The role of platelets in sepsis

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Chapter 3

Thrombocytopenia impairs host defense against *Burkholderia pseudomallei* (melioidosis)

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ABSTRACT

Infection with the Gram-negative bacillus *Burkholderia pseudomallei* (melioidosis) is an important cause of pneumonia and sepsis in Southeast Asia and has a mortality of up to 40% despite antibiotic treatment. As platelets are increasingly being recognized as key players in inflammation and immunity, we aimed to assess the role of platelets in the host response against *B. pseudomallei* infection. Admission platelet counts were determined in 1160 patients with culture-proven melioidosis. Thrombocytopenia, defined as platelets below $150x10^9$/L, was present in 31% of patients at admission and strongly correlated with mortality. During experimental murine melioidosis, induced by intranasal inoculation of *B. pseudomallei,* mice also developed thrombocytopenia, mimicking the clinical scenario. Mice treated with a platelet-depleting antibody (depletion to <5% of normal platelet counts) showed enhanced mortality during melioidosis compared to controls. Thrombocytopenic mice demonstrated enhanced bacterial growth in lungs, bronchoalveolar lavage fluid, and the liver. Platelets did not directly influence *B. pseudomallei* growth nor did they impact on neutrophil extracellular trap formation. Low platelet counts however did have a modest impact on early pulmonary neutrophil influx as well as local and systemic proinflammatory cytokine responses. Additional studies using platelet specific MyD88 deficient mice (lacking platelet Toll-like receptor signaling) and functional Glycoprotein (GP)Ibα deficient mice were conducted. This showed that mice lacking the platelet membrane glycoprotein GPIbα have reduced platelets counts during *B. pseudomallei* infections together with an impaired local host defense in the lung. Reminiscent of their role in hemostasis, platelet depletion impaired vascular integrity, resulting in early lung bleeding during experimental melioidosis. In summary, thrombocytopenia is associated with enhanced mortality in melioidosis patients and during experimental melioidosis, platelets play a protective role in both innate immunity and vascular integrity.

INTRODUCTION

Burkholderia pseudomallei is a Gram-negative environmental bacterium and the aetiological agent of melioidosis, a life-threatening infection that often presents with pneumonia and sepsis and mainly occurs in southeast Asia and northern Australia[1,2]. Despite appropriate antibiotic treatment mortality rates remain high, ranging from 10-40%[1]. Not surprisingly, melioidosis may be regarded as a good model to study Gram-negative sepsis[3,4]. A 2016 modelling study estimated that there are 165.000 cases, and 89.000 deaths annually. This global burden of melioidosis is much larger than previously thought[5]. Additionally, due to its high lethality, severity of disease, intrinsic resistance to common antibiotics and potential for easy dissemination, *B. pseudomallei* is declared as a Tier 1 biological threat agent. New insights in the pathogenesis of melioidosis are urgently needed in order to develop novel adjunctive treatment strategies.

Platelets, anuclear cells derived from megakaryocytes, are of vital importance for hemostasis. In recent years, it has become clear that platelets also play an important role in inflammation and immunity[6-8]. As an example, platelets have been described to express several immune related receptors such as Toll-like receptors which are of importance for microbial surveillance and regulation of inflammatory and immune responses[9-11]. Indeed, platelets can aid in the host defense against infection[6,9,12] and can influence inflammation both in the lung and the systemic compartment[6,13,14]. In murine studies, platelets have been shown to alter immune responses by influencing leukocyte functions and recruitment[6,15,16]. In patients with sepsis, low platelet counts could dysregulate immune responses by decreasing leukocyte adhesion signaling[17].

In line, we recently demonstrated that thrombocytopenia is a key feature of melioidosis and is correlated with mortality[18]. Von Willebrand factor (which can bind and activate platelets via platelet glycoprotein (GP)Ibα) levels are elevated in patients with melioidosis[18]. Apart from this data, the role of platelets in this disease is ill-defined. Therefore, we aimed to study the role of platelets in melioidosis, using both human and murine studies. By investigating a large cohort of melioidosis patients and a clinically relevant murine model of melioidosis, we were able to show that platelets aid in the local host defense during melioidosis.

METHODS

Cohort study

Patients presenting to Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand, with culture-confirmed melioidosis were prospectively included between 1 January 2002 and 31 December 2006. Patients were eligible if it was their first admission for culture-confirmed melioidosis, with patients younger than 15 years of age excluded (because pediatric cases have a different clinical presentation and prognosis). There were no other exclusion criteria. This cohort has been previously described elswhere[19]. Patients were stratified into three groups according to platelet counts at presentation; low platelets count (<100x10^9/L), intermediate low platelet counts (100-150x10^9/L), or normal platelet counts (>150x10^9/L). Boundaries were based on previous studies[17,20]. Outcome in the group with admission platelet counts <50x10^9/L and 50–100x10^9/L were not different (the confidence interval for one group was entirely contained within the confidence interval for the other). Analysis of other parameters did not reveal relevant differences between these groups. The results reported are therefore for the two groups combined for all analyses. Approval was obtained from the Ethical and Scientific Review subcommittee of the Thai Ministry of Public Health to use information collected during the cohort study. Written informed consent was obtained from all subjects. All procedures performed were in accordance with the the Helsinki Declaration of 1975 (revised 1983).

Animals

For platelet depletion experiment, specific pathogen-free C57Bl/6 mice (Charles River, France) were used. Platelet-specific MyD88 knock-out (Plt-MyD88$^{-/-}$) mice were generated as previously described [21]. IL4R/GPIbα mice (University of Arkansas for Medical Sciences, Little Rock, USA) are knock out for mouse GPIbα, without the associated macrothrombocytopenia that is prevented by transgenic expression of a protein chimera that consists of the extracellular part of the IL-4 receptor and the intracellular part of GPIbα[22]. Control mice were kept with co-housing. All genetically modified mice were backcrossed >6 times to a C57Bl/6 genetic background. Mice were housed in Animal Research Institute AMC facility under standard care and received standard rodent chow and water ad libitum. All experiments were conducted with mice between 8 and 12

weeks of age. Samples were randomized if applicable. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments. Experiments were carried out in accordance with the Dutch Experiments on Animals Act.

Experimental study design

Meliodosis was induced by intranasal inoculation with *B. pseudomallei* 350-500 colony forming units (CFU) in 50 µL (isotonic saline), as previously described[23-25]. 2 hours before infection C57Bl/6 mice were intravenously injected with platelet depleting antibody (polyclonal anti-mouse-GPIb alpha, 0.4 or 2 µg/g) or control immunoglobulin (Ig)G (both Emfret Analytics, Eibelstadt, Germany)[12]. To assess the effect of platelet depletion on survival, mice were observed for 10 days (n=20 per group) and clinical symptoms were scored with an independent animal biotechnician, unaware of group allocation, as previously described[12]. The clinical observation score consisted of the following parameters: solitude (0, absent; 1, present), posture (0, normal; 1, sphere), fur (0, normal; 1, pilo-erection), eyes (0, open; 1, closed; 2, dirty), alertness (0, normal; 1, slow; 2, apathetic; 3, non-responsive), pace (0, normal; 1, shaky; 2, collapse), respiration (0, normal; 1, heavy; 2, slow; 3, intermittent) and time to ascent when laid down (0, normal; 1, < 5 s; 2, > 5 s; 3, unresponsive). Mice were euthanized 24, 48 or 72 hours after induction of infection (n=8 per group); non-infected mice were sacrificed simultaneously (n=4 per group). Lungs for pathology and bronchoalveolar lavage fluid (BALF) were obtained in separate experiments to avoid dilution of samples.

