogen interactions has shed some light on pathogenic mechanisms, but many key questions await investigation (see textbox in chapter 17).

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Chapter 2

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Chapter 2


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Chapter 2


Chapter 3

Inflammation patterns induced by different *Burkholderia* species in mice


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Chapter 3

SUMMARY

*Burkholderia pseudomallei*, which causes melioidosis, a severe mainly pulmonary disease endemic in SE-Asia, is considered the most pathogenic of the *Burkholderia* genus. *B. thailandensis* however is considered avirulent. We determined differences in patterns of inflammation of *B. pseudomallei*-1026b (clinical virulent-isolate), *B. pseudomallei*-AJ1D8 (an *in-vitro* invasion deficient mutant generated from strain 1026b by Tn5-OT182-mutagenesis) and *B. thailandensis* by intranasally inoculating C57BL/6 mice with each strain. Mice infected with *B. thailandensis* showed markedly decreased bacterial outgrowth from lungs, spleen and blood 24-hours after inoculation compared to infection with *B. pseudomallei* and the invasion mutant AJ1D8. 48-hours after inoculation *B. thailandensis* was no longer detectable. This was consistent with elevated pulmonary cytokine and chemokine concentrations after infection with *B. pseudomallei* and the invasion mutant AJ1D8. 48-hours after inoculation *B. thailandensis* was no longer detectable. This was consistent with elevated pulmonary cytokine and chemokine concentrations after infection with *B. pseudomallei*-1026b and AJ1D8, and the absence of these mediators 48-hours, but not 24-hours, after inoculation with *B. thailandensis*. Histological examination however did show marked pulmonary inflammation in the mice infected with *B. thailandensis*, corresponding with substantial granulocyte-influx and raised myeloperoxidase-levels. Survival experiments showed that infection with 1x103 CFU *B. thailandensis* was not lethal, whereas inoculation with 1x106 CFU *B. thailandensis* was equally lethal as 1x103 CFU *B. pseudomallei*-1026b or AJ1D8. These data show that *B. pseudomallei*-AJ1D8 is just as lethal as wild-type *B. pseudomallei* in an *in-vivo* mouse model and *B. thailandensis* is perhaps more virulent than is often recognized.
INTRODUCTION

Burkholderia pseudomallei, a Gram-negative bacterium, is a potential bioterror agent and the causative agent of melioidosis, a severe disease that is endemic in the Far East and the most-common cause of pneumonia-derived sepsis in north-east-Thailand. The genus Burkholderia contains >30 species, of which B. pseudomallei and B. mallei are considered the most pathogenic. The closely related Burkholderia thailandensis, which has been first described only in 1998, is generally considered avirulent as it does not cause overt disease, and has previously been reported only in Southeast Asia. B. thailandensis can be distinguished from B. pseudomallei by its ability to assimilate arabinose. B. thailandensis coexists with B. pseudomallei in the soil in Thailand and is approximately >10^5-fold less virulent in Syrian hamsters and >10^7-fold less virulent in BALB/c mice than B. pseudomallei.

Although B. thailandensis has been considered harmless, this microorganism has been cultured from the purulent material of an amputated knee in Thailand and very recently, the first case of pneumonia and sepsis caused by B. thailandensis was described in the United States. Therefore, in the present study we determined the differences in patterns of inflammation of B. pseudomallei 1026b (clinical virulent isolate) and B. thailandensis by investigating the host response to these Burkholderia strains in mice in vivo and in different relevant cell lines in vitro. In addition, we aimed to compare the host response elicited by B. pseudomallei and B. thailandensis with that induced by B. pseudomallei AJ1D8, an in vitro invasion deficient mutant generated by Tn5-OT182-mutagenesis known to exhibit approximately 10% of the ability of the parental strain to invade a human pneumocyte cell line A549.

RESULTS

Equal responsiveness of murine alveolar macrophage and lung epithelial cell lines to stimulation with different Burkholderia strains in vitro

To first investigate the differences in inflammation patterns in the pulmonary compartment induced by infection with B. pseudomallei 1026b (clinical virulent isolate), B. thailandensis E264 (clinical avirulent isolate) and B. pseudomallei AJ1D8 (an in vitro invasion deficient mutant generated by Tn5-OT182 mutagenesis) we stimulated murine cell types considered important for the regulation of inflammation in the pulmonary compartment. We used the murine alveolar macrophage cell line, MH-S, because it has been shown to be a useful model for intracellular bacterial pathogens that cause respiratory diseases, including Mycobacterium tuberculosis and Chlamydia pneumophila. In addition, we chose to use the murine lung epithelial cell lines MLE-12 and MLE-15 as a model for B. pseudomallei stimulation of distal bronchiolar and alveolar epithelium cells respectively. Most surprisingly we saw no differences in the responsiveness of murine alveolar macrophage (MH-S) and lung epithelial cell lines (MLE-12 and MLE-15).
after stimulation with different the *Burkholderia* strains at various time points *in vitro* (Fig. 1). *B. thailandensis* proved to be equally capable of inflicting an inflammatory response upon stimulation of these pulmonary cell lines when compared to either *B. pseudomallei* 1026b or *B. pseudomallei* AJ1D8.

**Mice infected with *B. thailandensis* show a reduced bacterial outgrowth compared to mice intranasally infected with an equal dose of *B. pseudomallei* 1026b strain *in vivo***

To characterize the *in vivo* inflammatory response of these different *Burkholderia* strains, we infected mice with 1x10^3 CFU of each strain. In accordance with the notion that *B. thailandensis* is not virulent, *B. thailandensis* showed a markedly decreased bacterial outgrowth in lungs, spleen and blood 24 hrs after inoculation when compared to the groups infected with *B. pseudomallei* 1026b and the invasion mutant AJ1D8 (Fig. 2). 48 hours after inoculation *B. thailandensis* was...
no longer detectable in the pulmonary compartment. However, *B. thailandensis* was detectable in the spleen at this time point, indicating that *B. thailandensis* is capable of trespassing the pulmonary compartment and cause distant infection (Fig. 2). Bacterial loads became similar in mice infected with the three different strains when *B. thailandensis* was given in a dose 1000-fold higher than *B. pseudomallei* 1026b or *B. pseudomallei* AJ1D8 (Fig. 2).

**Pulmonary TNF-α, IL-6 and MCP-1 levels in mice infected with *B. pseudomallei* 1026b and *B. pseudomallei* AJ1D8**

The success of combating infections in the lung strongly depends on the efficacy of the local inflammatory response elicited 12. In order to study the extent and kinetics of the inflammatory response, we sacrificed mice at 24 and 48 hours after infection and measured the concentrations of the proinflammatory cytokines TNFα and IL-6 and the chemokine MCP-1 in lung homogenates. The enhanced bacterial outgrowth of the mice infected with the *B. pseudomallei* 1026b and *B. pseudomallei* AJ1D8 strains was consistent with a strong induction of TNF-α, IL-6 and MCP-1 in these two groups, whereas these inflammatory cytokines were absent or present at very low levels 48 hours after inoculation in the *B. thailandensis* group (Fig. 3). Remarkably however, at 24 hours after infection there were no significant differences in TNFα and MCP-1 levels between the three groups.

![Fig. 3. Pulmonary cytokine levels after intranasal infection with different *Burkholderia* strains.](image)

Mice were i.n. inoculated with 1x10³ CFU of *B. pseudomallei* 1026b, *B. thailandensis* or *B. pseudomallei* AJ1D8. Mice were sacrificed 24 or 48 hours after infection. TNFα (A), IL-6 (B) and MCP-1 (C) concentrations were measured in lung homogenates. Data are means ± SE of 8 mice per group per time point. No significant differences were observed between *B. pseudomallei* 1026b and *B. pseudomallei* AJ1D8. **p < 0.01; *** < 0.001 between *B. pseudomallei* and *B. thailandensis*; BD: below detection limit.

**Marked inflammation in the lungs of the mice infected with *B. thailandensis***

To obtain insight into the possible pathogenicity of *B. thailandensis* in the development of organ inflammation, lungs, spleen and liver were harvested at 24 and 48 hours after the induction of *B. pseudomallei* 1026b, *B. thailandensis* or *B. pseudomallei* AJ1D8 infection. Mild inflammation was observed in both spleens and livers of mice infected with *B. pseudomallei* 1026b or *B. pseudomallei* AJ1D8, whereas no liver or spleen pathology was seen in the *B. thailandensis* infected animals (data not shown). In addition, histological scores of the lung were similar in the *B. pseudomallei* 1026b and *B. pseudomallei* AJ1D8 strains (Fig 4G). Interestingly, however, histological examination did show major inflammation of the lungs of mice infected with *B. thailandensis*, as reflected by marked
pleuritis, peribronchial inflammation, oedema and endothelialitis (Fig. 4B and 4G). Granulocyte stainings of lung tissues revealed a similarly strong accumulation of neutrophils in lungs of the mice infected with \textit{B. thailandensis} (Fig. 4D-F). In line, MPO levels in lung homogenates were significantly raised in the mice infected with \textit{B. thailandensis} (Fig. 4H). Thus, pulmonary infection with \textit{B. thailandensis} in mice does cause significant inflammation of the lungs.

Fig. 4. Marked inflammation in the lungs of the mice infected with \textit{B. thailandensis}. Representative lung histology 48 hours after intranasal infection with $1 \times 10^3$ CFU of \textit{B. pseudomallei} strain 1026b (A), \textit{B. thailandensis} E264 (B) or \textit{B. pseudomallei} strain AJ1D8 (C). One sees significant inflammation, pleuritis, peribronchial inflammation, oedema and endothelialitis in all groups. Standardized pathology scores (means ± SEM) as described in the Methods section shows diminished, but still significant pathology in the mice infected with \textit{B. thailandensis} compared to the mice infected with \textit{B. pseudomallei} 1026b or \textit{B. pseudomallei} AJ1D8 (G). Furthermore, immunostaining for granulocytes (Ly-6G staining) show granulocytic infiltrations and granulocyte influx in all mice at 48 hours after inoculation with different \textit{B. pseudomallei} strain 1026b (D), \textit{B. thailandensis} E264 (E) or \textit{B. pseudomallei} strain AJ1D8 (F). Granulocyte influx corresponds with myeloperoxidase (MPO) activity levels in lung tissues which are depicted in graph (H). The dotted line represents the mean value obtained from uninfected mice. Data are mean ± SEM of 8 mice per group. Hematoxylin and eosin staining, magnification x 40; Ly-6G staining, magnification x 200. All data are 8 mice per group. ** p < 0.01 between \textit{B. pseudomallei} and \textit{B. thailandensis}.
Intranasal inoculation with high dose *B. thailandensis* is equally lethal as inoculation with low dose *B. pseudomallei* 1026b or *B. pseudomallei* AJ1D8

Having established that *B. pseudomallei* AJ1D8, which is known as an invasion-deficient mutant because of its *in vitro* characteristics, is capable of inflicting an equally strong pulmonary inflammatory response as the parent strain *B. pseudomallei* 1026b, we next investigated the virulence of these *Burkholderia* strains with regard to outcome in our model of experimentally induced melioidosis. Mice were intranasally infected with each *Burkholderia* strain and followed for maximal 6 weeks. These survival experiments showed that infection with $1 \times 10^3$ CFU *B. thailandensis* was not lethal, whereas inoculation with $1 \times 10^6$ CFU *B. thailandensis* was equally lethal as inoculation with $1 \times 10^3$ CFU *B. pseudomallei* 1026b (Fig. 5). No difference was observed in terms of outcome between mice infected with *B. pseudomallei* 1026b or *B. pseudomallei* AJ1D8 (Fig. 5).

![Fig. 5. Survival of mice intranasally infected with different *Burkholderia* strains. Survival after intranasal inoculation with $1 \times 10^3$ CFU *B. pseudomallei* 1026b (closed squares), compared to $1 \times 10^3$ CFU *B. thailandensis* (open squares) (A), $1 \times 10^6$ CFU *B. thailandensis* (open squares) (B) or $1 \times 10^3$ CFU *B. pseudomallei* AJ1D8 (open squares) (C). All mice were infected concurrently; graphs for different strains and/or doses are shown in separate panels for reasons of clarity. Mortality was assessed twice daily for 6 weeks. n = 12 per group; ns denotes not significant.](image-url)

**DISCUSSION**

In the present study we aimed to characterize the virulence of *B. thailandensis* and the invasion-deficient strain *B. pseudomallei* AJ1D8 in the pulmonary compartment by making use of both *in vitro* and *in vivo* experiments. Our experiments suggest that (1) *B. pseudomallei* AJ1D8 is just as lethal as the parent strain *B. pseudomallei* 1026b in a murine model of pulmonary melioidosis, and (2) *B. thailandensis* causes significant lung pathology in mice *in vivo*.

The mutant strain AJ1D8 has been shown to exhibit approximately 10% of the ability of the parental strain, 1026b, to invade A549 cells. We now show that there are no differences in the responsiveness of murine alveolar macrophage (MH-S) and lung epithelial cell lines (MLE-12 and MLE-15) in terms of cytokine release towards stimulation with either *B. pseudomallei* 1026b or *B. pseudomallei* AJ1D8. In addition, in our murine model of melioidosis, we found no difference in survival between mice infected with the parent strain *B. pseudomallei* 1026b and the so-called invasion deficient mutant *B. pseudomallei* AJ1D8. This is in accordance with the original report by Jones *et al.* who did not notice a survival difference in both infant diabetic rats and Syrian hamsters
infected with *B. pseudomallei* AJ1D8 or *B. pseudomallei* 1026b. Together with the fact that there were no differences in bacterial outgrowth from the lung and spleen, bacteremia, cytokine release and lung inflammation between *B. pseudomallei* 1026b and *B. pseudomallei* AJ1D8 in our murine model of melioidosis, we can conclude that the term invasion deficient for the AJ1D8 only refers to its *in vitro* characteristics of being less able to invade A549 cells.

For our *in vivo* infection models *Burkholderia* was administered via the airways. We consider this route of infection more clinically relevant than intravenous or intraperitoneal injection. Indeed, humans usually acquire melioidosis by inoculation through skin abrasions or inhalation and pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis. Different research groups have shown with survival experiments that *B. thailandensis* is less virulent than *B. pseudomallei* upon intraperitoneal administration. We now confirm and extend these findings in our murine model of melioidosis in which we infect the animals intranasally. In this model we were able to reproduce the major clinical characteristics of melioidosis, with rapid spread of bacteria to distant organs, multiple organ failure and abscess formation. In addition, we showed that, while infection with 1x10^3 CFU *B. thailandensis* was not lethal, inoculation with 1x10^6 CFU *B. thailandensis* is equally lethal as inoculation with 1x10^3 CFU *B. pseudomallei*. Of note, the differences in pathogenicity between both strains remain ill defined. It has been shown however that *B. thailandensis* is just as capable as *B. pseudomallei* to induce cell fusion and the formation of multinucleate giant cells after invasion of phagocytic cells. Furthermore, it was demonstrated recently that *B. pseudomallei* is more efficient than *B. thailandensis* in invasion of and adherence to cultured human epithelial A549 cells. However, after invasion, no differences in terms of survival and replication within these cultured A549 cells were observed between both *Burkholderia* strains. In terms of cytokine release, we now demonstrate that there are no differences in the responsiveness of murine alveolar macrophage (MH-S) and lung epithelial cell lines (MLE-12 and MLE-15) towards stimulation with either *B. pseudomallei* 1026 or *B. thailandensis*. Most remarkably, however, we did observe major pulmonary inflammation after intranasal inoculation of *B. thailandensis* could suggest that *B. thailandensis* is perhaps more virulent than is often recognized.

Although important genomic differences exist between the two closely related species *B. pseudomallei* and *B. thailandensis*, the genomes of these two species are regarded to be broadly similar. Because of this extensive genomic similarity between *B. pseudomallei* and *B. thailandensis* it has been suggested that *B. thailandensis* could be used as a possible model system for studying certain aspects of *B. pseudomallei* behaviour. Moreover, because of the assumption of its non-pathogenic nature, *B. thailandensis* has been suggested as a potential melioidosis vaccine (reviewed in). Iliukhin et al. showed that immunisation with live cultures of *B. thailandensis* resulted in approximately 50% protection against a subsequent *B. pseudomallei* challenge in guinea pigs. However, our data suggest that one should be cautious using live *B. thailandensis* as a vaccine. Our findings on the virulence of *B. thailandensis* in a murine model of melioidosis are in line with a recent report in the literature citing *B. thailandensis* as the cause of pneumonia-derived sepsis and call attention to the notion that *B. thailandensis* is perhaps not as avirulent as is often recognized.
EXPERIMENTAL PROCEDURES

Mice and experimental infection
The Animal Care and Use of Committee of the University of Amsterdam approved all experiments. Pathogen-free 8 to 10 week old C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). Age and sex-matched animals were used in all experiments. *B. pseudomallei* strain 1026b has been isolated in Sappasithiprasong hospital in 1993 from a blood culture from a septicaemic 39-year old female rice farmer presenting with bacteremia with soft tissue, skin, joint and splenic involvement. *B. thailandensis* E264 is an environmental isolate from north-east Thailand. For preparation of the inoculum, *B. pseudomallei* strain 1026b, *B. thailandensis* E264 and *B. pseudomallei* strain AJ1D8 were used and streaked from frozen aliquots into 50 ml Luria broth (Difco, Detroit, MI) for overnight incubation at 37°C in a 5% CO2 incubator. Thereafter, a 1 ml portion was transferred to fresh Luria broth and grown for approximately 5h to midlogarithmic phase. Bacteria were harvested by centrifugation at 1500 x g for 15 minutes, washed and resuspended in sterile isotonic saline at the desired concentration, as determined by plating serial 10-fold dilutions on blood agar plates. Pneumonia was induced by intranasal inoculation of 50 μl (1x10^3 CFU or 1x10^6 CFU) bacterial suspension. For this procedure mice were lightly anesthetized by inhalation of isofluorane (Upjohn, Ede, The Netherlands).

Determination of bacterial outgrowth
24 and 48 hrs after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium: active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Mijdrecht, The Netherlands) and sacrificed by bleeding from the inferior vena cava. The lungs and spleens were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of organ homogenates and blood, plated on blood agar plates and incubated at 37°C at 5% CO2 for 16 h before colonies were counted.

Preparation of lung tissue for cytokine measurements
For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% Triton X-100, and AEBSF (4-(2-aminoethylbenzenesulfonyl fluoride), EDTA-NA2, Pepstatin and Leupeptin (all 8 μg/ml; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed.

MH-S and MLE cells
The murine alveolar macrophage cell line MH-S (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% FCS, penicillin, streptomycin (GibcoBRL, Life Technologies, Rockville, MD) and 50 μM 2-βME (Sigma, Aldrich, St. Louis, MO). The murine transformed ATII respiratory epithelial cell lines MLE-12 and
Chapter 3

MLE-15 were generously provided by Jeffrey Whitsett, Cincinnati Children’s Hospital Medical Center, Cincinnati and were cultured in HITES medium (RPMI 1640 supplemented to 5 μg/ml insulin, 10 μg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone and 10 nM β-estradiol) supplemented with 2% FCS, penicillin and streptomycin (GibcoBRL, Life Technologies, Rockville, MD). In vitro stimulation was conducted in 96-well plates (Greiner, Alphen aan de Rijn, the Netherlands) at a density of 5x10^5 cells/ml. Following overnight culture at 37°C in 5% CO₂, adherent cells were washed twice and then stimulated with heat-killed *B. pseudomallei* strain 1026b (5x10^7 CFU/ml), *B. thailandensis* E264 (5x10^7 CFU/ml), *B. pseudomallei* strain AJ1D8 (5x10^7 CFU/ml) or RPMI for 4 or 24 hrs. Supernatant was harvested and stored at -20°C until assayed for cytokine release. In preliminary experiments we established that MH-S cells primarily release tumor necrosis factor (TNF-α) upon stimulation, whereas MLE-12 and MLE-15 predominantly release monocyte chemoattractant (MCP)-1 and cytokine-induced neutrophil chemoattractant (KC) upon stimulation (data not shown). Hence, these readouts were used for cellular responsiveness.

**Assays**

Mouse TNF-α, Interleukin (IL)-6 and MCP-1 were measured by cytometric bead array (CBA; BDBiosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. KC (R&D Systems, Minneapolis, Minnesota, MN) and myeloperoxidase (MPO) (HyCult Biotechnology, Uden, the Netherlands) were measured with commercially available kits according to the manufacturer’s instructions.

**Pathology**

Lungs and spleens to be examined for histology were harvested after infection, fixed in 10% formalin and embedded in paraffin. Four μm sections were stained with H&E, and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: surface with pneumonia, necrosis/abscess formation, interstitial inflammation, endothelialitis, bronchitis, edema, thrombus formation and pleuritis. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 32. Liver and spleen sections were scored on inflammation, necrosis/abscess formation and thrombus formation using the scale given above. The maximum total liver or spleen inflammation score was 12. Granulocyte staining was done exactly as described previously.

**Statistical analysis.**

Values are expressed as means ± SEM. Differences between groups were analyzed by Mann-Whitney U test or Kruskal-Wallis with Dunns post test analysis where appropriate. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Values of P<0.05 were considered statistically significant.
We are grateful to Marieke ten Brink and Joost Daalhuisen for expert technical assistance. We acknowledge the support of the Dutch Foundation for Tropical Research (WOTRO).
Chapter 3

REFERENCES


High throughput mRNA profiling characterizes the expression of inflammatory molecules in sepsis caused by *Burkholderia pseudomallei* (melioidosis)


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Chapter 4

**ABSTRACT**

Sepsis is characterized by an uncontrolled inflammatory response to invading microorganisms. We describe the inflammatory mRNA-profile of whole blood leukocytes, monocytes and granulocytes using a multigene-system for 35 inflammatory markers that included pro- and anti-inflammatory cytokines, chemokines and signal transduction molecules in a case-control study with 34 patients with sepsis caused by the Gram-negative bacterium *Burkholderia pseudomallei* (the pathogen causing melioidosis) and 32 healthy volunteers. Relative to healthy controls, septic patients showed increased transcription of a whole array of inflammatory genes in peripheral blood leukocytes, granulocytes and monocytes. Specific monocyte and granulocyte mRNA-profiles were identified. Strong correlations were found between inflammatory mRNA expression levels in monocytes and clinical outcome. These data underline the notion that circulating leukocytes are an important source for inflammatory mediators in patients with Gram-negative sepsis. Gene profiling such as done here provides an excellent tool to obtain insight in the extent of inflammation activation in patients with severe infection.
INTRODUCTION

Melioidosis is caused by the Gram-negative bacillus *Burkholderia pseudomallei* and is an important cause of severe sepsis in Southeast Asia and Northern Australia. The most feared clinical picture is melioidosis septic shock, often associated with pneumonia and bacterial dissemination to distant sites and a high mortality. Not surprisingly, melioidosis is regarded as an excellent model to study Gram-negative sepsis: melioidosis is prevalent among rice farmers in Southeast Asia - a relatively homogenous population – and is acquired in a community setting; furthermore patients present in large numbers to a single institution, melioidosis is caused by a single organism and is associated with a high mortality rate.

Sepsis is defined as the systemic inflammatory response to infection and is one of the leading causes of death in the western world. It has been well established that an uncontrolled activation of the inflammatory system to an invading pathogen can result in multiorgan failure and eventually death. Activation of leukocytes and the cytokine and chemokine networks are prominent features of the septic response. However, knowledge of the nature of this acute inflammatory state and the role of circulating leukocytes is limited. Most studies on the role of inflammation in sepsis have focused on the plasma levels of inflammatory mediators, most notably cytokines. However, plasma protein levels do not fully reflect the inflammatory signature of leukocytes in whole blood. Furthermore, tissue leukocytes and parenchymal cells may contribute to the plasma levels of inflammatory mediators. To date, little work has been devoted to leukocyte mRNA expression profiles in patients with sepsis. Preliminary studies using real-time RT-PCR or gene arrays have suggested activation of multiple pathways in whole blood leukocytes obtained from septic patients. These investigations did not provide insights into the relative contribution of monocytes and granulocytes in the systemic inflammatory response. To overcome these obstacles in assessing the inflammatory status of circulating leukocyte subsets, we made use of a sensitive quantitative assay that is capable of measuring a panel of mRNA levels in a large series of samples in a single reaction. Target genes included pro- and anti-inflammatory cytokines, chemokines, their receptors, and nuclear factor κB (NFκB) pathway components. We used this novel high throughput technology to characterize the inflammatory mRNA profile in monocytes and granulocytes of patients with sepsis caused by *B. pseudomallei*.

MATERIALS AND METHODS

Patients and control subjects

Patients were recruited prospectively at Sapprasitprasong Hospital, Ubon Ratchathani, northeast Thailand in 2004. Sepsis due to melioidosis was defined as culture positivity for *B. pseudomallei* from any clinical sample plus a systemic inflammatory response syndrome (SIRS). To meet the SIRS criteria patients had to meet at least three of the following four criteria: a core temperature of \( \geq 38^\circ\text{C} \) or \( \leq 36^\circ\text{C} \); a heart rate of \( \geq 90 \text{ beats/min} \); a respiratory rate of \( \geq 20 \text{ breaths/min} \) or a PaCO\(_2\) of \( \geq 32 \text{ mmHg} \) or the use of mechanical ventilation for an acute respiratory process; a white-cell
**Table 1. Inflammatory Genes as Analyzed by MLPA**

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<td></td>
<td>Glutathione S-transferase</td>
<td>GSTP1</td>
</tr>
<tr>
<td></td>
<td>V-myc oncogene homolog</td>
<td>MYC</td>
</tr>
<tr>
<td></td>
<td>Phosphodiesterase 4B, cAMP-specific</td>
<td>PDE4B</td>
</tr>
<tr>
<td></td>
<td>Platelet-derived growth factor, beta polypeptide</td>
<td>PDGFB</td>
</tr>
<tr>
<td></td>
<td>Protein-Tyrosine Phosphatase, type 4A, 2</td>
<td>PTP4A2</td>
</tr>
<tr>
<td></td>
<td>Protein-Tyrosine phosphatase, nonreceptor-type, 1</td>
<td>PTPN1</td>
</tr>
<tr>
<td></td>
<td>Serine proteinase inhibitor, clade B, member 9</td>
<td>SERPINB9</td>
</tr>
<tr>
<td></td>
<td>Thrombospondin 1</td>
<td>THBS1</td>
</tr>
<tr>
<td></td>
<td>Tissue Factor</td>
<td>TF</td>
</tr>
<tr>
<td>Control gene</td>
<td>Beta-2-microglobulin</td>
<td>B2M</td>
</tr>
<tr>
<td></td>
<td>Polyadenylate-specific ribonuclease</td>
<td>PARN</td>
</tr>
</tbody>
</table>
count of $\geq 12 \times 10^9/l$ or $\leq 4 \times 10^9/l$ or a differential count showing $> 10\%$ immature neutrophils. These definitions have been used in large clinical trials and were modified according to the latest revisions. Exclusion criteria were the use of dialysis and/or immunosuppressive therapy, known disorders of coagulation and concomitant infection with human immunodeficiency virus. Blood samples were drawn within 36 hours after start of therapy. Healthy blood donors recruited from the Sapprasithiprasong hospital blood bank served as a control population. The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, England and written informed consent was obtained from all study subjects.

RNA analysis using Multiplex ligation-dependent probe amplification (MLPA)

Heparin blood samples were drawn from the antecubital vein and immediately put on ice. Leukocytes were isolated using erylsis buffer. Monocyte and granulocyte enriched populations where isolated using Polymorphprep (Axis-Shield, Dundee, United Kingdom). Monocyte and granulocyte fractions were $> 98\%$ pure as determined by their scatter pattern on flow cytometry. After isolation leukocytes, monocytes and granulocytes were dissolved in Trizol and stored at $-80^\circ C$ until used for RNA isolation. RNA was isolated and analyzed by multiplex ligation-dependent probe amplification (MLPA) as previously described. The inflammatory genes as analyzed by MLPA are listed in table 1. This MLPA profiling method is insensitive to the total amount of mRNA that is included in the reaction; therefore, the profile is independent of the total cell count. All samples were tested with the same batch of reagents. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of the $\beta 2$ microglobulin control gene, resulting in the relative abundance of mRNAs of the genes of interest.

Statistical analysis

Values are expressed as means $\pm$ standard error of the mean (SEM). Differences between groups were analyzed by Mann-Whitney U test. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Patient characteristics

34 patients with sepsis caused by \textit{B. pseudomallei} (17 males) and 32 healthy control subjects (22 males) were enrolled. The mean age was 52 years (range, 18-86 years) and 41 years (range, 21-59) for patients and controls, respectively. \textit{B. pseudomallei} was cultured from body material from all patients: blood cultures were positive for \textit{B. pseudomallei} in 21 patients (61.7%), throat swab or tracheal suction in 13 patients (38.0%), sputum in 7 patients (21.0%), pus from abscess in 8 patients (23.5%) and urine in 5 patients (14.7%). After inclusion all patients received appropriate antimicrobial therapy. The overall in-hospital patient mortality was 44%.
mRNA profile encoding pro-inflammatory cytokines and mediators

To determine the inflammatory mRNA pattern in sepsis caused by \textit{B. pseudomallei}, MLPA was performed on mRNA isolated from unfractionated leukocytes and monocyte and granulocyte enriched populations. All the mRNAs analyzed are listed in table 1 (see also experimental procedures). Table 2 shows the mRNAs encoding pro-inflammatory cytokines and mediators. In whole blood leukocytes, sepsis caused by \textit{B. pseudomallei} was characterized by an increased expression of mRNAs for numerous cytokines (IL-1\(\beta\), IL-6, IL-15, IFN\(\gamma\), TNF\(\alpha\), TNF\(\beta\)) and chemokines (MIP-1\(\alpha\) and MIP-1\(\beta\)) (all \(p < 0.01-0.001\) versus controls), while not influencing the expression of IL-8 or IL-12. In order to determine the relative contribution of monocytes and granulocytes to this inflammatory mRNA profile, monocyte and granulocyte enriched populations were separately analyzed. Monocytes were shown to be the primary source for several cytokines and chemokines, most notably for TNF\(\alpha\), TNF\(\beta\), IL-15, MIP-1\(\alpha\) and MIP-1\(\beta\). In the monocyte population, there was no difference in expression of IL-6, IL-12, IL-1\(\beta\) and MCP-2 between patients and controls. In addition, reduced mRNA levels for IL-8, MCP-1 and TNF\(\beta\) in the monocytes of patients compared to controls were seen (all \(p < 0.05-0.01\) versus controls). The contribution of granulocytes to this inflammatory mRNA profile in whole blood was characterized by an increased expression of the IL-1\(\beta\), IL-6, IL-8 and IL-15 (\(p < 0.01-0.001\) vs controls), while not influencing the expression of the

\[\text{Table 2. Pro-inflammatory cytokines and mediators.}\]

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Leukocytes</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Patients</td>
</tr>
<tr>
<td>IFN(\gamma)</td>
<td>0.032 ± 0.002</td>
<td>0.083 ± 0.022 ***</td>
</tr>
<tr>
<td>IL12A</td>
<td>0.069 ± 0.003</td>
<td>0.145 ± 0.040</td>
</tr>
<tr>
<td>IL12B</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL15(1)</td>
<td>0.066 ± 0.004</td>
<td>0.275 ± 0.046 ***</td>
</tr>
<tr>
<td>IL15(2)</td>
<td>0.011 ± 0.001</td>
<td>0.060 ± 0.016 ***</td>
</tr>
<tr>
<td>IL1A</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL1B</td>
<td>0.191 ± 0.013</td>
<td>0.346 ± 0.030 ***</td>
</tr>
<tr>
<td>IL2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL6</td>
<td>0.018 ± 0.002</td>
<td>0.095 ± 0.046 ***</td>
</tr>
<tr>
<td>IL8</td>
<td>0.126 ± 0.012</td>
<td>0.112 ± 0.022</td>
</tr>
<tr>
<td>LTA</td>
<td>0.037 ± 0.002</td>
<td>0.084 ± 0.022 **</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MCP-2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MIP1A</td>
<td>0.020 ± 0.002</td>
<td>0.107 ± 0.018 ***</td>
</tr>
<tr>
<td>MIP1B</td>
<td>0.102 ± 0.007</td>
<td>0.201 ± 0.034 **</td>
</tr>
<tr>
<td>TNF</td>
<td>0.016 ± 0.001</td>
<td>0.078 ± 0.023 ***</td>
</tr>
</tbody>
</table>

Expression of inflammatory mRNAs of cases and control subjects in whole blood leukocytes, granulocytes and monocytes (normalized to B2M and in alphabetical order). Values given as mean ± SEM. P values are according to the Mann-Whitney test, \(\ast P<0.05\), \(\ast\ast P<0.01\), \(\ast\ast\ast P<0.001\) vs controls. ND indicates not detectable.
other analyzed pro-inflammatory cytokines and mediators. IFNγ mRNA levels were modestly increased in total leukocytes, but not in either monocyte or granulocyte enriched fractions.

**mRNA profile encoding anti-inflammatory cytokines and mediators**

Of note, together with the upregulation of the pro-inflammatory cytokines a similar upregulation of the anti-inflammatory cytokines was observed (table 3). Patients showed increased whole blood leukocyte IL-4, IL-10, IL-1RA and TNF-receptor-1 mRNA levels compared to controls (all \( p < 0.001 \) vs controls). The contribution of monocytes to this anti-inflammatory mRNA profile in whole blood was characterized by an increased expression of IL-4, IL-10, IL-1RA and TNF-receptor-1 (\( p < 0.01-0.001 \) vs controls), while the granulocytes showed increased mRNA levels of IL-1RA, IL-4 and TNF-receptor-1 (\( p < 0.01-0.001 \) vs controls).

**mRNA profile encoding signal transduction molecules and others**

Leukocytes in the blood of septic patients showed increased transcription of an array of genes involved in signal transduction and coagulation (table 4). Activation of leukocytes by *B. pseudomallei* was reflected by increased expression of mRNAs involved in the NFκB pathway (NFκB1, NFκB2, NFκB-inhibitor-1) and mRNAs for cyclin-dependent kinase inhibitor 1A, glutathione S-transferase, platelet-derived growth factor, protein-tyrosine phosphatases, serine proteinase inhibitor (SERPINB9), tissue factor and thrombospondin (\( p < 0.001 \) versus controls). The relative contribution of monocytes and granulocytes to the signal transduction and coagulation specific
Table 3. Anti-inflammatory cytokines and mediators.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Leukocytes</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Patients</td>
</tr>
<tr>
<td></td>
<td>IL1RA</td>
<td>0.307 ± 0.021</td>
</tr>
<tr>
<td></td>
<td>IL4(1)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>IL4(2)</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>IL10</td>
<td>0.014 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>IL13</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>TNFRSF1A</td>
<td>0.406 ± 0.019</td>
</tr>
</tbody>
</table>

Expression of inflammatory mRNAs of cases and control subjects in whole blood leukocytes, granulocytes and monocytes (normalized to B2M and in alphabetical order). Values given as mean ± SEM. P values are according to the Mann-Whitney test, ***P<0.001 vs controls. ND indicates not detectable.

Table 4. Signal transduction molecules and others.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Leukocytes</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Patients</td>
</tr>
<tr>
<td></td>
<td>B2M</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>BMI1</td>
<td>0.106 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>CDKN1A</td>
<td>0.048 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>GSTP1</td>
<td>0.029 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>MYC</td>
<td>0.174 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>NFKB1</td>
<td>0.183 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>NFKB2</td>
<td>0.025 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>NFKBIA</td>
<td>0.212 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>PARN</td>
<td>0.160 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>PDE4B</td>
<td>0.266 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>PDGFB</td>
<td>0.024 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>PTP4A2</td>
<td>0.709 ± 0.023</td>
</tr>
<tr>
<td></td>
<td>PTPN1</td>
<td>0.040 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>SERPINB9</td>
<td>0.201 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>THBS1</td>
<td>0.026 ± 0.002</td>
</tr>
</tbody>
</table>

Expression of inflammatory mRNAs of cases and control subjects in whole blood leukocytes, granulocytes and monocytes (normalized to B2M and in alphabetical order). Values given as mean ± SEM. P values are according to the Mann-Whitney test, *P<0.05, **P<0.01, ***P<0.001 vs controls. NA indicates not applicable; ND indicates not detectable.
### Inflammatory markers in Gram-negative sepsis

<table>
<thead>
<tr>
<th>Monocytes</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monocytes</strong></td>
<td><strong>Controls</strong></td>
<td><strong>Patients</strong></td>
</tr>
<tr>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0.234 ± 0.021</td>
<td>0.115 ± 0.009</td>
<td>0.149 ± 0.010</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>0.046 ± 0.004</td>
</tr>
<tr>
<td>0.000 ± 0.000</td>
<td>0.048 ± 0.022 ***</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>0.231 ± 0.011</td>
</tr>
<tr>
<td>0.004 ± 0.001</td>
<td>0.015 ± 0.005</td>
<td>0.192 ± 0.014</td>
</tr>
</tbody>
</table>

- **ND:** Not detected
Chapter 4

Table 5. Correlation between monocyte and granulocyte inflammatory mRNA expression and mortality in patients with sepsis caused by *B. pseudomallei*.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Leukocytes</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivors</td>
<td>Non-survivors</td>
</tr>
<tr>
<td>IL1B</td>
<td>0.318 ± 0.025</td>
<td>0.291 ± 0.050</td>
</tr>
<tr>
<td>IL1RA</td>
<td>0.562 ± 0.043</td>
<td>0.773 ± 0.135</td>
</tr>
<tr>
<td>IL15(1)</td>
<td>0.250 ± 0.073</td>
<td>0.266 ± 0.029 *</td>
</tr>
<tr>
<td>MIP1A</td>
<td>0.067 ± 0.009</td>
<td>0.110 ± 0.022</td>
</tr>
<tr>
<td>NFKB1</td>
<td>0.300 ± 0.016</td>
<td>0.378 ± 0.046</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>0.548 ± 0.054</td>
<td>1.089 ± 0.168 **</td>
</tr>
<tr>
<td>TNFRSF1A</td>
<td>0.718 ± 0.052</td>
<td>0.782 ± 0.106</td>
</tr>
<tr>
<td>SERPINB9</td>
<td>0.247 ± 0.029</td>
<td>0.402 ± 0.097</td>
</tr>
</tbody>
</table>

Expression of inflammatory mRNAs of survivors (n=20) and non-survivors (n=14) in whole blood leukocytes, granulocytes and monocytes (normalized to B2M and in alphabetical order). Only the inflammatory mRNAs with a significant relation to outcome are shown. Values given as mean ± SEM. P values are according to the Mann-Whitney test, *P<0.05, **P<0.01 vs survivors.

Inflammatory mRNA profile was separately analyzed. The majority of genes that were found upregulated in unseparated leukocytes was also upregulated in monocytes and granulocytes with a few exceptions. Increased expression of NFKB1 mRNA could only be detected in the granulocyte enriched population, while the monocytes appeared to be the source for the upregulated thrombospondin 1 mRNA levels (both p < 0.001 versus controls). Some genes (GSTP1, NFKB2, PDGFB, TF) showed a modestly enhanced expression in total leukocytes, which was not detectable in either monocyte or granulocyte enriched fractions.

**Correlation with mortality**

After characterizing the inflammatory mRNA profile we sought to examine differences in the mRNA profiles of leukocytes, monocytes and granulocytes between survivors and non-survivors of sepsis caused by *B. pseudomallei*. Table 5 shows the mRNAs of which the expression displayed an association with mortality. In particular, monocyte mRNA expression was associated with clinical outcome: IL-1β, IL-1RA, MIP-1α, NFKB1, NFKB1A and the TNF1-receptor-1 mRNAs were all significantly upregulated in the monocytes of non-survivors (p < 0.05-0.01 vs survivors). Interestingly, SERPINB9 mRNA upregulation was associated with a poor outcome only in granulocytes (p < 0.05 for the difference between survivors and non-survivors). There was no significant relation between the other analyzed inflammatory mRNA levels and outcome (data not shown).

**DISCUSSION**

The host response to sepsis is multifactorial and its pathogenesis complex. Analyses of gene expression patterns of blood leukocytes in human sepsis will provide useful information on
what has been called the "sepsis transcriptome" \cite{13,14}. Such clinical studies might help to gain new pathophysiological insights and help patient stratification, which in turn may result in more targeted and individualized therapeutic interventions. Numerous studies have already characterized the plasma protein concentrations of inflammatory mediators in sepsis. Our study is the first to perform an analysis of the inflammatory mRNA profile in sepsis, looking both at monocytes and granulocyte cell fractions.

Suzuki et al. were the first to comprehensively analyze the inflammatory gene expression profile of lipopolysaccharide-stimulated human macrophages as compared to resting monocytes by using serial analysis of gene expression (SAGE) \cite{15}. Nau et al. examined \textit{ex vivo} gene expression patterns in differentiated human macrophages in response to stimulation with different pathogens and showed that macrophages responded to a broad range of bacteria with a robust, shared pattern of gene expression \cite{16}. Furthermore, Feezor et al. characterized the inflammatory gene expression profile of leukocytes stimulated \textit{ex vivo} with Gram-negative and Gram-positive bacteria, demonstrating that the host inflammatory responses to Gram-negative and Gram-positive stimuli share some common response elements, but more important, exhibit distinct patterns of leukocyte gene expression \cite{14,17}. Lipopolysaccharide (endotoxin) is the major virulence factor in Gram-negative bacteria, and a recent study on the changes in blood leukocyte gene expression patterns in a human endotoxin model sheds lights on the effect this causes in humans \cite{13}. Calvano et al. intravenously administered endotoxin at a dose of 2 ng/kg in eight healthy volunteers and using a network-based analysis of systemic inflammation found that the human blood leukocyte response to acute systemic inflammation includes the transient dysregulation of leukocyte bioenergetics and modulation of translational machinery \cite{13}. In their discussion, Calvano et al stress the importance of the further identification of specific cell populations showing changes in inflammatory gene expression, which will require the isolation and enrichment of specific leukocyte subpopulations \cite{13}. Our study is the first of its kinds to use gene expression profiling to obtain insights into the systemic inflammatory status of both monocytes and granulocytes during human sepsis.
Part of our data confirm and extend previous studies. In a heterogeneous cohort of patients with septic shock, Pachot et al. also found increased levels of IL-1β mRNA in the leukocytes of patients with sepsis compared to healthy controls. In addition, the inflammatory gene expression profile of monocytes in our study confirmed the earlier ex vivo data by Suzuki et al. and Nau et al. who also showed an upregulation of genes encoding TNFα, IL-8, MCP-1, MIP-1α and MIP-1β in monocytes and macrophages stimulated with lipopolysaccharide or various bacteria. The marked pro-inflammatory response that occurs in sepsis is balanced by the activation of counter-regulatory pathways as illustrated by the release of anti-inflammatory cytokines. Indeed, in our patient population we observed a uniform upregulation of mRNAs encoding anti-inflammatory cytokines and cytokine antagonists such as IL-4, IL-10, IL-1RA, and TNF-receptor-1, in addition to upregulation of the pro-inflammatory cytokine mRNAs. This finding suggests that upregulation of IL-4 and IL-10 is insufficient to counter the pro-inflammatory consequences of sepsis caused by B. pseudomallei.

It is conceivable that in the not-too-distant future, mRNA expression profiles will be used for the identification of patients with sepsis who might benefit from a specific intervention. We show that high IL-1β, IL-1RA, MIP-1α, NFκB1, NFκB1A and TNF-receptor-1 mRNA levels in the monocytes are correlated with poor outcome. In a study that sought to determine gene expression in monocyte enriched populations in association with clinical outcome of patients with multiple blunt trauma, IL-1β was among the genes associated with a poor outcome. However, in our population of patients with sepsis due to melioidosis, we could not confirm the finding of another study, in which increased expression of IL-10 mRNA in leukocytes was correlated (and IL-1β not) with mortality in a heterogeneous population of patients with septic shock, highlighting the diverse nature of sepsis. The pattern of gene expression in patients who died suggests that in these individuals, there is a reprioritization of gene expression consistent with an early activation of selected genes involved in the initiation and propagation of a pro-inflammatory response. Given the fact that almost two-third of our patients had positive blood cultures for B. pseudomallei, it was possible to investigate potential differences in inflammatory mRNA profile between patients with bacteraemic and non-bacteraemic melioidosis. However, no significant difference of the inflammatory mRNA profile was seen between these two groups (data not shown), suggesting that the overall systemic inflammatory response as measured in whole blood leukocytes, granulocytes and monocytes is not greatly influenced by the detection of bacteria in the bloodstream in patients with sepsis.

The plasma levels of IL-6 and IL-8 are almost invariably strongly elevated in patients with severe sepsis (including melioidosis). Notably, using MLPA the mRNAs encoding these two cytokines were found to be not (IL-8) or only minimally (IL-6) increased in circulating leukocytes. These data strongly suggest that cells not present in blood are the major producers of IL-6 and IL-8 in sepsis, with endothelial cells and tissue macrophages being likely candidates. The expression of some genes was enhanced in the whole blood leukocytes but not in either monocyte or granulocyte enriched fractions. This suggests that lymphocytes are the source of some mRNAs in whole blood leukocytes. Indeed, lymphocytes are a major source for INFγ, of which modestly elevated mRNA levels were detected in blood leukocytes but not in monocytes or granulocytes.
The mRNA encoding tissue factor (TF), which is considered to be the initiator of coagulation activation in sepsis \(^{23}\), was detected at low levels in leukocytes of patients and controls, but was significantly higher in the former. The fact that TF mRNA levels were not detectable in monocytes or granulocytes may, especially in monocytes, be related to the limit of detection of the MLPA used. Of note, TF may in addition be produced by cell types not present in blood, in particular endothelial cells and macrophages \(^{23}\).

Our study has several limitations. Our observations were done in patients with culture proven melioidosis and caution is required when extending these findings to sepsis in general. In this respect it should be noted that Gram-positive and Gram-negative pathogens elicit distinct patterns of leukocyte gene expression \textit{in vitro} \(^{14, 17}\). In addition, during the inflammatory response to invading pathogens it is likely that the expression of inflammatory genes alters in time. We focused on the early phase of sepsis and only included patients as early as possible after hospital admission. Furthermore, it has to be noted that there was an age difference between the patient and control group which is due to the fact that it is difficult to obtain blood from healthy elder people living in Ubon Ratchathani, Thailand. We consider it unlikely, however, that this factor significantly influenced our main results considering the relatively large differences between patients and controls. Obtaining new biological insights from high-throughput genomic studies of human diseases is a challenge, limited by difficulties in recognizing and evaluating relevant biological processes from high quantities of experimental data \(^{13}\).

In conclusion, this study presents the direct measurement of molecular signatures from more than 30 inflammatory genes in the leukocytes, granulocytes and monocytes of a cohort of patients with sepsis caused by \textit{B. pseudomallei}. Our data underline the notion that circulating leukocytes are an important source for inflammatory mediators in patients with Gram-negative sepsis. Moreover, gene profiling such as done here by MLPA provides an excellent tool to obtain insight in the extent of inflammation activation in patients with severe infection.

**ACKNOWLEDGEMENTS**

We are grateful to the staff of Sapprasithiprasong Hospital, especially Professor Wipada Chaowagul. In addition, we thank Anita de Boer for expert technical assistance. This work was supported by the Dutch Foundation for Tropical Research (WOTRO). Allen Cheng was supported by a doctoral scholarship from the Australian National Health and Medical Research Council. Sharon Peacock is a Wellcome Trust Career Development Fellow.
Chapter 4

REFERENCES

Inflammatory markers in Gram-negative sepsis


Gene-expression profiles in murine melioidosis

Microbes Infect, 2008; 10(8); 868-877.

W. Joost Wiersinga, Mark C. Dessing, Tom van der Poll

Center for Infection and Immunity Amsterdam (CINIMA), Center for Experimental and Molecular Medicine, University of Amsterdam, Academic Medical Center, Amsterdam, the Netherlands
Melioidosis, caused by the bacterium *Burkholderia pseudomallei*, is a septicemic illness, often associated with pneumonia and bacterial dissemination to distant sites. Recently we reported the inflammatory mRNA-profile in blood leukocytes during human melioidosis. Knowledge of the inflammatory gene expression profile in the pulmonary compartment after infection with *B. pseudomallei* however is highly limited. We therefore aimed to characterize the inflammatory mRNA-profile in the pulmonary and systemic compartment during murine melioidosis. By using a newly developed mouse specific Multiplex-Ligation-dependent-Probe-Amplification (MLPA) assay we determined the expression profile of 33 genes encoding inflammatory proteins in lung tissue, leukocytes in bronchoalveolar-lavage-fluid (BALF) and blood leukocytes in mice before and at several time points after intranasal infection with *B. pseudomallei*. Relative to naïve mice, mice intranasally infected with *B. pseudomallei* showed increased transcription of a whole array of genes involved in inflammation, Toll-like receptor-signaling, coagulation, fibrinolysis, cell adhesion, tissue repair and homeostasis in the lung, BALF and blood compartment. Notably, many inflammatory genes showed to be differentially expressed during the course of infection. These data provide new information on compartmentalized inflammatory gene-expression profiles after infection with *B. pseudomallei*, increasing our insights into the extent of inflammation activation in the pulmonary and systemic compartment during melioidosis.
INTRODUCTION

Melioidosis, caused by the aerobic Gram-negative soil-dwelling bacillus *Burkholderia pseudomallei*, is a recognized biological threat agent and an important cause of severe sepsis in Southeast Asia and Northern Australia \(^1\). Pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis \(^1,2\). *B. pseudomallei* is able to survive and multiply in macrophages and can adhere and invade cultured human epithelial cell lines derived from alveolar and bronchial tissues \(^1,3\).

In recent years knowledge on the pathogenesis of this dreadful disease has increased considerably. It is now known that in the systemic compartment infection with *B. pseudomallei* affects a whole range of host mediator systems, including inflammatory modulators \(^1,4\), Toll-like receptor (TLR) signaling \(^5\) and coagulation \(^6\). In terms of host gene-expression profiles however data is still very limited. It was recently shown that *B. pseudomallei* rapidly modifies infected macrophage-like cells *in vitro* by upregulation of host cell inflammatory mRNAs such as the chemokines monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1 gamma (MIP-1\(\gamma\)) \(^7\). Mouse macrophages display an increased expression of ‘suppressor of cytokine signaling 3’ (SOCS-3) mRNA after stimulation with *B. pseudomallei* \(^8\). Furthermore, in order to analyze responses to immunisation, Ulett \textit{et al} made a first effort to investigate the gene profile of *B. pseudomallei* induced immunity using cDNA microarray in a mouse model of melioidosis highlighting parameters required for the generation of an adequate adaptive immunity \(^9\).

We recently reported the inflammatory mRNA-profile in blood leukocytes of patients with sepsis caused by *B. pseudomallei* \(^10\). Knowledge on the dynamics and extent of the inflammatory gene expression profile in the pulmonary and systemic compartment after infection with *B. pseudomallei* however is highly limited. Such knowledge may be important to obtain further insights into the immune response to infection with *B. pseudomallei*. Therefore, in the present study we aimed to characterize the inflammatory mRNA profile in the pulmonary and systemic compartment during melioidosis. We determined the relative expression of a set of 33 genes encoding cytokines, chemokines, proteins involved in coagulation and fibrinolysis, TLRs and associated proteins, and various other mediators implicated in the immune response to infection, in whole lung tissue, leukocytes harvested from broncho-alveolar lavage fluid (BALF) and whole blood leukocytes obtained from mice at various time points after intranasal infection with *B. pseudomallei*.
Chapter 5

MATERIALS AND METHODS

Mice
Pathogen-free 8 to 10 week old male wild-type C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). The Animal Care and Use of Committee of the University of Amsterdam approved all experiments.