Flow cytometry

Murine whole blood samples were assessed by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). Murine platelet counts were measured using hamster anti-mouse-CD61 mAb (BioLegend, San Diego, CA). GpIb expression was assessed by anti-mouse GPIba (Clone:Xia3, Emfret analytics). Platelet–neutrophil complex formation was determined by using rat anti-mouse-CD11b mAb (BD Biosciences, San Diego, CA, USA), rat anti-mouse-CD115 mAb (eBioscience, San Diego, CA, USA) and rat-anti-mouse Ly-6 mAb (BD Biosciences) in combination with anti-CD61 mAb.

Pathology

The paraffin embedded left lung lobe was cut into four-micrometer sections and stained with hematoxylin and eosin (H&E). Slides were coded and scoreed by a pathologist blinded for group identity as previously described[12,26]. In the lung bleeding was scored (0-4) and in the liver the following parameters were scored 0-4: inflammation, presence of necrosis/abscess formation, presence of thrombi and bleeding. The total histopathology score was calculated as the sum of the scores of all individual parameters. To determine neutrophil influx in the lung, sections were stained with anti-mouse Ly6G mAb (BioLegend). After staining, expression was quantified by digital image analysis: slides were scanned with the Olympus Slide system (Olympus dotSlide, Tokyo, Japan) to generate TIFF images of the full tissue section. Ly-6G positivity was measured using Image J (U.S. National Institutes of Health, Bethesda, MD); the amount of positivity was expressed as percentage of the total lung surface area.

Protein measurements

Interleukin (IL-)6, Tumor necrosis factor (TNF-)α, C-C motif ligand (CCL)2 and Interferon (IFN)-y were determined with a commercially available cytometric beads array multiplex assay (BD Biosciences). Myeloperoxidase (MPO) and C-X-C motif ligand (CXCL)2 were measured by ELISA (all R&D systems, Minneapolis, MN) as well as thrombin-antithrombin complex (TATc) levels (Affinity Biologicals Inc., Hamilton, Canada). Fibrin products were determined by western blot by using rabbit anti-mouse fibrinogen antibody (MyBioSource.com, San Diego, CA). Positive control for D-dimer was generated as previously described [27]. Hemoglobin concentrations were measured in 50-fold diluted lung homogenates by light density at 410 nm by NanoDrop spectrophotometer (Thermo Fisher Scientific). Aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a c702 Roche Diagnostics (Roche Diagnostics BV, Almere, the Netherlands). Cell free (cf)DNA was determined by diluting samples 50-100 fold with PBS containing 0.1% BSA and mixed with an equal volume of 1 µM SytoxGreen (Thermo Scientific, Waltham, MA, USA). Citrullinated histone 3 (CitH3) levels were determined by western blot using rabbit-anti-citH3 (Abcam, Cambridge, UK). Immunoreactive bands were visualized using an ImageQuant LAS 4000 (FujiFilm™ Corporation, Tokyo, Japan). For quantification, densitometry was performed with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

Statistical analysis

In the human cohort, analyses were performed using Stata/SE version 9 software (StataCorp). Differences between the 3 patient groups were compared using Fisher exact test for categorical variables and Mann-Whitney *U* test for continuous variables. Time to death to 28 days was analyzed using the Kaplan-Meier method; patients discharged alive from hospital within 28 days were assumed to have survived, but patients who self-discharged against medical advice were censored on the day of discharge. Logistic regression models were used to adjust for confounders identified using a conceptual framework (Figure S1)[28]. For murine studies, data are expressed as box and whisker plots or bars (mean with SEM). Comparisons between groups were first performed using a one-way analysis of variance on ranks (ANOVA); only when significant differences were present, groups at individual time points were tested using the Mann-Whitney U test. Survival was compared using the Kaplan-Meier method, followed by the log-rank test. Clinical observation scores were compared with a repeated measure analysis of variance. Analyses were done using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA). P-values < 0.05 were considered statistically significant.

RESULTS

Clinical melioidosis associates with thrombocytopenia

We analyzed 1160 patients with a first hospital admission for culture-positive melioidosis. All patients were prospectively identified, aged 15 years or older and presented to Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand as described [19]. The lungs were the most frequent organs infected (in 40% of patients). Patients were on average 51 years old (range 18-86 years) and 61% was male. In total, 362 patients (31%) showed thrombocytopenia, i.e. platelets counts below $150x10^9$/L. There were 199 patients (17%) with low platelet counts of $<100x10^9$/L, 163 patients (14%) with intermediate low counts between $100-149x10^9$/L, and 798 (69%) had normal platelet counts ($\geq150x10^9$/L). Baseline characteristics between groups were largely similar; in groups with low and intermediate low platelet counts there were slightly less median days of infective symptoms prior to presentation, slightly fewer patients with diabetes, and more patients with chronic kidney disease (Table 1).

61

	Platelets <100 x10^9/L	Platelets 100-149x10^9/L	Platelets ≥150x10^9/L	P-value
Baseline characteristics				
Patients, (n, %)	199 (17%)	163 (14%)	798 (69%)	
Age in years, (mean, SD)	51 [14]	53 [14]	50 [14]	0.20
Gender male (%)	126 (63%)	99 (61%)	437 (55%)	0.055
Days of infective symptoms prior to presentation (median, 95% CI)	7 [5-7]	7 [5-7]	10 [10–14]	0.027
Risk factors melioidosis				
Rice farmer (n, %)	159 (80%)	130 (80%)	616 (77%)	0.636
Known diabetes (n, %)	62 (31%)	46 (28%)	302 (38%)	0.042
Chronic kidney disease (n, %)	24 (12%)	24 (15%)	51 (6%)	<0.001
Nephrolithiasis (n, %)	8 (4%)	9 (6%)	39 (5%)	0.777
Corticosteroid use (n, %)	11 (6%)	6 (4%)	32 (4%)	0.576
Thalassemia (n, %)	3 (2%)	2 (1%)	12 (2%)	1.000
Malignancy (n, %)	0 (0%)	1 (1%)	2 (<1%)	0.434
Chronic liver disease (n, %)	3 (2%)	3 (2%)	6 (1%)	0.257
Outcome				
Hypotension (n, %)	126 (63%)	81 (50%)	214 (27%)	<0.0001
Respiratory failure (n,%)	118 (59%)	83 (51%)	196 (25%)	<0.0001
Acute kidney injury (n,%)	104 (52%)	67 (41%)	182 (23%)	<0.0001
Died in hospital (n,%)	155 (78%)	98 (60%)	246 (31%)	<0.0001

Table 1. Patient demographics and outcome. Age and days to infective symptoms prior to presentation were calculated using ANOVA, gender with chi-square test, and all risk factors were calculated using Fisher's exact

Dose response relationship between low platelet counts and mortality

Outcome was compared in melioidosis patients with different platelet counts. Thrombocytopenic patients developed more respiratory failure, hypotension and acute kidney injury during admission (Table 1, P<0.0001 vs. patients with normal platelet counts). Overall mortality was 43%, but patients with low (<100x10^9/L) and intermediate low (100-149x10^9/L) platelet counts on admission were associated with higher in-hospital mortality (Table 1, P<0.0001). In hospital mortality was highest in the low platelet count group (78%) followed by the intermediate-low group (60%) and normal platelet count group (31%). These findings were reproduced in the survival analysis up to 28 days post admission (P<0.001, Figure 1). Baseline odds ratio (OR) of death (*i.e.,* odds of death in the groups with platelets ≥150x10^9/L) were 0.45 [CI 0.4–0.5]. The OR in the intermediate platelet group was 3.4 (CI 2.4-4.8) and in the low platelet group 7.9 (CI 5.5-11.4). The correlations between platelet count and mortality remained after correcting for confounders sex, age, occupation, liver cirrhosis, malignancy and diabetes (OR 3.4 (CI 2.4– 4.9),

selected by hierarchical pathway analyses (Figure S1). These results show that low platelet counts are associated with poor outcome and higher mortality in melioidosis patients.
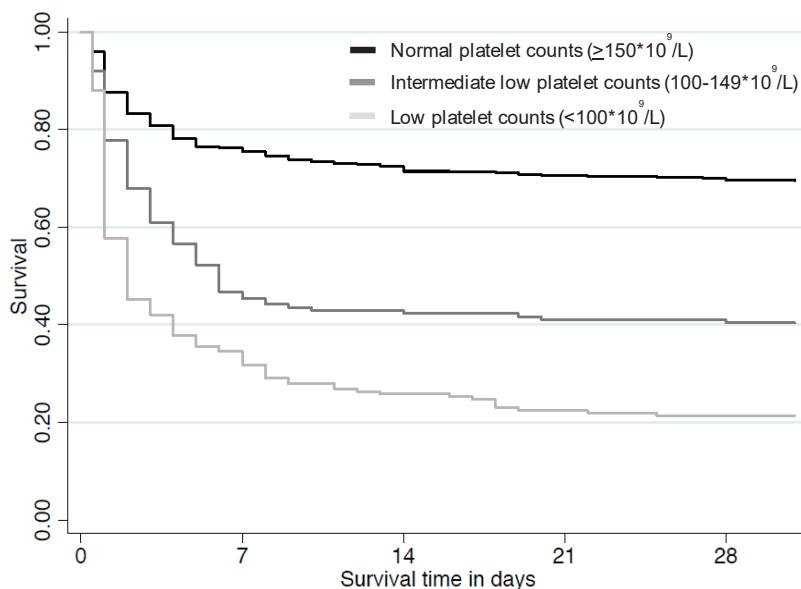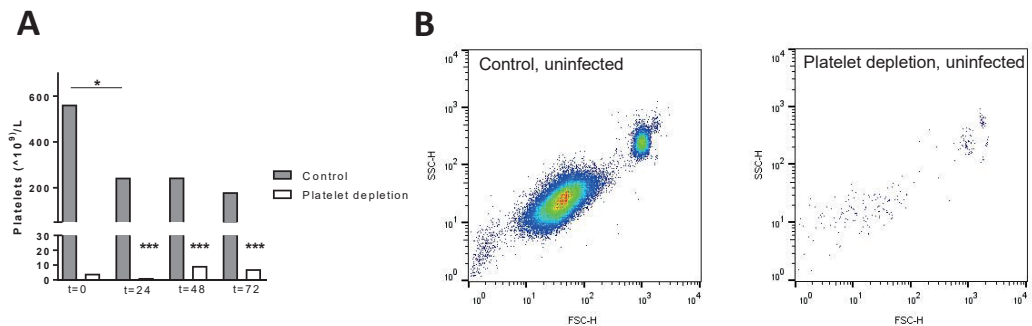

Figure 1. Thrombocytopenia is associated with increased mortality in melioidosis patients.

Kaplan Meier survival curves of 1160 patients with melioidosis stratified according to platelet counts on admission. Patient were stratified in groups with low platelets count ($<100x10^9$/L) in green, intermediate low platelet counts ($100-149x10^9$/L) in red, or normal platelet counts in blue ($\geq150x10^9$/L).

Experimental melioidosis induces thrombocytopenia and platelet depletion impairs survival and host defense

As human studies are limited in their ability to investigate causality, we conducted murine studies to further assess the direct contribution of platelets to the host response. We used a clinically relevant model, which starts with a low dose of *B. pseudomallei* given intranasally, with gradually increasing bacterial counts in the lung and disseminate to distant organs. Similar to patients with melioidosis, mice infected with *B. pseudomallei* developed thrombocytopenia (at 72 hours after infection $181*10^9$/L (95% CI 120-242)), in contrast to non-infected mice ($562*10^9$/L (95% CI 489-636)) as shown in Figure 2A (P<0.05 for infected vs. uninfected).

To investigate the role of platelets during melioidosis, mice were depleted of platelets towards levels of <5% of normal using anti-GPIbα antibody as described previously[12,20]. Mice remained thrombocytopenic during infection (Figure 2A-B). During murine melioidosis, platelet depleted mice showed increased mortality and an increased clinical observation score, a readout for disease severity (Figure 3A-B, P<0.001 vs. controls). To investigate if differences in outcome were mediated by changes in host defense, *B. pseudomallei* burden was assessed at multiple time points after infection. Platelet depletion increased bacterial dissemination in lung, broncho-alveolar lavage fluid (BALF), and liver (Figure 3C-E and Figure S2, P<0.05 vs. controls). These data indicate that during murine melioidosis, platelets are important for outcome and host defense.

Figure 2. Experimental melioidosis is associated with thrombocytopenia and effect of a-GPIbα on platelet counts. Mice were treated with a-GPIbα (platelet depletion) or IgG control (both 0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours or sacrificed uninfected. (A) Platelet counts before and after infection. (B) Representative log scale scatter plots of CD61 positive platelets in blood of naive mice and a-GPIbα treated mice. Data are expressed as bars (mean with SEM). *** P<0.0005, * P<0.05 versus IgG control or versus uninfected mice.