Experimental infection
The model of melioidosis has been described in detail \(^5,11\). Briefly, mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and inoculated intranasally with 50 μl isotonic saline containing 5 x 10^2 colony forming units (CFUs) of \(B.\) pseudomallei strain 1026b (kindly provided by Dr. Don Woods, University of Calgary, Canada). This bacterial dose induces 100% mortality within a five-day period \(^5,11\). Mice were sacrificed before and 24, 48 or 72 hrs after infection (n = 4 at each time point) for gene expression profiling as described below.

Preparation of mRNA from whole blood, whole lung homogenates and BALF
Mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium: active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Mijdrecht, The Netherlands) and sacrificed by bleeding from the vena cava inferior. Bronchoalveolar lavage (BAL) was performed unilaterally as described previously \(^12\). Briefly, the right lung was ligated after which the trachea was exposed through a midline incision and cannulated with a 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland) and lavage was performed of the left lung by instilling 2 times 0.5 mL aliquots of cold saline and collecting the fluid by aspiration. BAL fluid (BALF) was spun (300 rcf for 5 minutes) and the cell pellet was immediately dissolved in TRIzol (Invitrogen, Breda, the Netherlands). Right lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Hundred μl of lung homogenate was immediately dissolved in TRIzol. Whole blood was drawn from the inferior vena cava, immediately put on ice and spun (300 rcf for 5 minutes). Then, whole blood leukocytes were isolated using ice-cold erylysis buffer (active ingredients: 155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4). After a second centrifugation round (300 rcf for 5 minutes) leukocytes were dissolved in TRIzol. Thereafter, RNA from whole blood leukocytes, lung tissue and BALF leukocytes was prepared according to the manufacturer’s protocol.

RNA analysis using Multiplex ligation-dependent probe amplification (MLPA)
In collaboration with MRC-Holland (Amsterdam, the Netherlands) we have developed a mouse-specific kit for the simultaneous detection of 33 mRNA molecules. This set was designed to obtain a global insight into the induction of several inflammatory pathways implicated in the host response to infection. After isolation of RNA, we analyzed the inflammatory gene expression profile by multiplex ligation-dependent probe amplification (MLPA) \(^10,13\). The mRNA’s analyzed are listed in table 1. This MLPA profiling method is insensitive to the total amount of mRNA that is included in the reaction; therefore, the profile is independent of the total cell count. All samples
### Table 1. Genes analyzed by multiplex ligation-dependent probe amplification (MLPA)

<table>
<thead>
<tr>
<th>Functional category and gene product</th>
<th>Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines and chemokines</strong></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL-1b</td>
<td>Interleukin 1beta</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>Cxcl1 / KC</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Cd3 / Mip1a</td>
<td>Chemokine (C-C motif) ligand 3</td>
</tr>
<tr>
<td><strong>Toll-like receptor</strong></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TLR9</td>
<td>Toll-like receptor 9</td>
</tr>
<tr>
<td>Ly96 / MD2</td>
<td>Lymphocyte antigen 96</td>
</tr>
<tr>
<td>IRAK1</td>
<td>Interleukin-1 receptor-associated kinase 1</td>
</tr>
<tr>
<td>IRAK3</td>
<td>Interleukin-1 receptor-associated kinase 3</td>
</tr>
<tr>
<td>Nfkbia / IkBa</td>
<td>Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha</td>
</tr>
<tr>
<td><strong>Coagulation and fibrinolysis</strong></td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>F3 / TF</td>
<td>Coagulation factor III</td>
</tr>
<tr>
<td>Procr</td>
<td>Protein C receptor, endothelial / EPCR</td>
</tr>
<tr>
<td>Plaur / uPAR</td>
<td>Plasminogen activator, urokinase receptor</td>
</tr>
<tr>
<td>F2r / PAR1</td>
<td>Coagulation factor II (thrombin) receptor</td>
</tr>
<tr>
<td>F2rl1 / PAR2</td>
<td>Coagulation factor II (thrombin) receptor-like 1</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>Nos3 / eNOS</td>
<td>Nitric oxide synthase 3, endothelial cell</td>
</tr>
<tr>
<td>Icam1</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>Sele / E-selectin</td>
<td>Selectin, endothelial cell</td>
</tr>
<tr>
<td>Itga5</td>
<td>Integrin alpha 5 (fibronectin receptor alpha)</td>
</tr>
<tr>
<td>Itgav</td>
<td>Integrin alpha V</td>
</tr>
<tr>
<td>Itgb3</td>
<td>Integrin beta 3</td>
</tr>
<tr>
<td>Vcam1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>Hif1a</td>
<td>Hypoxia inducible factor 1, alpha subunit</td>
</tr>
<tr>
<td>Mmp2</td>
<td>Matrix metalloprotease 2</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Matrix metalloprotease 9</td>
</tr>
<tr>
<td>Ela2</td>
<td>Elastase 2, neutrophil</td>
</tr>
<tr>
<td>Hp</td>
<td>Haptoglobin</td>
</tr>
<tr>
<td><strong>Housekeeping gene</strong></td>
<td></td>
</tr>
<tr>
<td>B2M</td>
<td>Beta-2 microglobulin</td>
</tr>
</tbody>
</table>
Chapter 5

were tested with the same batch of reagents. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of the β2 microglobulin control gene, resulting in the relative abundance of mRNAs of the genes of interest 10, 13.

Cytokine protein measurements

Mouse interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations.

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). Differences between more than two groups were analyzed by Kruskall-Wallis test. In case of overall significance this was followed by Mann-Whitney test for the differences between two groups. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Values of \( P < 0.05 \) were considered statistically significant.

RESULTS

Induction of pneumonia-derived sepsis after inoculation with a lethal dose of \( B. \) pseudomallei.

A large number of severe melioidosis cases present with pneumonia with bacterial dissemination to distant body sites 1. Considering that it is not feasible to study inflammatory gene expression profiles at the tissue level in patients with melioidosis, we used a mouse model of this disease in which mice are intranasally infected with \( B. \) pseudomallei. In accordance with previous reports from our laboratory 5, 11, 14, intranasal infection with \( 5 \times 10^2 \) CFU of \( B. \) pseudomallei resulted in severe pneumonia with a logarithmic increase of bacterial loads in the lungs followed by an increase in bacterial numbers in blood and the appearances of pulmonary abscess formation analogous to that found in patients (data not shown). During experimental murine melioidosis an influx of granulocytes into both the lung and BALF is seen 15. Mice were killed 24, 48 and 72 hours after infection (i.e. directly before the first predicted death 5, 11) and inflammatory gene expression was determined in cells derived from lungs, BALF and blood.

Cytokine and chemokines mRNA gene-expression

To investigate the expression profile of several cytokines and chemokines we analyzed mRNA levels in lung tissue, leukocytes present in BALF and blood leukocytes. mRNA expression of Interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α and MIP-1α were detectable in all compartments studied. IL-10 and cytokine induced neutrophil chemoattractant (KC) were only detectable in lung tissue (Figure 1). IL-4 mRNA levels could not be detected. IL-6, IL-1β, IL-10, TNFα and MIP-1α mRNA levels all increased during the course of \( B. \) pseudomallei infection, although for IL-6 and TNFα mRNA the increase in blood leukocytes was variable and therefore
Gene-expression profiles in melioidosis

**Toll-like receptor and associated proteins**

The TLRs have emerged as the central line of defense against invading pathogens. They are the first to detect host invasion by pathogens and initiate the immune response. Figure 2 shows the mRNAs encoding TLRs and associated proteins in lung tissue, BALF and blood cells. TLR2 and TLR4 mRNAs were detectable in all three compartments studied, whereas TLR9 mRNA was only measurable in whole lungs and blood leukocytes. Of these only TLR2 showed a significant upregulation during the course of infection (Figure 2). In addition, we found that IRAK-3 mRNA was induced during experimental melioidosis, in particular in whole lungs, while MD-2 and

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**Fig. 1. Cytokine and chemokine mRNA expression profile during murine melioidosis.** Naive mice or mice infected with $5 \times 10^2$ CFU *B. pseudomallei* intranasally were sacrificed at 0, 24, 48 and 72 hours after inoculation and mRNA was extracted from lung tissue (white bars), bronchoalveolar-lavage fluid (BALF) (gray bars) or whole blood leukocytes (black bars). Interleukin (IL)-6 (A), IL-1β (B), IL-10 (C), tumor necrosis factor (TNF)-α (D), cytokine induced neutrophil chemoattractant (KC) (E) and inflammatory protein (MIP)-1α (F) mRNAs were measured by MLPA as described in the Methods section. Data are means ± SEM; statistical analysis per compartment: * p < 0.05 vs uninfected mice; ns: not significant.
Chapter 5

IRAK-1 transcription was down regulated at later time points. The observed pro-inflammatory status was reflected by increased levels of the nuclear transcription factor NFκBIA.

Coagulation and fibrinolysis

Knowledge of the involvement of coagulation and fibrinolysis in the pathogenesis of melioidosis is highly limited. mRNA levels of important mediators of the coagulation and fibrinolysis are...
displayed in Figure 3. mRNA levels of tissue factor (TF), tissue factor pathway inhibitor (TFPI), receptor for urokinase-type plasminogen activator (uPAR), protease-activated receptor (PAR)-1, PAR-2 and the endothelial protein C receptor (EPCR) were all detectable in whole lung homogenates; of these only uPAR was detectable in all three compartments studied. No significant rise in TF mRNA levels could be seen in the lung, although a marked decrease in TFPI was seen 48 hrs after infection. At 72 hours after infection mRNAs encoding TF and TFPI had decreased in BALF cells. PAR-1 mRNA levels in the lung decreased, while uPAR and EPCR mRNA increased during the course of infection. Lung PAR-2 mRNA levels displayed no significant alterations during murine melioidosis.

**Cell adhesion, tissue repair and homeostasis molecules**

Lung, BALF and blood cells of mice infected with *B. pseudomallei* showed altered transcription of an array of genes involved in cell adhesion, tissue repair and homeostasis. Whole lung tissue
Fig. 4. Integrins and tissue repair molecules mRNA expression profile during murine melioidosis. Naïve mice or mice infected with $5 \times 10^2$ CFU *B. pseudomallei* intranasally were sacrificed at 0, 24, 48 and 72 hours after inoculation and mRNA was extracted from lung tissue (white bars), bronchoalveolar-lavage fluid (BALF) (gray bars) or whole blood leukocytes (black bars). Intercellular adhesion molecule (ICAM)-1 (A), integrin alpha V (ITGAV) (B), hypoxia inducible factor (HIF)-1α (C), haptoglobin (HP) (D), integrine alpha 5 (ITGA5) (E), endothelial cell nitric oxide synthase (NOS)-3 (F), matrix metallopeptidase (MMP)-2 (G), MMP-9 (H) and integrin beta 3 (ITGB3) (I) mRNAs were measured by MLPA as described in the Methods section. Data are means ± SEM; statistical analysis per compartment: * $p < 0.05$ vs uninfected mice; ns: not significant.
Gene-expression profiles in melioidosis

Table 2. Elevated mRNA cytokine levels are associated with enhanced production at the protein level.

<table>
<thead>
<tr>
<th></th>
<th>Lung</th>
<th>BALF</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mRNA (rel.B2M)</td>
<td>Protein (pg/ml)</td>
<td>mRNA (rel.B2M)</td>
</tr>
<tr>
<td>IL-6</td>
<td>t = 0 0.57 ± 0.24 1872 ± 220 BD 737 ± 85 BD 150 ± 35</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>t = 24 0.54 ± 0.22 3959 ± 759 0.06 ± 0.03 4272 ± 605 BD 238 ± 30</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>t = 48 0.80 ± 0.27 9825 ± 175 0.41 ± 0.18 9459 ± 541 BD 10000 ± 250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>t = 0 0.08 ± 0.02 566 ± 83 0.76 ± 0.09 2830 ± 172 BD 11 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>t = 24 0.54 ± 0.08 1717 ± 253 1.19 ± 0.23 6954 ± 1164 BA 50 ± 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>t = 48 0.89 ± 0.16 4462 ± 349 0.91 ± 0.17 10000 ± 250 0.10 ± 0.09 2110 ± 273</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mice were intranasally inoculated with 5 x 10^2 CFU B. pseudomallei. After 0, 24, 48 and 72 h mice, levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured in lungs, bronchoalveolar lavage fluid (BALF) and blood, at both the mRNA and protein level. mRNA levels are expressed as the normalized ratio of the peak area divided by the peak area of the β2 microglobulin (B2M) control gene (see Materials and Methods). Data are expressed as mean ± SEM; BD: below detection.

Elevated mRNA cytokine levels are associated with enhanced production at the protein level

In order to analyze whether mRNA levels do correspond with protein concentrations, we measured the expression of the important pro-inflammatory cytokines IL-6 and TNFα both at the mRNA and protein levels in all compartments (Table 2). Indeed, the generally observed increase in IL-6 and TNFα levels over time in all three compartments was seen both at the mRNA and protein level. Of interest however, although both protein and mRNA levels of IL-6 and TNFα were highly elevated in both the pulmonary and BALF compartment, only the protein - and not the mRNA – levels where highly elevated in the systemic compartment, probably due to elevated synthesis in and consequent secretion from the lung.

DISCUSSION

Melioidosis is a debilitating septic disease with a mortality of up to 50% in endemic areas. As with other forms of sepsis, multiple organ failure and death are thought to result from an uncontrolled
inflammatory reaction after infection with *B. pseudomallei*. Severe pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis. Although several investigations have examined the host inflammatory response in melioidosis, our study is the first effort to evaluate the time-dependent expression of a set of inflammatory genes in the pulmonary (whole lung homogenates and BALF cells) and systemic (blood leukocytes) compartment after inoculation of *B. pseudomallei* via the airways in mice. For this we developed a mouse specific MLPA-kit that enables simultaneous quantitative measurement of a range of genes involved in the host response to infection. Here we demonstrate the expression profile of a whole array of genes involved in inflammation, TLR signaling, coagulation- and fibrinolysis pathways, cell adhesion, tissue repair and homeostasis during the course of *B. pseudomallei* infection in the lung, BALF and blood compartment. Notably, many inflammatory genes were shown to be differentially expressed during the course of infection.

Recently we have reported the inflammatory mRNA-profile in blood leukocytes of patients with sepsis caused by *B. pseudomallei*. This work was done by making use of the MLPA technique in which one can quantitate the relative expression of up to 40 different mRNAs in a single reaction using very small amounts of sample RNA. The benefit of MLPA over whole genome microarrays is that MLPA is a more targeted technique to analyze genes of interest. In addition, using MLPA one can (semi)quantify the extent of up/down regulation of genes. The disadvantage of MLPA, however, is that in contrast to whole genome microarrays, with MLPA altered expression of genes not included in the assay is missed. We now have extended the MLPA technique into the murine experimental set-up by making use of a newly developed mouse specific inflammatory MLPA kit.

In accordance with our data from patients with septic melioidosis, mice infected with *B. pseudomallei* demonstrated an upregulation of MIP-1α mRNA in the systemic compartment. Moreover, the observed increase in the expression of genes encoding IL-6, IL-1β, IL-10 and TNFα as seen in patients with melioidosis corresponded with a rise in the expression of these genes in the pulmonary compartment during the course of experimental murine melioidosis as seen in the current study. Of note, in general the elevated TNFα and IL-6 mRNA levels were associated with an enhanced production at the protein level. Our data extend earlier reports on cytokine mRNA expression in melioidosis. It has been shown in both BALB/c and C57BL/6 mice that infection with *B. pseudomallei* is associated with a rise in TNFα, IL-1β, IL-6 and IL-10 mRNA levels in the liver and spleen. Furthermore, it has been described that macrophage death induced by *B. pseudomallei* is associated with the release of IL-1β. Although it has been demonstrated KC mRNA is induced in the spleen during murine melioidosis, we could not demonstrate a significant upregulation of this chemokine in the pulmonary compartment after experimental infection of mice with *B. pseudomallei*. In sepsis, the marked pro-inflammatory response is balanced by the activation of counter-regulatory pathways as illustrated by the release of anti-inflammatory cytokines. In human melioidosis one sees elevated cytokine plasma levels of both IL-6 (a mixed pro- and anti-inflammatory cytokine) and IL-10 (the prototypic anti-inflammatory cytokine). In the present study, we observed an upregulation of mRNAs encoding the anti-inflammatory cytokine IL-10 only in lung tissue, whereas the anti-inflammatory cytokine IL-4 mRNA could
not be detected in any of the compartments analyzed. This finding suggests that the production of anti-inflammatory cytokines is relatively insufficient to counter the pro-inflammatory consequences of sepsis caused by *B. pseudomallei*.

The TLR detect host invasion by pathogens, initiate immune responses and form the crucial link between the innate and adaptive immune systems. In general, the interaction of a pathogen-related molecule to a TLR induces the recruitment of intracellular adaptor proteins to the TLR and the activation of several kinases, which ultimately leads to the translocation of nuclear transcription factors and the transcription of several genes encoding pro- and anti-inflammatory cytokines and chemokines. In the first publication on the role of the TLRs in melioidosis to date, we have shown recently that patients with melioidosis display an upregulation of multiple TLRs in peripheral blood monocytes and granulocytes. Although both TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei* in vitro, TLR2 detects the lipopolysaccharide (LPS) of *B. pseudomallei* and only TLR2 impacts on the immune response of the intact host in vivo. We now extend these findings by showing upregulation of TLR2 mRNA, and not of TLR4 or TLR9 mRNA, in the lung tissue of mice infected with *B. pseudomallei*. Furthermore, we found that IRAK-3 mRNA was induced during experimental melioidosis, while MD-2 IRAK-1 transcription was down regulated at later time points. The observed depletion of IRAK-1 mRNA is in line with studies in human volunteers injected with LPS and could reflect an early adaptation towards a state of immunotolerance during the course of melioidosis in order to dampen the overwhelming proinflammatory response. Of note, NFκB1a was upregulated in all compartments studied. Taken as a whole, it could well be that in melioidosis, the TLR-mediated inflammatory responses may exceed the threshold to maintain homeostasis of the immune system and cause harm by initiating an overwhelming inflammatory response upon the invasion of *B. pseudomallei*.

Activation of inflammatory and coagulation pathways is an important event in the pathogenesis of sepsis. Proteins involved in the coagulation system are TF, TFPI, PAR-1 and PAR-2, whereas uPAR and EPCR are members of the fibrinolysis and anti-coagulant pathways. In our model of murine melioidosis, TF mRNA did not change significantly in lung tissue although at 72 hours after infection a trend towards an increase was detected; in BALF cells however, TF mRNA declined during the course of the infection. Of interest, TFPI mRNA levels decreased in both lung and BALF cells, which may point to a diminished capacity to inhibit coagulation activation. PAR-1 mRNA levels in the lung decreased in lung tissue, which is expected to diminish the capacity of thrombin and TF-Factor VIIa mediated cell signalling.

Lung, BALF and blood cells of mice infected with *B. pseudomallei* showed altered transcription of an array of genes involved in cell adhesion, tissue repair and homeostasis. Of these factors, MMP-9 was increased in lung, BALF and blood cells, while MMP-2 was decreased in lung cells. MMP-2 and MMP-9 are both proteinases capable of degrading type IV collagen and have recently been implicated in the resolution of inflammatory cells in vivo. Haptoglobin, which is considered to be an acute phase protein, was upregulated in the systemic compartment. We did not observe any significant differences in eNOS3 expression during the course of infection, which is in line
with the finding that inducible nitric oxide synthase does not play a major role in the early control of *B. pseudomallei* infection in mice.\(^{29}\)

Our study has several limitations. It should be noted that, in order to avoid artificial alterations in gene expression profiles due to the purification procedures, we chose not to perform MLPA on purified cell populations from BALF or lung tissue. Furthermore, we focused on the acute septic form of melioidosis; therefore caution is required when extending these findings to the more undulant forms of less acute melioidosis. MLPA analyzes mRNA levels which may not directly correspond with protein concentrations. However, several previous studies, including investigations from our own laboratory, support the notion that the elevated mRNA described here are associated with enhanced production at protein level. For instance, during melioidosis, high pulmonary TLR2 mRNA levels are related to increased TLR2 protein levels.\(^{5}\) In addition, increased levels of several pro-inflammatory cytokine mRNAs in the systemic compartment go along with increase plasma levels of those cytokines.\(^{10}\) Our current study now shows that elevated pulmonary and BALF IL-6 and TNF\(\alpha\) protein levels correspond with increased mRNA expression levels. Lastly, obtaining new biological insights from high-throughput genomic studies of human diseases remains a challenge, limited by difficulties in recognizing and evaluating relevant biological processes from high quantities of experimental data.\(^{30}\)

In conclusion, we demonstrate the expression profile of a range of genes involved in inflammation, TLR signaling and the coagulation- and fibrinolysis pathway during experimental murine melioidosis. Our data show that throughout the course of infection a broad range of genes is differentially expressed. These data provide new information on compartmentalized inflammatory gene expression profiles after infection with *B. pseudomallei*, increasing our insights into the extent of inflammation activation in the pulmonary and systemic compartment during melioidosis.

**ACKNOWLEDGMENTS**

We are grateful to Anita de Boer, Marieke ten Brink en Joost Daalhuisen for expert technical assistance. We thank Michael W. Tanck for statistical advice.
REFERENCES


Chapter 5


Endogenous Interleukin-18 improves the early anti-microbial host response in severe melioidosis


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Chapter 6

ABSTRACT

Melioidosis is caused by the soil saprophyte *Burkholderia pseudomallei*, and is endemic in South East Asia. The pathogenesis of melioidosis is still largely unknown, although interferon (IFN)-γ seems to play an obligatory role in host defense. Previously, we have shown that IFN-γ production in melioidosis is controlled in part by interleukin (IL)-18. The aim of the present study was to determine the role of IL-18 in the immune response to *B. pseudomallei*. For this the following investigations were performed: (1) plasma IL-18 and blood monocyte IL-18 mRNA levels were elevated in 34 patients with culture proven melioidosis when compared to 32 local healthy controls; in addition, IL-18 binding protein levels were markedly elevated in patients, strongly correlating with mortality. (2) IL-18 gene-deficient (IL-18 KO) mice showed an accelerated mortality after intranasal infection with a lethal dose of *B. pseudomallei*, which was accompanied by an enhanced bacterial growth in their lungs, liver, spleen, kidneys and blood at 24 and 48 h post infection when compared to WT mice. In addition, IL-18 KO mice displayed evidence of enhanced hepatocellular injury and renal insufficiency. Together these data indicate that the enhanced production of IL-18 in melioidosis is an essential part of a protective immune response to this severe infection.
INTRODUCTION

Melioidosis is caused by the aerobic Gram-negative soil-dwelling bacillus *Burkholderia pseudomallei* and is an important cause of severe sepsis in Southeast Asia and Northern Australia. Humans acquire melioidosis by inoculation through skin abrasions or inhalation. More than half of patients with melioidosis present with pneumonia associated with bacterial dissemination to distant organs. Mortality due to melioidosis is high, varying from 50% in northeast Thailand to 20% in the higher technology setting of Northern-Australia. Interest in disease pathogenesis of melioidosis has increased following its classification as category B disease/agent of bioterrorism by the US Centers for Disease Control and Prevention.

*B. pseudomallei* is an intracellular pathogen that multiplies within macrophages. Although more is becoming known about the pathogenesis of melioidosis, host-pathogen interactions are still ill defined. Several investigations have implicated interferon (IFN)-γ as an important mediator in protective immunity against melioidosis. In mice infected with *B. pseudomallei* intraperitoneally, inhibition of IFN-γ lowered the LD50 from >5 x 10^5 to ~2 colony-forming units (CFUs) and was associated with an 8,500- and 4,400-fold increase in bacterial loads in liver and spleen respectively. Similarly, IFN-γ deficient mice displayed early mortality after intraperitoneal infection with *B. pseudomallei* together with strongly increased bacterial burdens in their spleen. Inhibition of interleukin (IL)-12 or IL-18, the predominant endogenous inducers of IFN-γ production, resulted in increased mortality in the same model. IL-18 is mainly produced by activated macrophages, and is first synthesized as a precursor protein (pro-IL-18, 24 kD), which requires splicing by caspase-1 to liberate the 18 kD mature active protein. The biological activity of IL-18 is further regulated by IL-18 binding protein (IL-18BP), which binds IL-18 thereby preventing the interaction with its cell-associated receptor. Besides its IFN-γ-inducing effect, IL-18 has many proinflammatory effects on T and natural killer (NK) cells, enhancing proliferation and cytotoxicity, activating nuclear factor-κB and stimulating the production of cytokines, including tumor necrosis factor (TNF)-α, IL-1, IL-2 and IL-6. Thus far only one investigation has directly addressed the role of endogenous IL-18 in experimental melioidosis. In that study administration of a blocking anti-IL-18 receptor antibody increased the early mortality after intraperitoneal injection of *B. pseudomallei*; the impact of IL-18 inhibition on antibacterial defense was not examined. The aim of the present study was to determine the role of IL-18 in the immune response to melioidosis. For this we measured the expression of IL-18 and IL-18BP in 34 Thai patients with culture proven melioidosis and assessed the function of endogenous IL-18 using IL-18 knockout (KO) mice and a model of intranasal infection with *B. pseudomallei* that mimics melioidosis with severe pneumonia and bacterial dissemination to distant body sites.
Chapter 6

MATERIALS AND METHODS

Patients study
34 patients with melioidosis (mean age 52 years, range 18-86 years; 50% male) were recruited prospectively at Sapprasithiprasong Hospital, Ubon Ratchathani, northeast Thailand in 2004. Sepsis due to melioidosis was defined as culture positivity for *B. pseudomallei* from any clinical sample plus systemic inflammatory response syndrome (SIRS)\(^6\). To meet the SIRS criteria patients had to meet at least three of the following four criteria: a core temperature of \(\geq 38^\circ C\) or \(\leq 36^\circ C\); a heart rate of \(\geq 90\) beats/min; a respiratory rate of \(\geq 20\) breaths/min or a PaCO\(_2\) of \(\geq 32\) mmHg or the use of mechanical ventilation for an acute respiratory process; and a white-cell count of \(\geq 12 \times 10^9/l\) or \(\leq 4 \times 10^9/l\) or a differential count showing \(> 10%\) immature neutrophils. These definitions have been used in large clinical trials and were modified according to the latest revisions\(^7, 8\). Exclusion criteria were the use of dialysis and/or immunosuppressive therapy, known disorders of coagulation and concomitant infection with human immunodeficiency virus. Blood samples were drawn within 36 hours of the start of appropriate antimicrobial therapy. 32 healthy blood donors (mean age 41 years, range 21-59 years; 71% male) recruited from the Sapprasithiprasong hospital blood bank served as a control population. The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, UK and written informed consent was obtained from all study subjects.

IL-18 and IL-18 BP measurements
Human IL-18 and IL-18BP\(_a\) were measured by ELISA (R&D, Minneapolis, MN). In addition, IL-18 mRNA levels were measured as follows. Heparin blood samples were drawn from the antecubital vein and immediately put on ice. Leukocytes were isolated using erylysis buffer. Monocyte and granulocyte enriched populations where isolated using Polymorphprep (Axis-Shield, Dundee, United Kingdom). Monocyte and granulocyte fractions were \(> 98\%\) pure as determined by their scatter pattern on flow cytometry. After isolation leukocytes, monocyte and granulocyte were dissolved in Trizol and stored at \(-80^\circ C\) until used for RNA isolation. RNA was isolated and analyzed by multiplex ligation-dependent probe amplification (MLPA) as described\(^9\) using an inflammatory-specific kit developed in collaboration with MRC-Holland (Amsterdam, the Netherlands). All samples were tested with the same batch of reagents. The levels of mRNA were expressed as a normalized ratio of the peak area divided by the peak area of the \(\beta 2\) microglobulin (B2M) gene, resulting in the relative abundance of mRNAs of the genes of interest\(^9\). Transcription of the B2M gene was not affected by *B. pseudomallei* infection.

Mouse infection
The Animal Care and Use of Committee of the University of Amsterdam approved all experiments. Pathogen-free 8 to 10 week old wild-type (WT) C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). IL-18 KO mice (backcrossed 6 times to a C57BL/6 background) were generated previously as described\(^10\) and generously provided by Dr. Shizuo Akira (Osaka University, Japan). Age and sex-matched animals were used in all experiments.
each timepoint one separate experiment was performed. The survival experiment was performed once. For preparation of the inoculum, *B. pseudomallei* strain 1026b (kindly provided by Dr. Don Woods, University of Calgary, Canada) was pipetted from frozen aliquots into 50 ml Luria broth (Difco, Detroit, MI) and placed overnight at 37°C in a shaking incubator. Thereafter, a 1 ml portion was transferred to fresh Luria broth and grown for ± 5h to midlogarithmic phase. Bacteria were harvested by centrifugation at 1500 x g for 15 minutes, washed and resuspended in sterile isotonic saline at a concentration of 5x10^2 CFUs/50 μl, as determined by plating serial 10-fold dilutions on blood agar plates. Pneumonia was induced by intranasal inoculation of 50 μl (5x10^2 CFU) bacterial suspension. For this procedure mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands).

**Determination of bacterial outgrowth**

24 and 48 hrs after infection, mice were anesthetized with Hypnorn (Janssen Pharmaceutica, Beerse, Belgium: active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Mijdrecht, The Netherlands) and sacrificed by bleeding from the vena cava inferior. The lungs, spleen, liver, kidneys and the brain were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of organ homogenates and blood, plated on blood agar plates and incubated at 37°C at 5% CO₂ for 16 h before colonies were counted.

**Preparation of lung tissue for cytokine measurements**

For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, and 8 μg/ml AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride), 100 μg/ml EDTA-NA₂, 20 μg/ml Pepstatin and 20 μg/ml Leupeptin (pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed.

**Assays**

Mouse IL-18 was measured by ELISA (MBL International, Woburn, MA). Mouse TNF-α, IFNγ, monocyte chemoattractant protein (MCP)-1 and IL-6 were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. Aspartate aminotransferase (ASAT), creatinine and urea were determined with commercially available kits (Sigma-Aldrich), using a Hitachi analyzer (Roche) according to the manufacturer’s instructions.

**Pathology**

Lungs, spleen, liver, kidneys and the brain of each mouse were harvested after infection, fixed in 10% formalin and embedded in paraffin. Four μm sections were stained with H&E, and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: % surface with pneumonia, necrosis/abscess formation, interstitial inflammation, endothelialitis, bronchitis, edema, thrombus formation and pleuritis. Liver and spleen sections were scored on inflammation, necrosis/
abscess formation and thrombus formation. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe.

Statistical analysis
Values are expressed as means ± SEM unless indicated otherwise. Differences between groups were analyzed by Mann-Whitney U test. Correlations were calculated using the Spearman’s rho test. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Values of $P<0.05$ were considered statistically significant.

RESULTS

Elevated plasma IL-18, IL-18 BP and blood leukocyte IL-18 mRNA levels in patients with culture proven melioidosis
To obtain a first insight into IL-18 and IL-18BP expression during septic melioidosis, we measured these cytokines in the plasma from patients with septic melioidosis and healthy controls. In addition we quantified IL-18 mRNA in their blood leukocytes (Figure 1). We first confirmed our earlier data on increased serum IL-18 concentrations in patients with melioidosis in the

![Fig. 1. Plasma IL-18, IL18 BP and blood leukocyte IL-18 mRNA levels in patients with culture proven melioidosis. Increased levels of IL-18 plasma (A), IL-18 mRNA in peripheral blood leukocytes (B) and IL-18 mRNA in peripheral blood monocytes (C) of patients (n=34) with septic melioidosis compared to healthy controls (n=32). The strongly increased levels of IL-18 BP (D) correlated with IL-18 levels (E). ** p < 0.01; *** p < 0.001.](image)
IL-18 in melioidosis

Current study population: relative to healthy controls (195 ± 13 pg/ml), patients displayed strongly elevated plasma IL-18 levels (4616 ± 601 pg/ml, \( P < 0.001 \); Figure 1A). We extended this finding by showing elevated plasma IL-18 mRNA levels in whole blood (unfractionated) leukocytes of patients with septic melioidosis (Figure 1B, \( P < 0.001 \) versus controls). Monocytes were at least in part responsible for this enhanced IL-18 mRNA production since patients had significantly elevated IL-18 mRNA levels in monocyte enriched cell fractions (Figure 1C, \( P < 0.01 \) versus controls). IL-18 mRNA was not detectable in granulocyte enriched cell fractions (data not shown). Of note, the difference in circulating IL-18 levels between patients and controls was much larger than the difference in IL-18 mRNA expression in peripheral blood leukocytes, suggesting that cell types not present in peripheral blood (e.g. tissue macrophages) contribute to circulating IL-18 levels and/or that more mature IL-18 is generated from pre-IL-18 in patients. Plasma IL-18BP levels were strongly elevated in patients (59 ± 3.7 ng/ml) relative to healthy controls (10 ± 0.5 ng/ml, \( P < 0.001 \); Figure 1D). In melioidosis patients, plasma IL-18 and IL-18BP levels showed a strong positive correlation (\( r = 0.70, P < 0.0001 \); Figure 1E).

Plasma IL-18 and IL-18BP correlate with mortality

High plasma levels of both IL-18 and IL-18BP on admission correlated with an adverse outcome. Patients who died during hospital admission had higher IL-18 (7552 ± 797 pg/ml vs 3014 ± 591 pg/ml) and IL-18BP (73 ± 2.6 vs 48 ± 4.7 ng/ml) concentrations than those who survived to discharge (both \( P < 0.001 \) for the differences between groups, Figures 2A and B). Further proof for a correlation between the plasma levels of IL-18 and IL-18BP and disease severity was obtained in 8 patients who survived and from whom a second blood sample was drawn after successful therapy. In these patients a strongly significant decrease in plasma IL-18 and IL-18BP concentrations was detected (both \( P < 0.001 \); Figures 2C and D).

![Fig. 2. Correlation of IL-18 and IL-18 BP levels with outcome in patients with severe melioidosis. Both IL-18 and IL-18 BP levels strongly correlated with outcome. Both IL-18 (A) and IL-18 BP (B) were strongly upregulated in patients who died in comparison to the patients who survived. Patients (n=8) that survived after two weeks of intensive treatment showed near normalization of IL-18 (C) and IL-18 BP (D) levels. ** \( p < 0.01 \); *** \( p < 0.001 \).]
Chapter 6

**IL-18 KO mice show accelerated mortality during experimental melioidosis**

Having established that IL-18 is upregulated in patients with severe melioidosis and correlates with mortality, we next investigated the involvement of IL-18 in the host response to *B. pseudomallei* infection in a murine model of melioidosis. Wild type (WT) and IL-18 KO mice were intranasally infected with *B. pseudomallei*. Inoculation with *B. pseudomallei* resulted in an increase in IL-18 concentrations in lung homogenates (from 44 ± 27 pg/ml to 269 ± 44 pg/ml; P = 0.0025) but not in plasma of WT mice at 48 hours after infection; in IL-18 KO mice IL-18 remained undetectable throughout. As a first experiment, WT and IL-18 KO mice were intranasally infected with a lethal dose of *B. pseudomallei* and followed until death. IL-18 deficiency had a negative effect on survival. Whereas all WT mice were dead after 123 hours (median survival time 90 hours), all IL-18 KO mice had died within 87 hours (median survival time 79 hours; P = 0.0052 for the difference between groups; Figure 3A).

IL-18 KO mice display an enhanced bacterial load of *B. pseudomallei*

To obtain an insight into the mechanisms underlying the accelerated mortality of IL-18 KO mice during experimental melioidosis, we infected WT and IL-18 KO mice with *B. pseudomallei* and sacrificed them after 24 and 48 hours (i.e. before the first deaths occurred) to determine
bacterial loads in lungs (the primary site of the infection), spleen, kidneys, brain and blood so as to evaluate bacterial loads and dissemination to distant body sites (Figure 3B to E). Relative to WT mice, IL-18 KO mice displayed strongly increased bacterial loads in the lungs and liver at 24 and 48 hours after infection, as well as in blood, kidneys (data not shown) and spleen at 48 hours (Figure 3B to E). There was no bacterial growth in the brains of either WT or IL-18 KO mice (data not shown).

**Lung histology and distant organ injury**

To further evaluate the role of IL-18 in the early antibacterial defense against *B. pseudomallei*, histological samples of lung, spleen, liver, kidney and brain obtained 24 and 48 hours after infection, were semi-quantitatively scored on the extent of inflammation. Pulmonary inflammation was characterized by significant inflammation, pleuritis, peribronchial inflammation, oedema and endothelialitis in both WT and IL-18 KO mice (Figure 4). Lung inflammation scores were similar at both 24 and 48 hours after infection in both mice strains (data not shown). In contrast, livers of IL-18 KO mice showed significantly more inflammation after 48 hours compared to WT mice after inoculation with *B. pseudomallei* (Figure 5A to C). Consistent with these pathology data, the plasma levels of ASAT were higher in IL-18 KO mice 48 hours post infection, reflecting increased hepatocellular injury in these animals (P < 0.05 versus WT mice, Figure 5D). In addition,
**Table 1.** Cytokine profile during experimental melioidosis in IL-18 KO mice.

<table>
<thead>
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<th></th>
<th>0 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
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<tr>
<td>Lung (pg/mL)</td>
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</tr>
<tr>
<td>INFγ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNFα</td>
<td>ND</td>
<td>ND</td>
<td>75 ± 24</td>
</tr>
<tr>
<td>IL-6</td>
<td>51 ± 11</td>
<td>373 ± 27</td>
<td>376 ± 65</td>
</tr>
<tr>
<td>MCP-1</td>
<td>300 ± 47</td>
<td>3278 ± 1084</td>
<td>1881 ± 894</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Plasma (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INFγ</td>
<td>ND</td>
<td>30 ± 5*</td>
<td>ND</td>
</tr>
<tr>
<td>TNFα</td>
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<tr>
<td>IL-6</td>
<td>ND</td>
<td>216 ± 14</td>
<td>154 ± 41</td>
</tr>
<tr>
<td>MCP-1</td>
<td>17 ± 2</td>
<td>572 ± 74</td>
<td>610 ± 79</td>
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</table>

IL-18 and IL-6 were measured in lung homogenates. Data are means ± SEM of 8 mice per group per time point. * p < 0.05; ND: not detectable or below detection limit.

**Fig. 5. Liver inflammation in IL-18 KO mice.** Livers of IL-18 KO mice showed more inflammation after 48 hours compared to WT mice after inoculation with *B. pseudomallei*. Representative hematoxilin and eosin stained liver histology slides (original magnification x 4, insets x 20) are shown of WT (A) and IL-18 KO (B) mice at 48 hours after inoculation with 5x10^2 CFU *B. pseudomallei*. Pathology scores (C) are given for 48 hrs after inoculation (means ± SE) as described in the Methods section. At 48 hrs after inoculation IL-18 KO mice also showed enhanced hepatic injury, as reflected by the plasma concentrations of ASAT (D) and a more renal failure, as reflected by plasma creatinine (E) and BUN (F). Data are expressed as mean ± SEM of 8 WT mice (open bars) and 8 IL-18 KO mice (black bars). U/L: units per liter; * p < 0.05 versus WT control.
IL-18 KO mice showed evidence of renal failure, as indicated by elevated plasma concentrations of urea (P < 0.05 versus WT mice, Figure 5F), whereas the increase in plasma creatinine did not reach significance (Figure 5H). On pathologic examination, renal histology was unremarkable in both IL-18 KO and WT mice. Spleen histology showed mild inflammation that was equally distributed in both mice strains. Moreover, histological examination of the harvested brains revealed no signs of inflammation.

**Enhanced inflammatory cytokine profile in of IL-18 KO mice infected with *B. pseudomallei***

To further examine the impact of IL-18 deficiency on the host response to melioidosis we measured the concentrations of IFN-γ, TNF-α, IL-6 and MCP-1 in lungs and plasma of IL-18 KO and WT mice 24 and 48 hours after infection with *B. pseudomallei* (Table 1). IFN-γ remained undetectable in lung homogenates of either IL-18 KO or WT mice; in plasma, however, WT but not IL-18 KO mice demonstrated elevated IFN-γ levels in plasma. TNF-α concentrations did not differ between the two mouse strains. IL-6 and MCP-1 levels were higher in IL-18 KO mice at 48 hours but not at 24 hours after infection (not significant for IL-6 in plasma).

**DISCUSSION**

In this study we sought to determine the role of IL-18 in the immune response to melioidosis. We demonstrate that the plasma concentrations of IL-18 and IL-18BP are elevated in patients with severe melioidosis and that high IL-18 and IL-18BP levels on admission are correlated with death. In experimental melioidosis induced by intranasal infection with a lethal dose of *B. pseudomallei*, IL-18 KO mice displayed an enhanced bacterial growth in lungs, blood and distant organs, accompanied by increased hepatocellular injury and renal insufficiency, and associated with an accelerated mortality. Together these data indicate that the enhanced production of IL-18 in melioidosis is an essential part of a protective immune response to this severe infection.

The current study builds on an earlier investigation from our group, reporting elevated circulating levels of IL-18 in patients with melioidosis. We here confirm these findings and in addition show that blood monocytes are a source for IL-18. IL-18 mRNA was also detectable in monocyte enriched cell fractions obtained from healthy controls, albeit at lower levels, which is consistent with a previous study showing constitutive IL-18 production by human monocytes in vitro. Circulating IL-18 levels showed a strong positive correlation with IL-18BP. This naturally occurring secreted protein has a high-affinity for binding to IL-18. IL-18BP is not a soluble receptor for IL-18 and is only distantly related to the cell-associated IL-18 receptor. IL-18BP exists as 4 isotypes (IL-18BPa,b,c,d) of which only IL-18BPa (measured in the present study) and IL-18BPc are able to neutralize IL-18. Both IL-18 and IL-18BP levels were higher in patients who eventually died and decreased in survivors after successful treatment. Our data are in line with other studies on IL-18 and IL-18BP performed in septic patients. In a heterogeneous group of septic patients IL-18 and IL-18BPa levels were both significantly elevated. At these observed high
levels, most IL-18 was bound to IL-18BP; however, the remaining free IL-18 was still higher than in healthy individuals. Furthermore, patients with septic shock who did not survive displayed higher IL-18 levels than patients who survived. In addition, in a small cohort of 13 patients with sepsis IL-18 levels correlated significantly with APACHE II scores.

One previous investigation studied the role of IL-18 in experimental melioidosis. In a recent study by Haque et al focusing on the role of T cells in the immune response against *B. pseudomallei*, blockade of the IL-18 receptor in a mouse model resulted in a decreased survival after intraperitoneal injection of *B. pseudomallei*; the effect of IL-18 inhibition on bacterial growth and the host inflammatory response was not investigated. Humans usually acquire melioidosis by inoculation through skin abrasions or inhalation. Pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis. We therefore used a model of melioidosis in which mice were infected with *B. pseudomallei* via the airways. In this model we found a strong protective role for IL-18: IL-18 KO mice were unable to control the infection, which resulted in increased distant organ injury and early mortality. The bacterial loads in particularly increased in the livers of IL-18 KO mice between 24 and 48 hours after infection, which was accompanied with significant pathology in this organ. The increased hepatocellular injury observed in IL-18 KO mice is in line with an earlier study from our laboratory, showing a similar response of these animals during abdominal sepsis caused by *Escherichia coli*. Of note, IL-18 KO mice had biochemical evidence of renal insufficiency, in particular elevated plasma urea concentrations, without histological changes in their kidneys, suggesting a pre-renal cause of the diminished kidney function.

IFN-γ release into the circulation was completely abrogated in IL-18 KO mice, indicating that IL-18 is an important factor in IFN-γ production induced by *B. pseudomallei*. In accordance, our group previously showed that the addition of anti-IL-18 to whole blood stimulated with heat-killed *B. pseudomallei* reduced IFNγ levels. Moreover, treatment with anti-IL-18 receptor antibody reduced the number of IFN-γ producing T and natural killer cells in mice injected with *B. pseudomallei* intraperitoneally. The protective role of IL-18 can be explained at least in part by reduced IFN-γ production, considering that elimination of IFN-γ rendered mice more susceptible to intraperitoneal infection with *B. pseudomallei*.

The present study adds to several previous investigations addressing the role of IL-18 in host defense against Gram-negative bacterial infection in vivo. Administration of anti-IL-18 to mice intravenously infected with *Salmonella typhimurium* resulted in enhanced bacterial growth in liver and spleen, similar to what was found here after infection with *Burkholderia*. In line, anti-IL-18 treatment during *Yersinia enterocolitica* infection was associated with a relatively enhanced growth of bacteria. In addition, IL-18 deficiency facilitated bacterial growth after intranasal infection with *Shigella flexneri* and intraperitoneal infection with *Escherichia coli*. IL-18 also contributed to an effective host defense against Gram-positive infection, including systemic infection with *Listeria monocytogenes* and pneumonia caused by *Streptococcus pneumoniae*. Remarkably, however, IL-18 deficiency was associated with an enhanced clearance of *Pseudomonas aeruginosa* from mouse lungs, and a diminished dissemination of the infection. In this respect it should be
noted that although *Pseudomonas* and *Burkholderia* share some common features, the diseases induced by intranasal infection of mice with either pathogen markedly differ. Indeed, *Pseudomonas* is cleared by immunocompetent mice \(^{24,25}\), whereas *Burkholderia* exponentially grows. Moreover, whereas experimental *Pseudomonas* pneumonia is associated with acute illness and acute lung inflammation, disease induced by *Burkholderia* develops more gradually, eventually resulting in disseminated abscess formation characteristic of human melioidosis. The differences are further illustrated by the fact that endogenous IFN-\(\gamma\), like IL-18, impaired host defense against experimentally induced *Pseudomonas* pneumonia \(^{25}\). In addition, Remick *et al* demonstrated that inhibition of IL-18 in septic peritonitis induced by cecal ligation and puncture had a variable effect on outcome dependent on the severity of the initial inflammatory response; IL-18 inhibition decreased mortality rates in mice with an increased risk of dying, but increased lethality in those mice with a predicted low mortality rate \(^{26}\). In acute shock produced by administration of LPS, IL-18 KO mice tolerated a 50% higher LPS dose than WT mice \(^{27}\), and treatment with an anti-IL-18 antiserum protected mice against the lethal effects of both *E. coli* and *Salmonella* LPS \(^{28}\). Taken together these findings are consistent with the concept of proinflammatory cytokines, like IL-18, acting as double-edged swords; inhibition of their activity during exaggerated inflammation in acute overwhelming infection or shock is beneficial for the host but their inhibition during more gradual infection facilitates bacterial growth \(^{29}\). The present investigations clearly identify IL-18 as a protective mediator during melioidosis by limiting replication and dissemination of *B. pseudomallei*.

**ACKNOWLEDGMENTS**

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Part II

Toll-like receptors and the innate immune response in melioidosis
The role of Toll-like receptors in sepsis


W. Joost Wiersinga, Tom van der Poll

Center for Infection and Immunity Amsterdam (CINIMA) and Department of Internal Medicine, Academic Medical Center, the Netherlands
INTRODUCTION

The recently discovered class of Toll-like receptors (TLRs) has emerged as the central line of defense against invading pathogens. They are the first to detect host invasion by pathogens, initiate immune responses and form the crucial link between the innate and adaptive immune systems. In general, the immune activation that follows TLR activation will be sufficient to combat the wide variety of pathogens that daily invade the human body. However, in the case of sepsis which can be defined as the disadvantageous systemic host response to infection, these TLR-mediated responses may exceed the threshold to maintain homeostasis of the immune system. This review focuses on the new insights in the pathogenesis of sepsis that is offered by the impressive amount of research that has been conducted in the TLR research field and their potential clinical implications for intensive care medicine.

THE TOLL-LIKE RECEPTOR FAMILY

The innate immune system discriminates potential pathogens from self through a series of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes. These motifs have been termed “pathogen-associated molecular patterns” or PAMPs, whereas their cognate binding partners on host cells involved in the innate immune response have been named “pattern-recognition receptors” or PPRs. Examples of PAMPs include lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, peptidoglycan (present in most bacteria), lipoteichoic acid (in many Gram-positive bacteria) and mannans in the yeast cell wall.

The Toll family of receptors, which is conserved throughout evolution from flies to humans, has been implicated to play a central role as PPRs in the initiation of cellular innate immune responses. First discovered in the fruit fly, at present 11 human homologs of Drosophila Toll have been identified. This human receptor family has been designated Toll-like receptors or TLRs. TLRs are distinguished from other PRRs by their ability to recognize, and more significantly, discriminate between different classes of pathogens. Ligands for 9 human TLRs have been described (see Table 1). Of note, one of the TLR mysteries relates to TLR11, a receptor present in mice, but not humans, and known to recognize uropathogenic Escherichia coli. Recently, the first defined ligand for TLR11 has been described as a profilin-like protein from Toxoplasma gondii. It has to be emphasized however that the TLRs function as one system; different components of one microorganism are recognized by different TLRs. Escherichia coli for example is a Gram-negative bacterium expressing several PAMPs (peptidoglycan, LPS, flagellin en bacterial DNA), which are all recognized by different TLRs (TLR2, TLR4, TLR5 and TLR9 respectively).

TLRs: the essential link between innate and adaptive immunity

It has become clear that activation of the innate immune system is a prerequisite for the induction of the adaptive immune system. TLRs form the bridge between these two systems and play an essential role in the coordination of the adaptive immune response. TLRs control induction of T cell responses at two levels, first by induction of co-stimulatory molecules which mark the
associated peptide as foreign and second by secretion of cytokines that are necessary to overcome
the peripheral tolerance induced by regulatory T cells. In addition, TLRs are responsible
for the induction of dendritic cell maturation, which is necessary to initiate adaptive immune
responses. It has to be said however, that other components of the innate immune system,
such as the complement system and natural killer (NK) cells, are also capable of influencing the
adaptive immune system.

Table 1. Toll-like receptor ligands in infectious diseases

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<tr>
<th>Receptor</th>
<th>Pathogen/PAMP</th>
<th>Origin of ligand</th>
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<td>TLR1 (with TLR2)</td>
<td>Triacyl lipopeptides</td>
<td>(Myco)bacteria</td>
</tr>
<tr>
<td></td>
<td>Soluble factors</td>
<td>Neisseria meningitides</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoproteins</td>
<td>Various pathogens</td>
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<td>Profilin-like protein</td>
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PAMP: pathogen-associated-molecular-patterns; LPS: lipopolysaccharide; CpG: cytosine phosphate guanine
dinucleotides.