A

B

C

D

E
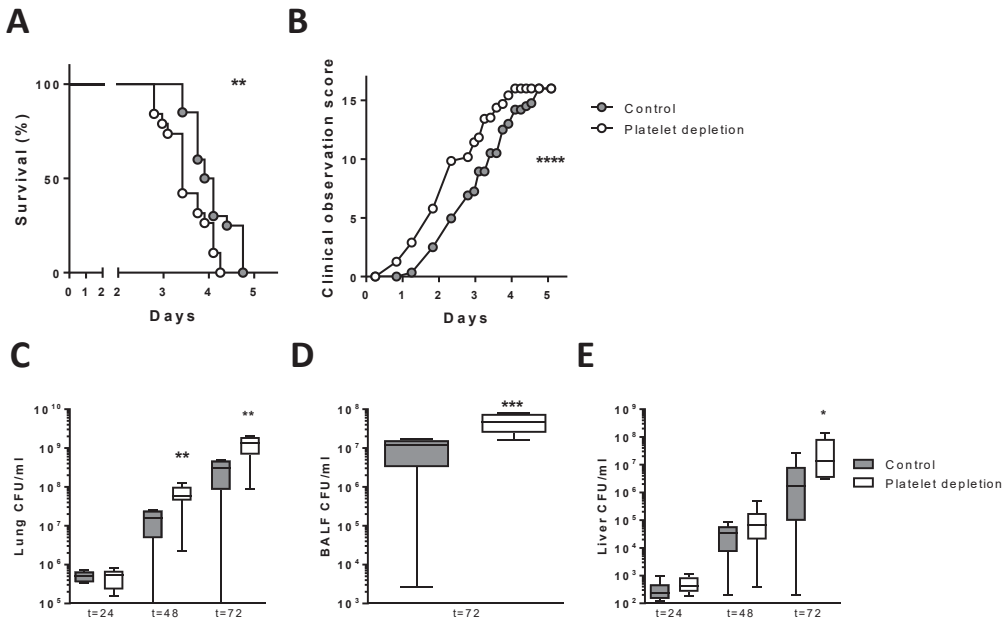
Figure 3. Thrombocytopenia impairs survival and enhances bacterial growth during *B. pseudomallei* pneumonia derived sepsis.
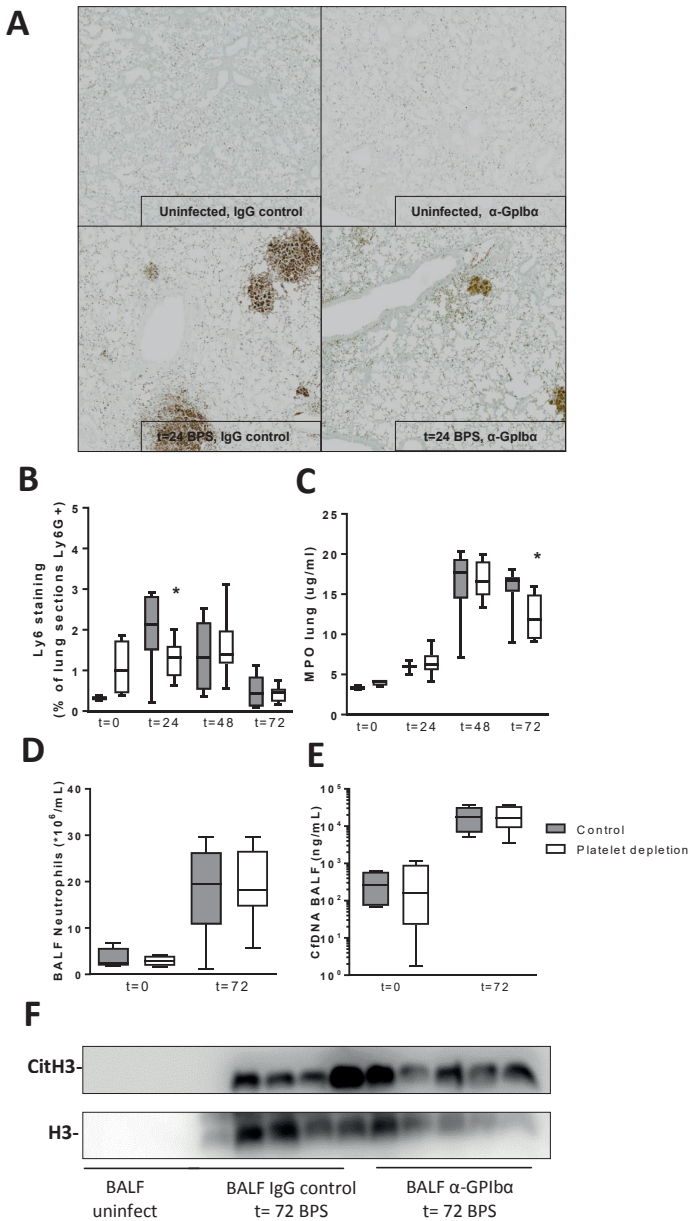
Mice were treated with a-GPIbα (platelet depletion) or IgG control (both 0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours or were observed in a survival experiment. Survival (A) and clinical observation score (B). (C-E) Bacterial quantification of indicated organs. n=20 per group for survival experiment and n=8 mice per group for bacterial quantification. *** P<0.0005, ** P<0.005, * P<0.05 versus IgG control. CFU = colony forming units. Abbreviations: BALF: broncheo-alveolar lavage fluid

No direct anti-bacterial effects of platetets on B. pseudomallei growth

To assess if platelets could directly influence bacterial growth, whole blood of platelet depleted and control mice was incubated with *B. pseudomallei* and *ex vivo* bacterial growth assessed. Blood of both groups showed similar bacterial growth, indicating that platelets do not directly influence *B. pseudomallei* growth (Figure S3). Also in human plasma, no differences were found in *B. pseudomallei* growth rate between plasma and platelet rich plasma (Figure S3).

Modest impact of platelets on early pulmonary neutrophil influx

As neutrophils influence antibacterial defense during melioidosis[21,22], we assessed if platelets mediate their protective effects via neutrophil recruitment. We therefore measured neutrophil influx in lungs by determining the number of Ly-6G-positive cells (Figure 4A-B) and by measuring myeloperoxidase (MPO) in whole lung homogenates in platelet depleted mice or controls at various time-points post infection (Figure 4C). Platelet depletion had a modest impact on early neutrophil recruitment, as reflected by reduced lung neutrophil Ly6G staining 24 hours after infection (Figure 4A-B, P<0.05 vs. controls). MPO levels were reduced at 72 hours post infection (Figure 4C, P<0.05 vs. controls), however neutrophil counts in the BALF and lung Ly6G staining did not show any differences at this late time point (Figure 4B,D and S4). Platelets are potent inducers of neutrophil extracellular traps (NETs) which are used by neutrophils to ensnare and kill *B. pseudomallei* [23,24]. To assess this, we determined CfDNA and CitH3 levels in BALF (Figure 4E-F). *B. pseudomallei* showed to be a potent inducer of NET formation, which was however similar between groups (Figure 4E-F). These data show that platelet depletion has a modest impact on early neutrophil recruitment, but not on NET formation during murine melioidosis.

Figure 4: Platelet depletion effects on early neutrophil recruitment but not NET formation.
Mice were treated with a-GPIbα (platelet depletion) or IgG control (both 0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours or sacrificed uninfected. (A-B) Ly6 stainings lung sections. (A) representative images Ly6 sections, (B) quantification. (C) MPO levels in lung (D) neutrophil counts in BALF. (E) Cell free DNA and Citrinullated histon 3 and histon 3 (F) levels in BALF. n=8 mice per group. * P<0.05 versus IgG control. Abbreviations: MPO=myeloperoxidase

*Platelet depletion increases local and systemic inflammatory responses*

Platelets can influence inflammatory responses of other cells, e.g. monocytes [25]. To investigate if this also mediated the observed protective effects of platelets during melioidosis, we assessed local and systemic cytokine and chemokine production. Platelet depletion increased cyto- and chemokines levels in both lung and plasma (Tumor necrosis factor (TNF-) and C-X-C motif ligand (CXCL)2), in part likely driven by higher bacterial loads (Table S1). This aggravated cytokine response in platelet depleted mice was not reflected in altered systemic organ damage, which is a hall mark feature of sepsis. There were no observed differences in liver damage (as scored by a blinded pathologist) or plasma Aminotransferase (AST) and alanine aminotransferase (ALT) levels between groups (Figure S5).

Platelet depletion towards a levels of <1% of normal also impairs host defense during meliodosis.

We have recently shown that during *K. pneumoniae* induced pneumosepsis, platelet depletion towards levels of <1% of normal has a more pronounced phenotype compared to platelet depletion towards levels of <5% of normal [12]. To investigate if platelet counts <1% would show similar effects on host defense, inflammatory responses and vascular integrity, we treated mice with a high dose (2 µg/g) platelet depleting antibody and assessed these parameters during melioidosis. Platelet counts <1% (Figure S6) also increased bacterial loads in lung and liver, but not in the blood (Figure S6). Additional, lung MPO levels were decreased, however Ly6G staining was similar between groups (Figure S6). Despite an increased local and systemic inflammatory response (Table S2), distant organ damage was similar (Figure S6). These data indicate that both platelet depletion <1% and <5% impair host defense during melioidosis.

Platelet depletion impairs vascular integrity during experimental melioidosis

Platelets are of vital importance for hemostasis and are known to prevent bleeding during pneumosepsis [12]. In line with this, we found that - where uninfected platelet depleted mice showed no signs of bleeding – platelet depletion (<5%) induced lung bleeding during melioidosis (Figure 5A). Bleeding was confirmed by increased lung and BALF hemoglobin measurements and lung pathology bleeding scores (Figure 5B-E, P<0.05 vs. control).
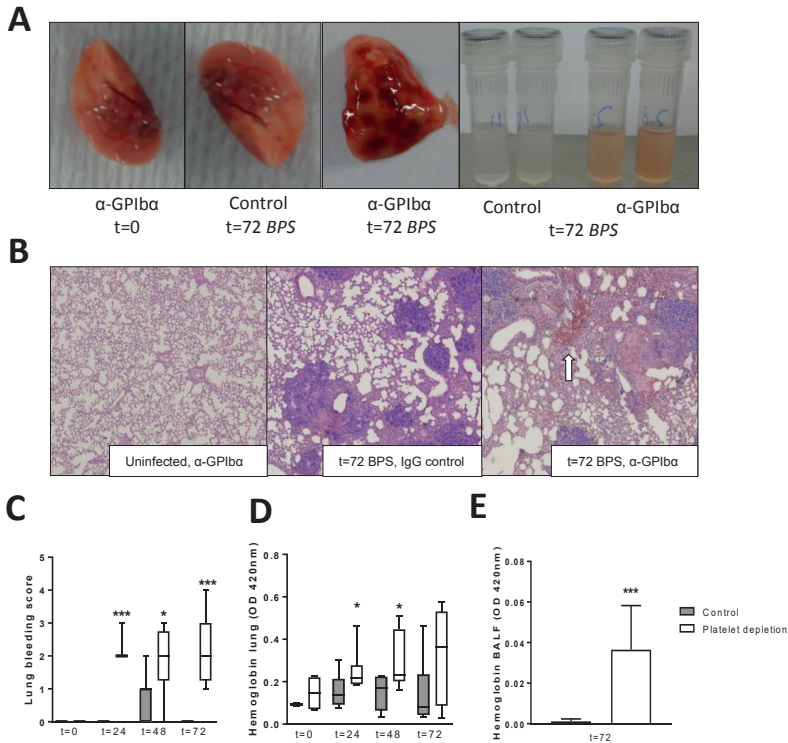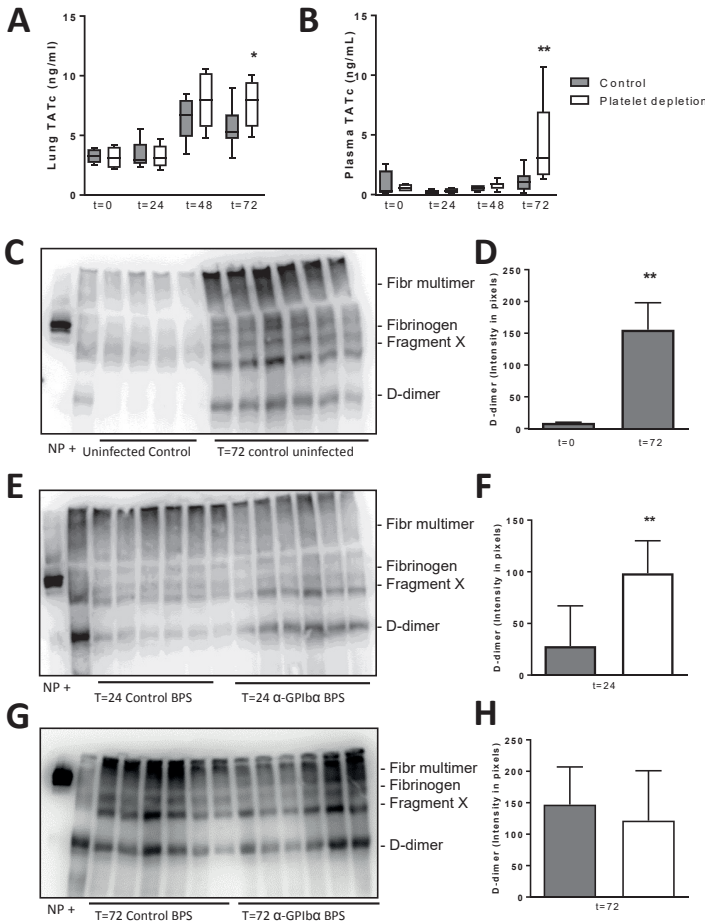
Figure 5: Thrombocytopenia results in lung bleeding at the site of infection.
Mice were treated with a-GPIbα (platelet depletion) or IgG control (both 0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours or sacrificed uninfected. (A) Representative photographs of naive or infected lungs and bronchoalveolar lavage fluid (BALF). (B-C) Lung bleeding. (B) Lung bleeding was scored on H&E stained tissue sections by a pathologist blinded for groups. (C) Representative microphotographs of H&E stained tissue sections (original magnification 40x). (D) Hb was measured in 50-fold diluted lung homogenates or (E) BALF. Data are represented as bars (mean with SEM). N=8 mice per group. OD = optical density. *** $P<0.0005$, * $P<0.05$ versus IgG control.