* heat shock proteins, or stress proteins, are present in all cells and function as transport proteins within the
cell. They are released under stressful conditions such as heat, cold or hypoxemia. When they are expressed at the
cell surface, they play a role as signaling proteins in the recognition of diseased cell by the immune system.
They are endogenous ligands of TLRs.
**TLR signaling**

The discovery that TLR4 is the long sought after LPS receptor was a major breakthrough in immunology. However, TLR4 showed to be not the only protein important in the recognition of LPS (see Figure). First, LPS binds to LPS-binding-protein (LBP), which transfers LPS to CD14. CD14 is expressed on the outer membrane of monocytes, some granulocytes and activated B-lymphocytes. MD-2, another TLR4-associated protein, is required to activate the CD14-MD-2-TLR4-complex (see figure 1). Binding of LPS to CD14 leads to the association of CD14 with MD-2 and TLR4. So far, TLR4 is the only known TLR that requires an extra protein next to the ligand to be activated. After stimulation of the TLR, the adapter molecule myeloid differentiation primary-response protein 88 (MyD88) is recruited. MyD88 associates with IL-1R-associated kinase (IRAK) 4. This results in the phosphorylation of IRAK-1 which forms a complex with TNF-receptor-associated-factor (TRAF) 6. This then interacts with another preformed complex (consisting of TAK1, TAB1 and TAB2 or TAB3) which leads to the activation of the inhibitor-of-nuclear-factor-κB (IKK) complex. The IKK complex phosphorylates the IkBs. Subsequent release of nuclear-factor-κB (NF-κB) results in the transcription of a whole range of inflammatory genes. In addition, it was recently shown that the transcription factor IRF-5, which forms a complex with MyD88 and TRAF6, also functions as a general signal transducer that mediates MyD88-dependent gene induction of proinflammatory cytokines.

Next to this so-called MyD88-dependent pathway, a MyD88-independent route, exists which is used only by TLR4 and TLR3. The MyD88-independent route will result in the delayed activation of NF-κB and the production of interferon-β. Furthermore, it is now known that different TLRs...
Chapter 7

use different adaptor molecules. This explains why various TLRs lead to different patterns of gene expression. Next to MyD88, the adapter molecules TIR-domain-containing-adaptor-protein (TIRAP), TIR-domain-containing-adaptor-protein-inducing-IFN-β (TRIF) and TRIF-related-adaptor-molecule (TRAM) are identified. TIRAP is essential for MyD88-dependent signaling through TLR2 and TLR4. TRIF is essential for the TLR3 and TLR4-mediated activation of the MyD88-independent pathway. TRAM is involved in TLR4 mediated MyD88-independent/TRIF-dependent signaling pathways.

At first sight, the TLR signaling pathway seems overwhelming by complexity. However, it is fascinating to see that the innate immune signals through a channel of relatively low complexity: only 11 TLRs, 4 adapter molecules and a couple of protein kinases are required for the recognition and response to a whole universe of often complex microbial molecules. After this narrow strait there are again thousands of different possible host responses. Beutler has called this the “hourglass” shape of the innate immune response.

Regulation of TLR signaling

In order to prevent strong uncontrolled inflammatory reactions tight regulation of the TLR signaling pathway is mandatory. Generally spoken, an encounter of the immune system with pathogens will result in the upregulation of a whole spectrum of different TLRs. For instance, in patients with sepsis caused by the Gram-negative bacterium *Burkholderia pseudomallei* increased expression of TLR1, TLR2 and TLR4 on the cell surface of circulating monocytes and granulocytes is seen, together with increased TLR1, TLR2, TLR4, TLR5 and TLR10 mRNA levels in blood cells. However, the exact consequences of these enhanced TLR expression profiles for host defense remain to be established. Furthermore, it has been shown that certain cytokines play essential roles in TLR regulation. Besides LPS, inflammatory cytokines, such as IL-2, IL-15, IL-1β, IFNγ and TNFα, are able to induce TLR2 gene expression in mouse macrophages. Interestingly, this did not hold true for TLR4 gene expression.

Negative regulation of the TLR signaling pathway is essential (see Figure and Table 2). In the cytoplasm, IRAK-M inhibits the dissociation of the IRAK1-IRAK4 complex from the receptor, suppressor-of-cytokines-signaling-1 (SOCS1) probably directly inhibits IRAK1 and a short form of MyD88 (MyD88s) blocks the association of IRAK4 with MyD88. On the cell membrane, other members of the TIR-superfamily, such as Single-immunoglobulin-IL-1R-related-molecule (SIGIRR) and ST2, also negatively modulate TLR signaling. More specifically, ST2 is an inhibitor of TLR2, TLR4 and TLR9 signaling. Lastly, the TLR-like molecule RP105, which surface expression is dependent on the co-expression of the MD-2 homolog MD-1, interacts directly with the TLR4 signaling complex, inhibiting its ability to bind a microbial ligand.

Other mechanisms by which TLR signaling can be controlled include the reduction of TLR expression by TLR degradation or inhibition by anti-inflammatory cytokines. Furthermore, it has become clear that TLRs can function as death receptors; this TLR-induced apoptosis may be important in the control of a dysregulated TLR response.
ARE THE TLRs CENTRAL IN THE HOST DEFENSE AGAINST SEPSIS?

Given their central role in the recognition of microbes, it is rational to hypothesize that TLRs play a central role in sepsis pathogenicity. Indeed, animals lacking the gene encoding TLR4 do not develop septic shock in response to LPS. Although LPS is the best studied and probably most important mediator of sepsis, peptidoglycan, lipoteichoic acid, bacterial CpG motifs and flagella are other important microbial products implicated in the pathogenesis of sepsis. All these PAMPs signal through different TLRs. As a result, the relationship between TLR expression and human sepsis may be complex.

In recent years TLR2 has been recognized as the Gram-positive TLR because of its ability to sense major Gram-positive cell wall components such as peptidoglycan and lipoteichoic acid, whereas TLR4 – the LPS receptor – is seen as the Gram-negative TLR. However, as more knowledge about the precise role TLRs in different bacteria becomes available, this concept has to be modified. For instance, *Streptococcus pneumoniae* is sensed by the innate immune system not only through TLR2 which recognizes lipoteichoic acid and peptidoglycan, but also through TLR4 with recognizes pneumolysin. It was recently showed that TLR2 is indispensable for alveolar macrophage responsiveness toward *S. pneumoniae*. However in the same study, TLR2 gene-deficient mice intranasally
inoculated with non-lethal to lethal doses of *S. pneumoniae* displayed only a modestly reduced inflammatory response in their lungs and showed an unaltered antibacterial defense and survival in comparison with wild-type mice. These data suggest that the function of TLR2 is limited in the innate immune response to *S. pneumoniae*. Clearly, other PRRs play an important role.

It seems to be obvious that TLR4 has an important role in Gram-negative infections. As mentioned, TLR4 deficient mice do not develop septic shock after administration of high doses of LPS. Furthermore, TLR4 deficiency resulted in diminished clearance of *H. influenzae* and *K. pneumoniae* in a mouse model of pneumonia, suggesting that the recognition of LPS by TLR4 contributes to an effective immune response during these infections. However, not all studies show the importance of TLR4 signaling in Gram-negative infections. When mice lacking the TLR4 gene were inoculated with the Gram-negative bacterium *B. pseudomallei* in a mice model of severe sepsis no differences are observed in terms of inflammatory response (cytokine production, histological organ damage) or outcome (survival) when compared to normal wild type mice (our own unpublished data). In the same model, TLR2 mutant mice show a clear survival advantage over wildtype mice. To make things more complicated, responsiveness of TLR2 to LPS has also been described. In this respect, it is interesting that pretreatment with bacterial lipoprotein, a TLR2 ligand, protected otherwise highly susceptible TLR4-deficient C3H/HeJ mice from *S. typhimurium* induced Gram-negative sepsis via enhanced bacteria clearance.

The relationship between TLR expression and human sepsis may be complex, as is suggested by some recent studies in septic patients. Increased cellsurface (neutrophils and monocytes) expression and increased mRNA levels of both TLR2 and TLR4 are seen in septic patients. No association with functional outcome could be determined. Interestingly, in one study increased levels of TLR2 mRNA were seen in both Gram-positive and Gram-negative sepsis, whereas TLR4 mRNA was only increased in Gram-positive sepsis.

**NEW TLR MEDIATED PLAYERS IN THE SEPSIS ARENA**

Some recently discovered mediators of sepsis are directly involved in TLR signaling, all of which are regarded as promising new therapeutic targets.

**Triggering Receptor Expressed on Myeloid cells-1 (TREM-1)**

TREM-1 amplifies the TLR-mediated inflammatory response to microbial products. TREM-1, which signals through the adapter protein DAP12, is strongly and specifically expressed on monocytes and neutrophils from patients with sepsis. In human endotoxemia, monocytes display a gradual up-regulation of TREM-1, whereas granulocyte TREM-1 expression was high at baseline and immediately down-regulated upon LPS exposure along with an increase in soluble TREM-1. Elevated concentrations of soluble TREM-1 in bronchoalveolar-lavage fluid can indicate ventilator-associated pneumonia in patients receiving mechanical ventilation, and high concentrations in plasma can indicate infection in patients with systemic inflammatory response syndrome. Excitingly, blockade of TREM-1 protected mice against LPS-induced shock.
as well as microbial sepsis caused by live *Escherichia coli* or cecal ligation and puncture (CLP) \(^24\). In addition, a synthetic peptide mimicking a short highly conserved domain of sTREM-1 protected septic animals from LPS hyper-responsiveness and death \(^{28}\).

Intriguingly, although TREM-1 signals through the adapter protein DAP12 \(^{24}\), a recent study showed that DAP12-deficient mice have – contrary to what would be expected - enhanced TLR responses *in vitro*, as indicated by an enhanced production of pro-inflammatory cytokines by DAP12-deficient macrophages in response to TLR agonists *in vitro* and *in vivo*, as indicated by an increased susceptibility to endotoxic shock \(^{29}\). Thus, perhaps certain DAP12-associated receptors function as negative regulators of TLR responses.

**Macrophage Migration Inhibitory Factor (MIF)**

In recent years MIF has emerged as a pivotal regulator of innate immunity that has been implicated in sepsis pathogenesis \(^{30,31}\). MIF regulates innate immune responses through modulation of TLR4 \(^{30}\); when MIF-deficient mice were challenged with LPS they showed a defective response as a direct result of decreased TLR4 expression \(^{30}\). In patients, MIF levels correlate with fatal outcome in sepsis \(^{32}\). MIF-directed therapies might offer a new treatment opportunity for sepsis. Inhibition of MIF activity with neutralizing anti-MIF antibodies protected mice from septic shock \(^{31}\). Furthermore, a specific small molecule inhibitor of MIF, named ISO-1, partially protects mice from sepsis induced by endotoxin or CLP \(^{33}\).

**High-Mobility Group Box 1 protein (HMGB-1)**

HMGB-1 is recognized as a cytokine and functions as a late mediator of sepsis and is elevated in the majority of septic patients \(^{34,35}\). It is secreted by activated immune cells and, along with the receptor for advanced glycation end products (RAGE), interacts with TLR2 and TLR4, which may provide an explanation for the ability of HMGB-1 to generate inflammatory responses that are similar to those initiated by LPS \(^{36}\). LPS stimulation was found to mediate the release of HMGB-1 from macrophages at a considerably later stage than the release of the pro-inflammatory cytokines TNF\(\alpha\) and IL-1 \(^{35}\). Administration of HMGB-1 itself was lethal to mice, whereas the administration of antibodies to HMGB-1 diminished endotoxin lethality \(^{35}\).

### TLR POLYMORPHISMS IN SEPSIS

Recent phenotype-genotype studies showed that TLR polymorphisms can alter both the susceptibility to and the clinical course of infectious diseases. Mutations in TLR encoding genes are not uncommon. For instance, the reported incidence of the TLR4 Asp299Gly polymorphism lies between 6 to 11% in the Caucasian population \(^{37,38}\).

It was first shown that TLR4 mutations (Asp299Gly and Thr399Ile) are associated with endotoxin hyporesponsiveness in humans \(^{39}\). Subsequently, it was reported that polymorphisms in TLR4 could predispose people to develop septic shock with Gram-negative microorganisms \(^{37,40}\). Most likely, the increased susceptibility to Gram-negative sepsis is caused by a diminished and thus inadequate response to LPS. Further associations have been found between the TLR2 Arg753GIn
polymorphism and increased susceptibility to sepsis caused by *Staphylococcus aureus* 41. On the other hand, in a series of 1047 patients with culture proven meningococcal disease, the TLR4 Asp299Gly polymorphism was – contrary to the hypothesis – not associated with host susceptibility or severity of disease 38. In a cohort of 252 critically ill patients, it was shown that single nucleotide polymorphisms (SNPs) in CD14 and TLR2 are associated with increased prevalence of sepsis, but not with altered prevalence of septic shock or decreased 28-day survival 42. In this study, as was expected, CD14 SNPs were associated with Gram-negative infections and TLR2 SNPs with Gram-positive infections 42. Interestingly, when these results are taken together, it could well be that certain SNPs in TLRs may alter recognition and clearance of bacteria, but they do not seem to change the outcome of patients with sepsis. In addition, one has to bear in mind the importance of ethnic differences in genetic variations; for example in a recent Japanese cohort of 197 critically ill patients and 214 healthy controls not one single participant carried a TLR4 polymorphism and no association was found between CD14 polymorphism and sepsis 43. New studies that investigate the role of TLR polymorphisms in sepsis are underway. SNP analyses can serve as both an important research tool to further elucidate the complex pathogenicity of sepsis and as a clinical instrument to predict the clinical course of ICU patients and to ultimately individualize treatment 44.

**THE TOLL-LIKE RECEPTORS AS A NEW TREATMENT TARGET IN SEPSIS**

Manipulation of TLR pathways has great therapeutic potential: novel TLR immune-regulatory drugs are being developed to treat a wide range of conditions, such as infectious diseases, asthma, inflammatory bowel disease and cancer 45. In the case of sepsis, one could think of TLR antagonists, TLR signaling pathway inhibitors or stimulators of the negative regulators of the TLR pathway as new treatment targets. Some examples. A recent mouse study in sepsis showed a marked reduction in the sepsis related mortality after selective blockage of TLR2 after inoculation with Gram-positive bacteria 46. Furthermore, studies in animal models have demonstrated the utility of anti-CD14 monoclonal antibody therapy in septic shock and these agents are currently being evaluated in clinical phase-2 trials 47, 48. Another strategy could involve TLR9. TLR9 recognizes CpG DNA, a specific pattern of nucleotides that is common in bacteria and viruses, but uncommon in humans. By using synthetic CpG sequences an innate and adaptive immune response could be generated involving cytotoxic T cells and disease-specific antibodies 49. In a mouse model of severe Gram-negative sepsis, CpG treatment one hour before inoculation with *B. pseudomallei* offered protection due to the rapid induction of proinflammatory cytokines 50. The treatment goal of TLR agents in sepsis should be to normalize and not to completely abolish the dysregulated and harmful inflammatory response. Maintaining a balance between host-defense functions and potentially harmful effects (e.g. tissue destruction and the induction of autoimmune disease) will be of vital importance in the development of TLR therapeutics 45.
CONCLUSION

The discovery of TLRs has been of enormous importance in both the field of microbiology and immunology and has shown that the innate immune system is not aspecific. TLRs form the crucial link between the innate and adaptive immune response. Surprisingly, these very complex immune responses are initiated by this family of only 11 different receptors. The first human studies on TLR expression in sepsis and experiments with mice lacking TLR genes have provided us new insights in the pathogenesis of sepsis and have underlined the importance of TLRs as the crucial first line of defense against microorganisms. Taken together, severe sepsis can probably be seen as the clinical manifestation of a TLR mediated dysregulation of the immune response to invasive pathogens \(^1\). Despite this significant progress in our understanding of the sepsis enigma many of the complex immune reactions during sepsis are still a mystery \(^1\). These outstanding questions on pathogenesis can be summarized by the fact that we need to know how dysregulation of the TLR system precisely results in clinical syndromes such as sepsis. In the end, it comes down to the question whether all this newly gained knowledge will help to improve the care of septic patients. Hopefully, in the not to distant future the TLR genotypic profiling of patients will help clinicians to make better treatment decisions. Most importantly, unraveling the role of TLRs in sepsis will provide new highly selective treatment targets in sepsis.

ACKNOWLEDGEMENT

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REFERENCES


Chapter 7


Chapter 8

Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by *Burkholderia pseudomallei* (melioidosis)

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Chapter 8

ABSTRACT

Background
Toll-like receptors (TLRs) are essential in host defense against pathogens by virtue of their capacity to detect microbes and initiate the immune response. TLR2 is seen as the most important receptor for Gram-positive bacteria while TLR4 is regarded as the Gram-negative TLR. Melioidosis is a severe infection caused by the Gram-negative bacterium *Burkholderia pseudomallei* that is endemic in SE-Asia. We aimed to characterize the expression and function of TLRs in septic melioidosis.

Methods and Findings
Patient studies: 34 patients with melioidosis demonstrated increased expression of CD14, TLR1, TLR2 and TLR4 on the cell surface of monocytes and granulocytes and increased CD14, TLR1, TLR2, TLR4, MD-2, TLR5 and TLR10 mRNA levels in purified monocytes and granulocytes when compared with healthy controls.

*In vitro* experiments: Whole blood and alveolar macrophages obtained from TLR2 and TLR4 knockout (KO) mice were less responsive to *B. pseudomallei* in vitro, whereas in the reverse experiment transfection of HEK 293 cells with either TLR2 or TLR4 rendered these cells responsive to this bacterium. In addition, the LPS of *B. pseudomallei* signals through TLR2 and not through TLR4.

Mouse studies: Surprisingly, TLR4 KO mice were indistinguishable from wild-type mice with respect to bacterial outgrowth and survival in experimentally induced melioidosis. In contrast, TLR2 KO mice displayed a markedly improved host defense as reflected by a strong survival advantage together with decreased bacterial loads, reduced lung inflammation and less distant organ injury.

Conclusions
Patients with melioidosis display an upregulation of multiple TLRs in peripheral blood monocytes and granulocytes. Although both TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei* in vitro, TLR2 detects the LPS of *B. pseudomallei* and only TLR2 impacts on the immune response of the intact host *in vivo*. Inhibition of TLR2 may be a novel treatment strategy in melioidosis.
INTRODUCTION

The recently discovered family of Toll-like receptors (TLRs) has emerged as an important first line of defense against invading pathogens. They detect host invasion by pathogens, initiate immune responses and form the crucial link between the innate and adaptive immune systems [1,2]. Upon the first encounter with a pathogen, TLRs recognize conserved motifs termed “pathogen-associated-molecular-patterns” or PAMPs [1,2]. In general, the immune activation that follows the interaction between TLR and PAMPs is sufficient to eliminate the wide variety of pathogens that daily invade the human body. However, in the case of sepsis, these TLR-mediated inflammatory responses may exceed the threshold to maintain homeostasis of the immune system and cause harm.

In Southeast Asia and northern Australia the Gram-negative soil dwelling bacillus *Burkholderia (B.) pseudomallei* is an important cause of community-acquired sepsis and sepsis-related mortality [3,4]. More than half of the cases of melioidosis, as this severe infection is named, habitually presents with pneumonia with bacterial dissemination to distant sites [5]. The mortality of primary disease is 50% in northeast Thailand and around 20% in the higher technology setting of northern Australia [6,7]. Reported cases are likely to represent ‘the tip of the iceberg’ since confirmation of disease depends on bacterial isolation, a technique that is not available in large areas of the world [3,8]. Interest in disease pathogenesis of *B. pseudomallei* has increased following its classification as category B disease/agents of bioterrorism by the US Centers for Disease Control and Prevention (www.bt.cdc.gov/agents/agentlist).

It is likely that TLRs contribute to host defense against *B. pseudomallei*. In general, TLR4 is considered of utmost importance for host defense against Gram-negative infection by virtue of its capacity to sense lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria and to relay LPS effects into the cellular interior [9]. Besides TLR4, TLR2 may play a role in the host response to *B. pseudomallei* considering that this bacterium expresses several PAMPs that can interact with TLR2, including lipopeptides and peptidoglycan. However, these assumptions remain speculative since the expression and function of these TLRs have not been studied in melioidosis thus far.

In the present study we aimed to characterize the expression and function of TLRs in sepsis caused by *B. pseudomallei* and found that, although both TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei in vitro*, only TLR2 impacts on the immune response of the intact host *in vivo*. 
Chapter 8

METHODS

Human studies

Patients
Blood samples were taken from 34 patients with sepsis caused by *B. pseudomallei*. Patients were prospectively recruited over a 4-month period after admission to Sapprasithiprasong hospital, a 1000-bed government hospital located in Ubon Ratchathani, northeast Thailand. Sepsis caused by melioidosis was defined by the presence of (1) culture-proven infection with *B. pseudomallei* and (2) a systemic inflammatory response syndrome (SIRS) as indicated by ≥ 3 of the following criteria: a core temperature of ≥ 38ºC or ≤ 36 ºC; a heart beat of ≥ 90 beats/min; a respiratory rate of ≥ 20 breaths/min or a PaCO₂ of ≥ 32 mmHg or the use of mechanical ventilation for an acute respiratory process; a white-cell count of ≥ 12 x 10⁹/l or ≤ 4 x 10⁹/l or a differential count showing > 10% immature neutrophils [10]. Exclusion criteria were the use of dialysis and/or immunosuppressive therapy, known disorders of coagulation and concomitant infection with human immunodeficiency virus. Blood samples were drawn within 36 hours after start of antimicrobial therapy. When possible, an additional blood sample was drawn from patients who recovered after two weeks of treatment. 32 healthy subjects, who were recruited from the Sapprasithiprasong Hospital blood bank as blood donors, served as a control population. The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, England and written informed consent was obtained from all study subjects or their attending relatives.

RNA analysis using Multiplex ligation-dependent probe amplification (MLPA)
Heparin blood samples were drawn from the antecubital vein and immediately put on ice. Leukocytes were isolated using erythrocyte lysis buffer. Monocyte and granulocyte enriched populations were isolated using Polymorphprep (Axis-Shield, Dundee, United Kingdom). Monocyte and granulocyte fractions were > 98% pure as determined by their scatter pattern on flow cytometry. After isolation leukocytes, monocytes and granulocytes were dissolved in Trizol (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C until used for RNA isolation. RNA was isolated and analyzed by multiplex ligation-dependent probe amplification (MLPA) as described [11-14] using a TLR-specific kit developed in collaboration with MRC-Holland (Amsterdam, the Netherlands) for the simultaneous detection of 17 mRNA molecules; 4 different probe sets were generated for TLR4 corresponding with 4 different TLR4 splice variants (R01 to R04) identified in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov); similarly 2 different probe sets for TLR8 were generated corresponding with 2 different TLR8 splice variants (R01 and R02). We recently published the specific oligonucleotides used for all TLR probe sets [15]. All samples were tested with the same batch of reagents. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of the E2 microglobulin gene, resulting in the relative abundance of mRNAs of the genes of interest [11-14].
TLRs in melioidosis

**FACS analysis**
CD14 and TLR cell surface expression on peripheral monocytes and granulocytes was determined by flow cytometry using fluorochrome-conjugated mouse anti-human CD14 (BD Biosciences, Mountain View, CA), TLR1, TLR2 and TLR4 (all Bioscience, San Diego, CA) antibodies in accordance with the manufacturer’s recommendations. Granulocytes were defined according to their scatter pattern and monocytes according to their scatter pattern and CD14 positivity. To correct for aspecific staining, appropriate isotype control antibodies (BD Biosciences) were used. Samples were analysed directly after sample collection by flow cytometry using FACScan (BD Biosciences).

**Mouse and in vitro studies**

*Mice*
Pathogen-free 8 to 10 week old wild-type (WT) C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). TLR2 knockout (KO) mice [16] and TLR4 KO mice [17] backcrossed 6 times to a C57BL/6 background were generously provided by Dr. Shizuo Akira, Osaka University, Japan. Age and sex-matched animals were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

*Preparation of alveolar macrophages*
Alveolar macrophages were harvested from TLR2, TLR4 KO and WT mice by bronchoalveolar lavage (BAL) (n= 5-8 per strain) as described [18,19]. The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). BAL was performed by instilling three 0.5 ml aliquots of sterile saline. Total cell numbers were counted from each sample using a hemocytometer. Cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin and 10% FCS in a final concentration of 1x10^4 cells/100 μl. Cells were then cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 2 h and washed with RPMI 1640 to remove non-adherent cells. Adherent monolayer cells were stimulated with washed (3 times in normal saline) heat-killed *B. pseudomallei* (clinical isolate strain 1026b [20,21]; 1x10^5 colony forming units (CFU)/ml) or RPMI 1640 for 16 h. Supernatants were collected and stored at –20°C until assayed for TNF-α.

*LPS extraction from B. pseudomallei 1026*
LPS was extracted from *B. pseudomallei* 1026b [22]. Bacteria from frozen stock were inoculated into 5ml of LB broth and incubated overnight at 37°C in air with shaking at 200 rpm. This starting culture was streaked onto 50 LB agar plates per strain. Plates were incubated at 37°C in air for 2 days, after which bacterial colonies were scraped from plates and resuspended in 100 ml sterile distilled water. LPS extraction using a hot aqueous-phenol method was performed as described by Brett et al [23]. Purified *B. pseudomallei* LPS was characterized by SDS-PAGE and silver staining [24].

*HEK cells*
Human embryonic kidney (HEK) 293 cells stably expressing CD14, CD14−TLR4, or CD14−TLR2, kindly provided by Dr. Douglas Golenbock, University of Massachusetts Medical School, Worcester,
have been described [25,26]. Cells were stimulated with LPS from *B. pseudomallei* 1026b (100 ng/ml), heat-killed *B. pseudomallei* (5x107 CFU/ml) or RPMI for 6 hrs. Supernatants were harvested and stored at -20oC until assayed for Interleukin (IL)-8.

**Experimental infection**

For preparation of the inoculum, *B. pseudomallei* strain 1026b was used (this strain has been isolated in Sappasithiprasong hospital in 1993 from a blood culture from a septic 29-year old female rice farmer presenting with bacteremia with soft tissue, skin, joint and splenic involvement [20,21]. Stock bacteria were streaked from frozen aliquots into 50 ml Luria broth (Difco, Detroit, MI) for overnight incubation at 37°C in a 5% CO2 incubator. Thereafter, a 1 ml portion was transferred to fresh Luria broth and grown for ±5h to midlogarithmic phase. Bacteria were harvested by centrifugation at 1500 x g for 15 minutes, washed and resuspended in sterile isotonic saline at a concentration of 5x10^2 CFUs/50 μl, as determined by plating serial 10-fold dilutions on blood agar plates. Pneumonia was induced by intranasal inoculation of 50 μl (5x10^2 CFU) bacterial suspension. For this procedure mice were lightly anesthetized by inhalation of isofluorane (Upjohn, Ede, The Netherlands).

**Determination of bacterial outgrowth**

24, 48 and 72 hrs after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium: active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Mijdrecht, The Netherlands) and sacrificed by bleeding from the inferior vena cava. The lungs and spleens were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of organ homogenates and blood, plated on blood agar plates and incubated at 37°C at 5% CO₂ for 16 h before colonies were counted.

**Preparation of lung tissue for cytokine measurements**

For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed.

**Assays**

Human IL-8 was measured by ELISA (Biosource, Etten-Leur, the Netherlands). Mouse TNF-α, Interleukin (IL)-6 and IL-10 were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined with commercially available kits (Sigma-Aldrich), using a Hitachi analyzer (Roche) according to the manufacturer’s instructions. Immunostaining for TLR4 on blood cells and whole lung cell suspensions was performed using directly labelled antibodies against GR-1 (GR-1 Fitc; Pharmingen, San Diego, CA) and TLR4 (TLR4-AlexaFluor, all Bioscience, San Diego, CA) and a biotin labeled antibody against F4/80 (Serotec, United Kingdom) in combination with streptavidin allophyco-
TTLs in melioidosis
cyanine (APC). All antibodies were used in concentrations recommended by the manufacturer. After staining, cells were fixed in 2% paraformaldehyde. TLR4 MFI was measured in the Gr-1-high gate (granulocytes), sidescatter low and F4/80 positive (monocytes) and sidescatter high and F4/80 positive (macrophages) gated populations.

Pathology
Lungs and spleens for histology were harvested after infection, fixed in 10% formalin and embedded in paraffin. Four μm sections were stained with H&E, and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: surface with pneumonia, necrosis/abscess formation, interstitial inflammation, endothelialitis, bronchitis, edema, thrombus formation and pleuritis. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe. The total “lung inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 32. Spleen sections were scored on inflammation, necrosis/abscess formation and thrombus formation using the scale given above. The maximum total spleen inflammation score was 12. Granulocyte staining was done exactly as described previously [27,28].

Statistical analysis
Values are expressed as means ± SEM. Differences between groups were analyzed by Mann-Whitney U test or Kruskal-Wallis analysis with Dunn's posthoc test where appropriate. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Values of P<0.05 were considered statistically significant.

RESULTS

Patient characteristics
34 patients with sepsis caused by \textit{B. pseudomallei} (mean age 52 years; range, 18-86 years; 50% male) and 32 healthy control subjects (mean age 41 years; range, 21-59 years; 71% male) were enrolled. \textit{B. pseudomallei} was cultured from body material from all patients: blood cultures were positive for \textit{B. pseudomallei} in 21 patients (61.7%), throat swab or tracheal suction in 13 patients (38.0%), sputum in 7 patients (21.0%), pus from abscess in 7 patients (21.0%) and urine in 5 patients (14.7%). The overall patient mortality was 44%.

Increased CD14, MD-2, TLR1, TLR2, TLR3, TLR4, TLR5, TLR8 and TLR10 mRNA levels in peripheral blood cells of patients with septic melioidosis
To obtain a first insight into TLR gene expression during melioidosis, we quantified TLR mRNA’s in blood leukocytes harvested from patients with septic melioidosis and healthy controls. MLPA was performed on RNA isolated from whole blood leukocytes as well as from monocyte and
Chapter 8

granulocyte enriched (> 98% pure) populations. Patients with melioidosis displayed elevated mRNA levels for most TLRs and TLR related proteins in unfractionated leukocytes, monocytes and granulocytes (Table 1, data of unfractionated leukocytes not shown). Specifically, melioidosis was associated with enhanced expression of monocyte and granulocyte mRNA expression of TLR1, TLR2, TLR4 (R01 and R04), TLR5, TLR8 (R01 and R02) and the TLR4 co-receptors MD-2 and CD14. Of note, TLR10 mRNA was only upregulated in unfractionated leukocytes (not shown) and granulocytes, but not in monocytes (Table 1). The mRNA’s encoding TLR3, TLR7 (both similar in patients and controls) and TLR9 (undetectable in patients and controls) were not influenced by melioidosis.

<table>
<thead>
<tr>
<th></th>
<th>Monocytes</th>
<th>Granulocytes</th>
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<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Patients</td>
</tr>
<tr>
<td>TLR1</td>
<td>1.49 ± 0.13</td>
<td>2.77 ± 0.36</td>
</tr>
<tr>
<td>TLR2</td>
<td>0.09 ± 0.01</td>
<td>0.70 ± 0.25</td>
</tr>
<tr>
<td>TLR3</td>
<td>0.01 ± 0.00</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>TLR4-R01</td>
<td>0.92 ± 0.08</td>
<td>1.98 ± 0.27</td>
</tr>
<tr>
<td>TLR4-R02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TLR4-R03</td>
<td>0.77 ± 0.06</td>
<td>1.38 ± 0.15</td>
</tr>
<tr>
<td>TLR-R04</td>
<td>0.28 ± 0.02</td>
<td>0.79 ± 0.11</td>
</tr>
<tr>
<td>TLR5</td>
<td>0.08 ± 0.01</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>TLR7</td>
<td>0.42 ± 0.04</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>TLR8-R01</td>
<td>0.20 ± 0.08</td>
<td>0.64 ± 0.12</td>
</tr>
<tr>
<td>TLR8-R02</td>
<td>1.87 ± 0.12</td>
<td>4.42 ± 0.64</td>
</tr>
<tr>
<td>TLR9</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>TLR10</td>
<td>0.26 ± 0.03</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>MD-2</td>
<td>1.31 ± 0.10</td>
<td>2.86 ± 0.40</td>
</tr>
<tr>
<td>CD14</td>
<td>2.31 ± 0.15</td>
<td>5.19 ± 0.62</td>
</tr>
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</table>

mRNA levels (means ± SE) for each gene are expressed as a normalized ratio of the peak area divided by the peak area of the β2 microglobulin gene, resulting in the relative abundance of mRNAs of the genes of interest [11-14]. ND: not detectable.

Increased expression of CD14, TLR1, TLR2 and TLR4 on the cell surface of circulating monocytes and granulocytes of patients with septic melioidosis

To determine the effect of *B. pseudomallei* sepsis on TLR protein expression at the surface of peripheral blood cells, we compared the expression of CD14, TLR1, TLR2 and TLR4 on circulating monocytes and granulocytes of patients with melioidosis and healthy controls using FACS analysis. Relative to controls, patients displayed higher cell-associated levels of CD14, TLR1, TLR2 and TLR4 on both monocytes and granulocytes (Fig. 1A). In addition, in five patients from whom blood could be obtained after recovery (14 days after admission), monocyte expression
Figure 1a. Increased expression of CD14, TLR1, TLR2 and TLR4 on peripheral blood monocytes and granulocytes of patients with melioidosis. CD14 (A,B), TLR1 (C,D), TLR2 (E,F) and TLR4 (G,H) on monocytes (A,C,E,G) and granulocytes (B,D,F,H) of patients (n=34) with severe melioidosis (triangles) and healthy controls (n=32; squares).
Chapter 8

TLR2 and TLR4 contribute to cellular responsiveness to \textit{B. pseudomallei} in vitro.

Having established that in particular TLR2 and TLR4 become upregulated at the surface of blood leukocytes in patients with severe melioidosis, we sought to obtain insights into the function of these TLRs in melioidosis by testing the requirement of TLR2 and TLR4 signaling upon first encounter between the bacterium and the host. We first tested the capacity of alveolar macrophages and whole blood harvested from WT, TLR2 KO or TLR4 KO mice to release TNF$\alpha$ upon stimulation with heat-killed \textit{B. pseudomallei} (effector:target ratio 1:10). Whole blood and alveolar macrophages obtained from TLR2 or TLR4 KO mice released less TNF$\alpha$ than alveolar macrophages and blood from WT mice upon stimulation with \textit{B. pseudomallei} in vitro (Fig. 2A and 2B). Consistent with these KO data, HEK 293 cells stably transfected with either CD14/TLR2 or CD14/TLR4 responded to \textit{B. pseudomallei} as measured by strong release of IL-8 into the supernatant (Fig. 2C). These data suggest that both TLR2 and TLR4 contribute to cellular responsiveness to \textit{B. pseudomallei} in vitro.

The LPS of \textit{B. pseudomallei} signals through TLR2

LPS has been implicated to play a major role in the induction of an innate immune response to Gram-negative bacteria. Although TLR4 is considered the LPS receptor, LPS derived from some bacteria are recognized by TLR2. We therefore were interested to determine whether LPS from \textit{B. pseudomallei} is recognized by TLR2 or TLR4. Remarkably, using HEK 293 cells stably transfected with either CD14/TLR2 or CD14/TLR4 we found that purified LPS of \textit{B. pseudomallei} 1026b signals through TLR2 and not through TLR4 (Fig. 2C).
TLRs in melioidosis

TLR2 KO, but not TLR4 KO mice are protected from *B. pseudomallei* induced lethality.

Having established that TLR2 and TLR4 take part in the recognition of *B. pseudomallei* by immune cells *in vitro*, we next investigated the involvement of these receptors in the outcome of melioidosis. TLR2 KO, TLR4 KO and WT mice were intranasally infected with *B. pseudomallei* and followed for 6 weeks. All WT and TLR4 KO mice died within 5 days after inoculation. However, mortality was delayed and reduced among TLR2 KO mice, of which 40% survived until the end of the 6 weeks observation period (*p* < 0.0001 for the differences between both mouse strains; Fig. 3).

**TLR2 KO, but not TLR4 KO mice show a reduced growth of *B. pseudomallei in vivo***

To obtain insight into the mechanisms underlying the reduced mortality of TLR2 KO mice during experimental melioidosis, we infected WT, TLR2 KO and TLR4 KO mice with *B. pseudomallei* and sacrificed the mice after 24, 48 and 72 hours (i.e. directly before the first predicted death in WT mice) to determine bacterial loads in lungs (the primary site of the infection), blood and spleen (to evaluate extent of bacterial disseminated) (Fig. 4). Relative to WT mice, TLR2 KO mice displayed strongly reduced bacterial loads in lungs, blood and spleen at 72 hours; this was also

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Figure 2. TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei in vitro*. Freshly isolated alveolar macrophages (A) and whole blood (B) of WT, TLR2 and TLR4 KO mice (*n* = 8 per group) were incubated with RPMI 1640 (control) or heat-killed *B. pseudomallei* (equivalent 10⁵ CFU; target:effector ratio 1:10) for 16 hours before TNFα production was measured. (C) HEK 293 cells stably transfected with CD14, CD14/TLR2 or CD14/TLR4 were incubated for 6 hours with medium, LPS of *B. pseudomallei* 1026 or HK *B. pseudomallei* before measurement of IL-8 in the supernatant. Data are means ± SE (*n* = 4). *** *p* < 0.001.
observed at the 48 hour time point, although the difference did not reach statistical significance. In TLR2 KO mice that survived 6 weeks after inoculation almost all bacteria were effectively cleared (data not shown). TLR4 KO mice demonstrated only higher bacterial loads in spleen (p < 0.001) and blood (p < 0.05) at 24 hours after infection; however, at later time points bacterial burdens did not differ significantly in TLR4 KO and WT mice in all body compartments except for the spleen at 72 hours, revealing modestly fewer *B. pseudomallei* CFU in TLR4 KO mice (p < 0.001).

**Figure 3. TLR2 KO, but not TLR4 KO mice show an enhanced survival during experimental melioidosis.** (A). Survival after intranasal inoculation with *B. pseudomallei* in WT (open circles), TLR2 KO mice (closed squares) and (B) TLR4 KO mice (closed squares). Mortality was assessed twice daily for 6 weeks. N = 12 per group. P value indicates the difference between TLR2 KO and WT mice. Ns = not significant.

**Figure 4. Bacterial loads in lungs, spleen and blood.** TLR2 KO mice demonstrate strongly reduced bacterial loads at 72 hours after infection in lungs (A), spleen (B) and blood (C). TLR4 KO mice show a modestly enhanced outgrowth at 24 hours after infection in spleen (E) and blood (F) but not in lungs (D). Data are mean ± SEM (n = 8 per group) at 24, 48 and 72h after inoculation with *B. pseudomallei* in WT mice (open bars), TLR2 KO mice (black bars) and TLR4 KO mice (chequered bars). * P < 0.05; ***P < 0.0001. Ns = not significant.
Figure 5. Reduced lung inflammation in TLR2 KO mice 72 hours after infection. Representative lung histology of WT (A,C,E) and TLR2 KO mice (B,D,F) at 24 (A,B), 48 (C,D) and 72 (E,F) hours after inoculation with $5 \times 10^2$ CFU B. pseudomallei, showing significantly less inflammation, pleuritis, peribronchial inflammation, oedema, endothelialitis and necrosis 72 hours after infection in the TLR2 KO mice (F,G) compared to WT (E-G). (G) shows pathology scores (means ± SE) calculated as described in the Methods section. The insets (A-F) are representative pictures of immunostaining for granulocytes, showing dense granulocytic infiltrations and confirming reduced inflammation and granulocyte influx in the TLR2 KO mice at 72 hours after inoculation with B. pseudomallei. TLR2 KO mice that survived the experiment shown in Fig. 3A were examined six weeks after inoculation; as would be expected they showed decreased organ inflammation as compared to the 72 time points. The abscesses that were still present were encapsulated (H). Magnification, x20. All data are from 8 mice per group at each time point, except for panel (H) (N = 5).
Together, these data suggest that TLR4 may play a minor role in limiting the early dissemination of *B. pseudomallei* from the lungs and, more strikingly, that the presence of TLR2 facilitates the growth of this pathogen in lungs and the subsequent spread to distant body sites.

**TLR2 KO mice demonstrate reduced lung inflammation**

To obtain further insight into the involvement of TLR2 and TLR4 in the inflammatory response after infection with *B. pseudomallei*, we semi-quantitatively scored lung histology slides generated from WT, TLR2 and TLR4 KO mice at various time points after infection. Infection with *B. pseudomallei* was characterized by severe pulmonary inflammation, with abscess formation.
necrosis, endothelialitis and thrombus formation. Consistent with the observed reduced bacterial outgrowth, TLR2 KO mice displayed reduced organ inflammation at 72 hours post-infection when compared with WT mice (Fig. 5). In accordance, the number of neutrophils present in the lung as analyzed by Gr1-immunostaining, was lower in the TLR2 KO mice (insets, Fig. 5). As expected, TLR2 KO mice that survived the 6-week survival study described above showed decreased lung inflammation as compared to the 72 time points; the abscesses that were still present were encapsulated in these animals (Fig. 5H). In contrast with the TLR2 KO mice, the TLR4 KO mice showed similar inflammation compared with WT mice (data not shown).

**Diminished late proinflammatory cytokine and increased early anti-inflammatory cytokine production in TLR2 KO mice**

The success of combating infections in the lung strongly depends on the efficacy of the local inflammatory response elicited [29,30]. In order to study the extent and kinetics of the inflammatory response, we sacrificed WT, TLR2 and TLR4 KO mice at multiple time points after infection and measured the concentrations of the proinflammatory cytokines TNFα and IL-6 and the anti-inflammatory cytokine IL-10 in lung homogenates (Fig. 6). TNFα and IL-6 levels were lower in TLR2 KO mice than in WT mice, especially at 72 hours after infection (p < 0.05 and p < 0.001, respectively). In addition, lung IL-10 concentrations were moderately lower in TLR2 KO mice at 24 and 48 hours after infection (p < 0.05). In contrast, the pulmonary levels of these mediators were similar in TLR4 KO and WT mice at all time points. In addition, TLR2 deficiency did not influence the expression of TLR4 on lung macrophages or blood monocytes or granulocytes throughout the infection (Fig. 7).

**TLR2 but not TLR4 KO mice demonstrate decreased distant organ injury**

To further evaluate the role of TLR2 and TLR4 in the systemic inflammatory response after infection with *B. pseudomallei*, we semi-quantitatively scored spleen histology slides generated from infected mice and performed routine clinical chemistry to evaluate hepatic and renal injury. No differences were observed between WT and TLR4 KO mice at any time point (data not shown).
Chapter 8

Figure 8. TLR2 KO mice demonstrate reduced distant organ injury. Mice were intranasally inoculated with 5x10^2 CFU of *B. pseudomallei*. After 72 hrs representative spleen histology of WT (A) and TLR2 KO (B) mice showed decreased inflammation and less thrombus formation in the TLR2 KO spleens. Magnification, x12.5. Arrows indicate increased thrombus formation in the WT mice as compared to TLR2 KO mice. The reduced inflammation and injury in the spleen at 72 hours after infection was confirmed by the semi-quantitative pathology score described in the Methods (C). At this time point TLR2 KO mice also showed less hepatic injury, as reflected by the plasma concentrations of ASAT (D) and ALAT (E), and less renal failure, as reflected by plasma creatinine (F) and BUN (G). Data are expressed as mean ± SEM of 8 WT mice (open bars) and 8 TLR2 KO mice (black bars). * P < 0.05; ** P < 0.01 versus WT control.

not shown). However, TLR2 KO demonstrated diminished inflammation in their spleens when compared to the WT mice (Fig. 8A-C). Furthermore, liver and kidney function were relatively preserved in TLR2 KO mice, as reflected by lower AST, ALT, BUN and creatinine plasma levels in these animals (Fig. 8D-G).

DISCUSSION

In the present study we aimed to characterize the expression and function of the TLRs in septic melioidosis, linking observational studies in patients with culture proven disease with functional studies using TLR deficient mice. We made the following key observations. 1) Patients with septic melioidosis have an increased expression of CD14, TLR1, TLR2 and TLR4 on the cell surface of circulating monocytes and granulocytes and increased CD14, MD-2, TLR1, TLR2, TLR3, TLR4, TLR5, TLR8 and TLR10 mRNA levels in peripheral blood cells. 2) Both TLR2 and TLR4 contribute to cellular responsiveness to *Burkholderia in vitro*, as reflected by reduced TNF-α release by alveolar macrophages and whole blood from TLR2 and TLR4 KO mice and by activation of HEK 293 cells stably transfected with either TLR2 or TLR4 upon incubation with *B. pseudomallei*. 3) The LPS of *B. pseudomallei* signals through TLR2 and not through TLR4. 4) TLR4 is not important for protective immunity against experimentally induced melioidosis, whereas TLR2 is associated
with the growth and dissemination of the infection, significantly contributing to distant organ injury and lethality.

Our study is the first to provide insights into the relative gene expression of all TLR family members in peripheral blood monocytes and granulocytes in patients with severe sepsis. In blood samples obtained from 34 prospectively enrolled patients with sepsis caused by *B. pseudomallei*, we showed that a whole repertoire of TLRs is upregulated at mRNA level in both monocytes and granulocytes. Of note, patients showed enhanced monocyte and granulocyte expression of three of four TLR4 splice variants. TLR4 variant 2 remained undetectable, which is consistent with our earlier study examining alveolar macrophages from healthy humans challenged with LPS via the airways [15]. TLR4 variant 1 is involved in LPS binding and triggering of intracellular signal transduction cascades, while variant 3 and 4 lack residues 24-34 which are essential for MD2 binding and LPS signalling [31]. The expression of TLR3 mRNA was very low in both patients and healthy controls, which can be explained by the fact that this intracellular receptor is primarily expressed by dendritic cells and to a lesser extent B and T cells [32-34]. In addition, we could not detect TLR9 mRNA in monocytes or granulocytes which is in line with studies showing that TLR9 mRNA in particular is expressed in plasmacytoid dendritic cells and B cells. The increase in mRNA’s for CD14, TLR1, TLR2 and TLR4 was accompanied by enhanced surface expression of the respective proteins circulating neutrophils and granulocytes. This is in accordance with two earlier clinical reports, which found increased expression of TLR2 and TLR4 on neutrophils and monocytes from septic patients [35,36].

Humans usually acquire melioidosis by inoculation through skin abrasions or inhalation [3,4]. Pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis [5]. We decided to develop a model in which *B. pseudomallei* was administered via the airways since this route may be more clinically relevant than the intraperitoneal route used in several previous studies. We reproduced the major clinical characteristics of melioidosis, with rapid spread of bacteria to distant organs, multiple organ failure and abscess formation. Among the TLRs with enhanced expression in patients with melioidosis, we considered TLR2 and TLR4 most interesting with respect to investigating the impact of their loss of function in experimentally induced melioidosis in mice. Indeed, of the other TLRs that showed increased expression, TLR1 has been implicated to function as a co-receptor together with TLR2 in the recognition of bacterial triacyl lipopeptides, whereas TLR8 senses single stranded viral RNA [1,2]. In addition, although TLR5, as the receptor that recognizes flagellin, in theory may be involved in host defense against *B. pseudomallei*, a recent report suggests that TLR5 especially or exclusively senses pathogenic flagellated bacteria present in the intestinal lumen, whereas the expression of this receptor was low in the mouse lung [37].

A remarkable and unexpected finding was that although TLR4 appeared very important for cellular responsiveness to *B. pseudomallei in vitro* (Figure 2), this receptor does not significantly contribute to host defense against melioidosis in vivo. In line with these in vivo observations, we demonstrated that purified LPS from *B. pseudomallei* 1026b (the strain also used in the in vivo infection studies) activates HEK293 cells via TLR2, not via TLR4. The evidence that TLR4 has
an important role in Gram-negative infections is exclusively derived from models using bacteria that express LPS recognized by TLR4 [9]. Indeed, TLR4 deficient mice are more susceptible to Gram-negative sepsis due to Salmonella typhimurium and Escherichia coli and to Gram-negative pneumonia due to Haemophilus influenzae, Klebsiella pneumoniae and Acinetobacter baumannii [27,38-41]. Of note, B. pseudomallei LPS differs in several aspects from the LPS of other Gram-negative organisms: B. pseudomallei LPS exhibits weaker pyrogenic activity in rodents compared with enterobacterial LPS [4,42] and LPS mediated activation of mouse macrophages in vitro is slower for LPS from B. pseudomallei than for E. coli LPS [43]. In this respect it is of interest that our laboratory previously established that TLR4 is not involved in host defense against pulmonary infection with Legionella pneumophila [44], a Gram-negative bacterium of which the LPS, like B. pseudomallei LPS, is a weak inducer of pro-inflammatory cytokine production by mononuclear cells [45]. Hence, it is conceivable that absent or insufficient LPS sensing by TLR4 makes this receptor redundant during infection in vivo with Legionella or Burkholderia. In addition, the recent finding that TLR4 does not mediate resistance against Pseudomonas aeruginosa acute lung infection suggests that for some Gram-negative pathogens signaling to other pattern recognition receptors may compensate for the loss of LPS – TLR4 signaling [46].

TLR2 is a promiscuous pattern recognition receptor recognizing multiple ligands expressed by a variety of microorganisms such as Mycoplasma (diacyl lipopeptides), mycobacteria (triacyl lipopeptides, lipoarabinomannan), fungi (zymosan, phospholipomannan), parasites (tGPI-mutin) and viruses (hemagglutinin protein from measles virus and unidentified structures of herpes simplex virus) [1,2]. In addition, TLR2 can signal a number of PAMPs expressed by bacteria, in particular lipoteichoic acid (exclusively expressed by Gram-positive bacteria), peptidoglycan and triacyl lipopeptides (found in most bacteria) and porins (Neisseria) [1,2]. Finally, TLR2 may be activated by LPS from some Gram-negative bacteria [47-49]. We here found that TLR2 contributes to the responsiveness of cells to B. pseudomallei using two complementary approaches: TLR2 KO macrophages and blood produced less TNF-α upon stimulation with Burkholderia, whereas TLR2 transfected HEK 293 cells released large amounts of IL-8 upon incubation with this bacterium. In addition, we identified B. pseudomallei LPS as a TLR2 ligand. Conceivably, B. pseudomallei expresses more TLR2 ligands with peptidoglycan and lipopeptides being possible candidates. The interaction between Burkholderia and TLR2 clearly impairs the protective immune response against this bacterium, as reflected by the fact that TLR2 KO mice displayed a strongly reduced lethality during experimental melioidosis, which was associated with a diminished bacterial growth and dissemination, and reduced distant organ injury. Previously, TLR2 KO mice were found less susceptible to lethal infections with Yersinia enterocolitica or Candida albicans through a mechanism that likely involved a stronger type 1 cytokine response due to diminished production of the prototypic type 2 cytokine IL-10 during infection [50,51]. In the case of Candida infection, TLR2 induced proliferation and survival of regulatory T cells, which appeared largely responsible for the increased IL-10 release induced by TLR2 [51]. Although a type 1 response is protective in melioidosis [52,53], a loss of type 2 cytokine stimulation in TLR2 KO mice can not explain the protection of these animals during B. pseudomallei infection. First, the differences in IL-10 concentrations between TLR2 KO and WT mice were minor and much less profound than the concurrent differences in the proinflammatory cytokine TNF-α. Second, the concentrations
of the prototypic type 1 cytokine interferon-\(\gamma\) remained very low or undetectable throughout the entire observation period (data not shown). Finally, antibody mediated depletion of regulatory T cells did not influence the outcome of melioidosis in our model (data not shown). More studies are warranted to dissect the exact mechanism by which TLR2 facilitates the growth and spread of \(B.\) pseudomallei \textit{in vivo}.

Melioidosis is a debilitating disease with an unacceptable high mortality rate \cite{3,4}. We here demonstrate that the expression of multiple TLRs is upregulated in peripheral blood leukocytes in septic patients with melioidosis. Most remarkable, although our results show that both TLR2 and TLR4 contribute to cellular responsiveness to \(B.\) pseudomallei \textit{in vitro}, TLR2 detects the LPS of \(B.\) pseudomallei and only TLR2 impacts on the immune response of the intact host \textit{in vivo}. Of note, TLR4 does not contribute to protective immunity in this severe Gram-negative infection. Taken together, these findings suggest that septic melioidosis can be seen as the clinical manifestation of a TLR mediated dysregulation of the immune response to invasive \(B.\) pseudomallei. In addition, this report further undermines the paradigm regarding TLR4 as “the Gram-negative receptor” and TLR2 as “the Gram-positive receptor”. Inhibition of TLR2 may be a useful adjunctive therapy for melioidosis.

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EDITORS’ SUMMARY

Background. Melioidosis is a severe tropical infection caused by the bacterium *Burkholderia pseudomallei*. This soil-dwelling pathogen (disease-causing organism) enters the body through cuts, by swallowed contaminated water, or by inhaled contaminated dust. Here, it can cause a severe lung infection or spread into the blood stream and around the body, where it causes widespread inflammation (sepsis) and organ failure. Untreated septic melioidosis is usually fatal. Even with antibiotic therapy, half the people who develop it in Thailand (a hot spot for melioidosis) die. *B. pseudomallei* is a “gram-negative” bacterium. That is, it is surrounded by a membrane that stops it taking up a stain used to detect bacteria. This membrane contains a molecule called lipopolysaccharide (LPS). Proteins on immune system cells called Toll-like receptors (TLRs), of which there are many, recognize LPS and other surface molecules common to different pathogens and tell the cells to make cytokines. These cytokines stimulate the immune system to kill the pathogen but also cause inflammation, the underlying problem in septic melioidosis and other forms of sepsis. In other words, TLRs are two-edged swords—they provide an essential first-line defense against pathogens, but cause life-threatening inflammation if overstimulated.

Why Was This Study Done? It isn’t known which TLRs are involved in melioidosis. TLR4 normally detects LPS, but the surface of *B. pseudomallei* also carries molecules that interact with TLR2. Understanding how *B. pseudomallei* interacts with TLRs might suggest new, more effective ways to treat septic melioidosis. Better remedies for this disease are badly needed because, as well as the infections it causes in the community, the US Centers for Disease Control and Prevention has identified *B. pseudomallei* as a potential bioterrorism agent. In this study, the researchers have characterized the expression and function of TLRs in septic melioidosis using human, in vitro (test tube), and animal approaches.

What Did the Researchers Do and Find? The researchers isolated monocytes and granulocytes (immune system cells involved in first-line defenses against pathogens) from patients with melioidosis and from healthy people. The patients’ cells made more TLR1, TLR2, TLR4, and CD14 (a protein that enhances the activation of immune system cells by LPS) than those of the healthy controls and more of the mRNAs encoding several other TLRs. Next, the researchers tested the ability of heat-killed *B. pseudomallei* to induce the release of TNFα (a cytokine produced in response to TLR signaling) from macrophages (immune system cells that swallow up pathogens) isolated from wild-type mice and from mice lacking TLR2 or TLR4. Macrophages
isolated from wildtype mice made more TNFα than those from TLR2- or TLR4-deficient mice. In addition, a human kidney cell line engineered to express CD14/TLR2 or CD14/TLR4 but not the parent cell line released IL8 (another cytokine) when stimulated with heat-killed *B. pseudomallei*. Other experiments in these human cell lines showed that LPS purified from *B. pseudomallei* signals through TLR2 but not through TLR4. Finally, the researchers tested the ability of TLR2- and TLR4-deficient mice to survive after infection with live *B. pseudomallei*. Compared with TLR4-deficient or wild-type mice, the TLR2-deficient mice had a strong survival advantage, a lower bacterial load, reduced lung inflammation, and less organ damage.

**What Do These Findings Mean?** These findings show that people with melioidosis have increased expression of several TLRs, any one of which might cause the sepsis associated with *B. pseudomallei* infection. The in vitro findings indicate that TLR2 and TLR4 both contribute to the responsiveness of immune cells to *B. pseudomallei* in test tubes, but that only TLR2 detects the LPS of this bacterium. This unexpected result—TLR4 normally responds to LPS—might indicate that there is something unique about the LPS of *B. pseudomallei*. Finally, the survival of TLR2-deficient mice after infection with *B. pseudomallei* suggests that TLR2-mediated dysregulation of the immune system in response to invasive *B. pseudomallei* might cause septic melioidosis. Although these results need confirming in people, they suggest that inhibition of TLR2 in combination with antibiotic therapy might improve outcomes for people with melioidosis.

**Additional Information.**
- Information is available from the US Centers for Disease Control and Prevention on melioidosis (in English and Spanish)
- The UK Health Protection Agency provides information for the public and health professionals on melioidosis
- Wikipedia has pages on melioidosis and on Toll-like receptors (note: Wikipedia is a free online encyclopedia that anyone can edit; available in several languages)
- The MedlinePlus encyclopedia contains a page on sepsis (in English and Spanish)
Chapter 8

REFERENCES


Chapter 8


Chapter 9

MyD88 dependent signaling contributes to protective host defense against *Burkholderia pseudomallei*

Submitted

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Chapter 9

ABSTRACT

Toll-like receptors (TLRs) have a central role in the recognition of pathogens and the initiation of the innate immune response. Myeloid differentiation primary-response gene 88 (MyD88) and TIR-domain-containing adaptor protein inducing IFNβ (TRIF) are regarded as the key signaling adaptor proteins for TLRs. Melioidosis, which is endemic in SE-Asia, is a severe infection caused by the gram-negative bacterium *Burkholderia pseudomallei*. We here aimed to characterize the role of MyD88 and TRIF in host defense against melioidosis. First, we found that MyD88, but not TRIF, deficient whole blood leukocytes released less TNFα upon stimulation with *B. pseudomallei* compared to wild-type (WT) cells. Thereafter we inoculated MyD88 knock-out (KO), TRIF mutant and WT mice intranasally with *B. pseudomallei* and found that MyD88 KO, but not TRIF mutant mice demonstrated a strongly accelerated lethality, which was accompanied by significantly increased bacterial loads in lungs, liver and blood, and grossly enhanced liver damage compared to WT mice. The decreased bacterial clearance capacity of MyD88 KO mice was accompanied by a markedly reduced early pulmonary neutrophil recruitment and a diminished activation of neutrophils after infection with *B. pseudomallei*. MyD88 KO leukocytes displayed an unaltered capacity to phagocytose and kill *B. pseudomallei* in vitro. In conclusion, MyD88 dependent signaling, but not TRIF dependent signaling, contributes to a protective host response against *B. pseudomallei* at least in part by causing early neutrophil recruitment towards the primary site of infection.
INTRODUCTION

Innate immune recognition is based on the detection of molecular structures that are unique to microorganisms. The Toll family of receptors (TLRs) has a central role as pattern recognition receptors (PRRs) in the initiation of cellular innate immune responses. TLRs can activate tissue-resident macrophages to produce pro-inflammatory cytokines, including TNF-α and IL-6, which coordinate local and systemic inflammatory responses. TLR signalling depends on the selective use by different TLRs of five different adaptor molecules: myeloid differentiation primary-response gene 88 (MyD88), TIR-domain-containing adaptor protein inducing IFNβ (TRIF), MyD88-adaptor-like (MAL), TRIF-related adaptor molecule (TRAM) and sterile armadillo-motif-containing protein (SARM). MyD88 and TRIF are regarded as the main adaptor proteins. MyD88 is the key signalling adaptor for all TLRs - with the exception of TLR3 and certain TLR4 signals –, the IL-1-receptor (IL-1R) and IL-18R; its main role is the activation of nuclear factor-κB (NF-κB). MyD88 is directly recruited to the TIR (Toll/IL-1R) domains in certain TLRs and acts to recruit IL-1R-associated kinase (IRAK). TRIF is now known to control the TLR4-induced MyD88-independent pathway, and also to be the exclusive adaptor used by TLR3. MAL and TRAM both act as bridging adaptors, with MAL recruiting MyD88 to TLR2 and TLR4, and TRAM recruiting TRIF to TLR4 to allow for IFN regulatory factor (IRF)-3 activation. SARM has recently been shown to function as a negative regulator of TRIF.

Given their central role in the recognition of microbes, TLR signalling is likely to play a crucial role in the event of sepsis: on the one hand TLR signalling is essential for the early detection of pathogens, but on the other hand can cause excessive inflammation after uncontrolled stimulation. *Burkholderia pseudomallei*, a gram-negative bacterium that causes melioidosis and a recognized biological threat agent, is one of the most important causes of pneumonia-derived and community-acquired sepsis in South-East Asia and northern-Australia. We have recently shown that both TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei* in vitro, while only TLR2 impacts on the immune response of the intact host in vivo. In the present study we sought to determine the contribution of MyD88 and TRIF in the innate immune response to *B. pseudomallei* and found that MyD88, but not TRIF, signalling plays a crucial protective role in experimental melioidosis at least in part by causing early neutrophil recruitment to the site of infection.

MATERIALS AND METHODS

Mice

Pathogen-free 10 week old C57BL/6 wild-type (WT) mice were purchased from Harlan Sprague Dawley Inc. (Horst, the Netherlands). MyD88 knockout (KO) mice backcrossed 6 times to a C57BL/6 genetic background were generously provided by Dr. Shizuo Akira (Osaka University.)
Mice deficient in TRIF, generously provided by Dr. Bruce Beutler (Scripps Research Institute, La Jolla, CA), were obtained by inducing random germline mutations in C57BL/6 mice by using N-ethyl-N-nitrosourea. Age and sex-matched animals were used in all experiments. The Animal Care and Use of Committee of the University of Amsterdam approved all experiments.

**In vitro stimulation**

Whole blood, obtained from uninfected WT, MyD88 KO and TRIF mutant mice by bleeding from the inferior vena cava, was stimulated with heat-killed *B. pseudomallei* strain 1026b (5x10⁷ CFU/ml) or RPMI for 16 hrs as described. Supernatants were harvested and stored at -20°C until assayed for TNF release.

**Experimental infection**

For preparation of the inoculum, *B. pseudomallei* strain 1026b was used and streaked from frozen aliquots into 50 ml Luria broth (Difco, Detroit, MI) for overnight incubation at 37°C in a 5% CO₂ incubator. Thereafter, a 1 ml portion was transferred to fresh Luria broth and grown for ± 5h to midlogarithmic phase. Bacteria were harvested by centrifugation at 1500 x g for 15 minutes, washed and resuspended in sterile isotonic saline at a concentration of 5x10² CFUs/50 μl, as determined by plating serial 10-fold dilutions on blood agar plates. Pneumonia was induced by intranasal inoculation of 50 μl (5x10² CFU) bacterial suspension. For this procedure mic

**Determination of bacterial outgrowth**

At designated time points after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium: active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Mijdrecht, The Netherlands) and sacrificed by bleeding from the inferior vena cava. The lungs and liver were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of organ homogenates and blood, plated on blood agar plates and incubated at 37°C at 5% CO₂ for 16 h before colonies were counted.

**Assays**

Lung homogenates were prepared as described earlier. Mouse TNF-α, IFN-γ, monocyte chemoattractant protein (MCP)-1, IL-6 and IL-10 were measured by cytometric bead array (CBA) multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. Myeloperoxidase (MPO; HyCult Biotechnology, Uden, the Netherlands), lipopolysaccharide-induced CXC chemokine (LIX; R&D Systems, Minneapolis, MN), KC and macrophage-inflammatory protein-(MIP)-2 (both R&D Systems, Minneapolis, MN) were measured using commercially available ELISAs or antibody pairs. Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined with commercially available kits (Sigma-Aldrich), using a Hitachi analyzer (Roche) according to the manufacturer’s instructions.
Pathology
Lungs and liver for histology were prepared and analyzed as described earlier. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: surface with pneumonia, necrosis/abscess formation, interstitial inflammation, endothelialitis, bronchitis, edema, thrombus formation and pleuritis. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 32. Liver sections were scored on inflammation, necrosis/abscess formation and thrombus formation using the scale given above.

Flow cytometry
Lung cell suspensions were obtained by passing the lungs through a 40-μm cell strainer (BD, Franklin Lakes, NJ) as described previously. Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4); the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium), and counted by using a hemocytometer. The percentages of macrophages, neutrophils and lymphocytes were determined using a FACSCalibur (BD, San Jose, CA). Cells were brought to a concentration of 1 x 10⁷ cells/ml in FACs buffer (PBS supplemented with 0.5% PBS, 0.01% NaN₃ and 0.35 mM EDTA). Immunostaining for cell surface molecules was performed for 30 minutes at 4ºC using directly labeled antibodies (abs) against GR-1 (GR-1 FITC, BDPharmingen, San Diego, California), CD11b (CD11b-phycoerythrin, BDPharmingen) and a biotin labeled antibody against F4/80 (Serotec, Oxford, United Kingdom) in combination with streptavidin allophycocyanine. All abs were used in concentrations recommended by the manufacturer (BD Pharmingen, San Diego, CA). After staining, cells were fixed in 2% paraformaldehyde. Neutrophils were counted using the scatter pattern and GR-1 high gate, monocytes in the sidescatter low and F4/80 positive gate and macrophages in the sidescatter high and F4/80 positive gate.

Phagocytosis and bacterial killing assays
Phagocytosis was evaluated in essence as described before. Concentrated B. pseudomallei preparation was treated for 90 minutes at 38ºC with 100 μg/ml mitomycine-C (Sigma-Aldrich, Zwijndrecht, the Netherlands) to prepare alive but growth-arrested bacteria. To determine the neutrophil phagocytosis capacity, 50 μl of whole blood was incubated with carboxyfluorescein-diacetate-succinimidy-ester (CFSE dye, Invitrogen, Breda, the Netherlands) labeled, growth-arrested bacteria (1x10⁷ CFU/ml) and incubated for 10, 60 or 120 minutes at 37ºC. Cells were suspended in Quenching solution, incubated in FACS lysis/fix solution (BD, Mountain View, CA) and neutrophils were labeled using anti-Gr-1-PE (BD Pharmingen, San Diego, CA). Cells were washed with ice-cold FACS buffer after which the degree of phagocytosis of neutrophils was determined using FACSCalibur (Becton Dickinson). The phagocytosis index of each samples was calculated as follows: (mean fluorescence x % positive cells at 37ºC) minus (mean fluorescence x % positive cells at 4ºC). Bacterial killing was determined as described. In brief, peritoneal macrophages were harvested from WT and MyD88 KO mice and plated as described above (n=5 per strain). B. pseudomallei was added at a multiplicity of infection (MOI)
of 50 and spun onto cells at 2000 rpm for 5 minutes, after which plates were placed at 37°C for 10 minutes. Each well was then washed 5 times with ice-cold PBS to remove extracellular bacteria. To determine bacterial uptake after 10 minutes, wells were lysed with sterile dH2O and designated as t=0. RPMI was added to remaining wells and plates were placed at 37°C for 10 or 60 minutes after which cells were again washed 5 times with ice-cold PBS and lysed with dH2O. Cell-lysates were plated on blood agar plates and bacterial counts were enumerated after 16h. Bacterial killing was expressed as the percentage of killed bacteria in relation to t=0.

Statistical analysis
All data are expressed as mean ± SEM. Difference between groups were analyzed with Mann-Whitney U test or Kruskal-Wallis analysis with Dunn post hoc test where appropriate. For survival analyses, Kaplan-Meier analyses followed by log-rank test was performed. These analyses were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Values of \( p < 0.05 \) were considered statistically significant.

RESULTS

MyD88 but not TRIF is required for cellular responsiveness to \textit{B. pseudomallei} \textit{in vitro}

To obtain a first insight into the function of the TLR adaptor molecules MyD88 and TRIF in melioidosis, we tested the requirement of MyD88 and TRIF signaling upon first encounter between \textit{B. pseudomallei} and the host. Therefore, we tested the capacity of whole blood harvested from WT, MyD88 KO and TRIF mutant mice to release TNF\( \alpha \) upon stimulation with \textit{B. pseudomallei}. MyD88 deficient leukocytes, but not TRIF deficient leukocytes, released less TNF\( \alpha \) than WT leukocytes upon stimulation with \textit{B. pseudomallei} \textit{in vitro} (Fig. 1), suggesting that MyD88, and not TRIF, contributes to cellular responsiveness towards \textit{B. pseudomallei} \textit{in vitro}.

![Figure 1. MyD88, but not TRIF, is required for cellular responsiveness to \textit{B. pseudomallei} \textit{in vitro}.](image-url)
MyD88 KO, but not TRIF mutant, mice show an accelerated mortality during experimental melioidosis

We next investigated the involvement of MyD88 and TRIF in the host response to *B. pseudomallei* infection *in vivo*. As a first experiment WT, MyD88 KO and TRIF mutant mice were intranasally infected with a lethal dose of *B. pseudomallei* and followed for one week. MyD88 deficiency had a markedly negative influence on survival: whereas all WT mice were dead after 160 hours (median survival time 118 hours), all MyD88 KO mice died within 88 hours (median survival time 76 hours; \( P < 0.001 \) for the difference between groups; Fig. 2A). TRIF deficiency on the other hand did not impact on survival during experimental melioidosis (Fig. 2B).

Figure 2. MyD88 KO, but not TRIF KO, mice show an accelerated mortality during experimental melioidosis. Survival of wild-type (WT, open rounds) and MyD88 KO mice (black squares) (A) or TRIF mutant (black squares) mice (B) intranasally infected with 5 x 10^2 CFU *B. pseudomallei*. Mortality was assessed twice daily for one week. \( n = 8-10 \) per group; ns denotes not significant; \( P \) value indicates the difference between MyD88 KO and WT mice.

Figure 3. MyD88 KO mice show increased bacterial outgrowth during experimental melioidosis. WT and MyD88 KO mice were intranasally infected with *B. pseudomallei* (5x10^2 CFU). Bacterial loads were measured 24 h and 72 h after inoculation in lungs (A) and liver (B). BC+ indicates the number of positive blood cultures. Data are mean ± SEM (n = 6-7 per group at each time point). ** \( P < 0.01 \).
Chapter 9

**MyD88 but not TRIF contributes to bacterial clearance of B. pseudomallei in vivo**

To obtain insight into the mechanisms underlying the accelerated mortality of MyD88 KO mice during experimental melioidosis, we infected WT and MyD88 KO mice with *B. pseudomallei* and sacrificed them after 24 (i.e. just before the first symptoms of illness occurred) and 72 hours (i.e. before the first deaths occurred) to determine bacterial loads in lungs (the primary site of the infection), liver and blood (to evaluate dissemination to distant body sites; Fig. 3). Relative to WT mice, MyD88 KO mice displayed strongly increased bacterial loads in their lungs at 24 and 72 hours after infection, as well as in their livers at 72 hours (Fig. 3). This was in line with an increase in the number of positive blood cultures (Fig. 3). Conversely, similar bacterial loads in the lungs, liver and blood of WT and TRIF mutant mice were observed 72 hours after infection with *B. pseudomallei* (Fig. 4).

![Graph A: Trif deficiency does not impact on bacterial outgrowth during experimental melioidosis.](image)

**Figure 4.** Trif deficiency does not impact on bacterial outgrowth during experimental melioidosis. WT and TRIF mutant mice were intranasally infected with *B. pseudomallei* (5x10² CFU). Bacterial loads were measured 72h after inoculation in lungs (A) and liver (B). BC+ indicates the number of positive blood cultures. Data are mean ± SEM (n = 8 per group at each time point); ns denotes not significant.

**Role of MyD88 and TRIF in B. pseudomallei induced pulmonary inflammation**

The success of combating infection strongly depends on the generation of an effective inflammatory response at the primary site of infection. Therefore, to further investigate the role of MyD88 and TRIF in the inflammatory response towards infection with *B. pseudomallei*, we first measured cytokine levels in whole lung homogenates harvested from WT, MyD88 KO and TRIF mutant mice after intranasal infection with *B. pseudomallei* (Table 1). 24 h after infection, pulmonary TNFα, MCP-1 and IL-10 were lower in MyD88 KO mice relative to WT mice (Table 1). At 72 h after infection TNFα levels remained lower in MyD88 KO mice, while MCP-1 and IL-6 levels tended to be higher in these animals (Table 1). In addition, histological samples of lungs obtained 24 and 72 hours after infection were semi-quantitatively scored on the extent of inflammation. Pulmonary inflammation was characterized by significant inflammation, pleuritis, peribronchial inflammation, oedema and endothelialitis in both WT and MyD88 KO mice (Fig. 5). MyD88 KO mice tended to have more lung injury compared to WT mice, however lung inflammation scores were not significantly different at 72 hours after infection between both
mice strains (data not shown). Pulmonary cytokine levels and lung inflammation scores were similar in TRIF mutant and WT mice at all time points (data not shown).

Enhanced liver damage in MyD88 KO mice

After the lung, the liver is one of the most commonly affected organs in melioidosis. Considering this and the observed increased bacterial loads in the liver, we examined the influence of MyD88 deficiency on liver damage 72 h post infection. Upon histopathological examination, both WT and MyD88 KO mice showed mild inflammation of liver tissue as characterized by the influx of leukocytes into the hepatic parenchyma (Fig. 6A-B). In contrast to WT mice, MyD88 KO mice showed foci of liver necrosis and the formation of small abscesses (Fig. 6B). In line, the extent of hepatic inflammation as quantified according to the scoring system described in Materials and Methods section was significantly increased in MyD88 KO mice compared to controls (Fig. 6C). The increased hepatocellular injury in MyD88 KO mice after infection with B. pseudomallei was further underscored by clinical chemistry: MyD88 KO mice displayed strikingly higher plasma levels of ASAT and ALAT compared to WT mice (Fig. 6D-E).

Table 1. Role of MyD88 in B. pseudomallei induced pulmonary cytokine levels

<table>
<thead>
<tr>
<th></th>
<th>WT t = 24</th>
<th>MyD88 KO</th>
<th>WT t = 72</th>
<th>MyD88 KO</th>
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<tr>
<td>TNF-α</td>
<td>50 ± 18</td>
<td>10 ± 2*</td>
<td>1048 ± 266</td>
<td>314 ± 33**</td>
</tr>
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<td>IFN-γ</td>
<td>BD</td>
<td>BD</td>
<td>11 ± 3</td>
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<tr>
<td>MCP-1</td>
<td>993 ± 255</td>
<td>245 ± 46*</td>
<td>6354 ± 1379</td>
<td>9950 ± 32</td>
</tr>
<tr>
<td>IL-6</td>
<td>487 ± 122</td>
<td>85 ± 15</td>
<td>1418 ± 869</td>
<td>2990 ± 456</td>
</tr>
<tr>
<td>IL-10</td>
<td>120 ± 48</td>
<td>28 ± 11*</td>
<td>278 ± 53</td>
<td>68 ± 16</td>
</tr>
</tbody>
</table>

Groups of 5-7 mice were intranasally inoculated with 5 x 10² CFU B. pseudomallei. After 24 and 72 h mice were sacrificed, lungs were removed, homogenized and cytokines were measured. Data are expressed as mean ± SEM; BD: below detection; * P < 0.05, ** P < 0.01 versus WT controls.
Figure 6. Enhanced liver damage in MyD88 KO mice. Representative hematoxylin- and eosin-stained liver histology slides for WT (A) and MyD88 KO (B) mice at 72 h after inoculation with 5x10^2 CFU B. pseudomallei show more inflammation and foci of necrosis (as indicated by the asterisk) in the livers derived from MyD88 KO animals compared to WT controls (original magnification, x 400) corresponding with higher pathology scores (see Methods section) (C). At 72 h after inoculation MyD88 KO mice also showed enhanced hepatic injury, as reflected by the plasma concentrations of aspartate aminotransferase (ASAT) (D) and alanine aminotransferase (ALAT) (E). Data are mean ± SEM (n = 5-6 per group); U/L, units per liter; * P < 0.05; ** P < 0.01.

Figure 7. No difference in B. pseudomallei phagocytosis or killing capacity between WT and MyD88 KO cells. (A) Peripheral blood neutrophils were incubated at 37°C with CFSE-labeled growth-arrested B. pseudomallei (1x10^7 CFU/ml) after which time-dependent phagocytosis was quantified (see Methods section). (B) Killing capacity of peritoneal macrophages are shown as percentage of killed B. pseudomallei compared to t = 0. Data are mean ± SEM; n= 5 per mouse strain. Open rounds represent WT cells, while black squares represent MyD88 KO mice; ns denotes not significant.
No role of MyD88 in phagocytosis of *B. pseudomallei*

The experiments described above established the key role of MyD88 in the antibacterial defense towards *B. pseudomallei* infection. MyD88 has been described to play an important role in phagocytosis and killing of invading bacteria \(^25\) and since *B. pseudomallei* is a facultative intracellular bacterium \(^12\), \(^28\)-\(^30\) we next determined whether MyD88 contributes to phagocytosis and/or killing of *B. pseudomallei*. Interestingly, we found that MyD88 deficient neutrophils demonstrated an unaltered capacity to phagocyte *B. pseudomallei* (Fig. 7A). In addition, no difference in the killing capacity between WT and MyD88 deficient macrophages was observed (Fig. 7B).

MyD88 plays an important role in the early neutrophil recruitment after infection with *B. pseudomallei*

To further dissect the mechanism for the strong protective role of MyD88 during experimental melioidosis and in light of recent data suggesting that MyD88 is necessary for early neutrophil recruitment during hypersensitivity pneumonitis \(^31\) as well as pulmonary infection with *Pseudomonas aeruginosa* \(^32\), we investigated the role of MyD88 in neutrophil recruitment in our model. Therefore, we compared the pulmonary cell influx in WT and MyD88 KO mice after inoculation with *B. pseudomallei*. At 24 hours after infection, MyD88 KO mice had significantly fewer neutrophils in their lungs compared with WT mice (Table 2 and Fig. 8A). In addition to a lower absolute amount of neutrophils in MyD88 KO mice, the neutrophils that were recruited were also less activated as demonstrated by both diminished CD11b positivity (Fig. 8B) and decreased pulmonary MPO content (Fig. 8C). In line, local levels of the neutrophil attracting chemokines MIP-2 and KC tended to be lower or were significantly reduced in MyD88 KO mice at 24 hours after infection (Fig. 8D-E). The pulmonary levels of LIX, a murine neutrophil-chemoattractant CXC chemokine \(^33\), did not differ between WT and MyD88 KO mice at this early time point (Fig. 8F). Together these data suggest that the protective effect of MyD88 in the host defense against melioidosis can at least in part be explained by MyD88 dependent neutrophil recruitment following exposure to *B. pseudomallei*.

**Table 2.** Effect of MyD88 deficiency on total and differential lung cell counts.

<table>
<thead>
<tr>
<th></th>
<th>Leukocytes</th>
<th>Macrophages</th>
<th>Monocytes</th>
<th>Neutrophils</th>
<th>Lymphocytes/Other</th>
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<tbody>
<tr>
<td></td>
<td>x 10^5/ml</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>23 ± 1</td>
<td>20 ± 1</td>
<td>13 ± 1</td>
<td>25 ± 3</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>MyD88 KO</td>
<td>10 ± 1</td>
<td>21 ± 1</td>
<td>12 ± 1</td>
<td>6 ± 1**</td>
<td>60 ± 1**</td>
</tr>
</tbody>
</table>

Total leukocyte counts (x 10^5/ml) and differential cell counts in lungs of wild-type (WT) and MyD88 knock-out mice 24 hours after intranasal infection with 5 x 10^2 CFU of *B. pseudomallei*. Data are mean ± SEM (n = 6-7/group); ** P < 0.01 versus WT.
DISCUSSION

The identification of the TLR family in conjunction with their specific signaling pathways has led to an explosion of knowledge in understanding both the physiological and pathophysiological role of these innate immune signaling pathways in the event of sepsis. Although our knowledge on the role of the innate immunity in the pathogenesis of sepsis caused by *B. pseudomallei* has progressed rapidly over the past years, much is unclear about which TLR signaling pathways are activated and how these TLR initiated signals are integrated into the more general infrastructure of the host defense in which they operate. The melioidosis mouse model in which *B. pseudomallei* is delivered per the intranasal route has been proven successful in mimicking pneumonia-derived septic melioidosis and the availability of MyD88 and TRIF deficient mice enabled us to further elucidate the predominant TLR signaling routes involved in melioidosis. We found that MyD88, but not TRIF, plays an important protective role in the host defense against *B. pseudomallei in vivo*. MyD88 deficient, but not TRIF mutant whole blood leukocytes released...
MyD88 and TRIF in melioidosis

less TNFα upon stimulation with *B. pseudomallei* compared to WT cells. In addition, during experimental melioidosis MyD88 KO mice, but not TRIF mutant mice, demonstrated strongly increased lethality, accompanied by significantly increased bacterial loads when compared to WT mice. The decreased bacterial clearance capacity of MyD88 KO mice was accompanied by markedly reduced early pulmonary neutrophil recruitment and a diminished activation of neutrophils. These results further contribute to our understanding of the pathogenesis of sepsis in general and severe melioidosis in particular by revealing the important protective role of the MyD88 dependent signaling pathway by causing early neutrophil recruitment following *B. pseudomallei* exposure.

Our data are in line with other studies showing the crucial role of MyD88 in host defense against infection with both gram-positive and gram-negative bacteria. More specifically, our current results now further add to accumulating evidence that MyD88 is necessary for neutrophil recruitment during pneumonia. Neutrophils are known to play a critical role in the host defense against *B. pseudomallei*. During murine melioidosis activated neutrophils are rapidly recruited to the lungs after intranasal infection with *B. pseudomallei*. In addition, depletion of neutrophils with anti-GR-1+ antibody severely exacerbated disease and was associated with a 1000-fold increase in pulmonary bacterial loads within 4 days. We now show that during experimental melioidosis MyD88 is crucially involved in protective neutrophil recruitment. We further extend these findings by showing the importance of MyD88 in the activation of neutrophils as demonstrated by reduced CD11b expression on MyD88 KO neutrophils. The early diminished neutrophil recruitment was accompanied by reduced levels of the CXC chemokines KC and MIP-2, but unaltered early LIX levels, suggesting that MyD88 deficiency at least in part diminishes neutrophil influx during pneumonia derived melioidosis due to an attenuated production of CXC chemokines at the primary site of the infection. Interestingly, we also show that MyD88 does not play a role in phagocytosis or killing of the facultative intracellular *B. pseudomallei*. This is in contrast with one earlier report describing MyD88 to be important in phagocytosis and killing of invading bacteria, but does correspond with more recent evidence arguing against a role of TLR-signalling in phagocytosis. Notably, the observed decrease in neutrophil recruitment in the infected MyD88 KO mice was associated with decreased levels of TNFα, MCP-1 and IL-10 signifying that MyD88 is required for the induction of these cytokines.

Remarkably, MyD88 KO and WT mice displayed similar lung pathology at 72 hours after infection. Thus, neutrophil recruitment was relatively selectively influenced by MyD88 deficiency in the context of the lung inflammatory response as a whole. It should be noted that MyD88 KO mice had much higher bacterial loads in their lungs than WT mice, and one could therefore argue that MyD88 KO mice had a relatively attenuated inflammatory pulmonary response. Nonetheless, our data indicate that even in the absence of MyD88 signaling, which abrogates the function of all relevant TLRs as well as of the IL-1R and IL-18R, *B. pseudomallei* is still able to elicit profound inflammation in the pulmonary compartment. Further research is warranted which MyD88 independent components of the innate immune system contribute herein.
Chapter 9

Bacterial dissemination to the liver and the formation of liver abscesses is one of the hallmarks of melioidosis septic shock\(^{11,12}\). We now show that MyD88 KO mice display a markedly increased susceptibility to liver injury as indicated by increased histopathology scores and clinical chemistry levels. The MyD88 pathway has recently been shown to be essential for early liver restoration after partial hepatectomy\(^{46}\). Furthermore, in a model of sepsis caused by polymicrobial infection both systemic and hepatic inflammatory responses were strongly attenuated in the absence of MyD88\(^{47}\). Our current data indicate that in melioidosis MyD88 deficiency results in increased hepatocellular injury most likely due to the much higher bacterial loads in the liver. In addition, these data suggest that MyD88 dependent signaling is not required for the occurrence of liver inflammation and injury.

In addition of being the key signalling adaptor of virtual all TLRs, MyD88 also serves as the main adaptor molecule for the IL-1R and the IL-18R\(^{4}\). It has been shown \textit{in vitro} that \textit{B. pseudomallei} is capable of inducing caspase-1 dependent death in macrophages which is accompanied by the release of IL-1β and IL-18\(^{48}\). Indeed, both IL-1β and IL-18 are known to be upregulated during melioidosis\(^{19,49,50}\). Our current data can now be brought in line with more recent findings showing the importance of IL-18 in the host defense against melioidosis: mice treated with anti-IL18R antibody\(^{51}\) or IL-18 KO mice\(^{19}\) have similar phenotypes as MyD88 KO mice as they all show a markedly increased susceptibility to infection with \textit{B. pseudomallei}. It remains to be established to what extent TLR dependent MyD88 signaling contributes to the phenotype of MyD88 KO mice in experimental melioidosis. We have previously reported on the role of TLR2 and TLR4 in the host defense against \textit{B. pseudomallei} and found that although both TLR2 and TLR4 contribute to cellular responsiveness to \textit{B. pseudomallei} \textit{in vitro} only TLR2 impacts on the immune response of the intact host \textit{in vivo}\(^{13}\). Specifically, TLR2 KO mice were strongly protected against \textit{B. pseudomallei} induced mortality, which was accompanied by much lower bacterial loads in lungs and livers\(^{13}\). Since TLR2 signaling fully relies on MyD88\(^{3,4}\), these results imply that complete MyD88 deficiency overrules the protective effect of TLR2 restricted MyD88 signaling. Our own preliminary results do not point toward an important role for TLR9 in host defense against \textit{B. pseudomallei} (Wiersinga WJ, et al, unpublished). A likely MyD88 dependent candidate within the TLR family as a mediator of protective immunity against melioidosis is TLR5, which recognizes flagellin, an important component of \textit{B. pseudomallei}\(^{12,52}\). The first studies using TLR5 KO mice are about to be initiated in our laboratory. Together, these data indicate that MyD88 deficiency results in a strongly impaired resistance against melioidosis likely due to a combined deficiency in IL-1R, IL-18R and possibly TLR5 signaling, and in spite of an interruption of harmful TLR2 signaling.

Two earlier studies documented a reduced innate immune response and a diminished bacterial clearance in TRIF deficient mice with experimentally induced gram-negative pneumonia caused by either \textit{Escherichia coli} or \textit{Pseudomonas aeruginosa}\(^{53,54}\). Our laboratory, however, reported that MyD88 but not TRIF is important in clearing non-typeable \textit{Haemophilus influenzae} from the mouse lung\(^{42}\). We now show that TRIF does not play a role in the host defense against \textit{B. pseudomallei}, which is in full correspondence with the earlier found limited role of TLR4 in this gram-negative infection\(^{13}\).
We conclude that MyD88 dependent signaling contributes to a protective host response against *B. pseudomallei* at least in part by causing early neutrophil activation and recruitment towards the site of infection. In addition our study reveals that the role of TRIF signaling is of minor importance for the clearance of infection with *B. pseudomallei*.

**ACKNOWLEDGMENTS**

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Chapter 9

REFERENCES

MyD88 and TRIF in melioidosis


Chapter 9


CD14 impairs host defense against gram-negative sepsis caused by *Burkholderia pseudomallei* in mice

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ABSTRACT

Background
CD14 is a pattern recognition receptor that can facilitate the presentation of bacterial components to either TLR2 or TLR4. We have recently shown that during melioidosis, a severe infection caused by the gram-negative bacterium *Burkholderia pseudomallei*, not TLR4 but TLR2 impacts on the immune-response of the intact host *in vivo*.

Methods
The function of CD14 in melioidosis was analyzed by means of both an *in vitro* and *in vivo* approach using wild-type (WT) and CD14 knock-out (KO) mice.

Results
CD14-deficient macrophages and whole blood leukocytes released less TNFα upon stimulation with *B. pseudomallei* or *B. pseudomallei* LPS *in vitro* compared to WT-cells. Strikingly, CD14 KO mice intranasally inoculated with *B. pseudomallei* demonstrated reduced lethality, accompanied by significantly decreased bacterial outgrowth compared to WT mice. Administration of recombinant soluble CD14 to CD14 KO mice partially reversed their phenotype into that of a WT-mouse. Lastly, CD14 deficiency did not alter the capacity of macrophages or neutrophils to phagocytose or kill *B. pseudomallei*.

Conclusion
CD14 is crucially involved in the recognition of *B. pseudomallei* by innate immune cells but plays a remarkable detrimental role in the host response against *B. pseudomallei*. Inhibition of CD14 may be a novel treatment strategy in melioidosis.
INTRODUCTION

Melioidosis, a recognized biological threat agent and caused by the soil-dwelling gram-negative bacillus *Burkholderia pseudomallei*, is an important cause of community-acquired sepsis in South-east Asia. Eradication of the organism following infection is difficult, with a slow fever-clearance time, the need for prolonged antibiotic therapy and a high rate of relapse if therapy is not completed. The mortality of primary disease varies between 20 to 50% [6,7]. Patients habitually present with pneumonia with bacterial dissemination to distant sites. We have previously developed a mouse model in which *B. pseudomallei* is administered via the airways, since this route may be more clinically relevant than the intraperitoneal route used in several previous studies.

CD14 and Toll-like receptors (TLRs) are key pattern-recognition-receptors of the innate immune system. These receptors are known to collaborate in order to detect host invasion by pathogens and form the crucial link between innate and adaptive immunity. The surface-anchored glycosyl-phosphatidylinositol CD14 acts as the ligand binding portion of the LPS-receptor complex, further consisting of TLR4 and the extracellular protein myeloid-differentiation-factor-2 (MD-2). Upon monocyte activation, membrane bound CD14 can be shed as a soluble form. Both membrane-bound and soluble (s)CD14 can interact with a variety of bacterial ligands from both gram-negative (e.g. LPS) and gram-positive bacteria (e.g. peptoglycan and lipoteichoic acid (LTA)).

Numerous studies have established the essential role of CD14 in systemic and pulmonary inflammation induced by LPS. In line, CD14 has found to play a key role in mounting an adequate innate immune response during gram-negative infection. Indeed, in *in vivo* models of infection inhibition or elimination of CD14 resulted in an increased outgrowth of different gram-negative bacterial species. We have previously documented a clear role for CD14 in improving the pulmonary clearance of clinically relevant gram-negative respiratory pathogens such as *Haemophilus influenzae* and *Acinetobacter baumannii*. In contrast, during murine pulmonary infection with the gram-positive pathogen *S. pneumoniae* it was shown that CD14 facilitates the dissemination of this pathogen from the respiratory tract, as reflected by the fact that CD14 KO mice - in contrast to wildtype controls - did not become bacteremic after intranasal infection.

We have recently characterized the expression and function of TLRs in septic melioidosis and found that although both TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei* in *vitro*, TLR2 detects the LPS of *B. pseudomallei* and only TLR2 impacts on the immune response of the intact host *in vivo*. In addition, increased CD14 expression was detected on circulating monocytes and granulocytes of patients with septic melioidosis. In the present study we aimed to characterize the function of CD14 in sepsis caused by *B. pseudomallei* using our established mouse model of melioidosis.
Mice
C57BL/6 wild-type (WT) mice were purchased from Harlan-Sprague Dawley Inc. (Horst, The Netherlands). CD14 knockout (KO) mice, backcrossed 6 times to C57BL/6 genetic background, were obtained from Jackson Laboratories (BarHarbor, ME). Age (10-12 weeks) and sex-matched animals were used in all experiments. The Animal Care and Use of Committee of the University of Amsterdam approved all experiments.

Preparation and stimulation of alveolar and peritoneal macrophages
Alveolar and peritoneal macrophages were harvested from WT and CD14 KO mice by bronchoalveolar and peritoneal lavage respectively (n=5–8/strain) as described 6, 25, 27. Adherent monolayer cells of alveolar and peritoneal macrophages and whole blood were stimulated with growth-arrested B.pseudomallei (clinical isolate strain 1026b 6, 28, 29; 1x10⁵ colony-forming-units (CFU)/ml), LPS from E.coli 055:B5 (100 ng/ml; Sigma-Aldrich, St. Louis, MO), LPS from B.pseudomallei (100 ng/ml) or RPMI-1640 for 16h. Due to a limited number of alveolar macrophages we chose to stimulate the alveolar macrophages only with B. pseudomallei. For in vitro experiments, we chose to use growth-arrested bacteria, in which only the replication mechanism of the bacteria is hampered, instead of heat-killed bacteria of which the whole bacterial structure is lost. For the preparation of growth-arrested B.pseudomallei, B.pseudomallei strain 1026b was cultured, thoroughly washed as described 4, 6 and dispersed in sterile PBS after the last centrifugation at a concentration of 1x10⁸ living bacteria/ml. Concentrated B.pseudomallei preparation was treated for 90 minutes at 38°C with 100 μg/ml mitomycine-C (Sigma-Aldrich, Zwijndrecht, the Netherlands) to prepare alive but growth-arrested bacteria. LPS from B.pseudomallei strain 1026b was prepared as described 6. Supernatants were collected and stored at −20°C until assayed for TNF-α.

Experimental infection and determination of bacterial outgrowth
Pneumonia was induced by intranasal inoculation with B.pseudomallei strain 1026b 6, 28, 29 (1 x 10² - 3 x 10³ CFUs/50 μl) as described 4-6. 1 x 10² represents the LD50. In some experiments, mice infected with B.pseudomallei received either saline of recombinant mouse sCD14 intranasally (1 μg; Biometec, Greifswald, Germany) intranasally at 0, 24 and 48 hrs relative to the time of infection 26. CFUs were determined as described 5, 26.

Assays
Lung homogenates were prepared as described 4-6. TNF-α, IL-6, IL-10, IL-12p70, interferon (IFN)-γ and monocyte-chemoattractant-protein (MCP)-1 were subsequently measured by cytometric-bead-array (CBA) multiplex-assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. Myeloperoxidase (MPO; HyCult-Biotechnology, Uden, the Netherlands) and sCD14 (Biometec, Greifswald, Germany) were measured with commercially available ELISA-kits. Alanine aminotransferase (ALAT), creatinine and BUN (urea) were determined with commercially available kits (Sigma-Aldrich), using a Hitachi analyzer (Roche).
CD14 in melioidosis

Pathology
Lungs, liver and spleen for histology were prepared and analyzed for lung inflammation as described 4-6. Granulocyte staining, using fluorescein isothiocyanate–labeled rat anti-mouse Ly-6G mAb (BDPharmingen, San Diego, CA), was done exactly as described previously 30.

Phagocytosis and bacterial killing assays
Phagocytosis was evaluated in essence as described before 27, 31. To determine the phagocytosis capacity of neutrophils or peritoneal macrophages, whole blood (50 μl) or peritoneal macrophages (resuspended in RPMI-1640 at a final concentration of 5x10^5 cells/ml) were incubated with carboxyfluorescein-diaceatate-succinimidyl-ester (CFSE-dye, Invitrogen, Breda, the Netherlands) labeled, growth-arrested bacteria (1x10^7 CFU/ml) and incubated for 10, 60 or 120 minutes at 37˚C. Cells were suspended in Quenching-solution and incubated in FACS lysis/fix-solution (BecktonDickinson, MountainView, CA). Neutrophils were labeled using anti-Gr-1-PE (BDPharmingen). Cells were washed with ice-cold FACS buffer after which the degree of phagocytosis was determined using FACSCalibur (BectonDickinson). Results are expressed as phagocytosis index, defined as the percentage of cells with internalized \textit{B. pseudomallei} times the mean fluorescence intensity 32, 33. Bacterial killing was determined as described 34. In brief, peritoneal macrophages were harvested from WT and CD14 KO mice and plated as described above (n=5/strain). \textit{B.pseudomallei} was added at a multiplicity-of-infection of 50 and spun onto cells at 2000rpm for 5min, after which plates were placed at 37˚C for 10min. Each well was then washed 5-times with ice-cold PBS to remove extracellular bacteria. To determine bacterial uptake after 10min., wells were lysed with sterile dH_2O and designated as t=0. RPMI was added to remaining wells and plates were placed at 37˚C for 10 or 60 minutes after which cells were again washed 5x with ice-cold PBS and lysed with dH_2O. Cell-lysates were plated on blood-agar-plates and bacterial counts were enumerated after 16h. Bacterial killing was expressed as the percentage of killed bacteria in relation to t=0.

Statistical analysis
Values are expressed as means ± SEM. Differences between groups were analyzed by Mann-Whitney U test or Kruskal-Wallis analysis with Dunn's posthoc-test where appropriate. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. All analysis with GraphPad Prism 4.00, GraphPad Software (SanDiego, CA). P<0.05 were considered statistically significant.

RESULTS

CD14 contributes to cellular responsiveness to \textit{B.pseudomallei in vitro}
To obtain a first insight into the function of CD14 in melioidosis, we tested the capacity of alveolar macrophages, peritoneal macrophages and whole blood harvested from WT and CD14 KO mice
to release TNFα upon stimulation with LPS derived from *B. pseudomallei* or intact growth-arrested *B. pseudomallei*. *E. coli* LPS was used as control stimulus expected to induce CD14-dependent TNFα release \(^{13,35}\). Macrophages and whole blood leukocytes obtained from CD14 KO mice released less TNFα than macrophages and blood leukocytes from WT mice upon stimulation with *B. pseudomallei* or *B. pseudomallei* LPS *in vitro*, although in blood stimulated with intact *B. pseudomallei* the difference with WT leukocytes did not reach statistical significance (Fig.1). Of note, *B. pseudomallei* was more potent than LPS in inducing TNFα release *in vitro*; this may have been related to the LPS dose used and/or the fact that *Burkholderia* harbors other proinflammatory components besides LPS. These data indicate that CD14 is involved in the recognition of *B. pseudomallei* and *B. pseudomallei* LPS by innate immune cells.

**CD14 KO mice are protected against *B. pseudomallei* induced lethality**

We next inoculated WT and CD14 KO mice with *B. pseudomallei* (either with 1x10^2 of 3x10^3 CFU in two independent experiments) and monitored them for 14 days (Fig.2). After infection with the lower dose, 50% of the WT mice had died after 6 days, while in sharp contrast all CD14 KO mice remained alive throughout the experiment (*P*<0.005 for the difference between both mouse strains; Fig.2A). A survival benefit for CD14 KO mice was also seen after infection with a high dose of *B. pseudomallei*. While in this experiment all WT mice had died after 4 days, mortality was delayed and reduced among CD14 mice, of which 10% survived until the end of the 6 weeks observation period (*P*<0.005 for the differences between both mouse-strains; Fig.2B).

**CD14 KO mice show a reduced growth of *B. pseudomallei* in vivo**

To gain insight into the mechanisms underlying the reduced mortality of CD14 KO mice during experimental melioidosis, we infected WT and CD14 KO mice with 5x10^2 CFU of *B. pseudomallei* and sacrificed the animals after 24, 48 or 72 hours to determine bacterial loads in lungs (the primary site of the infection), liver and blood (to evaluate the extent of bacterial dissemination)
CD14 in melioidosis

Figure 2. **CD14 KO mice show an enhanced survival during experimental melioidosis.** Survival of wild-type (WT, open rounds) and CD14 KO mice (black squares) intranasally infected with (A) 1 x 10^2 CFU or (B) 3 x 10^3 CFU *B. pseudomallei*. Mortality was assessed twice daily for 6 weeks. N = 10-12 per group. *P* value indicates the difference between CD14 KO and WT mice.

(Fig. 3). Relative to WT mice, CD14 KO mice displayed markedly reduced bacterial loads in their lungs and liver at 72 hours after infection. In addition, all WT mice had positive cultures from 48 hours onward, whereas *B. pseudomallei* could be cultured from blood at 72 hours from only three out of eight CD14 KO mice (Fig. 3). Hence, the presence of CD14 remarkably facilitates the growth of this pathogen in lungs and the subsequent spread to distant body sites. The same phenotype was seen in CD14 KO mice infected with a higher inoculum of 3 x 10^3 CFU of *B. pseudomallei*; in these latter experiments we also determined the bacterial loads in spleen homogenates, which – like liver – showed significantly fewer *B. pseudomallei* CFU in CD14 KO mice (data not shown).

Figure 3. **CD14 KO mice show a reduced growth of *B. pseudomallei* in vivo.** WT and CD14 KO mice were intranasally infected with *B. pseudomallei* (5 x 10^2 CFU). Bacterial loads were measured 24, 48 and 72h after inoculation in lungs (A) and liver (B). BC+ indicates the number of positive blood cultures. Data are mean ± SEM (n = 8 per group at each time point). *P* < 0.05.
Chapter 10

Reduced pulmonary influx of neutrophils in CD14 KO mice after infection with B. pseudomallei

Pneumonia results in inflammatory cell recruitment and local inflammation, which is an integral part of the host immune response. 72 hours after inoculation both WT and CD14 KO mice showed inflammatory infiltrates, characterized by interstitial inflammation together with endothelialitis, bronchitis, pleuritis and edema. No differences in total lung histopathological scores between CD14 KO and WT mice were seen at all timepoints. However, the lungs of CD14 KO mice contained fewer infiltrating neutrophils as visualized by Ly-6 staining 72h after inoculation. In accordance, measurements of MPO in lung homogenates showed reduced levels at 72h post infection in CD14 KO mice compared to controls.

Figure 4. Reduced pulmonary influx of granulocytes in CD14 KO mice after infection with B. pseudomallei. Both WT (A) and CD14 KO (B) mice infected with B. pseudomallei showed inflammatory infiltrates, characterized by interstitial inflammation together with endothelialitis, bronchitis, pleuritis and edema 72 hours after inoculation (hematoxylin and eosin stainings, magnification, x100). However, no differences in pathology scores calculated as described in the Methods section were seen between WT and CD14 KO mice (C). Representative pictures of immunostaining for granulocytes, showing reduced inflammation and granulocyte influx of WT (D) mice compared to CD14 KO (E) mice at 72 hours after inoculation with 5x10^3 CFU B. pseudomallei (magnification, x400). This corresponded with lower myeloperoxidase (MPO) levels in lung homogenates of CD14 KO mice (F). Data are means ± SEM (n = 8/strain). * P < 0.05.