We furthermore investigated if impaired coagulation might explain the effects of platelet depletion on host defense or vascular integrity, as platelets phosphatidylserine exposure aids in the conversion of coagulation factors [6]. A decrease in platelet counts can also indicate pathologic coagulation activation [8], which can contribute to complications in melioidosis such as diffuse intravascular coagulation and multiple organ failure[26,27]. Indeed, mice infected with *B. pseudomallei* demonstrated strong activation of the coagulation system, as reflected by high plasma levels of thrombin–antithrombin complexes (TATc) and elevated levels of D-dimer (Figure 6A-D). This is in line with findings in patients with melioidosis[28]. Platelet depletion (<5%) further increased lung D-dimer and TATc levels in lung and plasma (Figure 6A-H) when compared to

controls. The increased accumulation of fibrin products in the lung in the thrombocytopenic mice can be due to extravascular formation as a result of the bleeding or may be due to increased coagulation as a results of the increased bacterial burden and inflammatory response (Figure 3 and Table S1). Resonating with findings in platelet counts <5%, platelet depletion <1% also induced lung bleeding during melioidosis (Figure S6). Taken together, these results show that platelet depletion impairs vascular integrity during melioidosis.


Figure 6: Thrombocytopenia results in increased local and systemic coagulation
Mice were treated with a-GPIbα (platelet depletion) or IgG control (both 0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours or sacrificed uninfected. Plasma (A) and lung (B) Thrombin-anti-thrombin complex (TAtc) levels. (C,E,G) Lung Fibrinogen western blots showing D-dimer, Fragment X, Fibrinogen and Fibrin crosslinks in uninfected and infected mice, (D) semi quantification d-dimer. Lung fibrinogen western blots in mice infected for 24 hours (E) and 72 hours (G). Semi quantification of d-dimer in mice in mice infected for 24 hours (F) and 72 hours (H). N=8 mice per group. ** $P<0.005$, * $P<0.05$ versus IgG control. NP= naïve plasma, +=positive D-dimer control

Mice lacking platelet GPIbα, but not platelet TLR signalling, display impaired local host defense during melioidosis

To assess which platelet receptors were involved in the protective effect of platelets during melioidosis, we investigated both mice lacking platelet Toll-like Receptor (TLR) signaling or GPIbα during melioidosis. Platelet TLRs and GPIbα can both influence platelet leukocyte interactions and cytokine production [9,13,29]. Moreover, it was recently shown that platelet GPIbα can influence host defense during *Klebsiella pneumoniae* pneumosepsis[15]. To investigate platelet-specific TLR signaling, we used platelet-specific MyDd88 KO mice (Plt-MyD88^{-/-}), which lack the crucial TLR signaling protein MyD88 only in platelets and megakaryocytes[30]. Total MyD88^{-/-} mice have impaired host defense during *B. pseudomallei* infection[31]. Infected Plt-MyD88^{-/-} and littermates however had similar bacterial loads in all organs during *B. pseudomallei* infection (Figure S7). Moreover platelet activation, platelet-leukocyte interactions and thrombocytopenia were similar between both groups during experimental melioidosis (Figure S7). To investigate the role of platelet GPIbα, IL4R/GPIbα mice (that lack GPIbα, but without the associated macro-thrombocytopenia) [32] were infected with *B. pseudomallei*. As reported[32] IL4R/GPIbα mice had no GPIbα expression (Figure 7A-B, P<0.001 vs. controls) and reduced platelet counts during infection (Figure 7C, P<0.005 vs. controls). Platelet activation as measured by P-selectin on platelets was similar between mice strains during infection (Figure 7D), however platelet neutrophil-complexes were reduced in IL4R/GPIbα compared to controls (Figure 7E, P<0.005 vs controls). IL4R/GPIbα mice showed increased lung bacterial loads compared to controls during experimental melioidosis, but not to a similar extent as observed in the experiments in which platelets were depleted with anti-GPIbα antibody (Figure 7F, P<0.05 vs. controls) as bacterial loads in distant organs were unaffected (Figure 7G-H). These results show that GPIbα, but not platelet TLR signaling, contributes to the local host defense against *B. pseudomallei.*
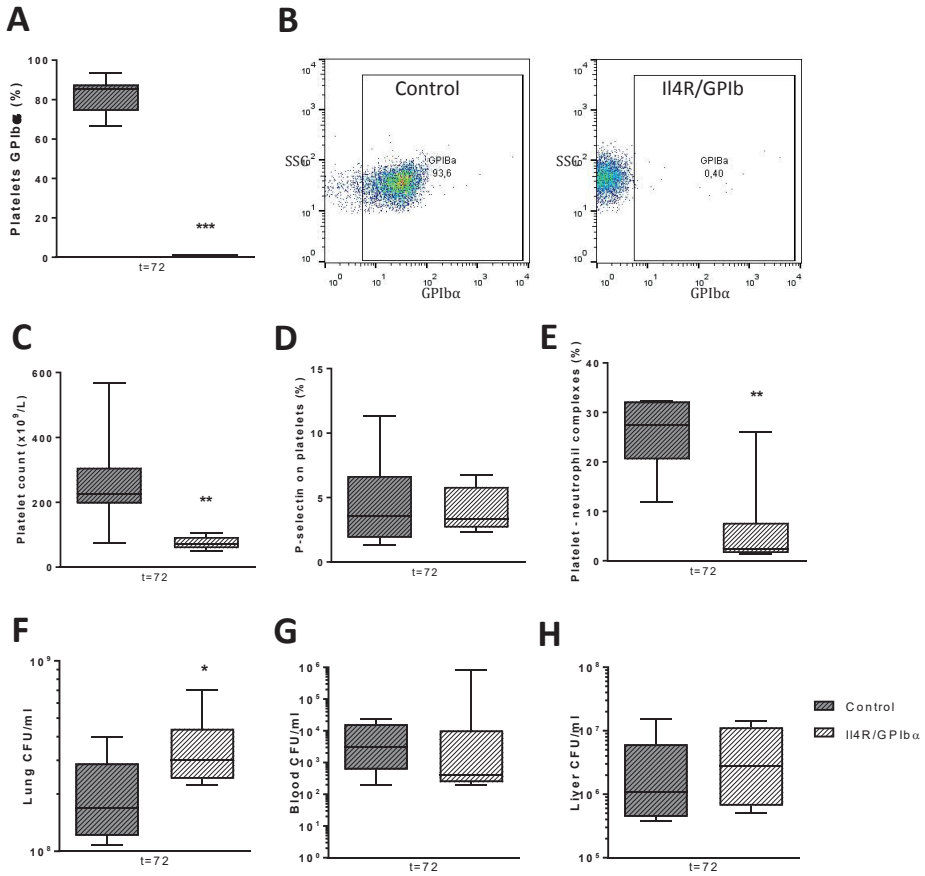
Figure 7: Platelet GPIb deficiency decreases platelet counts and leads increased bacterial growth in the lung during experimental melioidosis

Il4R/GPIbα or control mice were infected with *B. pseudomallei* via the airway and sacrificed after 72 hours. (A-B) GPIb expression on platelets; (C) Platelet counts (D) P-selectin expression (E) Platelet- neutrophil complex formation. (F-H) Bacterial quantification in indicated organs. n=8 mice per group. ***P<0.0005, ** P<0.005 , * P<0.05 versus IgG control.