Decreased systemic TNFα, and increased IFNγ cytokine levels in CD14 KO mice

Since cytokines are important regulators of the inflammatory response to bacterial pneumonia, we measured pulmonary and systemic TNFα, IL-6, IL-10, IL-12, IFNγ and MCP-1 levels at 24 hours after infection, all cytokine levels were low in both lungs and plasma, after which strong increases were found. In lungs, most differences between CD14 KO and WT mice did not reach statistical significance, although the concentrations of TNFα and IL-6 tended to be lower in the former mouse strain. Pulmonary IL-12 was low in both groups, but significantly higher in CD14 KO at 48 and 72 hours after infection. In plasma, CD14 KO mice showed decreased levels...
CD14 in melioidosis

Table 1. Cytokine concentrations in lung homogenates and plasma of WT and CD14 KO mice during melioidosis

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<th>T = 24 h</th>
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<td></td>
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<td></td>
<td>WT</td>
<td>CD14 KO</td>
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<td>pg/ml</td>
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<td>TNF-α</td>
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<td>353 ± 38</td>
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<tr>
<td>TNF-α</td>
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<tr>
<td>MCP-1</td>
<td>143 ± 19</td>
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Pulmonary and plasma cytokine levels after intranasal infection with 5 x 102 CFU B. pseudomallei. Wildtype (WT) and CD14 KO mice were sacrificed 24, 48 or 72 h after infection. Data are means ± SEM of eight mice per group per time point. IFN-γ = Interferon-γ; MCP-1 = monocyte chemoattractant protein-1. aP < 0.05; bP < 0.01; cP < 0.001.

of TNFα and IL-6, significantly so at 48 hours after infection. Plasma IFNγ and IL-12 plasma concentrations were slightly higher in CD14 KO mice at this time point (Table).

Effect of CD14 deficiency on distant organ injury

We next performed histopathological analyses of liver tissue and evaluated liver injury and kidney function by clinical chemistry at 72 hours after infection in both CD14 KO and WT mice. At this time point all mice showed evidence of hepatic injury, as characterized by inflammation of liver tissue, thrombi formation and foci of liver necrosis; the mean liver histological scores were similar in CD14 KO and WT mice (data not shown). However, the plasma levels of ALAT, indicative of hepatocellular injury, were decreased in CD14 KO mice compared to WT mice (132 ± 37 vs 264 ± 58 respectively, P<0.05). In addition, plasma creatinine and urea levels appeared to be lower in CD14 KO mice compared to WT mice, although these differences did not reach statistical significance due to a relatively large interindividual variation among WT mice (data not shown).

Role of sCD14 in host defense against B.pseudomallei

Having established the detrimental role of CD14 during experimental melioidosis, we next wondered whether soluble CD14 could compensate for CD14 gene deficiency during B.pseudomallei pneumonia. First, we measured sCD14 concentrations in BALF harvested from WT mice before and 24, 48 or 72 hours after infection. sCD14 was detectable in normal BALF and significantly
Figure 5. Effect of soluble CD14 on bacterial growth in CD14 KO mice. Concentrations of soluble CD14 in bronchoalveolar lavage fluid (BALF) (A) and plasma (B) of WT mice infected with 5x10^2 CFU *Burkholderia pseudomallei* show a significant increase during the course of pneumonia. Intranasal administration of recombinant mouse sCD14 (1 μg; 0, 24 and 48 h) to CD14 KO mice partially reverses their phenotype into that of WT mice: 72 hours after infection CD14 KO mice treated intranasally with recombinant CD14 (black bars) displayed a similar bacterial outgrowth in the bronchoalveolar lavage fluid (BALF) (C) and liver (E) as WT (white bars) mice. No change in the bacterial load in lungs of CD14 KO mice treated with recombinant sCD14 was seen (D). BC+ indicates the number of positive blood cultures. Data are means ± SEM (n = 5-8/strain). * P < 0.05; ** P < 0.01 versus t=0 or versus CD14 KO mice.
increased during the course of pneumonia (Fig.5A-B). We next administered CD14 KO mice intranasally with recombinant mouse sCD14, using a treatment and dosing schedule previously found to completely reverse the phenotype of CD14 KO mice in a model of *Streptococcus pneumoniae* pneumonia 26 (Fig.5C-E). Intranasal administration of recombinant mouse sCD14 to CD14 KO mice did not change the bacterial load in lungs of CD14 KO mice during experimental melioidosis (Fig.5D). However, in BALF sCD14 treatment of CD14 KO mice resulted in an enhanced bacterial growth that was indistinguishable from that in WT mice (Fig.5C). Furthermore, sCD14 treatment influenced the dissemination of *Burkholderia*: whereas all WT mice (8 out of 8) had a positive blood culture for *B. pseudomallei* and only 3 out of 8 CD14 KO mice, 6 out of 8 CD14 KO mice treated with sCD14 were bacteremic. In accordance, sCD14 administration resulted in higher bacterial loads in livers of CD14 KO mice, in essence reversing the phenotype of CD14 KO mice into that of WT mice (Fig.5E). In other words, sCD14 can in part replace cell-associated CD14 after infection with *B. pseudomallei* since sCD14 administration resulted in an enhanced bacterial growth in BALF of CD14 KO mice, which was accompanied by an increased dissemination to the circulation and liver. Together, these data suggest that sCD14 can in part compensate for cell bound CD14 in impairing host defense against *B. pseudomallei*.

**No role for CD14 in phagocytosis or killing of B. pseudomallei**

The experiments described above established that CD14 KO mice display a superior antibacterial defense towards *B. pseudomallei* infection. We next wished to determine whether CD14 contributes to phagocytosis and/or killing of *B. pseudomallei*. CD14 KO neutrophils (Fig.6A) and macrophages (not shown) demonstrated an unaltered capacity to phagocytose *B. pseudomallei*. In addition, no difference in the killing capacity between WT and CD14 KO cells was observed (Fig.6B).

![Figure 6. No difference in B. pseudomallei phagocytosis or killing capacity between WT and CD14 KO cells.](image-url)

**Figure 6. No difference in B. pseudomallei phagocytosis or killing capacity between WT and CD14 KO cells.** (A) Peripheral blood neutrophils were incubated at 37°C with CFSE-labeled growth-arrested *B. pseudomallei* (1x10<sup>7</sup> CFU/ml) after which time-dependent phagocytosis was quantified (see Method section). (B) Killing capacity of peritoneal macrophages are shown as percentage of killed *B. pseudomallei* compared to t = 0. Data are mean ± SEM; n= 5 per mouse strain. Open rounds represent WT cells, while black squares represent CD14 KO mice.
DISCUSSION

The primary objective of the present study was to examine the role of CD14 in the innate immune response during sepsis caused by *B. pseudomallei*. We here show that CD14 contributes to cellular responsiveness to *B. pseudomallei in vitro*. Strikingly, CD14 KO mice demonstrated a strongly reduced lethality after infection with *B. pseudomallei*, which was accompanied by significantly decreased bacterial loads, when compared to WT mice. Furthermore, the presence of sCD14 in the bronchoalveolar compartment of CD14 KO mice partially reversed the beneficial phenotype of these animals, as reflected by an increase in bacterial dissemination to the circulation and liver. These data indicate that CD14 plays a remarkable detrimental role in the host response against *B. pseudomallei*.

The essential role of CD14 in systemic and pulmonary inflammation induced by LPS is well established. Additionally, in *in vivo* models of infection with various gram-negative bacteria inhibition or elimination of CD14 resulted in increased bacterial outgrowth. However of interest, some studies have reported on the potential detrimental role of CD14 in the host defense against gram-negative bacteria: CD14 KO mice were reported to have an accelerated clearance of *E. coli* O111:B4 after intraperitoneal injection, although this could not be confirmed using *E. coli* O18:K1. Furthermore, in a model of chronic abscess-forming peritonitis induced by the gram-negative bacterium *Bacteroides fragilis*, reduced bacterial dissemination and liver injury was seen in CD14 KO mice compared to controls. These studies together with our current investigation challenge the paradigm that CD14 is required for mounting an effective innate immune response to gram-negative bacteria. One should be cautious however to extrapolate results obtained from studying one specific cause of sepsis towards the whole spectrum of sepsis. Indeed, *B. pseudomallei* is not a typical gram-negative bacteria as illustrated by the fact that the LPS of *B. pseudomallei* is recognized by TLR2 and not by TLR4. This only adds to the increasingly accepted opinion that sepsis cannot be seen as just one disease, and that in order to really understand its pathogenesis (and evaluate new therapies) one has to investigate every individual response elicited by every single pathogen. Of note, our experiments are derived solely from experiments performed with mice; therefore one should be careful when extrapolating the current results to humans.

The potential mechanism on how CD14 deficiency contributes to reduced bacterial outgrowth in experimental murine melioidosis remains to be elucidated. However, since the phenotype described here in CD14 KO mice strongly resembles the phenotype of TLR2 KO mice in melioidosis, we hypothesized that an interaction between CD14 and TLR2 could explain our observations. Indeed, both CD14 and TLR2 KO mice not only both display a reduced mortality and decreased bacterial growth *in vivo* during murine melioidosis, but CD14 and TLR2 are also both essential for the induction of TNFα release by macrophages stimulated with *B. pseudomallei* LPS. Since CD14 is known to be capable of facilitating the presentation of bacterial components to both TLR2 and TLR4, we therefore hypothesized that an interaction between CD14 and TLR2 could explain our observations. Preliminary experiments with confocal microscopy could...
however not support any physical association between CD14 and TLR2 in cells infected with *B. pseudomallei* *in vitro* (unpublished observations). We subsequently postulated that CD14, possibly in collaboration with TLR2, stimulates phagocytosis of the offending antigen, giving the facultative intracellular *B. pseudomallei* immediate access to a preferred environment. Would it be possible that *B. pseudomallei* deliberately uses CD14 as a cell entry receptor? Indeed, TLRs have been reported to be misused to facilitate intracellular invasion: MyD88 has been implicated in phagocytosis of *E. coli* and TLR2 deficiency possibly delays pneumococcal phagocytosis and impairs oxidative killing by granulocytes. Additionally CD14-dependent phagocytosis of gram-negative bacteria has been reported. However, our current data could not demonstrate a role for CD14 in either phagocytosis or killing of *B. pseudomallei*. In accordance, our laboratory earlier could not detect a role for CD14 in phagocytosis of *E. coli*. Of interest, recently substantive data has emerged arguing against a role of the TLRs in phagocytosis showing amongst others that TLR stimulation does not impact on phagosome maturation. Of note, at 48 h after infection CD14 KO mice displayed modestly increased pulmonary IL-12 and plasma IFN-γ concentrations. Although the differences with WT mice were only small, they could have contributed to the beneficial phenotype of CD14 KO mice considering that IFN-γ and its endogenous inducer IL-12, are known to play an essential role in the protective host defense against melioidosis. However, more studies are warranted to dissect the exact mechanism by which CD14 facilitates the growth and spread of *B. pseudomallei* *in vivo*.

We recently demonstrated that CD14 can facilitate dissemination of the gram-positive pathogen *S. pneumoniae* from the respiratory tract, as reflected by the fact that CD14 KO mice did not become bacteremic after intranasal infection. By using the same treatment schedule with sCD14 we here demonstrate that, like in pneumococcal pneumonia, sCD14 can in part replace cell-associated CD14 after infection with *B. pseudomallei*. sCD14 administration resulted in an enhanced bacterial growth in BALF of CD14 KO mice, which was accompanied by an increased dissemination to the circulation and liver. sCD14 treatment did not influence bacterial loads in whole lung homogenates of CD14 KO mice, suggesting that cell-associated rather than sCD14 is important for transition of *B. pseudomallei* from the bronchoalveolar space into lung tissue.

A recent phase 1 clinical trial with a recombinant anti-CD14 monoclonal antibody has shown that this therapy seems to be well tolerated in patients with severe sepsis and does not increase the incidence of secondary bacterial infection. We here show in mice that CD14 plays a crucial but detrimental role in the host response against gram-negative *B. pseudomallei*. This exceptional finding highlights the diverse nature of CD14 in the innate immune response against various pathogens. Inhibition of CD14 may be a novel treatment strategy in melioidosis.

**ACKNOWLEDGMENTS**

We are grateful to Marieke ten Brink and Joost Daalhuisen for expert technical assistance.
Chapter 10

REFERENCES

CD14 in melioidosis


Chapter 10


Immunosuppression associated with IRAK-M upregulation predicts mortality in gram-negative sepsis (melioidosis)


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ABSTRACT

Objective: Sepsis is associated with immunosuppression (characterized by a reduced capacity of circulating monocytes to release proinflammatory cytokines), which has been implicated in late mortality. Melioidosis, caused by the Gram-negative bacterium *Burkholderia pseudomallei*, is an important cause of community-acquired sepsis in SE-Asia with a mortality of up to 40%. Previous *in vitro* and murine studies have suggested a key role for so-called negative regulators of the Toll-like receptor (TLR) signaling pathway in immunosuppression. In this study, we investigated the expression of these negative TLR regulators in patients with septic melioidosis in association with the responsiveness of peripheral blood leukocytes of these patients to lipopolysaccharide (LPS) and *B. pseudomallei*.

Design: *Ex vivo* study.

Setting: Academic research laboratory

Patients: 32 healthy controls and 34 patients with sepsis caused by *B. pseudomallei*.

Intervention: None

Measurements: (1) Plasma cytokine levels, (2) *ex vivo* cytokine production capacity of whole blood and (3) purified mononuclear cell derived mRNA levels of key inhibitory molecules of the TLR-signaling cascade were investigated.

Main results: In accordance with an immunosuppressed state, whole blood of patients demonstrated a strongly decreased capacity to release the proinflammatory cytokines tumor necrosis factor-α, interleukin-1β and the chemokine interleukin-8 after *ex vivo* stimulation with LPS or *B. pseudomallei*. Analysis of MyD88-short, IRAK-M, IRAK-1, SOCS-3, SHIP-1, SIGIRR and A20 mRNA expression in purified mononuclear cells showed decreased IRAK-1 and elevated IRAK-M expression in patients with septic melioidosis. Immunosuppression was correlated with mortality; furthermore patients who went on to die had higher IRAK-M mRNA levels on admission than patients who survived.

Conclusions: Immunosuppression in sepsis caused by *B. pseudomallei* is associated with an up-regulation IRAK-M and an indicator of poor outcome.
INTRODUCTION

Sepsis is associated with a state of immunosuppression, which has been implicated as an important underlying cause of late sepsis mortality \(^1,^2\). Immunosuppression is characterized by a reduced capacity of circulating immune cells, most notably mononuclear cells, to release pro-inflammatory cytokines. Endotoxin tolerance is defined as a reduced responsiveness to a lipopolysaccharide (LPS) challenge following a first encounter with endotoxin \(^2\). Immunosuppression can be beneficial by preventing damage from an overwhelming inflammatory response. On the other hand, the observation of an altered immune status in patients may favor an increased risk of subsequent nosocomial infections \(^2^\text{-}^4\). There is however no clear demonstration that this phenomenon is directly responsible for poor outcome in patients. Of interest, in mice it has been shown that LPS-pretreatment leading to endotoxin tolerance protects against experimental infections \(^5^,^6\). Therefore, it seems that immunosuppression observed in septic patients may be different from endotoxin tolerance.

The molecular mechanisms underlying sepsis associated immunosuppression remains to be elucidated \(^1,^7,^8\). Recently, attention has been directed to a possible role for the negative regulators of Toll-like receptor (TLR) signaling \(^1,^9\). TLRs detect host invasion by pathogens, initiate immune responses and form the crucial link between the innate and adaptive immune systems \(^10,^11\). As the immune system needs to constantly strike a balance between activation and inhibition to avoid harmful and inappropriate inflammatory responses, TLR signaling must be tightly regulated \(^9\). Recent in vitro and murine studies have shown that immunosuppression is accompanied by the upregulation of inhibitory molecules that down-regulate TLR-signaling, suggesting a key role for these negative TLR regulators in immunosuppression. These include the transmembrane protein single-immunoglobulin-interleukin-1R-related-molecule (SIGIRR) and the intracellular molecules myeloid-differentiation-88-short (MyD88s), IL-1R-associated-kinase-M (IRAK-M), suppressor-of-cytokine signalling (SOCS), A20 and Src-homology-2-domain-containing inositol-5-phosphatase-1 (SHIP-1) \(^9\). Knowledge about the expression of these negative regulators of TLR signaling in sepsis in vivo is highly limited.

Melioidosis, caused by the Gram-negative bacillus *Burkholderia pseudomallei*, is an important cause of severe sepsis in Southeast-Asia \(^12,^13\). We have recently characterized the expression and function of the TLRs in septic melioidosis and found an up-regulation of multiple TLRs in peripheral blood mononuclear cells \(^14\). In the current study we aimed to investigate the expression of the negative TLR regulators in patients with septic melioidosis in association with the responsiveness of peripheral blood leukocytes of these patients to proinflammatory challenges.
Chapter 11

MATERIALS AND METHODS

Study population
34 patients with culture positive sepsis caused by *B. pseudomallei* (mean age 52 years; range, 18-86 years; 50% male) and 32 healthy control subjects (mean age 41 years; range, 21-59 years; 71% male) were prospectively recruited at Sapprasithiprasong Hospital, Ubon Ratchathani, northeast Thailand in 2004. *B. pseudomallei* was cultured in blood (n=21), throat swab or tracheal suction (n=13), sputum (n=7), pus from abscess (n=8) or urine (n=5). The overall patient mortality was 44%. Study design and subjects have been described in detail 14. Sepsis due to melioidosis was defined as culture positivity for *B. pseudomallei* from any clinical sample plus a systemic inflammatory response syndrome (SIRS) 15, 16. Blood was drawn within 36 hours of the start of appropriate antimicrobial therapy. The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, England and written informed consent was obtained from all study subjects.

Whole blood stimulation
Whole blood, anticoagulated with sodium heparin and diluted 1:1 (vol: vol) in pyrogen-free RPMI 1640 (Bio-Whittaker, Verviers, Belgium), was stimulated for 4 hours at 37ºC with LPS (final concentration 10 ng/ml; from *E. coli* serotype 055:B5; Sigma, St. Louis, MO), avirulent heat-killed *B. thailandensis* E264 (5x10^7 CFU/ml 17) or virulent *B. pseudomallei* strain 1026b (5x10^7 CFU/ml 18). In preliminary experiments we found that TNFα levels are at their maximum 4 hours after in vitro stimulation with LPS, while levels of IL-1β, IL-6 and IL-10 are already substantially elevated. Bacteria culture 19 and subsequent stimulation were done as described 20. After incubation, supernatant was obtained and stored at -20ºC until assayed for cytokine release. The amount of immunosuppression was determined by the capacity of whole blood sampled from patients and controls to release cytokines upon stimulation. The spontaneously released amounts of cytokines (e.g. pre-existing plasma cytokine + spontaneously released cytokines) were subtracted from the amount analyzed after stimulation (control stimulated – control spontaneous and patient stimulated – patient spontaneous).

Evaluation of mRNA levels by quantitative RT-PCR
Leukocytes were isolated from heparinized blood using lyysis buffer. Monocyte enriched populations were isolated using Polymorphprep (Axis-Shield, Dundee, UK). Purified mononuclear cells fractions were >98% pure as determined by their scatter pattern on flow cytometry. The lymphocyte:monocyte ratio did not differ between patients and controls in our population (data not shown). After isolation, mononuclear cells were dissolved in Trizol and stored at −80 ºC until used for RNA isolation. RNA was isolated as described 21. RT-PCR reactions were performed on cDNA samples that were 4-fold diluted in H₂O using FastStart DNA Master SYBR GreenI (Roche, Indianapolis, IN) with 2.5 mM MgCl₂ in a LightCycler (Roche, Indianapolis, IN) apparatus. PCR conditions were: 5 min 95ºC hot-start, followed by 40 cycles of amplification (95ºC for 15s, 60ºC for 5s, 72ºC for 20s). Quantification standard curves were constructed by PCR using serial dilutions.
of concentrated cDNA; data were analyzed using the LightCycler software. Gene expression is presented as a ratio of the housekeeping gene β2-microglobulin (B2M) \(^{21,22}\). No upregulation of B2M was seen in patients compared to controls (data not shown). Table 1 lists primers used for RT-PCR. Oligonucleotides were obtained from Eurogentec, Seraing, Belgium.

### Assays

Human tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-10, IL-8 and IL-12p70 were measured by cytometric-bead-array (CBA) multiplex-assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. The lower detection limit was 10 pg/ml for all cytokines analyzed.

### Statistical analysis

Values are expressed as means±SEM unless indicated otherwise. Differences between groups were analyzed by Mann-Whitney U test. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). A \( P < 0.05 \) was considered to represent a statistically significant difference.
RESULTS

Proinflammatory cytokine profile in patients
Consistent with the literature on sepsis caused by other pathogens 1, 23, 24, sepsis caused by *B. pseudomallei* was associated with elevated plasma levels of the cytokines IL-6, IL-8 and IL-10 (Table 2). IL-1β and IL-12p70 tended to be elevated in patients but the difference with controls was not significant.

Table 2. Patient cytokine profile

<table>
<thead>
<tr>
<th>Variables (pg/ml)</th>
<th>Healthy controls (n=32)</th>
<th>Patients (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>IL-6</td>
<td>37 ± 13</td>
<td>4501 ± 2964 *</td>
</tr>
<tr>
<td>IL-10</td>
<td>BD</td>
<td>67 ± 25 *</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>16 ± 6</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>IL-1β</td>
<td>78 ± 16</td>
<td>265 ± 142</td>
</tr>
<tr>
<td>IL-8</td>
<td>169± 17</td>
<td>2090 ± 787 *</td>
</tr>
</tbody>
</table>

TNF-α, tumor necrosis factor-α; IL, Interleukin; BD: below detection limit; Cytokine values given as mean ± SEM. P values are according to the Mann-Whitney test, *P < 0.05 vs healthy controls.

Figure 1. Sepsis caused by *Burkholderia pseudomallei* (melioidosis) is characterized by immunosuppression. Whole blood obtained from patients (n=34, gray bars) and healthy controls (n=32, white bars) was stimulated with lipopolysaccharide (LPS), heat-killed *B. thailandensis* E264 or heat-killed *B. pseudomallei* strain 1026b for 4 hours before measurement of TNF-α (A), IL-1β (B), IL-6 (C), IL-8 (D) and IL-10 (E) in the supernatant (see Materials and Methods for details). Data are means ± SEM; **P < 0.01, ***P < 0.001 versus healthy controls; ns denotes not significant.
Immunosuppression in Gram-negative sepsis

did not reach statistical significance, in part due to a large inter-individual variation. TNF-α was undetectable in both patients and controls.

**Sepsis caused by B. pseudomallei is characterized by immunosuppression, with decreased pro-inflammatory cytokine levels upon stimulation of whole blood**

To determine the amount of immunosuppression during severe melioidosis, we tested the capacity of whole blood sampled from patients and controls to release pro- and anti-inflammatory cytokines upon stimulation with LPS or *Burkholderia* species. Whole blood obtained from patients with sepsis caused by *B. pseudomallei* released less TNF-α, IL-1β and IL-8 than blood from controls upon *ex vivo* stimulation with either LPS, *B. thailandensis* or *B. pseudomallei* (Fig. 1). In contrast, a trend towards an enhanced production of IL-10 was observed in patients compared to controls after *ex vivo* LPS and bacterial stimulation although these differences did not reach statistical significance (Fig. 1).

**No difference in SOCS-3, A20 and MyD88s mRNA expression between septic patients and controls**

Having established that sepsis caused by *B. pseudomallei* is characterized by immunosuppression, we sought to obtain insights into the expression profiles of molecules known to be important negative regulators of the TLR-signaling cascade in purified peripheral mononuclear cells. We first determined the mRNA levels of SOCS-3, A20 and MyD88s in patients and controls. SOCS-3 is an inhibitor of the IL-6 receptor. The feedback inhibition by SOCS-3 of IL-6 signaling plays an important role in the differential outcome of IL-10 versus IL-6 signaling. Furthermore, it is known that SOCS-3 is expressed in monocytes from septic mice. However, in our patient population we did not observe a significant upregulation of SOCS-3 mRNA expression in peripheral blood mononuclear cells (Fig. 2A). A20, a classic negative feedback inhibitor induced directly by NF-κB, inhibits the generation of active NF-κB by blocking at the TRAF/IKK level. In a murine study it was shown that A20 is required for termination of TLR responses. In patients with septic melioidosis A20 mRNA expression levels were not significantly altered from controls (Fig. 2B).

**Figure 2. Expression of SOCS-3, A20 and MyD88s in septic patients.** In patients with septic melioidosis no differences were observed in mRNA levels of SOCS-3 (A), A20 (B) and MyD88s (C). mRNA levels were measured in purified peripheral mononuclear cells. After quantitative RT-PCR the copy numbers for all genes were normalized against β2-microglobulin, which was also amplified by RT-PCR. Mean ± SEM are shown for patients with septic melioidosis (n=34, gray bars) and healthy controls (n=32, white bars); ns denotes not significant.
Chapter 11

MyD88 plays a key role in TLR-signaling as all TLRs (except TLR3) signal through this adaptor protein. MyD88s is an inhibitory splice variant of MyD88 and has been postulated to be involved in LPS tolerance. We did however not see a difference between MyD88s mRNA expression in septic patients and controls (Fig. 2C).

**Decreased SHIP-1 and SIGIRR mRNA expression in septic melioidosis**

Since the SHIP-1, a PI3-kinase inhibitor, is known to be involved in the induction of endotoxin tolerance in mice, we determined SHIP expression and found that SHIP-1 mRNA expression was significantly lower in patients compared to controls (Fig. 3A). Mononuclear cell mRNA levels of SIGIRR, which has been shown to be a negative modulator of LPS-induced signaling, was also significantly reduced in our cohort of patients with septic melioidosis compared to controls (Fig. 3B).

![Figure 3. Expression of SHIP-1 and SIGIRR mRNA in septic patients.](image)

**Figure 3. Expression of SHIP-1 and SIGIRR mRNA in septic patients.** In purified mononuclear cells from patients with septic melioidosis strongly decreased mRNA levels were seen of both SHIP-1 (A) and SIGIRR (B). mRNA levels were measured in purified peripheral mononuclear cells. After quantitative RT-PCR the copy numbers for all genes were normalized against β2-microglobulin, which was also amplified by RT-PCR. Mean ± SEM are shown for patients with septic melioidosis (n=34, gray bars) and healthy controls (n=32, white bars); **P < 0.01, ***P < 0.001 versus healthy.

![Figure 4. Increased IRAK-M and decreased IRAK-1 mRNA expression in septic patients.](image)

**Figure 4. Increased IRAK-M and decreased IRAK-1 mRNA expression in septic patients.** Strongly increased levels of IRAK-M mRNA (A) were observed in the mononuclear cells from septic patients (n=34; gray bars) compared to controls (n=32; white bars), while IRAK-1 mRNA expression (B) was significantly decreased in patients. After quantitative RT-PCR the copy numbers for all genes were normalized against β2-microglobulin, which was also amplified by RT-PCR. Mean ± SEM; *P < 0.05, ***P < 0.001 versus healthy controls.
Increased IRAK-M and decreased IRAK-1 mRNA expression in septic melioidosis

Another factor that has been implicated in immunosuppression is IRAK-M, an inhibitor of IRAK-1/IRAK-4 signaling. Importantly, IRAK-M is produced exclusively by monocytes/macrophages. We found that IRAK-M mRNA was strongly induced in blood mononuclear cells of septic patients, while IRAK-1 transcription was downregulated (Fig. 4). Thus in whole blood mononuclear cells an upregulation of IRAK-M is accompanied by a drop in IRAK-1 levels, indicating that immunosuppression is potentially ruled by a change in the IRAK-1/IRAK-M-ratio.

Table 3. Association between mortality and levels of (A) ex vivo induced cytokines and (B) regulators of the TLR cascade in patients with sepsis caused by *B. pseudomallei*

<table>
<thead>
<tr>
<th>(A) Ex vivo induced cytokine production</th>
<th>Unit</th>
<th>Survivors</th>
<th>Non-survivors</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS-induced TNFα pg/ml</td>
<td>1975 ± 381</td>
<td>473 ± 158</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td><em>B. thailandensis</em> induced TNFα pg/ml</td>
<td>7352 ± 728</td>
<td>3462 ± 1008</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td><em>B. pseudomallei</em> induced TNFα pg/ml</td>
<td>8696 ± 434</td>
<td>3976 ± 925</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>LPS-induced IL-10 pg/ml</td>
<td>21 ± 10</td>
<td>50 ± 18</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td><em>B. thailandensis</em> induced IL-10 pg/ml</td>
<td>48 ± 13</td>
<td>73 ± 24</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td><em>B. pseudomallei</em> induced IL-10 pg/ml</td>
<td>45 ± 12</td>
<td>74 ± 20</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>LPS-induced IL-1β pg/ml</td>
<td>970 ± 484</td>
<td>14 ± 13</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td><em>B. thailandensis</em> induced IL-1β pg/ml</td>
<td>4632 ± 950</td>
<td>831 ± 467</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td><em>B. pseudomallei</em> induced IL-1β pg/ml</td>
<td>5043 ± 969</td>
<td>904 ± 644</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>LPS-induced IL-6 pg/ml</td>
<td>4743 ± 1095</td>
<td>1025 ± 491</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td><em>B. thailandensis</em> induced IL-6 pg/ml</td>
<td>7425 ± 1326</td>
<td>1214 ± 592</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td><em>B. pseudomallei</em> induced IL-6 pg/ml</td>
<td>7386 ± 1366</td>
<td>2311 ± 1320</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>LPS-induced IL-8 pg/ml</td>
<td>1256 ± 530</td>
<td>102 ± 102</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td><em>B. thailandensis</em> induced IL-8 pg/ml</td>
<td>4537 ± 631</td>
<td>2995 ± 548</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td><em>B. pseudomallei</em> induced IL-8 pg/ml</td>
<td>3641 ± 581</td>
<td>2862 ± 548</td>
<td>Ns</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Regulators of the TLR cascade</th>
<th>Unit</th>
<th>Survivors</th>
<th>Non-survivors</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS-3</td>
<td>0.23 ± 0.08</td>
<td>0.20 ± 0.06</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>A20</td>
<td>2.33 ± 0.63</td>
<td>2.87 ± 1.33</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>MyD88s</td>
<td>0.77 ± 0.14</td>
<td>0.82 ± 0.12</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>SHIP-1</td>
<td>0.39 ± 0.10</td>
<td>0.54 ± 0.10</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>SIGIRR</td>
<td>0.80 ± 0.17</td>
<td>0.72 ± 0.13</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>IRAK-M</td>
<td>0.51 ± 0.09</td>
<td>0.80 ± 0.12</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>IRAK-1</td>
<td>0.64 ± 0.22</td>
<td>0.62 ± 0.25</td>
<td>Ns</td>
<td></td>
</tr>
</tbody>
</table>

Correlation between mortality and levels of (A) ex vivo induced cytokines and (B) regulators of the TLR cascade in survivors (n=20) and non-survivors (n=14) of sepsis caused by *B. pseudomallei*. Ex vivo induced cytokine production was analyzed by stimulating whole blood obtained from septic patients with lipopolysaccharide (LPS), heat-killed *B. thailandensis* or heat-killed *B. pseudomallei* after which cytokine production was measured. IRAK-M, IRAK-1, SOCS-3, MyD88s, SHIP-1, SIGIRR and A20 values represent mRNA levels of purified peripheral mononuclear cells (see Methods section for details). Values given as mean ± SEM. P values are according to the Mann-Whitney test. Ns denotes not significant.
Correlation with clinical outcome

Having characterized the expression of a spectrum of important negative regulators of the TLRs in septic melioidosis patients with immunosuppression, we next sought to examine differences in these profiles in terms of clinical outcome. Therefore we compared the levels of both the ex vivo induced cytokines and the regulators of the TLR cascade between survivors (n=20) and non-survivors. (n=14) (Table 3) We found that immunosuppression was strongly associated with poor outcome. Ex vivo LPS, B. thailandensis or B. pseudomallei-induced TNF-α secretion were all significantly lower in non-survivors than in survivors (P<0.05-0.001, table 3). Equally strong associations where seen for IL-1β and IL-6: whole blood from survivors stimulated with either LPS, B. thailandensis or B. pseudomallei produced significantly more of these cytokines than whole blood derived from non-survivors (P<0.05-0.001, table 3). Of note, no association with outcome was observed for the ex vivo stimulus-induced capacity of whole blood to secrete the anti-inflammatory cytokine IL-10. Of all the measured modulators of the TLR-cascade, only IRAK-M mRNA levels at admission were associated with outcome: patients who went on to die had significantly higher IRAK-M mRNA concentrations than those who survived (P<0.05, table 3). Further evidence for an association between IRAK-M mRNA levels and disease severity was obtained in patients who survived and from whom a second blood sample could be obtained after successful therapy. Although we were able to collect a follow-up sample in only 7 patients, a trend towards lower mononuclear cell IRAK-M mRNA levels was seen in these patients (Fig. 5, P=0.05) Levels of SOCS-3, A20, MyD88s, SHIP-1, SIGIRR and IRAK-1 did not differ between survivors and non-survivors (table 3).

DISCUSSION

Although it has been known for over one and a half decades that sepsis is associated with immunosuppression, the mechanism underlying this phenomenon has been a puzzle for as many years. Recent data derived from both in vitro and mouse studies have identified the negative regulators of the TLR cascade as potential players in the initiation and regulation of
Immunosuppression in Gram-negative sepsis

Immunosuppression \(^1\)\(^,\)\(^8\). In the present study we aimed to investigate both the responsiveness of peripheral blood leukocytes to relevant bacterial stimuli together with the expression patterns of negative TLR regulators in mononuclear cells derived from patients with septic melioidosis. In accordance with an immunosuppressed state, whole blood of patients demonstrated a strongly decreased capacity to release proinflammatory cytokines after \textit{ex vivo} stimulation with LPS or \textit{Burkholderia} species. These alterations coincided with decreased IRAK-1 and elevated IRAK-M mRNA expression in purified mononuclear cells from septic patients. The clinical importance of these findings is underscored by the fact that immunosuppression and IRAK-M mRNA levels correlated with mortality. These results may shed light on one of the possible underlying mechanisms responsible for the high mortality observed in severe melioidosis (in our cohort 44%).

Immunosuppression in sepsis is characterized by a reduced capacity of circulating monocytes to release proinflammatory cytokines \(^2\)\(^,\)\(^34\)\(^-\)\(^38\). Of note, we used the term “immunosuppression” throughout the manuscript to indicate the fact that blood leukocytes were less responsive to \textit{ex vivo} stimulation with \textit{Burkholderia} and LPS. It should be noted, however, that the nomenclature on this topic is rather confusing, and the term “endotoxin tolerance” could have been used here as well. We now corroborate the findings on immunosuppression in sepsis for melioidosis and confirm that the phenomenon of immunosuppression is characterized not only by a diminished capacity of whole blood to produce TNF-\(\alpha\), but also by decreased IL-1\(\beta\) and IL-8 production. More over, one could argue that whole blood leukocytes were completely endotoxin tolerant because there was really no further increase in cytokine levels over those measured in plasma alone when whole blood was stimulated \textit{ex vivo} with LPS or \textit{Burkholderia}. The capacity of immune cells to produce IL-10 in sepsis has been the subject of conflicting reports. Our data are in line with most studies by demonstrating an enhanced or unchanged IL-10 production of circulating leukocytes in response to LPS \(^2\)\(^,\)\(^8\)\(^,\)\(^39\)\(^,\)\(^40\). Absolute IL-10 levels in patients however were not much increased compared to plasma levels. Hence, our data suggest that whole blood leukocytes from patients with melioidosis can still sense \textit{Burkholderia}, but that the intracellular signalling has been modified to limit the production of pro-inflammatory cytokines and to maintain or favor that of the anti-inflammatory \(^2\).

Interestingly, stimulation with non-pathogenic \textit{B. thailandensis} produced the exact same degree of immunosuppression as seen with the virulent \textit{B. pseudomallei} isolate. This might indicate that the observed immunosuppressive effect is a result from TLR stimulation and may be less related to pathologic sepsis. One should keep in mind however that, since we have not performed direct cytokine stimulation or stimulation with other specific TLR ligands next to LPS, we can not state whether the observed phenomenon is TLR specific or more a general functional deficit. In addition, in our \textit{ex vivo} stimulation assays of whole blood with LPS or \textit{Burkholderia}, different immune cells, such as T-cells, B-cells, natural killer (NK) cells as well as monocytes, will all contribute to the release of cytokines. Since we subsequently studied the expression of the negative regulators of the TLRs only in the mononuclear cells one cannot directly correlate these levels with whole blood cytokine levels. We however primarily wondered how these key inhibitory molecules of the TLR-signaling cascade were expressed in the mononuclear cells of patients with septic melioidosis.
Chapter 11

The TLR ligands LPS and CpG are potent inducers of SOCS1 and SOCS3; not surprisingly therefore the role of the SOCS proteins in the proinflammatory TLR responses have been extensively investigated \(^{41, 42}\). Of interest however, the role of SOCS-3 in the anti-inflammatory action of IL-10 has been refuted and SOCS-3 does not seem to be involved in the downregulation of LPS signaling \(^{43}\). This seems to be in line with our data since we did not observe a significant change in SOCS-3 mRNA expression levels in patients relative to controls, suggesting that SOCS-3 is not directly involved in immune dysregulation. One should keep in mind however, that T-cell SOCS-3, a known regulator of T-cell cytokine levels \(^{41}\), has not been analyzed in our study and might as well have been contributing to the observed immunosuppression. We also found unchanged MyD88s mRNA mononuclear cell expression septic melioidosis patients when compared with controls. This is in contrast to an earlier study reporting increased MyD88s levels in 16 sepsis patients suffering from different underlying infections \(^8\). Several factors may have played a role in the discrepant MyD88s mRNA results in the latter investigation \(^8\) and our present study, including differences in patient populations (our population was twice as large and involved severe infection by a single pathogen), severity of disease (44% mortality in our study, mortality not listed in the previous study), differences in used primer sequences and employed housekeeping genes and the method used to purify mononuclear cells.

SHIP is increased in macrophages upon LPS stimulation and involved in the induction of endotoxin tolerance in mice \(^{30}\). In accordance with studies in LPS-injected volunteers who displayed a downregulation of SHIP-1 mRNA levels in peripheral blood leukocytes \(^{44, 45}\), we observed decreased SHIP-1 mRNA levels in septic patients compared to controls. A limitation of our study however is that we did not analyze the number of T and B cells in patients and controls; this could have influenced our data since SHIP-1, but also SOCS-3 are known to be highly expressed in immunosuppressed T cells. SIGIRR is thought to inhibit LPS signaling by attenuating the recruitment of MyD88 and IRAK-1 to TLR4 through TIR-TIR domain interactions between SIGIRR and TLR4. In mice it has been shown that SIGIRR functions as an inhibitor of IL-1 and TLR signaling \(^{31}\). In a cohort of 16 septic patients SIGIRR mRNA levels were reported to be upregulated in isolated monocytes \(^8\). We can not confirm this finding in our cohort of patients with sepsis caused by \textit{B. pseudomallei}. Factors discussed above for differential results for MyD88s mRNA levels in this earlier study \(^8\) and our investigation may have again contributed to the discrepant data on SIGIRR mRNA. In addition, it should be noted that several reports have suggested that SIGIRR is downregulated after induction of inflammation. For instance, in mice SIGIRR expression was shown to be reduced at 6 and 12 hrs after injection of LPS in many tissues, including lung and kidneys, after which the expression returned to baseline by 24 hrs, at which time the mice recovered from the LPS challenge \(^{31}\). Furthermore, children with asymptomatic bacteriuria displayed lower SIGIRR expression on their neutrophils than children without bacteriuria \(^{46}\). Taken together these data suggest that neither MyD88s nor SIGIRR play a significant role in the immunosuppression accompanying melioidosis.

IRAK-M is an inhibitor of IRAK-1/IRAK-4 signaling that is specifically expressed by monocytes/macrophages. Recently, in line with our current data our laboratory recently reported that immunosuppression in healthy humans following intravenous injection of LPS was associated...
with increased whole blood IRAK-M mRNA and decreased IRAK-1 mRNA levels. In addition, in cultured monocytes derived from septic patients IRAK-M was expressed more rapidly upon exposure to LPS when compared to controls. In contrast to our data however, significant upregulation of IRAK-M could not be detected in directly harvested uncultured monocytes, possibly related to the small sample size, the use of a different primer set, and/or different causative organisms. Interestingly, immunosuppression is significantly reduced in IRAK-M deficient mice. Deng et al. recently showed that IRAK-M mediates the sepsis-induced suppression of innate immunity in the pulmonary compartment. Together these data suggest that IRAK-M is a pivotal mediator of immunosuppression accompanying sepsis.

The association between immunosuppression and mortality in patients with melioidosis described here is in keeping with published findings in sepsis patients. Septic patients who eventually died were found to have a sustained decreased production of cytokines by monocytes upon LPS stimulation. Of interest, ex vivo LPS-induced TNFα production was shown to be a better indicator of clinical outcome in patients with sepsis than monocyte HLA-DR expression and constitutive IL-6 secretion. Furthermore, immunosuppression has been implicated in late mortality of surgical ICU patients. We now confirm and extend these findings by showing that this holds true for the capacity of whole blood not only to secrete TNF-α upon stimulation with LPS or Burkholderia species but also to secrete IL-1β and IL-6. To our knowledge we now show for the first time that on admission, patients who went on to die had higher levels of a negative regulator of the TLR cascade, namely IRAK-M, than patients who survived. IRAK-1 levels however did not differ between survivors and non-survivors, suggesting the existence of other additional effector mechanisms not reflected in the measured mononuclear cells mRNA. For instance, LPS activated mononuclear cells are able to suppress T- and B-cell and NK-cell responses by both TGFβ and PGE2 related mechanisms. Further research is needed to extract these underlying mechanisms.

CONCLUSIONS

In conclusion, we found profound immunosuppression in sepsis caused by B. pseudomallei associated with an up-regulation of mononuclear cell IRAK-M mRNA levels. Immunosuppression and high levels of IRAK-M are indicators of poor outcome. In light of the unacceptably high mortality among patients with septic melioidosis it is worthwhile to consider strategies based on inhibiting IRAK-M.
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REFERENCES

Chapter 11


Immunosuppression in Gram-negative sepsis

Expression profile and function of triggering receptor expressed on myeloid cells (TREM)-1 in melioidosis


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ABSTRACT

Background
Triggering-receptor-expressed-on-myeloid-cells-(TREM)-1 amplifies Toll-like-receptor-initiated responses against pathogens. We aimed to characterize TREM-1-expression and function in sepsis caused by Burkholderia pseudomallei (melioidosis).

Methods
TREM-1-expression was determined in leukocytes and plasma from 34 melioidosis patients and 32 controls, and in mice with experimentally-induced melioidosis. Responsiveness toward B. pseudomallei of TREM-1-positive and TREM-1-negative leukocytes was tested in vitro. TREM-1 function was inhibited in mice using a synthetic peptide mimicking the ectodomain of this receptor.

Results
Patients demonstrated increased soluble-TREM-1 plasma levels and TREM-1 surface-expression on monocytes, but not granulocytes. Similarly, mice inoculated with B. pseudomallei displayed a gradual rise in soluble-TREM-1 and increase in blood monocyte but not granulocyte TREM-1 expression. At the primary infection-site, however, granulocyte TREM-1 expression was enhanced and the rise in soluble-TREM-1 occurred earlier. Additionally, purified human TREM-1 granulocytes showed reduced responsiveness to B. pseudomallei relative to TREM-1+ granulocytes; a difference not detected for TREM-1- and TREM-1+ monocytes. Treatment with a peptide mimicking a conserved-domain of soluble-TREM-1 partially protected mice from B. pseudomallei induced lethality.

Conclusions
During melioidosis TREM-1 expression is differentially regulated on granulocytes and monocytes; measurements of TREM-1-expression on blood granulocytes may not provide adequate information on granulocyte TREM-1-expression at the infection site. TREM-1 may be a therapeutic target in melioidosis.
INTRODUCTION

Triggering-receptor-expressed-on-myeloid-cells (TREM)-1 is a new mediator in the inflammatory arena that amplifies Toll-like receptor (TLR)-initiated responses against microbial challenges by potentiating the secretion of proinflammatory cytokines \(^1\). TREM-1, which signals through the adaptor protein DAP12, is strongly and specifically expressed on monocytes and neutrophils from patients with sepsis \(^1,2\). Elevated concentrations of soluble-TREM-1 in bronchoalveolar-lavage (BAL) fluid can indicate ventilator-associated-pneumonia in patients receiving mechanical ventilation \(^3\), and high concentrations in plasma can indicate infection in patients with systemic inflammatory response syndrome \(^4\). Excitingly, blockade of TREM-1 protects mice against LPS-induced shock, as well as sepsis caused by *Escherichia coli* or cecal ligation and puncture (CLP) \(^1\).

In Southeast-Asia and northern-Australia the Gram-negative bacillus *Burkholderia (B.) pseudomallei* is an important cause of community-acquired sepsis \(^5,6\). More than half of these cases of melioidosis, as this severe infection is named, habitually present with pneumonia, frequently associated with bacterial dissemination to distant sites \(^6,7\). In the present study we aimed to characterize the expression and function of TREM-1 in melioidosis. For this we examined TREM-1 expression in peripheral blood leukocytes obtained from patients with severe melioidosis and in different compartments of mice infected with a lethal dose of *B. pseudomallei*. The functional role of TREM-1 during melioidosis was studied *in vitro* by investigating the responsiveness toward *B. pseudomallei* of TREM-1-positive and TREM-1-negative leukocytes as well as *in vivo* by treating mice infected with *B. pseudomallei* with a synthetic peptide mimicking a short highly conserved domain of soluble-TREM-1.

MATERIALS AND METHODS

Patients

34 patients with melioidosis (mean age 52 years, range 18-86 years; 50% male) were recruited prospectively at Sapprasithiprasong Hospital, Ubon Ratchathani, northeast-Thailand in 2004. Sepsis due to melioidosis was defined as culture positivity for *B. pseudomallei* from any clinical sample plus a systemic-inflammatory-response-syndrome (SIRS) \(^8\). To meet the SIRS-criteria patients had to meet ≥3 of the following 4 criteria: core-temperature of ≥38°C or ≤36 ºC; heart-rate of ≥90 beats/min; respiratory-rate of ≥20 breaths/min or PaCO\(_2\) of ≥32 mmHg or use of mechanical ventilation for an acute respiratory process; and white-cell-count of ≥12x10\(^9\)/l or ≤4x10\(^9\)/l or differential count showing >10% immature neutrophils \(^8,9\). Exclusion criteria were the use of dialysis and/or immunosuppressive therapy, known coagulation-disorders and concomitant infection with human-immunodeficiency-virus. Blood samples were drawn within 36 hours after start of appropriate antimicrobial therapy. 32 healthy blood donors (mean age 41 years, range 21-59 years; 71% male) recruited from the Sapprasithiprasong hospital bloodbank...
Chapter 12

served as a control-population. The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, UK; written informed consent was obtained from all study subjects.

**Flow cytometric-analysis**

All samples were analyzed directly after sample collection by flow cytometry using FACSCalibur (BectonDickinson, San Jose, CA). In patients and controls, the cell-surface expression of the molecules of interest was determined on peripheral monocytes and granulocytes by using fluorochrome-conjugated mouse anti-human CD14 (BD Biosciences, Mountain View, CA), TREM-1 (R&D, Minneapolis, MN) and CD11b (Bioscience, San Diego, CA) antibodies in accordance with the manufacturer’s recommendations. Granulocytes were defined according to their scatter-pattern and monocytes according to their scatter-pattern and CD14-positivity. To correct for aspecific staining, appropriate isotype control antibodies (BD Biosciences) were used. Data on mean-cell-fluorescence-intensity (MFI) are presented as the difference between MFI intensities of specifically stained cells and non-specifically stained cells. Data on the number of positive cells were obtained by setting a quadrant marker for non-specific staining. In mice, immunostaining for TREM-1 on blood cells, cells obtained from BAL and whole lung cell suspensions was performed using directly labelled antibodies against GR-1 (GR-1 FITC; Pharmingen, San Diego, CA), TREM-1 (TREM-1-phycocerythrin; R&D, Minneapolis, MN) and a biotin-labeled antibody against F4/80 (Serotec, UK) in combination with streptavidin allophycocyanine (APC). Antibodies were used in concentrations recommended by the manufacturer. After staining, cells were fixed in 2%-paraformaldehyde. TREM-1 MFI was measured in the Gr-1-high gate (granulocytes), sidescatter low and F4/80 positive (monocytes) and sidescatter high and F4/80 positive (macrophages) gated populations.

**Evaluation of mRNA levels by quantitative RT-PCR**

Total RNA was isolated using the RNAeasy Mini-kit-system (Qiagen, Venlo, the Netherlands) and treated with RQ1 RNase-Free DNase (Promega, Leiden, the Netherlands) and reverse-transcribed using oligo(dT)primer and Moloney murine leukaemia-virus-reverse-transcriptase (Invitrogen Life Technologies) according to manufacturer’s recommendations. RT-PCRs were performed on cDNA samples that were 4-fold diluted in H$_2$O using FastStart DNA Master-SYBR-Green-I (Roche, Indianapolis, IN) with 2.5 mM MgCl$_2$ in a LightCycler (Roche) apparatus. PCR conditions were: 5 min 95°C hot-start, followed by 40 cycles of amplification (95°C for 15s, 60°C for 5s, 72°C for 20s). For quantification, standard curves were constructed by PCR on serial dilutions of concentrated cDNA; data were analyzed using LightCycler-software as described by the manufacturer. Gene expression is presented as a ratio of the housekeeping gene β2-microglobulin expression $^{10}$. All PCRs generated a single DNA-product of the expected length as judged by evaluation on ethidium bromide-stained 1.2% agarose gel-electrophoresis. Primers used for human TREM-1 were S485 CCTGGGCTCCATGAGAATT and AS717 CAGGACAGAGACCCAGGC. The forward-primer for the housekeeping gene was hB2M TCAATGGACTGGATATGGA and the reverse-primer hB2M AS231 GCCCTGTACCTTGATCGG. Oligonucleotides were purchased from Eurogentec, Seraing, Belgium.
TREM-1 in melioidosis

Murine melioidosis

The Animal Care and Use Committee of the University of Amsterdam approved all experiments. Pathogen-free 8 to 10 week old C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). For preparation of the inoculum, B. pseudomallei strain 1026b, kindly provided by Dr. Don Woods, University of Calgary, Canada \(^{11,12}\), was streaked from frozen aliquots into 50 ml Luria-broth (Difco, Detroit, MI) and incubated overnight at 37°C in 5% CO\(_2\). Thereafter, a 1 ml portion was transferred to fresh Luria-broth and grown for ± 5h to midlogarithmic phase. Bacteria were harvested by centrifugation at 1500xg for 15 minutes, washed and resuspended in sterile isotonic saline at a concentration of 5x10\(^2\) or 7.5x10\(^2\) colony forming units (CFUs)/50 \(\mu\)l, as determined by plating serial 10-fold dilutions on blood-agar plates. Pneumonia was induced by intranasal inoculation of a 50 \(\mu\)l (5x10\(^2\) or 7.5x10\(^2\) CFU) bacterial suspension. This bacterial dose induces 100% mortality within a five-day period. 24, 48 and 72 hrs after infection, mice were sacrificed by bleeding from the inferior vena cava. BAL was performed as described previously \(^{13}\). Pulmonary cell suspensions obtained from infected mice were analyzed by FACS (BectonDickinson) as described previously \(^{14,15}\).

TREM-1 peptide (LP17)

LP17, a synthetic peptide mimicking a short highly conserved domain of sTREM-1, was chemically synthesized by (Pepscan Systems, Lelystad, the Netherlands) as described elsewhere \(^{16}\). The peptide with sequence order LQVTDSGLYRCVIYHPP was obtained in >99 yield and was endotoxin free. A control peptide was similarly synthesized and contained the same aminoacids as LP17 but in a different sequence order (TDSRCVIGLYHPPLQVY). Mice were given 100 \(\mu\)g of LP17 or control peptide via the intranasal route at different time points as indicated in the results section.

Assays

Human soluble-TREM-1 levels were measured with an immunoblot technique, as described previously \(^3\). Mouse soluble-TREM-1 was measured using a commercially available ELISA (R&D systems, Minneapolis, MN). Human tumor-necrosis-factor (TNF)-\(\alpha\), interleukin (IL)-1\(\beta\), IL-6 and IL-8 were measured by cytometric-bead-array (CBA) multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer’s recommendations.

Cell-sorting experiments

Leukocytes derived from 8 healthy male volunteers (mean age 30; range 28-41 years) were sorted into TREM\(^+\) and TREM\(^-\) monocytes and TREM\(^+\) and TREM\(^-\) granulocytes by FACS-sorting. Briefly, unstimulated whole blood leukocytes were labelled with TREM-1-PE and CD14-FITC. Subsequently, TREM-1\(^+\) and TREM-1\(^-\) monocytes and TREM-1\(^+\) and TREM-1\(^-\) granulocytes were separated with FACSArria (BDBiosciences, Mountain View, CA). All sorted subsets were >95% pure. 1x10\(^5\) isolated TREM\(^+\) and TREM\(^+\) monocytes and TREM\(^+\) and TREM\(^+\) granulocytes were stimulated with LPS (from E. coli 0111:B4 Sigma, St. Louis, MO, 10 ng/ml), heat-killed B. pseudomallei (clinical isolate strain 1026b) \(^{11,12}\) (1x10\(^5\) CFU/ml) or RPMI-1640 medium for four hours at 37°C after which supernatants were collected and stored at -20°C until assays were performed.

201
Chapter 12

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). Differences between groups were analyzed by Mann-Whitney U test or Kruskal-Wallis analysis with Dunn’s posthoc test where appropriate. Correlations were calculated using the Spearman’s rho test. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Values of P<0.05 were considered statistically significant.

Figure 1. Increased soluble, cell surface- and mRNA TREM-1 levels in melioidosis patients. In patients (n=34) with melioidosis, strongly increased plasma levels of soluble TREM-1 (A) were present on admission when compared to healthy controls (n=32). Patients who went on to die from melioidosis had higher soluble TREM-1 plasma levels on admission than patients who survived (B). TREM-1 mRNA was strongly upregulated in the peripheral blood leukocytes of melioidosis patients (n=34) compared to healthy controls (n=32) (C). In addition, increased cell surface expression of TREM-1 on peripheral blood monocytes (E), together with an increase of the percentage monocytes expressing TREM-1 (F) was seen in the melioidosis patients compared to controls. Graph D shows a representative histogram of IgG1 isotype control (thin line), monocytes TREM-1 expression of a healthy control (bold line) and the monocyte TREM-1 expression of a patient (filled gray). There was no difference in the TREM-1 cell surface expression on granulocytes (H) between cases and controls, while the percentage of granulocytes expressing TREM-1 (I) was lower in patients. Graph G shows a representative histogram of IgG1 isotype control (thin line), granulocyte TREM-1 expression of a healthy control (bold line) and the granulocyte TREM-1 expression of a patient (filled gray). MFI: fluorescence intensity; ns: not significant; ** p < 0.01; *** p < 0.001.
RESULTS

Increased TREM-1 expression in patients with severe melioidosis

To obtain an insight into TREM-1 expression during melioidosis, we first measured soluble-TREM-1 in plasma from 34 patients with culture proven *B. pseudomallei* infection and 32 local healthy controls. Fifteen patients (44%) with melioidosis died in hospital. Soluble-TREM-1 was profoundly elevated in melioidosis patients with mean plasma concentrations that were approximately 8-fold higher than in healthy subjects (Figure 1A, $P<0.0001$, for the difference between the two groups). On admission, patients who went on to die had higher soluble-TREM-1 plasma levels than patients who survived (Figure 1B; $P=0.01$). In eight patients in whom a second blood sample was obtained at the end of a two-week treatment period, plasma soluble-TREM-1 concentrations had not decreased compared to levels measured on admission to the hospital (data not shown). TREM-1 mRNA levels were significantly higher in peripheral blood leukocytes from patients than in leukocytes from healthy controls (Figure 1C, $P<0.001$). To determine the impact of melioidosis on TREM-1 protein expression at the surface of peripheral blood cells, we compared the TREM-1 expression on circulating monocytes and granulocytes of patients with melioidosis and controls using FACS analysis. Relative to controls, patients displayed higher cell

**Figure 2. Increased expression of CD11b on monocytes and granulocytes in melioidosis patients.** CD11b cell surface expression was strongly increased on monocytes (A) and granulocytes (B) of patients (n=34) with melioidosis compared to healthy controls (n=32). No correlation was seen between TREM-1 and CD11b cell surface expression on monocytes (C) or granulocytes (D). MFI: fluorescence intensity; ns: not significant; *** $p < 0.001$. 

203
surface levels of TREM-1 on their monocytes as reflected by higher MFI’s (Figure 1E, \(P<0.0001\)), as well as an overall increase in the percentage of monocytes expressing TREM-1 on their cell membranes (Figure 1F, \(P<0.0001\)). The mean intensity of cell-surface TREM-1 expression on peripheral granulocytes, as measured by MFI, was not different between cases and controls (Figure 1H, \(P=0.17\)); however, the percentage of TREM-1 positive granulocytes was lower in the patients compared to controls (Figure 1I, \(P<0.0001\)). Plasma soluble-TREM-1 levels were positively correlated with granulocyte cell-surface TREM-1 expression (\(r=0.40, P<0.05\)) but not with monocyte TREM-1 expression (\(r=0.30, P=0.07\)). The levels of neither cell-associated TREM-1 nor TREM-1 mRNA differed between survivors and non-survivors (data not shown).

**Cell-associated TREM-1 expression does not correlate with CD11b expression in patients with melioidosis**

To investigate whether TREM-1 expression is associated with the activation state of leukocytes, we analysed the expression of the activation marker CD11b. CD11b cell surface expression was strongly increased on monocytes (Figure 2A; \(P<0.0001\)) and granulocytes (Figure 2B; \(P<0.0001\)) of patients with severe melioidosis compared to healthy controls. However, no correlation was seen between TREM-1 and CD11b cell surface expression on monocytes (Figure 2C; \(r=0.23, P=0.21\)) or granulocytes (Figure 2D; \(r=0.08, P=0.66\)).

**Compartmentalized TREM-1 expression in a murine model of pneumonia-derived melioidosis**

A large part of severe melioidosis cases presents with pneumonia with bacterial dissemination to distant body sites. Considering that it is not feasible to study TREM-1 expression at the tissue level in patients with melioidosis, we used a mouse model of this disease in which mice are intranasally infected with *B. pseudomallei*. We used a bacterial inoculum that causes 100% mortality in C57BL/6 wild-type mice 4 days of infection. Mice were killed 24, 48 and 72 hours after infection (i.e. directly before the first predicted death) and TREM-1 expression was determined in lungs and blood together with bacterial load and lung histopathology. Mice developed severe pneumonia with an increase in bacterial load in the lungs (Figure 3A) followed by an increase in bacterial numbers in the blood (Figure 3B), liver and spleen (data not shown). During experimental murine melioidosis an influx of mainly granulocytes into both the lung and broncho-alveolar lavage fluid (BALF) was seen (Figure 3C–D). Lung histology showed abscess formation analogous to that found in patients (Figure 3E–G). Plasma soluble-TREM-1 levels remained constant during the first two days of infection, followed by a steep increase at 72 hours (Figure 4A, \(P<0.01\)). In BAL fluid (BALF), soluble-TREM-1 showed a much earlier rise: at 24 hours after infection, BALF soluble-TREM-1 concentrations were already markedly elevated and these levels remained relatively stable throughout the 72 hour period after inoculation (Figure 4B, \(P<0.01\)). Cell-associated TREM-1 expression in blood, BALF and whole lung cell suspensions was evaluated by FACS. Consistent with the data obtained from patients with melioidosis, infected mice showed an upregulation of TREM-1 expression on the blood monocytes (Figure 5A, \(P<0.001\)). Interestingly, the increase in TREM-1 on the monocytes occurred only at 72 hours after infection, thereby coinciding with the rise in plasma soluble TREM-1 concentrations. Again consistent with the patient data, granulocyte TREM-1 expression did not increase in blood of mice with melioidosis.
TREM-1 in melioidosis

Figure 3. Bacterial outgrowth and lung inflammation in mice infected with *B. pseudomallei*. C57BL/6 mice were intranasally infected with a lethal dose of *B. pseudomallei* (5x10^2 CFU). Bacterial loads were measured 24, 48 and 72h after inoculation in lungs (A) and blood (B). Data are mean ± SEM (n = 8 per group). Percentages of leukocyte cell populations infiltrating the lung (C) and BALF (D) during experimental murine melioidosis are depicted for monocytes (white bars), macrophages (gray bars) and granulocytes (black bars). Representative lung histology of WT mice at 24 (E), 48 (F) and 72 (G) hours after inoculation with 5x10^2 CFU *B. pseudomallei*, showing a steady increase in tissue damage over time as reflected by inflammation, pleuritis, peribronchial inflammation, oedema, endothelialitis and necrosis 72 hours after infection (G) compared to 24 hours after infection (D). Hematoxilin and eosin staining; original magnification x 40. Pictures taken by Dr. J.J. Roelofs, Department of Pathology, Academic Medical Center, Amsterdam.

Figure 4. Soluble TREM-1 levels in mice with melioidosis. Plasma soluble TREM-1 levels remained constant during the first two days of infection, followed by a steep increase at 72 hours (A). Increased levels of soluble TREM-1 in BALF (B) were observed already shortly after inoculation. BALF: broncho-alveolar-lavage-fluid. Data are means ± SEM of 8 mice per group at each timepoint. **P** TREM-1: soluble TREM-1. P values indicate statistical significance for changes between time points (Kruskal-Wallis test). Asterisks represent statistical significance for the difference with t = 0 (by posthoc test). **p < 0.01; ***p < 0.001 versus t=0.
Chapter 12

In the pulmonary compartment cell-associated TREM-1 expression was regulated differently. The most remarkable difference was that granulocytes recovered from BALF and lungs demonstrated an upregulation of TREM-1 (Figure 5B). Monocytes in BALF and lungs, like in blood, increased their TREM-1 expression during the course of the infection. Macrophages in BALF showed a strong constitutive TREM-1 expression, which was reduced after infection with *B. pseudomallei* (Figure 5E), whereas in lungs macrophage TREM-1 expression became upregulated during melioidosis (Figure 5F).

**Diminished pro-inflammatory response of TREM-1− granulocytes compared to TREM-1+ granulocytes.**

To investigate the function of the observed TREM-1 upregulation in patients and mice with melioidosis, we separated TREM-1+ and TREM-1− monocytes and TREM-1+ and TREM-1− granulocytes derived from healthy volunteers using a Cellsorter (Figure 6A). Thereafter freshly isolated TREM-1+ and TREM-1− cell populations were incubated with medium, LPS or heat-killed *B. pseudomallei*. Consistent with the hypothesis that TREM-1 is an amplifier of the inflammatory response...
response, TREM-1⁻ granulocytes released less IL-8 than TREM-1⁺ granulocytes after incubation with medium, LPS (Figure 6B, P<0.05) or B. pseudomallei (Figure 6B, P<0.01). The concentrations of other cytokines in the supernatants of purified granulocytes were either below the limit of detection (TNFα) or very low (IL-1β, IL-6) (data not shown). Purified monocytes released significant quantities of TNFα, IL-1β, IL-6 and IL-8; however, the supernatants of TREM-1⁺ and TREM-1⁻ monocytes contained equal concentrations of all cytokines under all incubation conditions (shown for IL-8 in Figure 6C).

TREM-1 peptide LP17 partially protects mice from B. pseudomallei induced lethality

To further investigate the in vivo role of TREM-1 in melioidosis we treated mice with a single dose of LP17 1 hour before a lethal dose of B. pseudomallei was given and found that LP17 pretreatment partially protected mice from B. pseudomallei induced lethality (Figure 7A, P< 0.01). Of interest, also the delayed treatment with LP17 at 1, 24 and 72 hrs after inoculation with B. pseudomallei caused a decrease in mortality (Figure 7A, P< 0.02). No difference on organ pathology or cytokine levels could be detected (data not shown) However, consistent with the protective effect, the LP17 treated mice showed less bacterial outgrowth in their spleen, although this could not be detected in the pulmonary compartment (Figure 7B-C).
Melioidosis is a debilitating septic disease with a mortality of up to 50% in endemic areas. As with other forms of sepsis, multiple organ failure and death are thought to result from an uncontrolled inflammatory reaction after infection with *B. pseudomallei*. Severe pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis. In light of the proinflammatory properties attributed to TREM-1 in pneumonia-derived sepsis, we studied the expression pattern of membrane bound TREM-1, TREM-1 gene expression and its soluble form in both humans and mice infected with *B. pseudomallei* in different cell types and in different body compartments. Our results demonstrate that during severe melioidosis expression of TREM-1 is differentially regulated on granulocytes and monocytes, and that in particular measurements of TREM-1 expression on granulocytes in the circulation may not provide adequate information on granulocyte TREM-1 expression at the site of the infection.

**DISCUSSION**

Melioidosis is a debilitating septic disease with a mortality of up to 50% in endemic areas. As with other forms of sepsis, multiple organ failure and death are thought to result from an uncontrolled inflammatory reaction after infection with *B. pseudomallei*. Severe pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis. In light of the proinflammatory properties attributed to TREM-1 in pneumonia-derived sepsis, we studied the expression pattern of membrane bound TREM-1, TREM-1 gene expression and its soluble form in both humans and mice infected with *B. pseudomallei* in different cell types and in different body compartments. Our results demonstrate that during severe melioidosis expression of TREM-1 is differentially regulated on granulocytes and monocytes, and that in particular measurements of TREM-1 expression on granulocytes in the circulation may not provide adequate information on granulocyte TREM-1 expression at the site of the infection.
Our data extend the findings of previous studies investigating TREM-1 expression in human endotoxemia and sepsis. Our laboratory previously showed that TREM-1 is upregulated on blood monocytes of human volunteers injected with LPS intravenously together with an increase in plasma soluble-TREM-1. In these volunteers LPS induced a down regulation of TREM-1 on circulating neutrophils, which is consistent with our current observations in melioidosis patients. Furthermore, plasma soluble-TREM-1 levels were elevated in patients with sepsis compared to patients with a systemic-inflammatory-response-syndrome without infection, and monocyte TREM-1 expression, but not granulocyte TREM-1 expression, was higher in septic shock patients than in healthy controls. Notably, in our patient population with severe melioidosis high plasma soluble-TREM-1 concentrations on admission were associated with death.

To investigate whether TREM-1 expression correlates with the activation state of leukocytes, we simultaneously analysed the cell-associated expression of the established activation marker CD11b in patients with melioidosis. Although CD11b expression was strongly enhanced on both monocytes and granulocytes in these patients, no association between CD11b and TREM-1 expression could be found, suggesting that TREM-1 expression is not strictly linked to cellular activation. Hence, TREM-1 likely is constitutively expressed on monocytes and granulocytes and may be further upregulated in the presence of microbes, such as bacteria or fungi.

In patients it was only feasible to study TREM-1 expression in cells drawn from peripheral blood. Since we were interested in the kinetics TREM-1 expression at the primary infection site, we made use of a mouse model of melioidosis in which mice are intranasally infected with a lethal dose of \textit{B. pseudomallei}. In view of the fact that pneumonia with bacterial dissemination to distant body sites is a common presentation of human melioidosis, we utilized a model in which \textit{B. pseudomallei} was administered via the airways. We reproduced the major clinical characteristics of melioidosis, with spread of bacteria to distant organs, multiple organ failure and abscess formation. By so doing we were able to precisely report the kinetics of membrane bound TREM-1 expression on monocytes and granulocytes together with its soluble form in both the lung and blood compartment. Notably, the differential regulation of TREM-1 expression on blood monocytes and granulocytes (with upregulation on the former cells and down modulation on the latter cells), as well as the increase in plasma soluble-TREM-1 concentrations were reproduced in mice with melioidosis. Of considerable interest, however, at the site of the infection granulocytes displayed enhanced TREM-1 expression, particularly in BALF. In mice, monocytes showed a similar up-regulation of TREM-1 in BALF and lungs as in blood. Macrophages in BALF, however, constitutively displayed strong TREM-1 expression, which diminished after infection, whereas in lungs a relatively low initial TREM-1 expression on macrophages became up-regulated during the course of infection. In addition, whereas soluble-TREM-1 rose relatively late in plasma (after 72 hours), in BALF soluble-TREM-1 concentrations were already markedly elevated at 24 hours post infection. Moreover, although the absolute concentrations of soluble-TREM-1 were higher in plasma at a late phase during the infection, soluble-TREM-1 levels likely were much higher in BALF in light of the dilution factor introduced by the lavage procedure. Together these data point strongly to local release of TREM-1, which is in line with a recent report on TREM-1 expression patterns in a murine model of septic shock induced by CLP, in which increased TREM-1 expres-
Chapter 12

Expression was seen on the phagocytic cells of the peritoneal compartment together with increased soluble-TREM-1 levels in peritoneal lavage fluid 22.

Although several studies have indicated that TREM-1 can amplify cytokine release induced by TLR ligands 1,21,23, to our knowledge the cytokine production capacity of TREM-1+ and TREM-1− cells has not been directly compared before. Our data reveal a functional difference between the observed TREM-1 upregulation on monocytes and granulocytes. In accordance with the hypothesis that TREM-1 is a pivotal amplifier of acute inflammation, TREM-1− granulocytes displayed a diminished pro-inflammatory response after LPS and *B. pseudomallei* stimulation. Interestingly however, there was no difference in LPS induced cytokine release between TREM-1+ and TREM-1− monocytes, suggesting the existence of additional amplifiers of the monocyte TLR-cascade. Further research is warranted to address this issue. The functional role of TREM-1 during melioidosis was further investigated *in vivo* by making use of a TREM-1 peptide, called LP17. Monocytes and macrophages produce a soluble form of TREM-1 and the administration of LP17, which mimics this soluble receptor, has been shown to reduce inflammatory hyper-responsiveness and mortality in both endotoxic shock in mice and *Pseudomonas aeruginosa* pneumonia in rats 16,24. By thus modulating the TREM-1 signaling, we found that treatment with LP17 partially protects mice from *B. pseudomallei* induced lethality. This finding further underscores the importance of TREM-1 signaling in melioidosis and highlights TREM-1 as a potential treatment target in melioidosis.

In conclusion, we have demonstrated that human melioidosis is associated with increased expression of TREM-1 in monocytes but not granulocytes, accompanied by elevated circulating levels of soluble-TREM-1. These findings were replicated in a mouse model of melioidosis, which further demonstrated that cell-associated TREM-1 expression at the primary site of infection was different from that in circulating blood cells. These results provide new information on TREM-1 regulation during severe infection, and give further support for the potential usefulness of TREM-1 as a diagnostic and therapeutic target.

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Chapter 12


Expression and function of macrophage migration inhibitory factor (MIF) in melioidosis

Submitted

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Chapter 13

ABSTRACT

Background
Macrophage migration inhibitory factor (MIF) has emerged as a pivotal mediator of innate immunity and has been shown to be an important effector molecule in severe sepsis. Melioidosis, caused by *Burkholderia pseudomallei*, is an important cause of community-acquired sepsis in Southeast-Asia. We aimed to characterize the expression and function of MIF in melioidosis.

Methods
MIF expression was determined in leukocytes and plasma from 34 melioidosis patients and 32 controls, and in mice infected with *B. pseudomallei*. MIF function was investigated in experimental murine melioidosis using anti-MIF antibodies and recombinant MIF.

Results
Patients demonstrated markedly increased MIF mRNA leukocyte and MIF plasma levels. Elevated MIF levels were associated with mortality. Mice inoculated intranasally with *B. pseudomallei* displayed a robust increase in pulmonary and systemic MIF expression. Anti-MIF treated mice showed lower bacterial loads in their lungs upon infection with a low inoculum. Conversely, mice treated with recombinant MIF displayed a modestly impaired clearance of *B. pseudomallei*.

Conclusions
MIF levels are markedly elevated during clinical melioidosis and correlate with patient’s outcome. In experimental melioidosis MIF modestly impaired antibacterial defense.
INTRODUCTION

Macrophage migration inhibitory factor (MIF) was one of the first cytokines to be discovered almost half a century ago. Since then MIF has emerged as a pivotal mediator of innate immunity in various inflammatory diseases such as rheumatoid arthritis and atherosclerosis and is considered to be an integral component of the host antimicrobial alarm system. MIF, a classical proinflammatory cytokine, is constitutively expressed by many tissues with environmental contact such as the lung and the gastrointestinal tract, and by numerous cell types, among others T- and B-lymphocytes, monocytes and macrophages. MIF-deficient macrophages are hyporesponsive to lipopolysaccharide (LPS) due to a down-regulation of Toll-like receptor (TLR)-4. In line, MIF knockout mice were resistant to LPS induced toxic shock. Recently it was shown that blood levels of MIF are elevated in patients with sepsis and able to predict early mortality. Similarly, MIF is increased in patients with meningococcal disease and highest in the presence of shock. Excitingly, treatment with anti-MIF antibodies protected mice from lethal peritonitis induced by Escherichia coli or cecal ligation and puncture (CLP). Furthermore, ISO-1 and OXIM-11, new small molecule inhibitors of MIF, offered significant protection to mice from CLP-induced sepsis. These data identified MIF as a potential mediator of lethality following abdominal sepsis.

In Southeast-Asia and Northern-Australia the gram-negative bacillus Burkholderia pseudomallei is an important cause of community-acquired sepsis. More than half of these cases of melioidosis, as this severe infection is named, habitually presents with pneumonia, frequently associated with bacterial dissemination to distant sites. In the present study we aimed to characterize the expression and function of MIF in melioidosis. For this we analysed MIF expression patterns in patients with melioidosis and in a mouse model of B. pseudomallei infection. MIF function was investigated in experimental murine melioidosis using anti-MIF antibodies and recombinant MIF.

MATERIALS AND METHODS

Patients

We included 34 individuals with sepsis caused by B. pseudomallei and 32 healthy controls in this study. Individuals were recruited prospectively at Sapprasithiprasong Hospital, Ubon Ratchathani, Thailand in 2004. Sepsis due to melioidosis was defined as culture positivity for B. pseudomallei from any clinical sample plus a systemic inflammatory response syndrome (SIRS). Study design and subjects have been described in detail. The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, England. We obtained written informed consent from all subjects before the study.
Chapter 13

Human plasma MIF and MIF mRNA measurements

Human MIF was measured by ELISA, as described elsewhere \(^{24}\). In addition, MIF mRNA levels were measured as follows. Heparin blood samples were drawn from an antecubital vein and immediately put on ice. Leukocytes were isolated using erylysis buffer, dissolved in Trizol and stored at \(-80^\circ C\). Thereafter, RNA was isolated and analyzed by multiplex ligation-dependent probe amplification (MLPA) using an inflammatory-specific kit as described \(^{25,26}\) (MRC-Holland, Amsterdam, the Netherlands). Levels of mRNA were expressed as a normalized ratio of the peak area divided by the peak area of the \(\beta_2\) microglobulin (B2M) gene, resulting in the relative abundance of mRNAs of the genes of interest \(^{25}\).

Murine melioidosis

The Animal Care and Use of Committee of the University of Amsterdam approved all experiments. Male C57BL/6 mice (age 8-10 weeks) were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). Age-matched animals were used in each experiment. For the inoculum, \(B.\ pseudomallei\) strain 1026b, kindly provided by Dr. Don Woods \(^{27,28}\), was used and prepared as described \(^{23,29-31}\). Pneumonia was induced by intranasal inoculation of a 50 μl (5 x 10\(^4\), 2.5 x 10\(^5\) or 7.5 x 10\(^2\) colony forming units (CFU)/ 50 μl) bacterial suspension. 48 hours after infection, mice were anesthetized with Hypnorm\textsuperscript{®} (Janssen Pharmaceutica, Beerse, Belgium: active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Mijdrecht, The Netherlands) and sacrificed by bleeding from the vena cava inferior. Lungs and livers were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of organ homogenates and blood, plated on blood agar plates and incubated at 37°C at 5% CO\(_2\) for 16 h before colonies were counted. In some experiments mice were injected intraperitoneally with 2 mg of anti-MIF or non-immune IgG 2 hours before bacterial inoculation or with 50 μg recombinant mouse MIF or control buffer at the onset of infection as described previously \(^{16,32,33}\). For anti-MIF treatment, mice were treated 2 hours before bacterial inoculation with 2 mg anti-MIF mAb or non-immune IgG intraperitoneally exactly as described previously \(^{16,32}\). Rabbit polyclonal anti-MIF and recombinant MIF were generated as described \(^{16,33}\).

Murine assays

The ELISA for mMIF developed according to the 4-span approach was used as described in detail \(^{34}\). Tumor necrosis factor (TNF)-\(\alpha\), interferon (IFN)-\(\gamma\), interleukin (IL)-6, IL-10 and IL-12p70 were determined using a cytometric bead array (CBA) multiplex assay in accordance with the manufacturer’s instructions (BD Biosciences, San Jose, CA).

MIF immunochemistry and histologic examination

Four-μm thick lung tissue sections were sampled 48 hours after infection and mounted on aminopropylmethoxysilane-coated glass slides, deparaffinized in xylol, taken through to absolute alcohol, blocked for endogenous peroxidase with 0.1% hydrogen peroxide in methanol (45 minutes) and rehydrated through graded alcohols. They were boiled for 15 minutes in 10 mM citrate buffer (containing 2.94 g sodium citrate trisodium salt dihydrate per liter H\(_2\)O, pH 6.0) in a
MIF in melioidosis

microwave oven and rinsed in Tris-buffered saline (TBS). To reduce non-specific binding, sections were incubated for 10 minutes in normal goat serum (Pel-Freez Biologics, Rogers, AK) 1:30 in TBS. After 40-minutes incubation with polyclonal rabbit anti-MIF purified IgG diluted 1:200 in TBS containing 2% bovine serum albumin (final immunoglobulin concentration: 25mg/l), the sections were incubated for 30 minutes with biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) diluted 1:400, then for 30 minutes with ABC-peroxidase complex solution (Vector) prepared according to the manufacturer’s instructions. Peroxidase activity was revealed with 5-5'-diaminobenzidine as chromogen and the sections were counterstained in Meyer’s acid-free hematoxylin. As a negative control, the primary antibody was replaced by pre-immune rabbit purified IgG Furthermore, to score inflammation, lung and livers from infected mice were harvested 48 hours after infection, fixed in 10%-formalin and embedded in paraffin. Four µm sections were stained with hematoxylin and eosin and analyzed by a pathologist blinded for groups exactly as described previously.

Statistical analysis
Values are expressed as means ± standard error of the mean (SEM). Differences between groups were analyzed by Mann-Whitney U test or Kruskal-Wallis analysis with Dunn’s posthoc test where appropriate. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Values of \( P < 0.05 \) were considered statistically significant.

RESULTS

MIF expression is elevated in patients with melioidosis and correlates with poor outcome
To obtain an insight into MIF expression during melioidosis, we first measured MIF in plasma from 34 patients with culture proven \( B. \) pseudomallei infection and in plasma from 32 local healthy controls. The mortality rate in this cohort of patients was 44%. MIF was markedly elevated in melioidosis patients with mean plasma concentrations that were approximately 2-fold higher than in those of healthy subjects (Figure 1A, \( P < 0.01 \). Plasma levels of MIF were associated with an adverse outcome: on admission patients who went on to had higher MIF concentrations than those who survived (Figure 1B, \( P < 0.01 \)). In line, MIF mRNA levels were significantly higher in peripheral blood leukocytes from patients than in leukocytes from healthy controls (Figure 1C, \( P < 0.001 \)).

Increased MIF expression in the pulmonary compartment during experimental pneumonia-derived melioidosis
Since the majority of severe melioidosis cases presents with pneumonia with bacterial dissemination to distant body sites \(^{19-21}\) and considering the fact that it is not feasible to study MIF expression at tissue level in patients with melioidosis, we used a well-established murine model

217
Chapter 13

of pneumonia-derived melioidosis in which mice are intranasally infected with *B. pseudomallei* 23, 29, 31. In agreement with the data obtained in patients with melioidosis, infected mice showed an abundant upregulation of MIF expression, both in the pulmonary and systemic compartment (Figure 2, both *P* < 0.01). Immunohistochemical staining of lung tissue was performed to further identify the distribution of MIF expression during melioidosis. Positive immunostaining for MIF was observed in untreated control animals in alveolar macrophages and within the bronchial epithelium (Figure 3A). Granulocytes did not stain positive for MIF. After infection with *B. pseudomallei* there was a marked increase in immunostaining of the epithelial submucosa, bronchial epithelial cells and inflammatory cells, most notably of alveolar macrophages (Figure 3B).

**Effect of recombinant MIF treatment on bacterial outgrowth**

To obtain a first insight into the function of MIF during experimental melioidosis, we treated mice infected with 2.5 x 10^2 CFU *B. pseudomallei* mice with 50 μg recombinant MIF using a dose similar to that used previously in an experimental septic shock model 16, 32. Treatment of mice...
with recombinant MIF at the time of infection resulted in increased MIF concentrations in lung homogenates 48 hours later (from 33 ± 1.3 to 729 ± 56.6 ng/ml; P < 0.001). Mice were sacrificed 48 hours after inoculation to determine bacterial loads in lungs (the primary site of the infection), liver and blood (to evaluate to which extent the infection disseminated to distant body sites) (Figure 4). Relative to infected but non-treated controls, mice treated with recombinant MIF displayed almost 10-fold higher bacterial loads in the liver (Figure 4, P < 0.01). In addition, a clear trend was seen towards higher bacterial loads in the pulmonary and systemic compartments of recombinant MIF treated mice, although the differences with control mice did not reach statistical significance (Figure 4).

**Effect of anti-MIF treatment on bacterial outgrowth**

Having found that administration of supra physiological doses of MIF results in a partially impaired bacterial clearance during experimental melioidosis, we next hypothesized that treatment with anti-MIF antibodies would result in decreased bacterial outgrowth and performed the reverse

**Figure 3. Immunostaining for MIF in the lungs of mice.** Positive immunostaining for MIF in lung tissue was observed in non-infected control animals of bronchial epithelial cells and alveolar macrophages (A). 48 hours after infection with *B. pseudomallei* there was a marked increase in immunostaining of the epithelial submucosa, bronchial epithelial cells and inflammatory cells, most notably of alveolar macrophages (B). Magnification x10, insets x20.

**Figure 4. Effect of recombinant MIF on bacterial clearance.** Mice, treated with control buffer (white bars) or recombinant MIF (grey bars) and inoculated with 2.5 x 10^2 CFU *B. pseudomallei* intranasally, were analysed for bacterial outgrowth in the lungs (A), liver (B) and blood (C) 48 hours later. Data represent mean ± SEM of n=8 mice per group; ** p < 0.01.
experiment by examining the effect of anti-MIF treatment. Therefore, before inoculating mice with *B. pseudomallei*, we injected mice with anti-MIF antibodies using a dosing schedule previously found to be protective in a mouse model of *E. coli* or CLP-induced peritonitis. To evaluate whether anti-MIF treatment interferes with bacterial clearance, we first determined bacterial loads 48 hours after infection with an inoculum of 2.5 x 10² CFU *B. pseudomallei* (Figure 5). At this dose no significant differences in bacterial outgrowth in either lungs, liver or blood were observed. To determine whether the effect of anti-MIF therapy is dependent on the size of the infectious dose, we next infected mice with a higher (5 x 10² CFU *B. pseudomallei*) and lower (5 x 10¹ CFU *B. pseudomallei*) inoculum (Figure 6). At the highest dose no effect of anti-MIF treatment was seen on the bacterial outgrowth in the lungs, liver or blood (Figure 6B). However, at the lowest inoculum mice treated with anti-MIF had almost 10-fold less *B. pseudomallei* CFU in their lungs compared to control mice (Figure 6A, P < 0.05). With this low inoculum, none of the mice showed positive *Burkholderia* cultures in liver or blood. Together, these data suggest that anti-MIF treatment inhibits the growth of *B. pseudomallei* in the lungs after infection with a relatively low bacterial dose.

![Figure 5. Effect of anti-MIF antibodies on bacterial clearance.](image)

![Figure 6. Effect of anti-MIF treatment on bacterial clearance is dependent on the inoculum size.](image)
Influence of anti-MIF and recombinant MIF on the inflammatory response

Since cytokines are important regulators of the inflammatory response to acute lower respiratory tract infection and given the observation that protective anti-MIF treatment reduced TNFα concentrations in mouse model of sepsis induced by *E. coli* or CLP, we measured the levels of TNFα, IL-6, IL-10, IL-12 and IFNγ in lung homogenates and plasma obtained 48 hours after infection with 2.5 x 10^2 CFU *B. pseudomallei* (Table 1). Anti-MIF treatment did not influence pulmonary cytokine levels in our model of experimental melioidosis (Table 1). Previously it was shown that plasma TNFα levels induced by LPS were lower in MIF-deficient mice compared to wild-type mice. However also in plasma no differences in TNFα or IL-6, IL-10, IL-12 and IFNγ levels were seen between anti-MIF treated and control mice after inoculation with *B. pseudomallei* (data not shown). In addition treatment with 50 µg recombinant MIF did not influence cytokine levels in either the pulmonary (Table 1) or systemic compartment (data not shown). Considering that MIF is regarded as an important proinflammatory mediator, we determined whether modulation of MIF levels could have an effect on organ inflammation during experimental melioidosis.

Therefore, we performed histopathological analyses of lung and liver tissues in control mice and mice treated with anti-MIF or recombinant MIF and infected with *B. pseudomallei*. Although all mice showed evidence of inflammation as characterized by diffuse infiltrates, interstitial inflammation and bronchitis there were no differences in total organ histopathological scores between groups (data not shown).

**Table 1.** Cytokine concentrations in lung homogenates of mice treated with anti-MIF antibodies or recombinant MIF during experimental melioidosis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Non-immune IgG</th>
<th>Anti-MIF mAb</th>
<th>Control</th>
<th>Recombinant MIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>2632 ± 524</td>
<td>1217 ± 475</td>
<td>1910 ± 154</td>
<td>2827 ± 652</td>
</tr>
<tr>
<td>IL-6</td>
<td>2421 ± 255</td>
<td>2461 ± 242</td>
<td>1530 ± 203</td>
<td>2105 ± 459</td>
</tr>
<tr>
<td>IL-10</td>
<td>97 ± 17</td>
<td>100 ± 8.3</td>
<td>79 ± 13</td>
<td>125 ± 15</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>91 ± 8.6</td>
<td>108 ± 7.4</td>
<td>116 ± 7.6</td>
<td>116 ± 14</td>
</tr>
<tr>
<td>IFNγ</td>
<td>432 ± 198</td>
<td>218 ± 78.4</td>
<td>366 ± 37</td>
<td>311 ± 151</td>
</tr>
</tbody>
</table>

Pulmonary cytokine levels after intranasal infection with 2.5 x 10^2 CFU *B. pseudomallei*. Mice were sacrificed 48 hours after infection. Data are means ± SEM of eight mice per group per time point. TNFα: tumor-necrosis-factor (TNF)α; IL = interleukin; IFNγ = Interferon-γ.

**DISCUSSION**

In the present study we aimed to characterize the expression and role for MIF in melioidosis, linking observational studies in patients with culture-proven disease with functional studies in mice in which we modulated the levels of MIF during experimentally induced melioidosis. Our study shows that patients with severe melioidosis have strongly increased MIF plasma and MIF mRNA leukocyte levels. High plasma MIF levels were markedly associated with mortality. Similarly, mice intranasally inoculated with *B. pseudomallei* displayed a strong increase in pulmonary and systemic MIF expression. The functional role of MIF in our model of experimental melioidosis...
Chapter 13

however was modest given the fact that modulation of MIF levels only moderately influenced the innate immune response towards *B. pseudomallei*. Anti-MIF treatment only decreased bacterial outgrowth when mice were inoculated with a low dose of *B. pseudomallei* whereas - conversely - mice treated with recombinant MIF displayed a modestly impaired clearance of *B. pseudomallei*. These data are the first to report on the expression and function of MIF during melioidosis.

MIF expression is increased in a wide variety of infectious diseases, ranging from viral infections, such as Dengue, HIV and West Nile virus infection, malaria, tuberculosis and various forms of sepsis. Our study further extends these findings by demonstrating increased plasma and blood leukocyte mRNA levels of MIF in patients with severe melioidosis. Importantly, we demonstrated a strong association between elevated MIF levels and increased mortality. This is in line with a recent study among pediatric and adult patients with severe sepsis or septic shock caused predominantly by *Neisseria meningitides* and other gram-negative bacteria in which elevated MIF levels were shown to be predictive of early mortality. MIF, however, is not always upregulated after acute infection or inflammation. For instance, in children with acute malaria circulating MIF levels were significantly lower compared with healthy, malaria-exposed children. Furthermore, MIF release could not be detected in a human endotoxemia model and is not produced by whole blood cells incubated with LPS. Also in HIV seropositive patients low serum MIF levels were associated with a high 1-month mortality. This further highlights the potential diverse roles MIF can play in the host response against various invading pathogens.

Melioidosis, which is the most common form of community-acquired sepsis in Northern-Australia and Eastern-Thailand, is associated with a mortality of up to 50% in endemic areas. Multiple organ failure and death are thought to result from an uncontrolled inflammatory reaction after infection with *B. pseudomallei*. Severe pneumonia with bacterial dissemination to distant body sites is a common presentation of melioidosis. In light of the proinflammatory properties attributed to MIF in sepsis, we studied the expression and function of MIF in a well-established mouse model of melioidosis. In line with our patient data and in line with various other murine models of sepsis induced by LPS, *E. coli* or CLP, we observed a strong upregulation of MIF expression in both the lungs and blood of mice inoculated with *B. pseudomallei*. However, MIF seems to play a less important role in the innate immune response in melioidosis, which is in contrast with previous studies pointing towards a central role of MIF in other forms of infection. With regard to bacterial infection, the role of MIF has been studied in abdominal sepsis caused by either intraperitoneal injection of *E. coli* or CLP. In these models, anti-MIF from the same source and administered in the exact same dose protected mice from mortality, reduced TNFα levels and diminished bacterial growth. The fact that anti-MIF did not have a major impact on the immune response to *B. pseudomallei* could be related to differences in the primary site of infection and/or differences in the pathogens involved. In this respect it is worthwhile noting that MIF regulates innate immune responses in gram-negative infections through modulation of Toll-like receptor. We have recently shown - counter intuitively for a gram-negative infection - that TLR2 detects the LPS of *B. pseudomallei* and that only TLR2 impacts on the immune response of the intact host in vivo, whereas TLR4 does not contribute to protective immunity in melioidosis. As such, the minor role of TLR4 in the innate immune...
response towards *B. pseudomallei* could be an explanation for our present findings revealing an equally limited role for MIF in melioidosis. Interestingly, during murine *Listeria monocytogenes* infection, the elimination of bacteria from the spleen and liver was not affected by anti-MIF antibody although this treatment was able to rescue mice from lethal infection. In contrast, we did not observe a significant effect of anti-MIF treatment relative to control antibody treatment on survival using a lethal model of melioidosis (data not shown). The role of MIF has further been studied in models of viral and parasitic infections. In a mouse model of acute West Nile virus infection, MIF deficiency lead towards a reduced viral load and inflammatory response in the brain compared to wild-type mice. In addition, MIF deficient mice had an enhanced susceptibility towards *Trypanosoma cruzi* infection associated with decreased TNFα, IL-12 and IFNγ levels. In a model of malaria, infection of MIF knockout mice with *Plasmodium chabaudi* resulted in increased survival compared to control mice.

In reverse experiments we found that treatment of *B. pseudomallei* infected mice with recombinant MIF caused impairment of the bacterial clearance capability. Earlier studies showed that recombinant MIF increased mortality during *E. coli* sepsis when co-injected with bacteria in mice. In these investigations the effect of recombinant MIF on bacterial loads was not reported. These findings imply that increased levels of MIF can be harmful in the acute host response against invading bacteria. In this respect it is of interest that during the immune suppressed state which occurs in the late phase of the septic response and which is characterized by a reduced capacity of immune cells to produce proinflammatory cytokines such as TNFα, it was shown that treatment with recombinant MIF could protect animals from bacterial superinfection in a mouse model of CLP-induced peritonitis. This further highlights the potential diverse nature of MIF function during the course of sepsis.

Our study has several limitations. Our observations were done in patients with sepsis caused by *B. pseudomallei* and caution is required when extending these findings to less severe or chronic melioidosis, since we focused on the early acute phase of melioidosis. Furthermore, although our *in vivo* model of melioidosis has been important in elucidating the role of other inflammatory mediators in melioidosis, data obtained from a mouse model by definition should be extrapolated to patients with melioidosis with great caution. In addition, it would be of interest to confirm our results in MIF knockout mice, although we consider it less likely that the use of these mice will yield strongly different data in light of the modest differences observed in the different treatment groups. Lastly, obtaining new biological insights from studies using antibodies and recombinant proteins of interest remains a challenge, limited by the notion of considerable cooperation between inflammatory factors involved and extensive redundancy in the host response against invading pathogens.

In conclusion, MIF levels are markedly increased during melioidosis, and elevated levels correlate with mortality. Although mice with experimentally induced melioidosis showed strongly upregulated expression of MIF in lungs and blood, inhibition of MIF with a specific antibody only modestly influenced the host response. Similarly, administration of recombinant MIF did not
strongly impact on the immune response to *B. pseudomallei* infection. These data argue against an important role for MIF in the pathogenesis of melioidosis.

**ACKNOWLEDGMENTS**

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Chapter 13


Part III

Coagulation and fibrinolysis in melioidosis
Coagulation in sepsis


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INTRODUCTION

Activation of inflammatory and coagulation pathways is an important event in the pathogenesis of sepsis. In sepsis, which can be defined as the disadvantageous systemic host response to infection, the blood coagulation system is triggered. Activation of coagulation and deposition of fibrin as a consequence of inflammation can be considered instrumental in containing inflammatory activity to the site of infection. However, inflammation-induced coagulation may be detrimental in those circumstances when the triggered blood coagulation system is insufficiently controlled, which can lead to the clinical syndrome of disseminated intravascular coagulation and microvascular trombosis. In recent years, the vital roles of several elements of the hemostatic mechanism have, in part, been unraveled, including those of tissue factor (TF), thrombin, protease-activated cell receptors (PARs) and activated protein C (APC). Clinical trials of recombinant anticoagulants for sepsis have been conducted, of which only recombinant human APC reduced the 28-day mortality of sepsis patients. This chapter focuses on the new insights in the pathogenesis of sepsis offered by the impressive amount of research that has been conducted in recent years on the coagulation system in sepsis and their potential clinical implications for intensive care medicine.

COAGULATION AND TISSUE FACTOR

TF is regarded as the one of the primary initiators of the inflammation-induced coagulation cascade. TF is constitutively expressed by different cell types in the extravascular compartment, including pericytes, cardiomyocytes, smooth muscle cells and keratinocytes. As a consequence of a disruption of the vascular integrity, TF-expressing cells located in the underlying cell layers will get into contact with bloodstream. In addition, during severe inflammation cells present in or lining the circulation, in particular monocytes and endothelial cells, will also start expressing TF. Interaction of TF with factor VIIa, which circulates at low levels in the bloodstream, results in the activation of factor X either directly, or indirectly through the activation of factor IX. Activated factor X converts prothrombin (Factor II) to thrombin, which finally induces the conversion of fibrin to fibrinogen, thereby inducing the formation of a blood clot. Amplification is required for adequate clot formation, which in particular takes place on phospholipid surfaces presented by activated platelets. Besides this more traditional role for cell-associated TF, more recent evidence points to a role for blood-borne TF in blood clotting. Indeed, microparticles bearing TF and the P-selectin glycoprotein ligand-1 (PSGL-1, a protein expressed by leukocytes) have been found essential for the formation of thrombi at sites of injury (Figure 1). Such microparticles, that can be released by monocytes upon activation by bacterial agonists or cytokines, readily bind to activated platelets through an interaction between PSGL-1 within the particle and its natural counter receptor P-selectin expressed by platelets. As a consequence, at sites of injury activated platelets and TF rich microparticles assemble, allowing for a potent and concentrated procoagulant response. Hence, activation of platelets may accelerate fibrin formation in several ways: by providing a phospholipid surface at which amplification of coagulation is facilitated and by concentrating TF rich microparticles.
The pivotal role of TF in activation of coagulation during a systemic inflammatory response syndrome, such as produced by endotoxemia or severe sepsis, has been established by many different experiments. Generation of thrombin in humans intravenously injected with a low dose of endotoxin, documented by a rise in the plasma concentrations of the prothrombin fragment F1+2 and of thrombin-antithrombin (TAT) complexes, was preceeded by an increase in TF mRNA levels in circulating blood cells, enhanced expression of TF on circulating monocytes and the release of TF containing microparticles. In line with this observation, baboons infused with a lethal dose of *Escherichia coli* demonstrated a sustained activation of coagulation, which was associated with enhanced expression of TF on circulating monocytes and the release of TF containing microparticles. In line with this observation, baboons infused with a lethal dose of *Escherichia coli* demonstrated a sustained activation of coagulation, which was associated with enhanced expression of tissue factor on circulating monocytes, and patients with severe bacterial infection have been reported to express TF activity on the surface of peripheral blood mononuclear cells. More importantly, 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 endotoxin, intravenous infusion of recombinant TF pathway inhibitor (TFPI) at two different doses caused a dose-dependent inhibition of coagulation activation. Strategies that potently inhibited coagulation activation in endotoxemic or bacteremic primates include antibodies directed against TF or factor VIIa/VIIa, active site inhibited factor VIIa (Dansyl-Glu-Gly-Arg chloromethylketone or DEGR-VIIa) and TFPI.

**ANTICOAGULANT MECHANISMS**

Blood clotting is controlled by three major anticoagulant proteins: TFPI, antithrombin and APC. TFPI is an endothelial cell derived protease inhibitor that inactivates factor VIIa bound to TF. Antithrombin inhibits factor Xa, thrombin and factor IXa, as well as factor VIIa bound to TF; these anticoagulant activities of antithrombin are accelerated by vascular heparin-like
Coagulation in sepsis

proteoglycans. The protein C system provides important control of coagulation by virtue of the capacity of APC to proteolytically inactivate factors Va and VIIIa, thereby preventing the procoagulant activities of factors Xa and IXa. In the protein C system thrombin functions as an anticoagulant; this pathway is triggered when thrombin binds to thrombomodulin on the vascular endothelium (Figure 2)\textsuperscript{11, 12}. Thrombomodulin-bound thrombin mediates the activation of protein C, an event that is augmented by the endothelial protein C receptor (EPCR). Thrombin bound to thrombomodulin is efficiently inhibited by antithrombin and protein C inhibitor. Hence, thrombomodulin inhibits coagulation in various ways: by conversion of thrombin into an activator of protein C and by accelerating the inhibition of thrombin. Moreover, the thrombin-thrombomodulin complex can activate thrombin-activatable fibrinolysis inhibitor (TAFI), an endogenous fibrinolysis inhibitor that removes C-terminal lysine residues from fibrin thereby rendering fibrin less sensitive to the action of plasmin. Protein S serves as an essential cofactor for APC. Hemostasis is further controlled by the fibrinolytic system. Plasmin is the key enzyme of this system, which degrades fibrin clots. Plasmin is generated from plasminogen by a series of proteases, most notably tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The main inhibitor of PAs is PA inhibitor-1 (PAI-1), which binds to t-PA and u-PA.

Several preclinical studies have supported the anticoagulant potencies of TFPI, antithrombin and the protein C system \textit{in vivo}. As discussed above, exogenous TFPI attenuated consumptive coagulopathy in septic primates \textsuperscript{8}. Similarly, antithrombin treatment inhibited the procoagulant response during severe sepsis in baboons \textsuperscript{13}. 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 \textit{Escherichia coli} infusion, and converted a sublethal

\textbf{Figure 2: Multiple functions of the thrombomodulin-thrombin complex.} Thrombomodulin is essential for thrombin (IIa)-mediated activation of PC (protein C), a step that is further amplified by the endothelial cell protein C receptor (EPCR). APC inactivates coagulation cofactors Va and VIIIa, thereby reducing thrombin generation, and also directly impacts on inflammation (see text). Thrombomodulin also more directly suppresses inflammation. In addition, thrombomodulin is a cofactor for thrombin-mediated activation of TAFI. Activated TAFI (TAFIa) cleaves basic C-terminal amino acid residues of its substrates, including fibrin, and thereby impairs efficient transformation of plasminogen to plasmin. TAFIa also inactivates the proinflammatory factors C3a, C5a and bradykinin. Lines ending with an arrow indicate activation/generation. Lines ending with a bullet indicate inhibition.
model produced by a LD$_{10}$ dose of *Escherichia coli* into a severe shock response associated with disseminated intravascular coagulation and death $^{14}$. Furthermore, treatment of baboons with an anti-EPCR monoclonal antibody, 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 *Escherichia coli* infection to lethal sepsis with disseminated intravascular coagulation $^{15}$. Furthermore, interference with the bioavailability of protein S by administration of C4b binding protein, causing a decrease in free protein S levels, resulted in similar changes $^{16}$.

Severe sepsis is characterized by activation of TF-dependent coagulation with concurrent inhibition of anticoagulant mechanisms: while TF procoagulant activity is markedly enhanced, the activities of TFPI, antithrombin, the protein C – APC system and fibrinolysis are all impaired, resulting in a shift toward a net procoagulant state $^{17}$. During a severe systemic inflammatory response syndrome, antithrombin levels are markedly decreased due to impaired synthesis (as a result of a negative acute phase response), degradation by elastase from activated neutrophils, and –quantitatively most importantly– consumption as a consequence of ongoing thrombin generation $^{1}$. Pro-inflammatory cytokines can also cause reduced synthesis of glycosaminoglycans on the endothelial surface, which will also contribute to reduced antithrombin function, since these glycosaminoglycans can act as physiological heparin-like cofactors of antithrombin. 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, thereby reducing the biological availability of this important cofactor for protein C. In patients with severe meningococcal sepsis this downregulation of thrombomodulin and consequent impaired activation of protein C was confirmed *in vivo* $^{18}$. Finally, fibrinolysis is impaired in sepsis, primarily due to exaggerated release of PAI-1 $^{1, 17}$.

**INTERACTION BETWEEN COAGULATION AND INFLAMMATION**

It is now generally accepted that bidirectional interactions exists between coagulation and inflammation $^{1, 2}$. Cytokines are crucial soluble mediators of inflammation. Several proinflammatory cytokines can activate the coagulation system *in vivo*, including tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-1, IL-6 and IL-12 $^{19-22}$. Importantly, although anti-TNF-$\alpha$ treatment is highly protective against mortality in experimental sepsis induced by intravenous administration of live bacteria $^{23}$, elimination of TNF-$\alpha$ does not influence activation of coagulation in models of endotoxemia and sepsis $^{24, 25}$. These data indicate that mortality and activation of coagulation are not necessarily linked phenomena. Endogenous IL-6 may be involved in coagulation activation considering that in chimpanzees injected with low dose endotoxin, treatment with an anti-IL-6 antibody prevented coagulation activation $^{26}$, although this IL-6 mediated procoagulant effect could not be confirmed in healthy humans challenged with endotoxin using another anti-IL-6 antibody $^{27}$.  