Chapter 3

DISCUSSION

Here, we show that during clinical melioidosis there is a strong association between low platelet counts and mortality, and that in murine melioidosis platelet depletion reduces survival and impairs host defense.

Our associations between thrombocytopenia and mortality in melioidosis patients are in line with our previous findings in a much smaller patient cohort [18] as well as other studies looking at sepsis patients in general [17,33,34]. The correlations between platelet counts and mortality might be a reflection of disease severity. This is underlined by the finding that melioidosis patients with low platelet counts developed more hypotension, respiratory failure and kidney failure. However, thrombocytopenia is associated with altered immune responses independent of disease severity, as we have shown previously in another cohort of sepsis patients [17]. Disease severity scores of our patients were however not collected, as a result of patient inclusion in a low resource setting, hampering our ability to correct for this important confounder.

In contrast to observational cohort studies, murine studies can be used to investigate the direct contributing effect of platelets on melioidosis outcome. Similar to the human setting, murine melioidosis led to marked thrombocytopenia and platelet depletion (<5%) was associated with increased mortality. Platelets moreover directly contributed to host defense against *B. pseudomallei*, both at the local site of infection as well as distant organs, such as the liver. Neutrophils recruited to the site of infection can influence outcome during melioidosis[21], however mostly during late stage infection. Additionally, interactions between platelets and neutrophils can influence bacterial killing[9]. During melioidosis, platelet depletion modestly impaired early neutrophil recruitment to the lung. These findings are in line with previous studies showing that platelets can influence recruitment of neutrophils to the site of infection[25,35]. Interestingly, we already observed an effect of platelets on antibacterial defense 48 hours after infection, whereas previous studies showed an effect of neutrophils 72 hours after *B. pseudoma*llei infection[21]. Platelet induced NET formation has been shown to influence bacterial growth[9], however we did not observe an effect on cfDNA and CitH3 levels (a marker of NET formation[36]). Earlier, we reported that compromised NETs (by DNase treatment) also did not affect murine melioidosis[23]. Previous studies have also shown that platelet TLR signaling was important for restriction of bacterial growth[9], but during melioidosis, platelet TLR signaling did not contribute to host defense.

These differences might be explained by differences in bacteria, the intra-cellular nature of *B. pseudomallei* or the model (acute high dose vs. slowly growing).

In contrast, mice lacking platelet GPIbα did show impaired host defense in the lung. Il4R/GPIbα mice showed increased bacterial burden in the lung after 3 days of infection, but not to the similar extent as platelet depleted mice. Il4R/GPIbα mice are GPIbα deficient mice without the associated macrothrombocytopenia[32], however we found that during melioidosis, platelet counts were still reduced in Il4R/GPIbα mice compared to controls. The contribution of the lower platelet counts on the phenotype seen, remains to be established. In line with a protective effect of platelet GPIbα, a recent study also found a protective role for platelet GPIbα during gram-negative pneumosepsis by *K. pneumoniae*, using a GPIbα blocking antibody[15].

Platelets protect against bleeding, specifically at the site of infection and inflammation[12,37]. Platelet depletion resulted in bleeding in the lung during *B. pseudomallei* infection, this is consistent with previous reports of other gram negative pneumosepsis models. In contrast to *Klebsiella* infection however, melioidosis also induced severe bleeding when platelet counts were <5%. Possibly, *B. pseudomallei* infection of cells causes more severe damage to tissue and vascular integrity, which renders more platelets needed to prevent bleeding. Also, in platelet depleted mice lung bleeding was already seen at an early time point (24 hours after infection) in *B. pseudomallei* infection. It is possible that this early lung bleeding influences adequate host defense and thereby contributes to the differences in antibacterial response seen between control and platelet depleted mice. In human melioidosis it is not well known that *B. pseudomallei* infection results into bleeding. However most melioidosis cases are occurring in low resource countries, with the exception of Australia, and therefore this clinical sign might be missed. The importance of the risk of bleeding in melioidosis patients remains to be elucidated.

In conclusion, we found that in melioidosis patients, low platelet counts are associated with increased mortality and that in murine melioidosis, platelet depletion severely hampered survival, host defense and vascular integrity.

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Chapter 3

SUPPLEMENTAL MATERIAL

Supplemental tables

ng/mL	Lung homogenates		Plasma	
	Control	Platelet depletion	Control	Platelet depletion
24 h				
TNF-α	3.8 (0.1-8.5)	3.3 (0.2-9.3)	0.2 (0.1-0.2)	0.1 (0.1-0.2)
IL-6	23.1 (6.6-36.9)	41.0 (30.6-43.5)	0.2 (0.2-0.2)	0.2 (0.2-1.0)
IFN-γ	1.2 (0.8-2.1)	2.3 (1.0-3.0)	b.d.	b.d
CXCL2	20.3 (13.5-26.7)	24.1 (14.1-31.4)	n.d.	n.d.
CCL2	n.d.	n.d.	3.1 (2.8-3.7)	2.8 (2.1-3.4)
48 h				
TNF-α	20.6 (12.7-23.6)	23.8 (23.8-31.1)*	0.6 (0.4-1.0)	1.2 (1.0-1.4)*
IL-6	82.0 (41.6-10.8)	113.3 (91.4-135.0)	2.4 (1.6-13.1)	18.6 (10.3-32.7)
IFN-γ	2.7 (2.5-2.9)	2.5 (2.1-3.1)	0.1 (0-0.2)	0.3 (0.2-0.6)*
CXCL2	166.2 (112.6-194.5)	310.8 (215.6-343.1)**	n.d.	n.d.
CCL2	n.d.	n.d.	5.3 (2.3-11.7)	4.0 (1.5-12.3)
72 h				
TNF-α	23.2 (4.1-27.3)	40.0 (27.7-41.4)**	2.1 (0.7-7.1)	7.1 (4.7-10.9)*
IL-6	77.3 (35.0-88.9)	79.0 (7.6 -112.5)	50.1 (6.9-157.1)	145.1 (100.6-277.1)
IFN-γ	2.3 (1.8-2.6)	2.3 (2.1-2.8)	0.09 (0-0.3)	0.5 (0.2-2.0)
CXCL2	294.8 (200.5-465.6)	737.4 (417.3-873.3)*	n.d.	n.d.
CCL2	n.d.	n.d.	9.6 (2.9-16.2)	12.2 (10.3-19.0)

Table S1. Cytokine and chemokine levels

Cytokine and chemokine levels in lung homogenate and plasma from platelet depleted (0.4 ug/g anti-GPIbα) or control mice (0.4 ug/g IgG control) inoculated with *B. pseudomallei*, sacrificed 24, 48 or 72 hours post infection. Values are in ng/mL and presented as median (interquartile range). * P<0.05, **P<0.01 vs. control. Abbreviations: b.d.= below detection, n.d.= not determined