236
Interestingly, inhibition of coagulation by some but not all interventions also influence the inflammatory response during experimental bacteremia. Interventions inhibiting the TF pathway in lethal *Escherichia coli* sepsis in baboons not only prevented disseminated intravascular coagulation, but also resulted in an increased survival. These findings contrast with interventions that block the coagulation system more downstream: administration of factor Xa blocked in its active center (DEGR-Xa), failed to influence lethality of bacteremic baboons, while completely inhibiting the development of disseminated intravascular coagulation. Moreover, administration of exogenous APC or interference with the bioavailability of endogenous APC also impacts on survival in this model. In line, heterozygous protein C deficient mice demonstrated higher levels of proinflammatory cytokines and increased neutrophil invasion in their lungs after intraperitoneal injection of endotoxin. These observations have led to the hypothesis that inhibition of the VIIa-TF pathway and exogenous or endogenous APC protect against death not merely by an effect on the coagulation system, but (at least in part) through effects on inflammatory responses different from the procoagulant response.

**Table 1.** Proteases that activate PARs

<table>
<thead>
<tr>
<th>PAR</th>
<th>Proteases that activate PARs</th>
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<tbody>
<tr>
<td>PAR-1</td>
<td>thrombin, factor Xa, APC, granzyme A, trypsin</td>
</tr>
<tr>
<td>PAR-2</td>
<td>trypsin, tryptase, factor VIIa, factor Xa, proteinase 3, Der P3 D9, acrosien</td>
</tr>
<tr>
<td>PAR-3</td>
<td>thrombin</td>
</tr>
<tr>
<td>PAR-4</td>
<td>thrombin, trypsin, cathepsin G</td>
</tr>
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Proteases of the coagulation system as well as anticoagulant proteins can directly influence inflammatory processes. In this respect, PARs seem to play a pivotal role in linking coagulation and inflammation. A typical feature of PARs is that they serve as their own ligand. Proteolytic cleavage by an activated coagulation factor, including thrombin, factors VIIa and Xa and APC (Table 1), leads to exposure of a neo-amino terminus, which activates the same receptor, initiating transmembrane signalling (Figure 3). Conversely, cathepsin G from granulocytes cleaves PAR-1 at a different site from thrombin to generate a disabled receptor that cannot respond to thrombin anymore. The PAR family consists of four members, PAR-1 to PAR-4, that are localized in the vasculature on endothelial cells, mononuclear cells, platelets, fibroblasts and smooth muscle cells. Low concentrations of thrombin activate PAR-1, whereas high concentrations are required to activate PAR-3 and PAR-4. In humans, thrombin activates platelets by cleavage
of PAR-1 and PAR-4, whereas thrombin activates mouse platelets by cleavage of a PAR-3–PAR-4 complex. In primary endothelial cells, APC signalling is mediated through PAR-1, whereas TF-factor VIIa-factor Xa can signal when PAR-1 is blocked, indicating signalling through PAR-2. PAR-1 dependent APC signalling induces a number of genes that are known to down regulate proinflammatory signalling pathways and that inhibit apoptosis. Thrombin activation of PAR-1 has been shown to induce the expression of proinflammatory cytokines and chemokines in vitro. In addition, endotoxin and TNF-α induction of IL-6 expression by cultured endothelial cells is enhanced by the activation of PAR-1 and PAR-2. Endotoxin and inflammatory cytokines also induce PAR-2 and PAR-4 expression in cultured endothelial cells. Most probably, the activation of multiple PARs by coagulation proteases enhances inflammation during sepsis. This is further underscored by a recent study showing that genetically modified mice expressing low levels of TF exhibited reduced IL-6 expression and increased survival in a mouse model of endotoxemia compared with control mice. In contrast, hirudin inhibition of thrombin or a deficiency in either PAR-1 or PAR-2 did not affect IL-6 expression or mortality in their model. However, combining hirudin treatment to inhibit thrombin signaling through PAR-1 and PAR-4 with PAR-2 deficiency reduced endotoxin-induced IL-6 expression and increased survival. Taken together, these studies suggest that activation of multiple PARs by coagulation proteases may contribute to inflammation in endotoxemia and sepsis. In vivo evidence for a role of coagulation-protease stimulation of inflammation comes from recent experiments showing that the administration of recombinant factor VIIa to healthy human subjects causes a small but significant 3 to 4-fold rise in plasma levels of IL-6 and IL-8.

Antithrombin has also been found to impact on inflammation. For example, antithrombin can diminish the expression of β2 integrins on leukocytes and by binding to syndecan 4 (a proteoglycan on neutrophils) can inhibit chemokine-induced neutrophil migration. In addition, antithrombin can enhance prostacyclin formation and inhibit nuclear factor κB signaling in endothelial cells and can decrease TF expression and IL-6 production by monocytes and endothelium. Much effort has been done to elucidate the mechanisms by which APC exerts its anti-inflammatory properties. APC inhibits inflammation indirectly through reducing thrombin generation and thereby thrombin-induced inflammation via PARs. However, APC also directly attenuates inflammation by inhibiting monocyte expression of TF and TNF-α, nuclear factor κB translocation, cytokine signaling, TNF-α induced upregulation of cell surface leukocyte adhesion molecules and leukocyte-endothelial cell interactions. Thrombomodulin exerts anti-inflammatory effects at multiple levels (Figure 2). First, thrombomodulin is essential for the activation of protein C to APC; as such, thrombomodulin is key to the anti-inflammatory properties of APC. Second, the activation of TAFI requires the thrombomodulin-thrombin complex and activated TAFI has been demonstrated to suppress bradykinin activity and complement activation. Furthermore, the lectin domain of thrombomodulin likely plays a direct role in the orchestration of inflammatory reactions. Indeed, genetically modified mice that lack the N-terminal lectin-like domain of thrombomodulin displayed a reduced survival after systemic endotoxin administration, showed increased neutrophil recruitment to the lungs and responded with larger infarcts after myocardial ischemia/reperfusion injury. Importantly, deletion of the lectin-like domain of thrombomodulin did not influence the capacity of thrombomodulin to activate protein C.
indicating that the anti-inflammatory effects of this part of thrombomodulin are not mediated by APC. Finally, multiple interactions exist between inflammation and mediators of the fibrinolytic system. Fibrinolytic activators and inhibitors may modulate the inflammatory response by their effect on inflammatory cell recruitment and migration. For instance, uPAR (the receptor for u-PA) mediates leukocyte adhesion to the vascular wall or extracellular matrix components and its expression on leukocytes is strongly associated with their migratory and tissue-invasive potential. This is illustrated in a mouse model of bacterial pneumonia where uPAR deficient mice displayed a profoundly reduced neutrophil influx in the pulmonary compartment. The plasma concentrations of PAI-1 are strongly elevated in patients with sepsis, and such elevated circulating PAI-1 levels are highly predictive for an unfavorable outcome in sepsis patients. It remains to be established whether the elevated PAI-1 levels merely are indicative of a strong inflammatory response of the host, rather than bearing any pathophysiological significance. Recent findings that a sequence variation in the gene encoding PAI-1 influences the development of septic shock in patients and relatives of patients with meningococcal infection has provided circumstantial evidence that PAI-1 might play a functional role in the host response to bacterial infection.

**Figure 4: Proposed bidirectional relation between inflammation and coagulation in sepsis.** (1) Invading pathogens are recognized by the immune system through the Toll-like receptors (TLRs). After recognition, the coagulation cascade is activated by inducing tissue factor (TF) expression on monocytes and granulocytes. In sepsis decreased levels of free protein S and activated protein C (APC) are seen, ultimately leading to enhanced thrombin formation. (2) A fibrin clot with activated mononuclear cells is formed. In severe cases this may lead to disseminated intravascular coagulation. (3) The activated counteracting plasmin-mediated fibrinolysis leads to the formation of fibrin degradation products (FDP). (4) Furthermore, after binding to uPA the upregulated uPAR on monocytes and granulocytes will enhance the fibrinolytic pathway. PAI-1, which is strongly upregulated in sepsis, inhibits these fibrinolytic events. (5) Binding of among others TF and thrombin to specific PARs on inflammatory cells may affect inflammation by inducing release of proinflammatory cytokines, which will further modulate coagulation and fibrinolysis. Straight and dashed arrows indicate stimulatory and inhibitory effects, respectively.
Chapter 14

infection 40. Figure 4 presents a global overview of the bimodal interactions between coagulation and inflammation in sepsis.

COAGULATION ACTIVATION AND ORGAN FAILURE

Patients with sepsis almost invariably show evidence for activation of the coagulation system. Although the majority of these patients do not have clinical signs of disseminated intravascular coagulation, patients with a laboratory diagnosis of this syndrome are known to have a worse outcome than patients with normal coagulation parameters. A number of small clinical studies have suggested that sepsis-related disseminated intravascular coagulation is associated with not only a high mortality but also organ dysfunction and that attenuation of coagulation may ameliorate organ failure in this condition. In the placebo group of the PROWESS study (which addressed the efficacy of recombinant human APC in severe sepsis, see below) patients with disseminated intravascular coagulation displayed a mortality rate of 43% versus 27% in patients without disseminated intravascular coagulation 41. Similarly, in the KyberSept trial (addressing the efficacy antithrombin in severe sepsis, see below) 28-day mortality among placebo-treated patients with disseminated intravascular coagulation was 40% versus 22% in patients without this syndrome 42. Data obtained from a large clinical study in 840 patients with severe sepsis have further suggested a direct relationship between coagulopathy and organ failure and death 43. In this cohort both baseline coagulation abnormalities (on admission) and first-day changes in the coagulation biomarkers antithrombin, prothrombin time and D-dimer correlated with 28-day mortality. In addition, shifts in these coagulation markers during the first day of severe sepsis correlated with new organ dysfunctions, progression from single to multiple organ failure, and delayed resolution of existing organ dysfunction 43. Thrombocytopenia is a common feature of disseminated intravascular coagulation and in sepsis the extent of thrombocytopenia is correlated with an adverse outcome 44. These findings have led to the hypothesis that systemic activation of coagulation can contribute to organ failure by inducing tissue hypoxia. However, the causal link between disseminated intravascular coagulation and organ failure is still a matter of debate, arguing that if microvascular thrombosis contributes to organ dysfunction in sepsis, anticoagulants would reduce organ failure and improve outcome 45; as already alluded to above, down stream intervention in the coagulation cascade by DEGR-Xa did not influence lethality of bacteremic baboons, while completely inhibiting coagulation activation 28.

CLINICAL TRIALS WITH ANTIMOCOAGULANTS IN SEPSIS

Following from the above many new potential anti-sepsis targets have been identified. After promising results from animal studies and small Phase II trials, three specific anticoagulant
proteins were evaluated in large multinational clinical trials to test their efficacy in the treatment of severe sepsis: recombinant human APC, antithrombin and TFPI. As discussed above, during inflammation-induced activation of coagulation such as seen in severe sepsis, the function of all of these endogenous anticoagulant pathways is impaired, which provides a clear rationale for exogenous administration of these agents.

Recombinant human APC, also known as drotrecogin alfa (activated), possesses anti-inflammatory, antithrombotic, and profibrinolytic properties. In the PROWESS study, in which 1690 patients with severe sepsis were randomized to receive APC or placebo, APC significantly reduced morbidity and mortality. Patients treated with APC had a statistically and clinically significant reduction in 28-day mortality (24.7% in the treatment group and 30.8% in the placebo group; relative risk reduction, 19.4% [95% CI, 6.6%-30.5%]), representing the first published trial in sepsis showing a clear survival benefit. Importantly, APC is not effective in reducing mortality in patients with severe sepsis and a low risk of death (defined by an Acute Physiology and Chronic Health Evaluation [APACHE II] score <25 or single-organ failure).

Antithrombin has been studied in a large phase 3 clinical trial involving 2314 patients (the KyberSept study). Despite encouraging results obtained in earlier smaller trials, the phase 3 study failed to demonstrate a benefit of the use of exogenous antithrombin concentrate in patients with severe sepsis. After completion of this trial questions were raised about the dose of antithrombin used, which was lower than used in preclinical animal studies which may have influenced the possible anti-inflammatory effects of antithrombin. In addition, the concurrent use of heparin may have affected the trial results: there was a trend toward a survival benefit in patients who received antithrombin without heparin. A recent posthoc analysis of this trial has indicated that antithrombin may improve outcome in patients with disseminated intravascular coagulation not receiving heparin. In these patients antithrombin reduced 28-day mortality by 14.6% when compared with placebo. These highly interesting findings await confirmation in a prospectively designed clinical trial.

TFPI (recombinant human TFPI, tifacogin) is the third anticoagulant that was tested in a large multicenter phase 3 trial in patients with severe sepsis (the OPTIMIST trial). In spite of promising phase 2 data, treatment with TFPI failed to affect all-cause 28-day mortality. Remarkably, TFPI appeared to be relatively effective in the first half of the trial, but ineffective in the second half. Several post-hoc exploratory analyses were unable to explain this changing mortality pattern. Heparin had a clear beneficial impact on the outcome in all the three phase 3 trials described. However, a firm conclusion can not be drawn from these data since the administration of heparin was a postrandomisation event. However, the use of heparin in sepsis remains an appealing (and cheap) treatment option. It was recently shown in a murine model of disseminated intravascular coagulation that low molecular weight heparin could attenuate LPS-induced multiple organ failure. Therefore, a well-conducted randomized, placebo-controlled trial of heparin for severe sepsis is warranted.
Severe sepsis triggers clotting, diminishes the activity of natural anticoagulant mechanisms and impairs the fibrinolytic system. Augmented interactions between inflammation and coagulation can give rise to a vicious cycle, eventually leading to dramatic events such as manifested in severe sepsis and disseminated intravascular coagulation. Unraveling the role of coagulation and inflammation in sepsis will pave the way for new treatment targets in sepsis that can modify the excessive activation of these systems. At present it remains unclear whether anticoagulant therapy improves survival in severe sepsis; in addition, it remains uncertain whether the beneficial effect of recombinant human APC derives from its anticoagulant properties. It is without doubt that our knowledge on the interplay between coagulation and inflammation during sepsis will further increase in the very near future.
REFERENCES


Chapter 14

Coagulation in sepsis


Activation of coagulation with concurrent impairment of anticoagulant mechanisms correlates with a poor outcome in severe melioidosis


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Chapter 15

ABSTRACT

Background
Melioidosis, which is caused by infection with the Gram-negative bacterium *Burkholderia pseudomallei*, is an important cause of sepsis in SE-Asia with a mortality of up to 40%. Knowledge of the involvement of coagulation and fibrinolysis in the pathogenesis of melioidosis is highly limited.

Objective
To define the involvement of the coagulation and fibrinolytic systems in patients with severe melioidosis.

Methods
Parameters of coagulation and fibrinolysis were measured in 34 patients with culture proven septic melioidosis and 32 healthy controls.

Results
Patients demonstrated strong activation of the coagulation system, as reflected by high plasma levels of soluble tissue factor, the prothrombin fragment F1+2 and thrombin-antithrombin complexes (TATc), and consumption of coagulation factors resulting in a prolonged-prothrombin-time (PT) and activated-partial-thromboplastin-time (PTT). Concurrently, anticoagulant pathways were downregulated in patients: protein C, protein S and antithrombin levels were all decreased when compared to controls. Patients also demonstrated evidence of activation and inhibition of fibrinolysis, as reflected by elevated concentrations of tissue-type plasminogen activator (tPA), plasminogen-activator-inhibitor-type-1 (PAI-1), plasmin-α2-antiplasmin complexes (PAPc) and D-dimer. High TATc/PAPc-ratios in patients pointed to a predominance of the prothrombotic pathway in melioidosis. Furthermore, soluble thrombomodulin levels were increased. The extent of coagulation activation correlated with mortality; patients who went on to die had higher TATc, F1+2, tPA and PAPc and lower protein C and antithrombin levels on admission than patients who survived.

Conclusions
The coagulation system is strongly activated during melioidosis. A high degree of activation of the coagulation system is an indicator of poor outcome in patients with melioidosis.
INTRODUCTION

Melioidosis is caused by the Gram-negative bacillus *Burkholderia pseudomallei* and is an important cause of community-acquired sepsis in Southeast Asia and Northern Australia. Sepsis almost invariably leads to hemostatic abnormalities, ranging from insignificant laboratory changes to severe disseminated intravascular coagulation. Activation of inflammatory and coagulation pathways is an important event in the pathogenesis of sepsis and is known to be a significant contributor to mortality.

In recent years, the vital roles of several elements of the hemostatic mechanism have, in part, been unraveled, including those of tissue factor (TF), thrombin and the protein C pathway. As a result many new potential anti-sepsis targets have been identified. Three specific anticoagulant proteins were evaluated in large multinational clinical trials to test their efficacy in the treatment of severe sepsis: recombinant human activated protein C (APC), antithrombin and TF pathway inhibitor (TFPI), of which recombinant human APC was shown to be effective in reducing 28-day mortality of sepsis patients with a high likelihood of dying.

The underlying mechanisms responsible for the high mortality observed in severe melioidosis remain poorly understood. Similarly, our knowledge of the involvement of coagulation and fibrinolysis in the pathogenesis of melioidosis is highly limited. The aim of this study was to obtain new insights into the pathogenesis of this infection by defining the involvement of the coagulation and fibrinolytic systems in patients with severe melioidosis. We focused on the three major pathways – procoagulant, anticoagulant and fibrinolytic – that together determine the overall balance of clot formation and resolution. Furthermore, we investigated whether the extent of coagulation activation was associated with patient outcome.

MATERIALS AND METHODS

Study population

34 patients with septic melioidosis (mean age 52 years, range 18-86 years; 50% male) were recruited prospectively at Sapprasithiprasong Hospital, Ubon Ratchathani, northeast Thailand in 2004. The overall mortality rate in this group was 44%. Study design and subjects have been described in detail. Sepsis due to melioidosis was defined as culture positivity for *B. pseudomallei* from any clinical sample plus a systemic inflammatory response syndrome (SIRS). To meet the SIRS criteria, patients had to meet at least three of the following four criteria: a core temperature of ≥ 38ºC or ≤ 36 ºC; a heart rate of ≥ 90 beats/min; a respiratory rate of ≥ 20 breaths/min or a PaCO₂ of ≥ 32 mmHg or the use of mechanical ventilation for an acute respiratory process; and a white-cell count of ≥ 12 x 10⁹/l or ≤ 4 x 10⁹/l or a differential count showing > 10% immature neutrophils. Blood was drawn within 36 hours of the start of appropriate antimicrobial therapy.
32 healthy blood donors (mean age 41 years, range 21-59 years; 71% male) recruited from the Sapprasithiprasong hospital blood bank served as a control population. The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, England and written informed consent was obtained from all study subjects.

**Flow cytometric analysis**

Heparin blood samples of patients and controls were drawn from the antecubital vein and immediately put on ice. For determination of the cell surface expression of tissue factor (TF) on peripheral monocytes and granulocytes, all samples were analyzed directly by flow cytometry using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) by using fluorochrome-conjugated mouse anti-human TF antibody (BD Biosciences, San Diego, CA) in accordance with the manufacturer’s recommendations. Granulocytes were defined according to their scatter pattern and monocytes according to their scatter pattern and CD14 (BD Biosciences) positivity. To correct for non specific staining, appropriate isotype control antibodies (BD Biosciences) were used. Data on mean cell fluorescence intensity (MFI) are represented as the difference between MFI intensities of specifically stained cells and nonspecifically stained cells. Data on the number of positive cells were obtained by setting a quadrant marker for nonspecific staining.

**Evaluation of mRNA levels by quantitative RT-PCR**

Leukocytes were isolated from heparinized blood using erylysis buffer. Monocyte and granulocyte enriched populations where isolated using Polymorphprep (Axis-Shield, Dundee, United Kingdom). Monocyte and granulocyte fractions were > 98% pure as determined by their scatter pattern on flow cytometry. After isolation, leukocytes, monocytes and granulocytes were dissolved in Trizol and stored at –80 °C until used for RNA isolation. RT-PCRs were performed on cDNA samples in a LightCycler apparatus (Roche, Indianapolis, IN) as described 13. Data were analyzed using the LightCycler software as described by the manufacturer. Gene expression is presented as a ratio of the expression of the housekeeping gene β2-microglobulin 14. Primers used for human TF were S410 TGAAGGATGTGAAGCAGACG and AS583 TTGTTCCCACCTGTTC AAAA. The forward primer for the housekeeping gene was hB2M TCAATAGGACTGGATATGCGA and the reverse primer hB2M AS231 GCCCTGTACCTTGATCCTTG. Oligonucleotides were obtained from Eurogentec, Seraing, Belgium.

**Assays**

All assays were done in citrated plasma. Plasminogen activator inhibitor type 1 (PAI-1) and soluble thrombomodulin were measured using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s instructions (PAI-1: TintElize PAI-1, Biopool, Umea, Sweden; soluble TM: Diagnostica Asnieres-sur-Seine, France). The plasma concentrations of prothrombin fragment F1+2 and thrombin-antithrombin complexes (TATc) were measured by ELISA (Dade Behring, Marburg, Germany). Plasmin-α2-antiplasmin complexes (PAPc) were determined by ELISA (DRG Instruments GmbH, Marburg, Germany). Tissue-type plasminogen activator (tPA) was assayed by ELISA (Innotest, Hyphen BioMed, Andrézy, France). Soluble tissue factor (sTF)
Coagulation in melioidosis

was determined by ELISA (American Diagnostica, Stamford, CT). TFPI activity was measured on a Behring Coagulation System (Dade Behring, Marburg, Germany) according to a method described by Sandset et al\textsuperscript{15}. Protein C was determined using the Coamatic protein C activity kit from Chromogenix (Mölndal, Sweden). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Coagulation assays (prothrombin time (PT) and activated partial thromboplastin time (PTT)) were performed on an automated coagulation analyzer (MDA, bioMérieux, Durham, NC) with reagents and protocols from the manufacturer (bioMérieux). D-dimers were measured with an automated quantitative latex particle immunoassay (MDA, bioMérieux). The results of the MDA D-dimer assay are expressed as micrograms of human fibrinogen equivalent units (FEU) per millilitre of plasma. Antithrombin activity was determined with Berichrom Antithrombin (Dade Behring, Marburg, Germany) on a Behring Coagulation System (Dade Behring).

Statistical analysis

Values are expressed as means ± SEM unless indicated otherwise. Differences between groups were analyzed by Mann-Whitney U test. These analyses were performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). \( P < 0.05 \) was considered statistically significant.

RESULTS

Activation of coagulation

In patients with septic melioidosis, coagulation was significantly enhanced as reflected by elevated plasma concentrations of TATc and prothrombin fragment F1+2 compared to healthy controls (both \( P < 0.001 \) for the differences between groups, Figures 1A and B). In addition, both PT and PTT were prolonged in patients compared to controls, pointing to an increased consumption of clotting factors (both \( P < 0.001 \) for the differences between groups, figures 1C and D).

Enhanced expression of TF

TF is regarded as the primary initiator of the inflammation-induced coagulation cascade\textsuperscript{7,8}. In patients with sepsis caused by \textit{B. pseudomallei} strongly increased plasma levels of soluble TF were seen when compared to healthy controls (\( P < 0.001 \) for the differences between groups, figure 2A). Using flow cytometry, we found an upregulation of TF on the cell surface of granulocytes in patients (99.8 ± 8.4 MFI) compared to controls (63.2 ± 2.4 MFI, \( P < 0.001 \)), but not on the monocytes of patients (73.1 ± 17.1 MFI) compared to controls (57.4 ± 4.8 MFI, \( P = 0.46 \)). However, TF was found to be expressed on only 1.0 ± 0.2% of the granulocytes of patients versus on 3.1 ± 0.6% of the granulocytes of controls (\( P < 0.001 \)). Equally low numbers of TF positive cells were found in the monocyte populations: only 2.6 ± 0.3% vs 2.1 ± 0.2% vs of the monocytes of patients and controls respectively expressed TF (\( P = 0.24 \)). To gain further insights into TF expression by circulating monocytes and granulocytes during melioidosis we analyzed TF mRNA expression in isolated
Chapter 15

blood monocytes and granulocytes. We were, however, unable to detect TF mRNA expression in either cell type (data not shown). TFPI, which forms a complex with TF and blood protease factors leading to inhibition of thrombin generation and fibrin formation, was significantly upregulated in patients compared to controls ($P < 0.001$, figure 2B).

Activation and inhibition of fibrinolysis

Patients demonstrated evidence of activation and inhibition of fibrinolysis, as reflected by elevated plasma concentrations of TPA and PAI-1 ($P < 0.001$ for the differences between groups, figures 3A and B). In addition, the plasma levels of D-dimer and PAPc complexes were strongly elevated

Fig. 1. Activation of coagulation in severe melioidosis. Significant increases in plasma levels of thrombin-antithrombin complex (TATc) (A) and prothrombin fragment F1+2 (B) were seen in patients (black bars; n=34) compared to healthy controls (white bars; n=32). In addition, increases in PT (C) and PTT (D) were detected in patients compared to controls. Data are means ± SEM. *** $P < 0.001$.

Fig. 2. Enhanced expression of tissue factor TF. In patients with melioidosis increased plasma levels of soluble TF (A) were seen when compared to healthy controls. In addition, tissue factor pathway inhibitor (TFPI) was also significantly enhanced in patients (black bars) compared to controls (open bars) (B). MFI: fluorescence intensity. Data are means ± SEM. *** $P < 0.001$.

blood monocytes and granulocytes. We were, however, unable to detect TF mRNA expression in either cell type (data not shown). TFPI, which forms a complex with TF and blood protease factors leading to inhibition of thrombin generation and fibrin formation, was significantly upregulated in patients compared to controls ($P < 0.001$, figure 2B).
Coagulation in melioidosis

Fig. 3. Activation and inhibition of fibrinolysis. Activation and inhibition of fibrinolysis in melioidosis was reflected by elevated plasma concentrations of tissue-type plasminogen activator (tPA) (A) and plasminogen activator inhibitor type I (PAI-1) (B) in patients (black bars) compared to controls (white bars). In addition, patients showed strongly increased plasma levels of D-Dimer (C) and plasmin-α2-antiplasmin complex (PAPc) (D) when compared to healthy controls. To assess coagulation—fibrinolysis (im) balance, the TATc/PAPc-ratios were calculated, which were significantly higher in patients (E). Data are means ± SEM. * \( P < 0.05; *** \ P < 0.001.

in patients compared to controls (\( P < 0.001\), figures 3C and D). In order to determine whether the procoagulant or fibrinolytic pathway was dominant we analyzed the TATc/PAPc-ratios \(^{16,17}\). We found that patients displayed significantly higher TATc/PAPc ratios compared to controls pointing to a predominance of the procoagulant pathway (\( P < 0.001\), figure 3E).

Downregulation of anticoagulant pathways

The increase in coagulation and fibrinolysis factors was paralleled by a downregulation of the anticoagulant pathways: protein C, free protein S and antithrombin levels were all decreased in patients with melioidosis compared with healthy controls factors (all \( P < 0.001\) for the differences between groups, Figures 4A-C). In patients decreased protein C concentrations coincided with elevated soluble thrombomodulin levels (\( P < 0.001\), figure 4D).

Activation of coagulation and inadequate fibrinolysis correlate with clinical outcome

Having characterized the spectrum of coagulation activation in patients with melioidosis, we next sought to examine differences in the coagulation profiles between survivors and non-survivors of sepsis caused by \( B. \) pseudomallei. Table 1 shows the association between markers of coagulation and mortality in survivors (n=20) and non-survivors (n=14). In particular, patients who went on to die had significantly higher TATc, F1+2, TFPI, tPA and PAPc concentrations than those who survived (\( P < 0.05-0.001\), table 1). The ratio between coagulation activation and fibrinolysis activation (TATc/PAPc) was higher in non-survivors than survivors, suggesting inadequate fibrinolysis in patients who died (\( P < 0.01\); table 1). Moreover, non-survivors had lower levels of the anticoagulants protein C and antithrombin than survivors (\( P < 0.05-0.01\), table 1). Further
Fig. 4. Downregulation of anticoagulant pathways and upregulation of soluble thrombomodulin (sTM). Anticoagulant pathways were downregulated in patients with melioidosis (black bars) compared to controls (white bars): protein C (A), free protein S (B) and antithrombin (AT) (C) levels were decreased in patients compared to healthy controls. Moreover, sTM was upregulated in patients (D). Data are means ± SEM. *** P < 0.001.

TABLE 1. Association between markers of coagulation and mortality in patients with sepsis caused by B. pseudomallei.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Units</th>
<th>Survivors</th>
<th>Non-survivors</th>
<th>P-value</th>
</tr>
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<tr>
<td>TATc</td>
<td>μg/L</td>
<td>9.5 ± 2.2</td>
<td>54.7 ± 16.2</td>
<td>&lt; 0.001</td>
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<tr>
<td>F1+2</td>
<td>nmol/l</td>
<td>1.4 ± 0.2</td>
<td>3.2 ± 1.1</td>
<td>&lt; 0.05</td>
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<tr>
<td>sTF</td>
<td>pg/ml</td>
<td>165 ± 4</td>
<td>170 ± 10</td>
<td>Ns</td>
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<tr>
<td>TFPI</td>
<td>%</td>
<td>150 ± 9</td>
<td>292 ± 51</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>tPA</td>
<td>ng/ml</td>
<td>16.7 ± 1.5</td>
<td>41.0 ± 8.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PAI-1</td>
<td>ng/ml</td>
<td>271 ± 34</td>
<td>1140 ± 396</td>
<td>Ns</td>
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<tr>
<td>PAPc</td>
<td>μg/l</td>
<td>731 ± 56</td>
<td>1180 ± 178</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>TATc/PAPc</td>
<td>ratio</td>
<td>0.016 ± 0.004</td>
<td>0.093 ± 0.004</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Prot. C</td>
<td>%</td>
<td>66.9 ± 5.0</td>
<td>40.4 ± 5.9</td>
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</tr>
<tr>
<td>Prot. S free</td>
<td>%</td>
<td>35.4 ± 2.6</td>
<td>30.9 ± 2.5</td>
<td>Ns</td>
</tr>
<tr>
<td>AT</td>
<td>%</td>
<td>86.1 ± 3.9</td>
<td>73.2 ± 6.9</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>sTM</td>
<td>ng/ml</td>
<td>131 ± 6</td>
<td>136 ± 6</td>
<td>Ns</td>
</tr>
<tr>
<td>D-dimer</td>
<td>μg/ml</td>
<td>5.7 ± 2.4</td>
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<tr>
<td>PTT</td>
<td>sec</td>
<td>20.6 ± 0.9</td>
<td>26.5 ± 3.9</td>
<td>Ns</td>
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</table>

Correlation between markers of coagulation and mortality in survivors (n=20) and non-survivors (n=14) in patients with sepsis caused by B. pseudomallei. Values given as mean ± SEM. P values are according to the Mannn-Whitney test. Ns denotes not significant.
evidence for an association between the plasma levels of coagulation markers and disease severity was obtained in 8 patients who survived and from whom a second blood sample was drawn after successful therapy. In these patients strong decreases in plasma TATc, tPA, PAPc, sTM and D-Dimer concentrations were detected, while PTT and protein C levels both normalized (P < 0.05-0.001 for the difference between admission and discharge values; figure 5). However, plasma levels of F1+2, soluble TF, TFPI, PAI-1, free protein S and antithrombin were not yet normalized at the time of discharge (data not shown).

**Fig. 5. Normalisation of parameters of coagulation and fibrinolysis in patients that survived.** Patients (n=7-8) that survived after two weeks of intensive treatment showed near normalization of thrombin-antithrombin complex (TATc) (A), tissue-type plasminogen activator (tPA) (B), plasmin-α2-antiplasmin complex (PAPc) (C), protein C (D), soluble thrombomodulin (sTM) (E), D-Dimer (F) and PTT (G). Lines connect paired data from individual patients. * P < 0.05; ** P < 0.01; *** P < 0.001.
DISCUSSION

In the present study, we demonstrate that the coagulation system is strongly activated in severe melioidosis, involving all three major pathways – procoagulant, anticoagulant and fibrinolytic. The clinical importance of these findings is underscored by the notion that the extent of coagulation activation correlates with mortality. This sheds light on one of the possible underlying mechanisms responsible for the high mortality observed in severe melioidosis (in our cohort 44%). On admission, patients who went on to die had higher TATc, F1+2, TFPI, tPA and PAPc and lower protein C and antithrombin levels than patients who survived. Moreover, the TATc/PAPc-ratio, which provides insight into the coagulation—fibrinolysis (im)balance, was higher in patients, especially in those who did not survive. Together these data point to an overall prothrombotic state in patients with melioidosis.

Several lines of evidence indicate that TF is the primary initiator of coagulation activation in severe infection. Patients with severe bacterial infection have been reported to express TF activity on the surface of peripheral blood mononuclear cells. Importantly, inhibition of TF activity attenuated coagulation activation in models of systemic inflammation and infection. Although in our study cell-associated TF was expressed only to a very modest extent on peripheral blood granulocytes of patients with melioidosis, this does not exclude an important role for this protein in activation of coagulation. TF is constitutively expressed by different cell types in the extravascular compartment, including pericytes, cardiomyocytes and smooth muscle cells, which as a consequence of a disruption of the vascular integrity during severe infections, may get into contact with the bloodstream. Additionally, endothelial cells may express TF upon activation. Whether or not TF is expressed on neutrophils continues to be the subject of intense debate. However, neutrophils may acquire circulating TF derived from other cell types. Furthermore, one could imagine that neutrophils that express or acquire TF upon activation and will move out of the circulation towards the place of infection.

Of note, recent evidence points to a role for blood-borne TF in blood clotting: microparticles bearing TF and the P-selectin glycoprotein ligand-1 have been found important for the formation of thrombi at sites of injury. In our cohort of patients the observed upregulation of TF on granulocytes could reflect the state in which activated granulocytes instantly bind soluble TF (mainly derived from TF rich microparticles). As a consequence, the observed TF expression on the cell surface of granulocytes is far from indicative for de novo TF synthesis. Of note LPS stimulation of whole blood is associated with a direct transfer of TF from monocytes to platelets in the absence of free TF-rich microparticles, which probably is accounted for by the fusion of TF-rich microparticles with activated platelets exposing P-selectin. Indeed, a significant part of the active TF observed in patients with septic melioidosis will probably be associated with the platelets. Circulating microparticles are found in septic patients and are derived from monocytes, platelets and endothelial cells. The extent to which they contribute to the disease process is not known: recent evidence suggests that the presence of microparticles and microparticle-cell conjugates may predict a more favourable outcome in severe infections.
Coagulation in melioidosis

In our cohort of patients we did observe that the sTF levels in blood of the survivors and the non-survivors were almost identical. This could indicate that TF-rich microparticles are associated with blood cells and not abundantly available in its free form in plasma. Recently, there has been considerable interest into whether or not microparticles might have any diagnostic potential. One could question however whether or not measuring microparticles in plasma will have any diagnostic value in patients with septic melioidosis. It has been reported that increased TF production which is not balanced by TFPI promotes a poor prognosis in sepsis patients. Indeed, the increased levels of TFPI, the physiologic inhibitor of TF, were apparently relatively incapable of blocking the TF-driven activation of coagulation in our cohort of patients with severe melioidosis.

Fibrinolysis was grossly activated in our cohort of patients as shown by increased tPA, PAPc and D-Dimer levels. However, the strong release of PAI-1 probably results in a net suppression of fibrinolysis. Of note, the assay used for tPA also detects tPA complexed to its inhibitor PAI-1, which further adds to the observed high tPA antigen levels. Although it has been reported that high PAI-1 levels correlate with adverse outcome in severe sepsis, in our patient population only a trend towards higher PAI-1 values was identified in those patients who did not survive.

Our data collected in a cohort of melioidosis patients are consistent with results obtained in patients with severe sepsis caused by various organisms, confirming the notion that the vast majority of septic patients have increased markers for systemic coagulation activation, decreased anticoagulant proteins and depressed fibrinolysis. In addition, our data on reduced expression of anticoagulant mechanisms are consistent with a previous investigation reporting low circulating levels of protein C, protein S and antithrombin in patients with suspected melioidosis. Notably, activation of protein C requires binding to the thrombomodulin-thrombin complex. We here demonstrate that patients with melioidosis have decreased plasma protein C concentrations together with elevated circulating levels of soluble thrombomodulin; a further indication of a prothrombotic state in melioidosis.

This study adds to accumulating evidence demonstrating a bidirectional interaction between coagulation and inflammation. Several proinflammatory cytokines are capable of activating the coagulation system in vivo, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-12. We have recently shown that melioidosis is characterized by an increased expression of TNFα, IL-1, IL-6 and IL-12 mRNAs in whole blood leukocytes. We now show that in severe melioidosis the blood coagulation system is triggered. As a consequence, one could postulate that in melioidosis activation of coagulation and deposition of fibrin as a consequence of inflammation can be considered instrumental in containing inflammatory activity to the site of infection. However, inflammation-induced coagulation may be detrimental in those circumstances when the triggered blood coagulation system is insufficiently controlled, which can lead to the overwhelming infection with B. pseudomallei subsequently leading towards the clinical syndrome of disseminated intravascular coagulation, microvascular thrombosis and multi-organ failure. Indeed, post-mortem examinations in patients who died from acute septicaemic melioidosis have demonstrated fibrin clots as part of the pathology seen with this disease.
In conclusion, we characterize for the first time the disturbances in both the coagulation and fibrinolysis system as seen in severe melioidosis and demonstrate that strong activation of the coagulation system is an indicator of a poor outcome in patients with melioidosis. Our results can open a new window of research in the melioidosis field focusing on the interplay between coagulation and inflammation. Promising treatment strategies could include those in which the inflammatory reaction during severe melioidosis is suppressed while the activated coagulation system is inhibited. This could include combined plasma- and leukopheresis in which inflammatory cells and products are removed while new plasma provides essential inhibitors of the coagulation system.

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REFERENCES

Chapter 15

Urokinase receptor is necessary for bacterial defense against pneumonia-derived septic melioidosis by facilitating phagocytosis

Submitted

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Chapter 16

ABSTRACT

Urokinase receptor (uPAR, CD87), a glycosylphosphatidylinositol-anchored protein, is considered to play an important role in fibrinolysis and inflammation. *B. pseudomallei*, a gram-negative bacillus that is able to survive and replicate within leukocytes, causes melioidosis, which is an important cause of community acquired sepsis in Southeast-Asia. We here investigated the expression and function of uPAR both in patients with septic melioidosis and in a murine model of experimental melioidosis.

uPAR mRNA and surface expression was increased in patients with septic melioidosis in/on both peripheral blood monocytes and granulocytes as well as in the pulmonary compartment during experimental pneumonia-derived melioidosis in mice. uPAR deficient mice intranasally infected with *B. pseudomallei* showed an enhanced growth and dissemination of *B. pseudomallei* when compared to wild type mice, corresponding with increased pulmonary and hepatic inflammation. uPAR knock-out mice showed significantly reduced neutrophil migration towards the pulmonary compartment after inoculation with *B. pseudomallei* and uPAR deficient macrophages and granulocytes displayed a markedly impaired phagocytosis of *B. pseudomallei*. uPAR deficiency did not influence hemostatic and fibrinolytic responses during severe melioidosis.

In conclusion, uPAR is crucially involved in the host defense against sepsis caused by *B. pseudomallei* by facilitating the migration of neutrophils towards the primary site of infection and subsequently facilitating the phagocytosis of *B. pseudomallei*. 
Urokinase receptor (uPAR, CD87) is a glycosylphosphatidylinositol-anchored protein that functions as the receptor for urokinase-type plasminogen activator (uPA) (1). Although uPAR lacks a cytosolic domain it has the ability to transmit intracellular signals through interaction with other transmembrane proteins such as integrins, caveolin and G-protein-coupled receptors (1, 2). uPAR, which is expressed by a wide variety of cells, including monocytes, macrophages and neutrophils (1, 3), has important roles in both physiological and pathological processes: in addition to its regulatory role in fibrinolysis and inflammation, it has been implicated in tumor invasion, metastasis, urinary protein loss as well as the development of protective immunity in infections (1, 2, 4-6). Importantly, uPAR has been shown to contribute to activation and mobilisation of leukocytes (2, 7) and to play a protective role in murine pulmonary infection (7, 8). In humans, intravenous injection of lipopolysaccharide (LPS) was associated with an upregulation of uPAR on circulating monocytes and granulocytes (9, 10). However, the precise role of uPAR in human sepsis is still largely undefined.

Melioidosis, caused by the Gram-negative bacillus *Burkholderia pseudomallei*, is an important cause of community-acquired sepsis in Southeast-Asia and northern-Australia (11, 12). We recently reported that patients with melioidosis demonstrate evidence for both activation and inhibition of fibrinolysis, as reflected by concurrently elevated concentrations of tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor type I (PAI-1), accompanied by a net increase in plasmin generation as indicated by elevated plasma concentrations of plasmin-α2-antiplasmin complexes and D-dimer (13). More than half of the cases of melioidosis habitually presents with pneumonia, frequently associated with bacterial dissemination to distant sites (12, 14). Pulmonary exposure to *B. pseudomallei* rapidly elicits the recruitment of activated neutrophils (15, 16). Neutrophils play a crucial role in the host defense against various pathogens, both by direct antimicrobial activity (17) and through the production of immunoregulatory cytokines and chemokines (18). Indeed, the depletion of neutrophils in a murine model of pneumonia-derived melioidosis caused a marked exacerbation of infection (15). In *vitro* models indicate that *B. pseudomallei* survives and replicates within neutrophils and monocytes (12, 19-21). *B. pseudomallei* is very capable of escaping from phagocytosis and consequent killing. On the one hand, the capsular polysaccharide of *B. pseudomallei* contributes to resistance to phagocytosis by reducing the deposition of complement factor C3b (22), while on the other hand *B. pseudomallei* is able to quickly escape from endocytic vesicles into the cytoplasm by lysing the endosome membranes (12, 23). There are no data yet however on ways used by the host to enable effective phagocytosis of invading *B. pseudomallei*.

With the aim of clarifying the role of uPAR in pneumonia-derived melioidosis, we here investigate the expression and function of uPAR in both patients with septic melioidosis and in a murine model of experimental melioidosis. Our results show that uPAR is upregulated in melioidosis and, while its *in vivo* role in fibrinolysis is limited, it does play a key role in bacterial clearance. This is explained by both *in vitro* and *in vivo* experiments in which we show that neutrophil
recruitment and phagocytosis are uPAR-dependent mechanisms in melioidosis. Activation of uPAR and its favourable effects on antibacterial host defense represent a new host defense mechanism in melioidosis.

**METHODS**

**Human subjects**

34 patients with sepsis caused by *B. pseudomallei* and 32 healthy controls from the same area were studied. All subjects were recruited prospectively at Sapprasithiprasong Hospital, Ubon Ratchathani, Thailand in 2004. Sepsis due to melioidosis was defined as culture positivity for *B. pseudomallei* from any clinical sample plus a systemic inflammatory response syndrome (SIRS) (24). Study design and subjects have been described in detail (25). The study was approved by both the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, England. We obtained written informed consent from all subjects before the study.

**FACS-analysis**

In humans, expression of uPAR and CD14 on monocytes and neutrophils in whole blood was determined with a FACSCalibur (BectonDickinson, San Jose, CA) using fluorochrome-conjugated mouse anti-human uPAR (Pharmingen, San Diego, CA) and CD14 antibodies (BD Biosciences, Mountain View, CA) in combination with the appropriate isotype control antibodies. Granulocytes were identified according to their scatter pattern and monocytes according to their scatter pattern and CD14 positivity. In mice, blood and whole lung cell suspensions were obtained as described previously (16, 25). Immunostaining was performed using directly labelled antibodies against GR-1 (GR-1 FITC; Pharmingen), F4/80 (Serotec, Oxfordshire, UK), and a biotin-labeled antibody against uPAR (R&D Systems, Minneapolis, MN) in combination with streptavidin conjugated peridinin chlorophyll protein (Strep-PerCP). After staining, cells were fixed in 2%-paraformaldehyde. uPAR mean fluorescence intensity (MFI) was measured in the Gr-1-high gate (granulocytes), sidescatter low and F4/80 positive (monocytes) and sidescatter high and F4/80 positive (macrophages) gated populations. Antibodies were used in concentrations recommended by the manufacturer.

**Quantitative real-time-PCR**

Leukocytes were isolated from heparinised blood using erythrocyte lysis buffer. Monocyte and granulocyte enriched populations were isolated using Polymorphprep (Axis- Shield, Dundee, United Kingdom) as described (25). Monocyte and granulocyte fractions were > 98% pure as determined by their scatter pattern on flow cytometry. After isolation leukocytes, monocytes and granulocytes were dissolved in Trizol (Invitrogen, Carlsbad, CA) and stored at −80 °C until used for RNA isolation. Real-time RT-PCR was performed using LightCycler (Roche, Woerden, the Netherlands) apparatus as described (16). Gene expression is presented as a ratio of the housekeeping gene β2-microglobulin expression (hB2M) (26). Primers, purchased from Eurogentec,
Seraing, Belgium, used for human uPAR were S606 AATCCTGGAGCTTGAAAATCT and AS875 CCACCTTTATACAGGAGA.

Murine melioidosis
The Animal Care and Use of Committee of the University of Amsterdam approved all experiments. All mice were on a C57BL/6 background. Pathogen-free 8 to 10 week old wild-type mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). uPAR knock-out [KO] mice were purchased from Jackson Laboratories (Bar Harbor, ME) (27). Age- and sex-matched animals were used in each experiment. For the inoculum, B. pseudomallei strain 1026b, kindly provided by Dr. Don Woods (28, 29), was used and prepared as described (16, 25). Pneumonia was induced by intranasal inoculation of a 50 μl (5x10^2 colony forming units (CFU)/50 μl) bacterial suspension. 24, 48 and 72 hrs after infection, mice were sacrificed by bleeding from the inferior vena cava. Pulmonary homogenates, cell suspensions, peritoneal lavage and bronchoalveolar lavage fluid (BALF) were obtained from infected mice as described previously (16, 25, 30).

Assays
Tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-10 and IL-12p70 were determined using a cytometric bead array (CBA) multiplex assay (BD Biosciences). Myeloperoxidase (MPO; HyCult Biotechnology, Uden, the Netherlands), thrombin-antithrombin complexes (TATc; Dade Behring, Marburg, Germany) and D-dimer (Diagnostics Stago, Asnières-sur-Seine, France) were measured with commercially available ELISA kits. Plasminogen activator activity (PAA) was measured by an amidolytic assay. Briefly, diluted euglobulin-precipitated fractions of plasma were incubated with 0.30 mmol/l S-2251, 0.13 mol/l plasminogen and 0.12 mg/ml CNBr fragments of fibrinogen (all obtained from Chromogenix, Sweden). Conversion of plasminogen to plasmin was detected by subsequent conversion of the chromogenic substrate S-2251 and was detected with a spectrophotometer. Results are expressed in percentage increase as compared to base-line values of normal plasma (31). Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), BUN (urea) and creatinin were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO) using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Histologic examination
Organs were harvested at indicated time points, fixed in 10%-formalin and embedded in paraffin. Four μm sections were stained with hematoxylin and eosin and analyzed by a pathologist blinded for groups. To score inflammation and damage, the entire organ surface was analyzed regarding the presence of the following: necrosis/abscess formation, interstitial inflammation, endothelialitis, bronchitis, edema, thrombus formation and, when applicable, pleuritis (25, 32). Neutrophils were counted in 6 randomly chosen fields (x100 magnification) as described (33). Granulocyte staining was done as described earlier (8). Fibrin(ogen) stainings were performed as earlier described (34) after which digital images were captured of three non overlapping areas (20x objective) using a DFC500 digital camera mounted on a DM5000B microscope (both from Leica Microsystems, Wetzlar, Germany). The area for positive for fibrin(ogen) was determined with Image Pro Plus software (Media Cybernetics, Silver Spring, MD) and expressed as the percentage of the total surface area.

265
Chapter 16

Cell culture and stimulation

Whole blood and peritoneal macrophages from untreated uPAR KO and WT mice (n = 5–8/strain) were harvested as described (16, 25, 30). Cells and heparinized whole blood were stimulated with lipopolysaccharide (LPS) from *B. pseudomallei* 1026b (25) (500 ng/ml), Mitomycin-C (0.2 mg/ml) (Sigma-Aldrich, Zwijndrecht, the Netherlands) treated growth-arrested *B. pseudomallei* (5 x 10^6 CFU/ml), or RPMI 1640 medium for 16h. Supernatants were collected and stored at –20°C until assayed.

Bacterial killing and phagocytosis

Bacterial killing was determined as described previously (30, 35). In brief, *B. pseudomallei* was spun onto a monolayer of peritoneal macrophages (derived from 5 different mice per group), after which plates were placed at 37°C for 10 minutes. After washing 5 times with ice-cold PBS to remove extracellular bacteria, bacterial uptake after 10 minutes was determined by lysing the wells with sterile dH_2O. This was designated as t=0. RPMI was added to remaining wells and plates were placed at 37°C for 5 and 30 minutes after which cells were washed and lysed with dH_2O. Cell-lysates were plated on blood agar plates and bacterial counts were enumerated after 16h. Bacterial killing was expressed as the percentage of killed bacteria in relation to t=0. Phagocytosis was evaluated essentially as described before (30, 36). Growth-arrested *B. pseudomallei* was labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE dye, Invitrogen, Breda, The Netherlands). Peritoneal macrophages (derived from 5 different mice per group) were incubated with growth-arrested CFSE-labeled *B. pseudomallei* (2.5x10^7 CFU/ml) for 0, 15 and 60 minutes. Phagocytosis was stopped by placing cells on ice; thereafter cells were washed in PBS and suspended in Quenching solution (Orpegen, Heidelberg, Germany). To determine the neutrophil phagocytosis capacity, 50 μl of whole blood was incubated with bacteria after which cells were suspended in Quenching solution, incubated in FACS lysis/fix solution (BecktonDickinson) and neutrophils were labeled using anti-Gr-1-PE (Pharmingen). Phagocytosis was determined using FACS. Phagocytosis index of each sample was calculated: (mean fluorescence x % positive cells at 37ºC) minus (mean fluorescence x % positive cells at 4ºC).

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). Differences between groups were analyzed by Mann-Whitney U test or Kruskal-Wallis analysis with Dunn’s posthoc test where appropriate. These analyses were performed using GraphPad Prism version 4.03, GraphPad Software (San Diego, CA). Values of *P* < 0.05 were considered statistically significant.

RESULTS

Increased uPAR expression in patients with severe melioidosis

We quantified uPAR mRNA and surface expression on both peripheral blood monocytes and granulocytes in 34 individuals with culture-proven severe melioidosis and 32 healthy controls.
Patients showed profoundly elevated levels of both uPAR mRNA and cell surface expression compared to controls (Fig. 1). In addition, an overall increase in the percentage of monocytes expressing uPAR on their cell membranes was seen in patients compared to controls (97.7 % ± 2.3 vs 80.1 % ± 3.1, \(P < 0.0001\)), together with a modest decline in the percentage of uPAR positive granulocytes (74.6 % ± 4.6 vs 89.9 % ± 2.3, \(P < 0.05\)). In this cohort of patients, in which the mortality rate was 44%, the levels of either cell-associated uPAR or uPAR mRNA did not differ between survivors and non-survivors (data not shown).

**Increased uPAR expression in the pulmonary compartment during experimental pneumonia-derived melioidosis**

Since the majority of severe melioidosis cases present with pneumonia with bacterial dissemination to distant body sites (11, 12, 14) and considering the fact that it is not feasible to study uPAR expression at tissue level in patients with melioidosis, we used a murine model of pneumonia-derived melioidosis in which mice are intranasally infected with *B. pseudomallei* (16, 25, 37). In line with the data obtained in patients with melioidosis, 48 hours after infection mice showed an upregulation of uPAR expression on their granulocytes, monocytes (Fig. 2) and alveolar macrophages (data not shown). The increase in uPAR expression was much more pronounced at the primary site of infection, the pulmonary compartment, when compared...
Chapter 16

Figure 2. Urokinase receptor (uPAR) is upregulated in murine melioidosis. Cell-surface uPAR expression on blood monocytes (A), blood granulocytes (B) and pulmonary monocytes (C) and pulmonary granulocytes (D) 48 hours after intranasal inoculation with *B. pseudomallei* (gray bars) compared to saline treated control mice (white bars); n = 8 per group; MFI, mean fluorescence intensity; *P < 0.05.

Figure 3. uPAR deficiency results in an enhanced growth and dissemination of *B. pseudomallei in vivo.* uPAR knock-out (KO) mice (gray bars) demonstrate strongly increased bacterial loads at 48 and 72 hours after infection in their lungs (A), liver (B) and blood (C) compared to wildtype (WT) mice (white bars; n = 8 per group per time point). CFU: colony forming unit; *P < 0.05; **P < 0.01; ***P < 0.001.
to the systemic compartment where the observed increase in uPAR expression did not reach statistical significance (Fig. 2).

**uPAR deficient mice show an enhanced growth and dissemination of B. pseudomallei in vivo**

To obtain insights into the functional role of uPAR in melioidosis, we infected uPAR KO and WT mice with *B. pseudomallei* and performed quantitative cultures of lung, liver and blood at various time points thereafter. Relative to WT mice, uPAR KO mice displayed strongly increased bacterial loads in the lungs, liver and blood at 48 and 72 hours after intranasal infection with a lethal dose of *B. pseudomallei* (Fig. 3).

**uPAR KO mice display increased late lung inflammation, but decreased early neutrophil migration during melioidosis**

To further evaluate the role of uPAR in antibacterial defense against *B. pseudomallei*, pulmonary inflammation and granulocyte recruitment into lung tissue were assessed. Consistent with the observed enhanced growth of *B. pseudomallei* in uPAR KO mice, uPAR KO mice showed increased late pulmonary inflammation, which was characterized by significantly more inflammation, pleuritis, peribronchial inflammation, oedema and endothelialitis when compared to control mice (Fig. 4). Strikingly however, early pulmonary neutrophil recruitment was impaired in uPAR KO mice.
Chapter 16

KO mice in response to intranasal infection with \textit{B. pseudomallei} as visualized by Ly-6 staining and confirmed by lower MPO concentrations in lung homogenates at 24 hours after infection (Fig. 5).

Limited effect of uPAR deficiency on cytokine response and distant organ injury

Because the localized production of cytokines is an important part of host defense against infection (38), we measured the concentrations of these mediators in the pulmonary and systemic compartment. Overall, uPAR deficiency did not have a major impact on cytokine concentrations after infection with \textit{B. pseudomallei} (Table I). The most notable difference was a mean 50% reduction in TNF\(\alpha\) levels in lung homogenates of uPAR KO mice at 24 hours after inoculation in uPAR KO mice when compared to WT mice (C), corresponding with decreased early MPO activity levels in lung tissues in uPAR KO mice (D). Magnification, x400. White bars represent WT mice, gray bars represent uPAR KO mice (n=8 per group at each time point). Number of granulocytes expressed as mean number of granulocytes per field ± SEM. MPO, myeloperoxidase. * \(P < 0.05\); ** \(P < 0.01\).

Figure 5. Decreased early granulocyte migration in urokinase receptor (uPAR) KO mice infected with \textit{B. pseudomallei}. Representative of Ly6G-immunostaining for granulocytes of WT (A) and uPAR KO mice (B) showing significantly less neutrophil influx at 24 hours post inoculation in uPAR KO mice when compared to WT mice (C), corresponding with decreased early MPO activity levels in lung tissues in uPAR KO mice (D). Magnification, x400. White bars represent WT mice, gray bars represent uPAR KO mice (n=8 per group at each time point). Number of granulocytes expressed as mean number of granulocytes per field ± SEM. MPO, myeloperoxidase. * \(P < 0.05\); ** \(P < 0.01\).

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Table 1. Cytokine response in lung homogenates and plasma of WT and urokinase receptor (uPAR) KO mice during melioidosis

<table>
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<th>T = 24 h</th>
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<th>T = 72 h</th>
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<tr>
<td></td>
<td>WT</td>
<td>uPAR KO</td>
<td>WT</td>
</tr>
<tr>
<td><strong>Lung homogenate</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TNF-α</td>
<td>850 ± 133</td>
<td>418 ± 107*</td>
<td>1326 ± 241</td>
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<tr>
<td>IL-6</td>
<td>1472 ± 128</td>
<td>1484 ± 343</td>
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<td>120 ± 25</td>
<td>102 ± 21</td>
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<tr>
<td>IL-12p70</td>
<td>32 ± 7</td>
<td>26 ± 7</td>
<td>38 ± 10</td>
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<tr>
<td>IFN-γ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>TNF-α</td>
<td>20 ± 3</td>
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<tr>
<td>IFN-γ</td>
<td>ND</td>
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Pulmonary and systemic cytokine levels after intranasal infection with $5 \times 10^9$ CFU *B. pseudomallei*. Wildtype (WT) and uPAR KO mice were sacrificed 24, 48 or 72 h after infection. Data are means ± SEM of eight mice per group per time point. TNF-α = Tumor necrosis factor-α; IL = Interleukin; IFN-γ = Interferon-γ; ND = not detectable. * P < 0.05; ** P < 0.01; *** P < 0.001.

With these pathology data, the plasma levels of ASAT (1284 ± 457 vs 183 ± 45, P < 0.05) and ALAT (814 ± 351 vs 86 ± 21, P < 0.05) were higher in uPAR KO mice 72 hours post infection compared to WT mice, reflecting increased hepatocellular injury in these animals. Additionally, all mice showed evidence of renal failure, as indicated by elevated plasma concentrations of urea (12.5 ± 4.4 vs 7.5 ± 0.6, not significant) and creatinine (19.2 ± 9.3 vs 8.5 ± 0.5, not significant), however no differences were seen between uPAR KO and WT mice.

**uPAR does not contribute to cellular responsiveness to *B. pseudomallei* in vitro**

To obtain additional insights into the function of uPAR in the host defense against *B. pseudomallei*, we started to analyse the requirement of uPAR signaling upon first encounter between the bacterium and the host. Therefore, we tested the cytokine production capacity of macrophages and whole blood harvested from WT and uPAR KO mice upon stimulation with *B. pseudomallei* LPS or growth-arrested *B. pseudomallei* (effector:target ratio 1:10). Whole blood and peritoneal macrophages obtained from uPAR KO mice released equal amounts of TNF-α, IFN-γ, IL-6, IL-10 and IL-12p70 upon stimulation with *B. pseudomallei in vitro* as compared to WT mice (data not shown). These data suggest that uPAR does not contribute to cellular responsiveness to *B. pseudomallei in vitro*. 

271
Chapter 16

uPAR deficiency does not influence hemostatic and fibrinolytic responses during severe melioidosis

Since uPAR is thought to play a regulatory role in fibrinolysis (1, 6), we measured pulmonary TATc, D-dimer, PAA and fibrin levels in both WT and uPAR KO mice after intranasal inoculation with \( B. \) pseudomallei. No differences in thrombin generation, as reflected by TATc plasma levels between WT and uPAR KO mice were seen (Fig. 6). To investigate whether the upregulated uPAR expression influenced the fibrinolytic activity, we measured D-dimer and PAA levels. No differences were observed in D-dimer and PAA levels between uPAR KO and WT mice (Fig. 6). Finally, measurement of the extent of fibrin deposition in lung tissue showed increased fibrin accumulation after infection but again no differences between uPAR KO and WT mice (Fig. 6). Together these data do not support a major role of uPAR in fibrinolysis and coagulation during melioidosis.

Impaired phagocytosis of \( B. \) pseudomallei in uPAR deficient macrophages and granulocytes

The experiments described above established that uPAR KO display a diminished antibacterial defense towards \( B. \) pseudomallei infection, characterized by increased bacterial loads, accompanied by diminished early recruitment of neutrophils to the primary site of infection and reduced
neutrophil MPO levels. We next wished to determine whether uPAR contributes to phagocytosis and/or killing of *B. pseudomallei*. No difference in the killing capacity between WT and uPAR KO cell was observed (Fig. 7). However, both uPAR KO macrophages and uPAR KO granulocytes demonstrated a markedly diminished capacity to phagocytose *B. pseudomallei* (Fig. 7). Taken together, the observed impairment of bacterial clearance in uPAR KO mice can be explained by both a reduction of early neutrophil recruitment in addition to a diminished phagocytosis capacity of these recruited uPAR deficient immune cells.

**DISCUSSION**

We here show that uPAR is upregulated in severe melioidosis and plays a major role in the antibacterial innate immune response. During melioidosis, uPAR contributes to the recruitment of neutrophils to the primary site of infection and the capacity of neutrophils to phagocytose *B. pseudomallei*. As a consequence, uPAR KO mice displayed a markedly impaired clearance of *B. pseudomallei* in the pulmonary and systemic compartment upon intranasal infection together with increased lung and liver inflammation. uPAR did not impact on the fibrinolytic response to infection with *B. pseudomallei*. These data are the first to describe a role for uPAR in melioidosis and further add to our understanding of how infection with this facultative intracellular organism can lead to a full blown septic illness.

Our study is the first to provide insights into the expression of both mRNA and protein cell surface uPAR expression in a cohort of patients with sepsis. In blood samples obtained from 34 prospectively enrolled patients with sepsis caused by *B. pseudomallei*, we showed that the increased uPAR mRNA expression is accompanied by enhanced uPAR surface expression on both monocytes and granulocytes. Since we were also interested in uPAR expression at the primary infection site and given the fact that pneumonia with bacterial dissemination to distant body sites is a common presentation of human melioidosis (11, 12, 14), we made use of a mouse model
of melioidosis in which mice are intranasally infected with a lethal dose of *B. pseudomallei* (16, 25, 37). By doing so we were able to demonstrate increased expression of uPAR on both monocytes and granulocytes at the primary place of infection. These data are in accordance with previous data showing an increased release of uPAR in both plasma and urine of patients with urosepsis (39) and increased uPAR cell surface expression on monocytes of healthy volunteers injected with endotoxin (9, 10).

Leukocyte trafficking into pulmonary tissue and airspaces is a critical component of an adequate host defense response (40) and uPAR has been shown to play a key role in both neutrophil migration and activation (2, 7, 41). uPAR can facilitate cell migration in two ways: first, after binding to uPA it facilitates the generation of plasmin at the cell surface, resulting in degradation of the extracellular matrix and the induction of cell migration (1, 2); second, uPAR causes the activation and mobilisation of leukocytes through interaction with β2-integrins, most notably CD11b/CD18 (2, 7). In the event of critical illness due to invasion of pathogens, uPAR has been shown to be important in the recruitment of leukocytes towards the primary site of infection in pneumococcal meningitis (42) and both *Pseudomonas aeruginosa* and pneumococcal pneumonia (7, 8). We now underwrite these earlier reports by showing that uPAR KO mice have an impaired host defense against *B. pseudomallei* as indicated by increased bacterial outgrowth and increased organ inflammation accompanied by a reduced early neutrophil migration towards the primary site of infection. This fully underscores the emerging insight that neutrophils play a vital role in host defense against *B. pseudomallei* (15).

Our results showing a markedly impaired host defense in mice lacking uPAR after inoculation with *B. pseudomallei* can be explained not only by a diminished neutrophil recruitment towards the pulmonary compartment but also by the finding that uPAR is crucially involved in phagocytosis of *B. pseudomallei*. Since *B. pseudomallei* is a very virulent and facultative intracellular organism (12, 19-21) effective killing and phagocytosis are of paramount important during melioidosis. We and others have previously reported on the potential role of uPAR in the phagocytosis of *E. coli* and *P. aeruginosa* (41, 43). We now extend these findings by showing that uPAR is necessary for effective phagocytosis of *B. pseudomallei* by both macrophages and neutrophils. This newly described mechanism provides new insights of how the immune system is capable of mounting an effective host response against *B. pseudomallei*. Interestingly, uPAR does not play a direct role in the killing of *B. pseudomallei*. Furthermore, in preliminary experiments we were not able to show a direct interaction between uPAR and *B. pseudomallei* (data not shown), suggesting the existence of additional important mediators in this uPAR phagocytosis pathway.

Our study not only demonstrates the important role of uPAR in neutrophil migration and phagocytosis during melioidosis but also reveals the relative unimportance of uPAR in the fibrinolytic response during sepsis caused by *B. pseudomallei*. In patients with melioidosis we have recently shown that the fibrinolytic system is both activated and inhibited as reflected by elevated concentrations of tPA, PAI-1, PAPc and D-dimer (13). Our current data argue against a major role of uPAR in fibrinolysis since there were no differences in D-dimer and PAA expression levels between uPAR KO and WT mice during experimental melioidosis. Moreover, fibrin deposition was equal in
both mice strains after infection. Interestingly, these data are in line with previous investigations (2). For instance, fibrin deposits were only found in the livers of adult mice with a dual deficiency in uPAR and tPA but not in uPAR KO mice (44). In animal models of lung injury and septic shock reduced uPA-mediated proteolysis correlated with excessive fibrin deposition, suggesting that uPA facilitates fibrinolysis by an uPAR independent mechanism (2, 45, 46). Clearly, the role of the fibrinolytic system in the host defense against \textit{B. pseudomallei} remains to be elucidated. Studies making use of uPA, tPA and PAI-1 deficient animal are underway in our laboratory.

In conclusion, we here show that uPAR is crucially involved in the host defense against \textit{B. pseudomallei} by facilitating the migration of neutrophils towards the primary site of infection and subsequently facilitating the phagocytosis of \textit{B. pseudomallei}. Activation of uPAR and its favourable effects on antibacterial host defense represent a new host defense mechanism in melioidosis. Manipulation of uPAR expression or function may be a target for immunomodulation in septic melioidosis.

**ACKNOWLEDGMENTS**

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Chapter 16

REFERENCES


Chapter 16


Chapter 17

Summary, general discussion and conclusion
Summary, general discussion and conclusion

SUMMARY

Melioidosis is caused by the aerobic gram-negative soil-dwelling bacillus *Burkholderia pseudomallei* and is an important cause of community-acquired sepsis in Southeast Asia and Northern Australia. The high associated mortality rate, wide availability from the environment in endemic areas, intrinsic resistance to many antibiotics and the potential for aerosol spread has made this organism a potential bioterror agent. **Chapter 2** gives a general introduction into the world of melioidosis and highlights recent advances in this research field. Recent advances include the description of the genome of *B. pseudomallei* which consists of a large chromosome (4.07 Mb) carrying genes mainly associated with cell growth and metabolism, and a smaller chromosome (3.17 Mb) which has a greater proportion of genes encoding accessory functions such as adaptation and survival in different environments. No single *B. pseudomallei* determinant has been shown to have a role in virulence during human disease, persistence or latency. However, putative virulence factors include quorum sensing, type III secretion system, capsular polysaccharide and, with less conclusive evidence, lipopolysaccharide (LPS) and flagella. *B. pseudomallei* is an intracellular pathogen that multiplies within macrophages. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α play important roles in early resistance against *B. pseudomallei* infection. Although more is becoming known about the pathogenesis of this bacterium in disease, host-pathogen interactions are still ill defined. This thesis aims to increase our insight into the pathogenesis of melioidosis with a strong focus on the innate immune response to *B. pseudomallei*.

In **part I** of this thesis we further characterize the innate immune response during melioidosis. **Chapter 3** gives an introduction to our murine model of melioidosis. In this article we determined differences in inflammatory patterns elicited by virulent and avirulent strains of *Burkholderia* by comparing differences in the host response of C57BL/6 mice after intranasal inoculation with either *B. pseudomallei*-1026b, a clinical virulent-isolate, *B. pseudomallei*-AJ1D8, an *in-vitro* invasion deficient mutant generated from strain 1026b by Tn5-OT182-mutagenesis or *B. thailandensis*, which is considered avirulent. Mice infected with *B. thailandensis* showed markedly decreased bacterial outgrowth from lungs, spleen and blood 24-hours after inoculation compared to animals infected with *B. pseudomallei* or the invasion mutant AJ1D8. 48-hours after inoculation *B. thailandensis* was no longer detectable. This was consistent with elevated pulmonary cytokine and chemokine concentrations after infection with *B. pseudomallei*-1026b and AJ1D8, and the absence of these mediators 48-hours, but not 24-hours, after inoculation with *B. thailandensis*. Histological examination however did show marked pulmonary inflammation in mice infected with *B. thailandensis*, corresponding with a substantial granulocyte-influx and raised myeloperoxidase (MPO)-levels. Survival experiments showed that infection with 1x10³ CFU *B. thailandensis* was not lethal, whereas inoculation with 1x10⁶ CFU *B. thailandensis* was equally lethal as 1x10³ CFU *B. pseudomallei*-1026b or AJ1D8. These data show that *B. pseudomallei*-AJ1D8 is just as lethal as wild-type *B. pseudomallei* in an *in-vivo* mouse model and that *B. thailandensis* is perhaps more virulent than is often recognized.
Chapter 17

Infection with virulent \textit{B. pseudomallei} can lead to overwhelming septic illness. Sepsis is characterized by an uncontrolled inflammatory response to invading microorganisms. In chapter 4 we describe the inflammatory mRNA-profile of whole blood leukocytes, monocytes and granulocytes using the multigene-system multiplex ligation-dependent probe amplification (MLPA) assay for 35 inflammatory markers that included pro- and anti-inflammatory cytokines, chemokines and signal transduction molecules in a case-control study with 34 patients with sepsis caused \textit{B. pseudomallei} and 32 healthy volunteers. Relative to healthy controls, septic patients showed increased transcription of a whole array of inflammatory genes in peripheral blood leukocytes, granulocytes and monocytes. Specific monocyte and granulocyte mRNA-profiles were identified. Strong correlations were found between inflammatory mRNA expression levels in monocytes and clinical outcome. These data underline the notion that circulating leukocytes are an important source for inflammatory mediators in patients with gram-negative sepsis such as melioidosis.

This chapter also shows that gene expression profiling such as done here provides an excellent tool to obtain insight in the extent of inflammation activation in patients with severe infection. Knowledge of the inflammatory gene expression profile in the pulmonary compartment after infection with \textit{B. pseudomallei} however is highly limited. This is important since septic melioidosis is often associated with pneumonia and bacterial dissemination to distant sites. Since it is not feasible to study the local inflammatory gene expression profile in humans with melioidosis, in chapter 5 we used our mouse model to characterize the inflammatory mRNA-profile in the pulmonary and systemic compartment during murine melioidosis. Using the MLPA assay we determined the expression profile of a whole range of genes encoding inflammatory proteins in lung tissue, leukocytes in bronchoalveolar lavage fluid (BALF) and blood leukocytes in mice before and at several time points after intranasal infection with \textit{B. pseudomallei}. Relative to naïve mice, mice intranasally infected with \textit{B. pseudomallei} showed increased transcription of a whole array of genes involved in inflammation, Toll-like receptor-signaling, coagulation, fibrinolysis, cell adhesion, tissue repair and homeostasis in the lung, BALF and blood compartment. Notably, many inflammatory genes showed to be differentially expressed during the course of infection. These data provide new information on compartmentalized inflammatory gene-expression profiles after infection with \textit{B. pseudomallei}, increasing our insight into the extent of inflammation activation in the pulmonary and systemic compartment during melioidosis. One of these mediators is IL-18, which is known to partially control the production of IFN-\(\gamma\). Therefore, we studied the role of IL-18 in the immune response to \textit{B. pseudomallei} (chapter 6). Plasma IL-18 and blood monocyte IL-18 mRNA levels were found to be markedly elevated in patients with septic melioidosis when compared to healthy controls. Additionally, IL-18 binding protein levels were markedly elevated in patients, strongly correlating with mortality. Furthermore, IL-18 knock-out (KO) mice showed an accelerated mortality after intranasal infection with a lethal dose of \textit{B. pseudomallei}, which was accompanied by an enhanced bacterial growth in their lungs, liver, spleen, kidneys and blood at 24 and 48 hours post infection when compared to wild type (WT) mice. Moreover, IL-18 KO mice displayed evidence of enhanced hepatocellular injury and renal insufficiency. Together these data indicate that the enhanced production of IL-18 in melioidosis is an essential part of a protective immune response to this severe infection.
The second part, part II, of this thesis focuses on the potential role of TLR in the host response against *B. pseudomallei*. TLRs are essential in host defense against pathogens by virtue of their capacity to detect microbes and initiate the immune response. TLR2 is seen as the most important receptor for gram-positive bacteria, while TLR4 is regarded as the gram-negative TLR. In general, the immune activation that follows TLR activation will be sufficient to combat the wide variety of pathogens that daily try to invade the human body. However, in the case of sepsis these TLR-mediated responses may exceed the threshold to maintain homeostasis of the immune system.

Chapter 7 provides an introduction to this part of the thesis and focuses on the new insights in the pathogenesis of sepsis that is offered by the impressive amount of research that has been conducted in the TLR research field and their potential clinical implications for intensive care medicine. In chapter 8 we aimed to characterize the expression and function of TLRs in septic melioidosis. We found that patients with melioidosis display an upregulation of multiple TLRs in peripheral blood monocytes and granulocytes. Patients had increased expression of CD14, TLR1, TLR2 and TLR4 on the cell surface of monocytes and granulocytes and increased CD14, TLR1, TLR2, TLR4, MD-2, TLR5 and TLR10 mRNA levels in purified monocytes and granulocytes when compared with healthy controls. With *in vitro* experiments we showed that whole blood and alveolar macrophages obtained from TLR2 and TLR4 KO mice are less responsive to *B. pseudomallei*, whereas in the reverse experiment transfection of HEK 293 cells with either TLR2 or TLR4 rendered these cells responsive to this bacterium. In addition, we found that the LPS of *B. pseudomallei* signals through TLR2 and not through TLR4. Surprisingly, TLR4 KO mice were indistinguishable from WT mice with respect to bacterial outgrowth and survival in experimentally induced melioidosis. In contrast, TLR2 KO mice displayed a markedly improved host defense as reflected by a strong survival advantage together with decreased bacterial loads, reduced lung inflammation and less distant organ injury. We here conclude that although both TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei in vitro*, TLR2 detects the LPS of *B. pseudomallei* and only TLR2 impacts on the immune response of the intact host *in vivo*. MyD88 (myeloid differentiation primary-response gene 88) and TIR domain-containing adaptor protein-inducing interferon-β (TRIF) are regarded as the key signaling adaptor proteins for TLRs.

Chapter 9 investigates the role of MyD88 and TRIF in the host defense against melioidosis. MyD88, but not TRIF, deficient whole blood leukocytes released less TNF-α upon stimulation with *B. pseudomallei* compared to WT cells. Subsequently we inoculated MyD88 KO, TRIF mutant and WT mice intranasally with *B. pseudomallei* and found that MyD88 KO, but not TRIF mutant mice demonstrated a strongly accelerated lethality, which was accompanied by significantly increased bacterial loads in lungs, liver and blood, and grossly enhanced liver damage compared to WT mice. The decreased bacterial clearance capacity of MyD88 KO mice was accompanied by a markedly reduced early pulmonary neutrophil recruitment and a diminished activation of neutrophils after infection with *B. pseudomallei*. MyD88 KO leukocytes displayed an unaltered capacity to phagocytose and kill *B. pseudomallei in vitro*. Taken together, MyD88 dependent signaling, but not TRIF dependent signaling, contributes to a protective host response against *B. pseudomallei* at least in part by causing early neutrophil recruitment towards the primary site of infection. Finally, chapter 10 shows that CD14 is crucially involved in the recognition of *B. pseudomallei* by innate immune cells but plays a remarkable detrimental role in the host response against *B. pseudomallei*. CD14-deficient macrophages and whole blood leukocytes released less TNF-α.

283
Chapter 17

upon stimulation with *B. pseudomallei* or *B. pseudomallei* LPS *in vitro* compared to WT-cells. Strikingly, CD14 KO mice intranasally inoculated with *B. pseudomallei* demonstrated reduced lethality, accompanied by significantly decreased bacterial outgrowth compared to WT mice. Administration of recombinant soluble CD14 to CD14 KO mice partially reversed their phenotype into that of a WT-mouse. Lastly, CD14 deficiency did not alter the capacity of macrophages or neutrophils to phagocytose or kill *B. pseudomallei*.

The next set of papers focuses on the question how the TLR-signaling cascade is regulated during melioidosis. Sepsis is associated with immunosuppression (characterized by a reduced capacity of circulating monocytes to release proinflammatory cytokines), which has been implicated in late mortality. Previous *in vitro* and murine studies have suggested a key role for so called negative regulators of the TLR-signaling pathway in immunosuppression. In *chapter 11*, we investigated the expression of these negative TLR regulators in patients with septic melioidosis in association with the responsiveness of peripheral blood leukocytes of these patients to LPS and *B. pseudomallei*. In accordance with an immunosuppressed state, whole blood of patients demonstrated a strongly decreased capacity to release the proinflammatory cytokines TNF-α, IL-1β and the chemokine IL-8 after *ex vivo* stimulation with LPS or *B. pseudomallei*. Analysis of MyD88-short, IRAK-M, IRAK-1, SOCS-3, SHIP-1, SIGIRR and A20 mRNA expression in purified mononuclear cells showed decreased IRAK-1 and elevated IRAK-M expression in patients with septic melioidosis. Immunosuppression was correlated with mortality; furthermore patients who went on to die had higher IRAK-M mRNA levels on admission than patients who survived. To sum up, immunosuppression in sepsis caused by *B. pseudomallei* is associated with an up-regulation of IRAK-M and an indicator of poor outcome. *Chapter 12* in contrast focuses on an amplifier of the TLR-cascade, named triggering receptor expressed on myeloid cells (TREM)-1. In patients with melioidosis, we found increased soluble-TREM-1 plasma levels and TREM-1 surface-expression on monocytes, but not granulocytes. Similarly, mice inoculated with *B.pseudomallei* displayed a gradual rise in soluble-TREM-1 and an increase in blood monocyte but not granulocyte TREM-1 expression. At the primary infection-site, however, granulocyte TREM-1 expression was enhanced and the rise in soluble-TREM-1 occurred earlier. Additionally, purified human TREM-1-`granulocytes showed reduced responsiveness to *B.pseudomallei* relative to TREM-1-`granulocytes, a difference not detected for TREM-1-` and TREM-1-`monocytes. Treatment with a peptide mimicking a conserved-domain of soluble-TREM-1 partially protected mice from *B.pseudomallei* induced lethality. This study shows that during melioidosis TREM-1 expression is differentially regulated on granulocytes and monocytes; measurements of TREM-1-expression on blood granulocytes may not provide adequate information on granulocyte TREM-1-expression at the infection site. Lastly, *chapter 13* describes studies on macrophage migration inhibitory factor (MIF), which has been shown to be a main effector molecule in severe sepsis. MIF levels are shown to be markedly elevated during clinical melioidosis and to correlate with patient’s outcome. In experimental melioidosis MIF modestly impaired antibacterial defense. Mice inoculated intranasally with *B. pseudomallei* displayed a robust increase in pulmonary and systemic MIF expression. Anti-MIF treated mice showed lower bacterial loads in their lungs upon infection with a low inoculum. Conversely, mice treated with recombinant MIF displayed a modestly impaired clearance of *B. pseudomallei*. 
In the last decade the bidirectional relation between inflammation and coagulation has been well established. Knowledge of the involvement of coagulation and fibrinolysis in the pathogenesis of melioidosis however is highly limited. Therefore, part III focuses on both coagulation and fibrinolysis in melioidosis. In sepsis the blood coagulation system is triggered. Chapter 14 focuses on the new insights in the pathogenesis of sepsis offered by the impressive amount of research that has been conducted in recent years on the coagulation system in sepsis. Chapter 15 defines the involvement of the coagulation and fibrinolytic systems in patients with severe melioidosis. Patients demonstrated strong activation of the coagulation system, as reflected by high plasma levels of soluble tissue factor, the prothrombin fragment F1+2 and thrombin-antithrombin complexes (TATc), and consumption of coagulation factors resulting in a prolonged PT and PTT. Concurrently, anticoagulant pathways were downregulated in patients: protein C, protein S and antithrombin levels were all decreased when compared to controls. Patients also demonstrated evidence of activation and inhibition of fibrinolysis, as reflected by elevated concentrations of tissue-type plasminogen activator (tPA), PAI-1 (plasminogen activator inhibitor type 1), PAPc (plasmin-α2-antiplasmin complexes) and D-dimer. High TATc/PAPc-ratios in patients pointed to a predominance of the prothrombotic pathway in melioidosis. Furthermore, soluble thrombomodulin levels were increased. The extent of coagulation activation correlated with mortality; patients who went on to die had higher TATc, F1+2, tPA and PAPc and lower protein C and antithrombin levels on admission than patients who survived. This shows that the coagulation system is strongly activated during melioidosis. A high degree of activation of the coagulation system is an indicator of poor outcome in patients with melioidosis. Chapter 16 further focuses on uPAR, a glycosylphosphatidylinositol-anchored protein, which is considered to play an important role in fibrinolysis and inflammation. uPAR mRNA and surface protein expression was found to be increased in patients with septic melioidosis in/on both peripheral blood monocytes and granulocytes as well as in the pulmonary compartment during experimental pneumonia-derived melioidosis in mice. uPAR deficient mice intranasally infected with B. pseudomallei showed an enhanced growth and dissemination of B. pseudomallei when compared to WT mice, corresponding with increased pulmonary and hepatic inflammation. uPAR KO mice showed significantly reduced neutrophil migration towards the pulmonary compartment after inoculation with B. pseudomallei and uPAR deficient macrophages and granulocytes displayed a markedly impaired phagocytosis of B. pseudomallei. Interestingly, uPAR deficiency did not influence hemostatic and fibrinolytic responses during severe melioidosis. Taken together, uPAR is crucially involved in host defense against sepsis caused by B. pseudomallei by facilitating the migration of neutrophils towards the primary site of infection and subsequently facilitating the phagocytosis of B. pseudomallei.

GENERAL DISCUSSION

Severe melioidosis can probably be seen as the clinical manifestation of a TLR mediated dysregulation of the immune response to invading B. pseudomallei. Sepsis has always been regarded as the result of an exacerbated detrimental inflammatory response to invading bacteria. However, recent
Figure. Proposed sequence of events in the pathogenesis of septic melioidosis. Invasion of *B. pseudomallei* and the septic response: the pro- and anti-inflammatory pathways in septic melioidosis. Invading bacteria are recognized by pathogen recognition receptors, such as the Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) proteins, after which a proinflammatory response is initiated via nuclear factor (NF)-κB. Next to the release of proinflammatory cytokines, the antibacterial response is characterized by activation of both the complement and coagulation systems. Triggering receptor expressed on myeloid cells (TREM)-1 serves as an amplifier of the TLR-initiated inflammatory response. The counteracting anti-inflammatory response is subsequently characterized by negative regulators of the TLRs such as ST2, the release of anti-inflammatory cytokines, apoptosis of lymphocytes, activation of the nicotinic anti-inflammatory pathway, and the release of anticoagulant proteins. Eventually, the balance between the pro- and anti-inflammatory reactions will determine the outcome of the septic response. Please note: the role of among others the NODs, the complement system, ST2, lymphocyte apoptosis and the nicotine pathway have not yet been studied in the context of melioidosis and still have to be elucidated for melioidosis. The here proposed sequence of events in the pathogenesis of septic melioidosis has partially been derived from research on sepsis caused by other bacteria. α7nAChR: α7-nicotinic acetylcholine receptor; APC: active protein C; HMGB-1: high-mobility group box 1 protein; IL: interleukin; IL-1RA: IL-1 receptor antagonist; IRAK-M: IL-1R-associated-kinase-M; MyD88: myeloid differentiation primary-response protein 88; ST2: TGF: transforming growth factor; TF: tissue factor; TFPI: tissue factor pathway inhibitor; TNF: tumor necrosis factor.

Figure adapted from: Wiersinga WJ and van der Poll T, Is the septic response good or bad, Current Infectious Disease Reports 2007, 9:366–373.
insights such as described in this thesis have forced us to reconsider this sepsis paradigm. Indeed, septic patients can die from the initial exacerbated hyperinflammatory response, but remarkably, most patients will succumb during the following extended period of immunodepression. Likewise, sepsis will cause triggering of the coagulation system while diminishing the activity of both natural anticoagulant mechanisms and the fibrinolytic system. Augmented interactions between inflammation and coagulation can give rise to a vicious cycle, eventually leading to dramatic events such as those manifested in septic melioidosis. A careful balance between the inflammatory and anti-inflammatory response is vital in order to survive a potential deadly invasion by *B. pseudomallei*. The Figure gives a proposed sequence of events in the pathogenesis of septic melioidosis. Please note however that the role of among others the nucleotide-binding oligodimerization domain (NOD) proteins, the complement system, the negative TLR-regulator ST2, lymphocyte apoptosis and nicotine pathway have not, or only to a very limited extent, been studied in the context of melioidosis. Their potential role in the host defense against *B. pseudomallei* has been based on research on sepsis caused by other pathogens and their role in melioidosis have to be elucidated\(^1\), \(^2\). Taken together, one could probably state that without a good inflammatory response to invading *B. pseudomallei* all infected patients will succumb, however too much of an inflammatory response will also lead to a fatal outcome.

### Table 1. Potential new immunomodulating treatment strategies for acute septic melioidosis

<table>
<thead>
<tr>
<th>Target or strategy</th>
<th>Potential mechanism</th>
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<tbody>
<tr>
<td>CpG</td>
<td>Unmethylated CpG motifs in synthetic oligodeoxynucleotide can enhance the uptake of bacteria by mouse macrophages. CpG treatment one hour before bacterial inoculation offers protection in a murine model of melioidosis(^6).</td>
</tr>
<tr>
<td>G-CSF</td>
<td>G-CSF increases neutrophil count and stimulates neutrophil function. Receipt of G-CSF is associated with a longer duration of survival but is not associated with a mortality benefit in patients with severe sepsis who are suspected of having melioidosis in Thailand(^7).</td>
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<tr>
<td>activated protein C</td>
<td>Recombinant human activated protein C (rhAPC) is a natural anticoagulant with potentially important anti-inflammatory properties. Treatment with recombinant human activated protein C reduces mortality in a cohort of adults with severe sepsis(^8), a finding that was not seen in children with severe sepsis(^9).</td>
</tr>
<tr>
<td>CD14</td>
<td>CD14 deficient mice are partially protected against a lethal dose of <em>B. pseudomallei</em>. Inhibition of CD14 in addition to antibiotics might give a survival advantage (this thesis).</td>
</tr>
<tr>
<td>TLR2</td>
<td>TLR2 deficient mice are partially protected against a lethal dose of <em>B. pseudomallei</em> through a yet unknown mechanism. Inhibition of TLR2 in addition to antibiotics might give a survival advantage (this thesis).</td>
</tr>
<tr>
<td>TREM-1</td>
<td>TREM-1 serves as an amplifier of the TLR-cascade. Treatment with a peptide mimicking a conserved-domain of soluble-TREM-1 partially protects mice from <em>B. pseudomallei</em> induced lethality (this thesis).</td>
</tr>
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G-CSF: granulocyte-colony stimulating factor; TLR: Toll-like receptor; TREM-1: Triggering receptor expressed on myeloid cells-1
### Chapter 17

**Table 2. Questions for future research**

<table>
<thead>
<tr>
<th><strong>Epidemiology</strong></th>
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<tr>
<td>To what extent will new global environmental sampling studies and improvements in diagnostic microbiology give a more complete picture of the geographical distribution of <em>Burkholderia pseudomallei</em>? What is the real burden of disease from melioidosis worldwide?</td>
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<tr>
<td>Are there any geographical or environmental differences that can account for the varying states of endemicity in regions with a climate that resembles highly endemic northeast Thailand, such as Vietnam and Laos?</td>
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<tr>
<th><strong>Virulence and pathogenesis</strong></th>
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<tr>
<td>What is the precise role of putative genomic islands in virulence?</td>
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<tr>
<td>Will the comparison of the genetic structure of isolates from the environment with those associated with invasive</td>
<td></td>
</tr>
<tr>
<td>Will the comparison of the genetic structure of isolates from the environment with those associated with invasive disease define whether some clones are more adept at causing melioidosis than others?</td>
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<tr>
<td>To what extent is LPS an important factor in the virulence and pathogenicity of <em>B. pseudomallei</em>?</td>
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<tr>
<td>Why is <em>B. pseudomallei</em> acute suppurative parotitis almost exclusively seen in Thai paediatric cases?</td>
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<tr>
<td>Why is person-to-person transmission so rare despite the high number of bacteria detected in the sputum of patients?</td>
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<tr>
<td>What is the mechanism that enables <em>B. pseudomallei</em> to hide from host defences for years or decades?</td>
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<tr>
<td>What mechanism renders diabetes such a disproportionately important risk factor for disease acquisition?</td>
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<tr>
<td>How is <em>B. pseudomallei</em> recognized by the innate immune system on first encounter?</td>
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<tr>
<td>What is the role of CD4+ lymphocytes in the control of infection by <em>B. pseudomallei</em>?</td>
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<tr>
<th><strong>Treatment and prevention</strong></th>
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<tr>
<td>Which treatment option is most effective at reducing the high disease-relapse rates? What combination and duration of antibiotics is optimal?</td>
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<tr>
<td>To what extent will better clinical care (including preventative measures, earlier clinical identification and better management of severe sepsis) improve the outcome of melioidosis in endemic areas?</td>
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<tr>
<td>What will be the optimal vaccine strategy? And to what extent will a vaccine prevent severe disease due to <em>B. pseudomallei</em>?</td>
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**FUTURE DIRECTIONS**

Despite several decades of clinical research, the mortality rate for septic melioidosis remains unacceptably high (20 to 50%, depending on regional differences). Current studies are underway to address whether meropenem is superior to ceftazidime during parenteral therapy, and whether doxycycline is a necessary component of oral treatment. Several possible vaccine candidates have been identified and studied in animals, but no vaccine will be available in the near future. Studies on host-pathogen interactions in melioidosis have not only shed light on pathogenic mechanisms, but have also identified potential new treatment targets (Table 1). However, many key questions await investigation. Table 2 summarizes some important questions for future research.
research on *B. pseudomallei* pathogenesis. The ongoing hunt to dissect pathways and discover new treatment targets for severe infections is intimidating and irresistible at the same time.

## CONCLUSION

These studies significantly contribute to our knowledge of the role of TLRs and the innate immune system in both sepsis and melioidosis. The current results have challenged the paradigm that TLR4 is the exclusive receptor for all gram-negative bacteria. Severe melioidosis can probably be seen as the clinical manifestation of a TLR mediated dysregulation of the immune response to invading *B. pseudomallei*. The characterization of the innate immune response to *B. pseudomallei* could well contribute to the development of new preventive and therapeutic strategies in patients with melioidosis: TLR2, CD14 and TREM-1 are identified as potential new treatment targets. “Ik heb gezegd en hierna is twijfel niet meer mogelijk (sic)”.

## REFERENCES

SAMENVATTING VOOR NIET-INGEWIJDEN

Dit proefschrift richt zich op de rol van de Toll-like receptoren (TLR) bij de afweer tegen binnendringende micro-organismen, in het bijzonder de Gram-negatieve bacterie Burkholderia pseudomallei, de verwekker van melioidosis. De TLR zijn een recent ontdekte klasse van receptoren (“voelsprieten”) op (maar ook in) allerlei cellen van het menselijk lichaam die onder andere betrokken zijn bij het herkennen bacteriën, schimmels en virussen. Melioidosis is een belangrijke oorzaak van sepsis, ook wel bloedvergiftiging genoemd, in zuidoost Azië en noord Australië. De hoge mortaliteit, de makkelijke toegankelijkheid, de intrinsieke resistentie tegen veel antibiotica en de mogelijkheid om het via aerosolen te kunnen verspreiden, hebben B. pseudomallei tot een potentieel bioterroristische wapen gemaakt. Het doel van de in dit proefschrift beschreven onderzoekslijn is om meer inzicht te verkrijgen in de pathogenese van melioidosis. De volgende samenvatting beschrijft kort wat de belangrijkste onderzoeksresultaten zijn zoals beschreven in dit boek en geeft tevens een overzicht van de mogelijke implicaties hiervan voor zowel het onderzoeksveld als de klinische praktijk.

Deel 1

In het eerste deel van dit proefschrift wordt de aangeboren afweer tijdens sepsis veroorzaakt door B. pseudomallei nader bestudeerd en bestaat onder meer uit een samenvattend overzichtsartikel van de bestaande wetenschappelijke literatuur over de bacterie B. pseudomallei en de ziekte melioidosis die het kan veroorzaken (hoofdstuk 2). Factoren die bijdragen aan het krijgen van de ziekte in endemische gebieden zijn o.a. de weersomstandigheden (bv. overstromingen) en de integriteit van het immuun systeem van de gastheer. Melioidosis kan zich uiten als een fulminant verlopend septisch ziektebeeld, maar ook al een meer op tuberculose lijkende chronische ziekte. Mogelijke virulentie factoren van B. pseudomallei zijn het type III secretie systeem, capsulaire polysacchariden, lipopolysaccharide (LPS) en flagella. B. pseudomallei is een intracellulair pathoogeen dat zichzelf kan vermeerderen in macr ofagen. Daarnaast weten we dat de cytokines IFN-γ en TNF-α belangrijk zijn voor een effectieve afweer tegen B. pseudomallei. Er is echter nog maar weinig bekend over de precieze interacties tussen gastheer en pathoogeen. Hoofdstuk 3 beschrijft het door ons opgezette muizen model voor melioidosis. C57BL/6 muizen worden hierbij via de luchtwegen geïnfecteerd met verschillende doseringen B. pseudomallei waarna de immuunrespons in kaart wordt gebracht door onder meer te kijken naar de bacteriële uitgroei, de cytokine response en de ontstane schade in verschillende organen. Meer specifiek wordt de immuun response beschreven die uitgelokt wordt door infectie met zowel virulente als avirulente Burkholderia species. Muizen die geïnfecteerd worden met de als niet-pathoogeen beschouwde B. thailandensis laten inderdaad minder bacteriële uitgroei en een verminderde cytokine response dan muizen geïnfecteerd met B. pseudomallei of B. pseudomallei-AJ1D8, een invasie deficiënte mutant. Histologische evaluatie laat echter zien dan de met B. thailandensis geïnfecteerde muizen wel degelijk ernstige longschade hebben, corresponderend met een forse toestroom en activatie van granulocyten. Overlevingsstudies laten zien dat inoculatie met 1 x 10^6 CFU B. thailandensis even lethaal is als inoculatie met 1 x 10^5 CFU B. pseudomallei of B. pseudomallei-
Deze data laten zien dat *B. thailandensis* waarschijnlijk meer virulent is dan vaak wordt aangenomen. Zoals gezegd kan een *B. pseudomallei* infectie tot een fulminante septische ziekte leiden. Sepsis wordt gekarakteriseerd door een ongecontroleerd inflammatoire respons op binnendringende micro-organismen. In *hoofdstuk 4* wordt het inflammatoire mRNA-profiel beschreven van circulerende leukocyten (witte bloedlichaampjes), monocyten en granulocyten met behulp van een *multigene-system multiplex ligation-dependent probe amplification (MLPA)* assay in een case-control studie met 34 patiënten met sepsis veroorzaakt door *B. pseudomallei* en 32 gezonde vrijwilligers. Vergeleken met controles, laten de leukocyten van septische patiënten een opregulatie zien van een heel scala aan proinflammatoire genen. Hierbij worden specifieke mRNA-profile voor de monocyten en granulocyten geïdentificeerd. Deze mRNA profielen blijken gecorreleerd te zijn aan ziekte mortaliteit. Verder laten deze data zien dat circulerende cellululaire leukocyten een belangrijke rol voor inflammatoire mediators zijn in patiënten met sepsis. Aangezien melioidosis vaak begint met een longontsteking wordt in *hoofdstuk 5* ons muizenmodel voor melioidosis gebruikt om met behulp van MLPA ook naar het inflammatoire genexpressie profiel te kijken in de longen en longlavaten. Vergeleken met naïeve muizen, laten muizen die geïnfecteerd waren met *B. pseudomallei* een opregulatie zien van genen die betrokken zijn bij inflammatie, de TLR, stolling, fibrinolysis en celades. Deze data geven ons nieuwe inzichten in de gecompartimentaliseerde inflammatoire genexpressie tijdens melioidosis. Een van deze mediators is IL-18, waarvan we weten dat het gedeeltelijk de productie van IFN-$\gamma$ controleert. Daarom wordt in *hoofdstuk 6* specifiek de rol van IL-18 tijdens melioidosis onderzocht. Vergeleken met gezonde controles zijn de plasma IL-18 en bloed monocyte IL-18 mRNA waarden opgereguleerd bij patiënten met melioidosis. IL-18 deficiënte muizen laten een verhoogde mortaliteit zien ten opzichte van wildtype muizen na infectie met *B. pseudomallei*. Dit komt overeen met meer bacteriële uitgroei in hun longen, lever, milt, nieren en bloed. IL-18 knock-out (KO) muizen hebben ernstiger nier- en leverfalen dan de controle muizen. Bij elkaar genomen laat dit zien dat een toegenomen productie van IL-18 een essentieel onderdeel is van een beschermende immuun response tijdens melioidosis.

**Deel II**

In het tweede deel wordt de rol van de TLR tijdens de immuun respons nader onderzocht. TLR zijn essentieel in de defensie tegen bacteriën omdat zij als eerste bacteriën herkennen en de immuun respons in gang zetten (*hoofdstuk 7*). TLR2 wordt gezien als de belangrijkste TLR voor Gram-positieve bacteriën en TLR4 voor Gram-negatieve bacteriën. In *hoofdstuk 8* laten we zien dat een heel scala aan TLR opgereguleerd worden bij patiënten met melioidosis. Zeer verrassend echter blijkt de virulentie factor LPS van deze Gram-negatieve bacterie niet via TLR4 te signaleren - wat men zo verwacht -, maar via TLR2. Muizen die het gen missen dat voor TLR2 codeert hebben een opvallend groot overlevingsdeel wanneer ze geïnfecteerd worden met *B. pseudomallei*. Dit haalt het paradigma onderuit dat TLR4 de belangrijkste TLR voor Gram-negatieve bacteriën is en identificeert TLR2 ook als aantrekkelijk aangrijpingspunt voor toekomstige therapieën tegen melioidosis. De andere hoofdstukken in dit deel (*hoofdstuk 9 t/m 13*) onderzoeken de rol van de aan de TLR geëxprimeerde eiwitten MyD88, TRIF, CD14, IRAK-4, TREM-1 en MIF. Hierin worden ook CD14 en TREM-1 geïdentificeerd als mogelijk nieuwe therapeutische targets. Opvallende genoeg is een negatieve regulator van de
Deel III
Het laatste deel, deel drie, van dit proefschrift gaat over de relatie tussen inflammatie en stolling tijdens sepsis. Na een inleiding (hoofdstuk 14), wordt de precieze activatie van het stollingsysteem tijdens melioidosis voor het eerst gekarakteriseerd (hoofdstuk 15). Patiënten met melioidosis laten een sterke activatie van het stollingsysteem zien (hoge plasma waarden van tissue factor, de prothrombine fragmenten F1+2 en TATc) en verbruik van stollingsfactoren resulterend in verlengde stollingstijden. Tegelijkertijd worden lagere waarden van de anticoagulante eiwitten (proteïne C, proteïne S en antithrombine) gezien in vergelijking met gezonde controle personen. Daarnaast wordt zowel activatie als remming van de fibrinolyse gezien: de waarden voor tPA, PAI-1, PAPc en D-dimer zijn verhoogd. De mate van stollingsactivatie blijkt te correleren met de klinische uitkomst: patiënten die overleden aan melioidosis hadden bij opname hogere waarden van TATc, F1+2, tPA en PAPc en lagere waarden van proteïne C en antithrombine. Er bestaan meerdere eiwitten die een te sterke activatie van de stolling kunnen verhinderen. uPAR zou een van deze eiwitten zijn en haar rol tijdens infectie met B. pseudomallei wordt nader onderzocht in hoofdstuk 16. Het blijkt echter dat uPAR niet zozeer voor de stolling en antistolling van belang is tijdens melioidosis, maar juist een belangrijke rol speelt bij het opruimen (phagocyteren) van binnendringende koloniën van B. pseudomallei.

Tot slot
Sepsis is altijd beschouwd als het resultaat van een te hevige, ontsproorde en dus schadelijke reactie van het immuunapparaat op een infectie. Nieuwe inzichten zoals ook beschreven in dit proefschrift dwingen ons dit beeld bij te stellen. Weliswaar kunnen patiënten met sepsis komen te overlijden tijdens de eerste fase van hyperinflammatie, maar de hoogste mortaliteit komt voor bij de overlevenden van deze periode waarin het immuunsysteem juist minder reactief is, de zogenaamde immunodepressieve fase. Een goede balans tussen pro- en anti-inflammatoire response blijkt essentieel om een potentieel dodelijke invasie van B. pseudomallei te overleven. Sepsis en ook melioidosis kunnen waarschijnlijk gezien worden als een door TLR gemedieerde dysregulatie van de immuun response op binnendringende bacteriën. Ondanks een paar decaden van wetenschappelijk onderzoek naar de ziekte melioidosis blijft de sterfte aan deze aandoening echter onacceptabel hoog (20 tot 50% afhankelijk van de regio). In de Algemene Discussie wordt een blik geworpen op de therapeutische mogelijkheden die op basis van de hier beschreven nieuwe (biochemische) inzichten ontwikkeld kunnen worden in de strijd tegen melioidosis en ernstige sepsis (hoofdstuk 17). Dit proefschrift identificeert TLR2, CD14 en TREM-1 als potentiële nieuwe therapeutische aangrijpingspunten voor de behandeling van melioidosis. Er blijven echter nog veel vragen onbeantwoord. Essentiële vragen voor toekomstig wetenschappelijk onderzoek op het gebied van melioidosis zijn onder te verdelen in de epidemiologie, pathogenese en behandeling van deze ziekte. De geografische distributie van B. pseudomallei in de wereld is nog onvoldoende onderzocht. Komt B. pseudomallei ook voor in tropisch Afrika? Waarom is de incidentie van melioidosis juist zo hoog in noordoost Thailand? De virulentie factoren van B. pseudomallei zijn ook nog niet goed gekarakteriseerd. In hoeverre is het LPS van B. pseudomallei belangrijk voor...
DANKWOORD

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