	Lung homogenates (t=72)		Plasma (t=72)	
ng/mL	Control	Platelet depletion (high dose)	Control	Platelet depletion (high dose)
TNF-α	30.7 (21.2-34.1)	64.2 (49.1-72.3)**	1.9 (1.4-3.4)	7.9 (7.1-9.6)**
IL-6	47.2 (35.3-51.0)	96.9 (89.2-130.3)**	22.0 (16.6-43.8)	241.8 (178.5-299.6)*
IFN-γ	1.7 (1.5-2.2)	2.4 (2.0-3.0)	0.1 (0.0-0.8)	0.0 (0.0-1.2)
CXCL2	271.3 (154.5-357.7)	658.8 (376.8-801.4)*	n.d.	n.d.
CCL2	n.d.	n.d.	3.3 (1.4-5.1)	1.2 (0.9-1.2)

Table S2. Cytokine and chemokine levels during experimental melioidosis in experiments in which the high dose of anti-GPIbα was used.
Cytokine and chemokine levels in lung homogenate and plasma from platelet depleted (mice injected with 2ug/g anti-GPIbα) or control mice (mice injected with 2ug/g IgG control) inoculated with *B. pseudomallei*, sacrificed 72 hours post infection. Values are in ng/mL and presented as median (interquartile range). *P<0.05 and **P<0.01 vs. control. Abbreviations: n.d.= not determined

Supplemental figures

| 1	Gender	Age		Occupation
2	Diabetes Mellitus	Cirrhosis/liver disease	Malignancy	Malnutrition*
4	Melioidosis	Thrombocytopenia		
5	Bleeding	immunothrombosis	Sepsis	
	Respiratory failure	Heart failure	Hypotension	
6		Mortality		

Figure S1. Conceptual hierarchical framework for risk factors for thrombocytopenia and mortality
Sex, age and occupation to healthcare occupy the highest level in the hierarchy because they are not dependent on any other factors. Factors in each level are dependent on factors in the level above and factors in lower levels cannot confound the effect of factors in higher levels because they occur later in time. Factors in level 4 are immediate proximate causes of death. *We used occupation as a proxy for malnutrition.
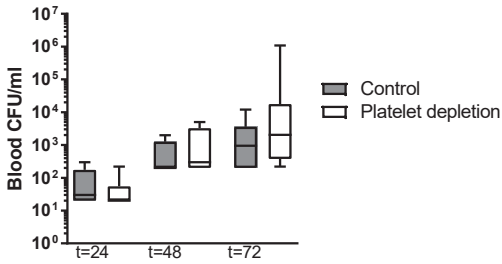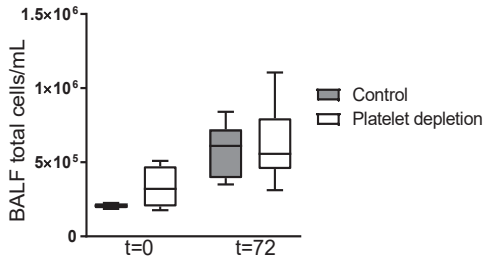
Figure S2. *In vivo B. pseudomallei* growth in blood during experimental melioidosis

Mice were treated with a-GPIbα (platelet depletion) or IgG control (0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours. Bacterial quantification in blood. n=8 mice per group.

A

B
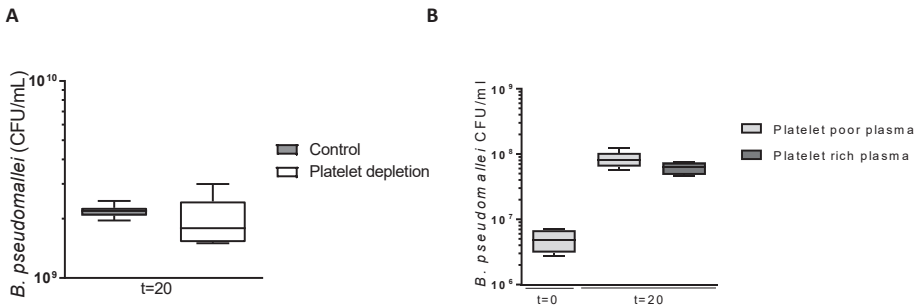
Figure S3. *Ex vivo B. pseudomallei* growth

(A) Mice were treated with a-GPIbα (0.4ug/g, platelet depletion) or IgG control sacrificed uninfected. Whole blood was incubated with viable *B. pseudomallei* (10^7CFU/mL) for 20 hours at 37 degrees after which bacterial counts were quantified. (B) Human platelet poor and platelet rich plasma was incubated with viable *Burkholderia* ($5*10^6$ CFU/mL) for 20 hours at 37 degrees after which bacterial growth was quantified. n=4 replicates or mice per group. Human experiments were performed at least twice with 2 independent donors.

Figure S4. Platelet depletion does not influence total cell influx in the broncheo-alveolar space during experimental melioidosis.

Mice were treated with anti-GPIbα (platelet depletion) or IgG control (both 0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrifice after 72 hours or uninfected. Total cell numbers in the broncho-alevolar lavage fluid in blood. n=8 mice per group.
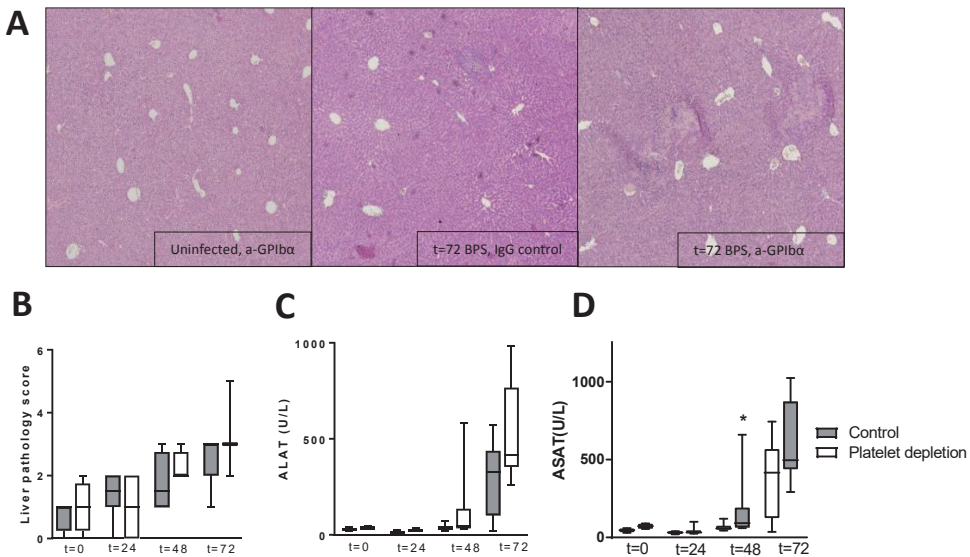

Figure S5. Platelet depletion does not influence liver damage

Mice were treated with anti-GPIbα (platelet depletion) or IgG control (both 0.4ug/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours. Representative images, original magnification 40x (A) and quantification (B) of liver damage. (C) ALAT (D) ASAT. n=8 mice per group. * P<0.05 versus IgG control
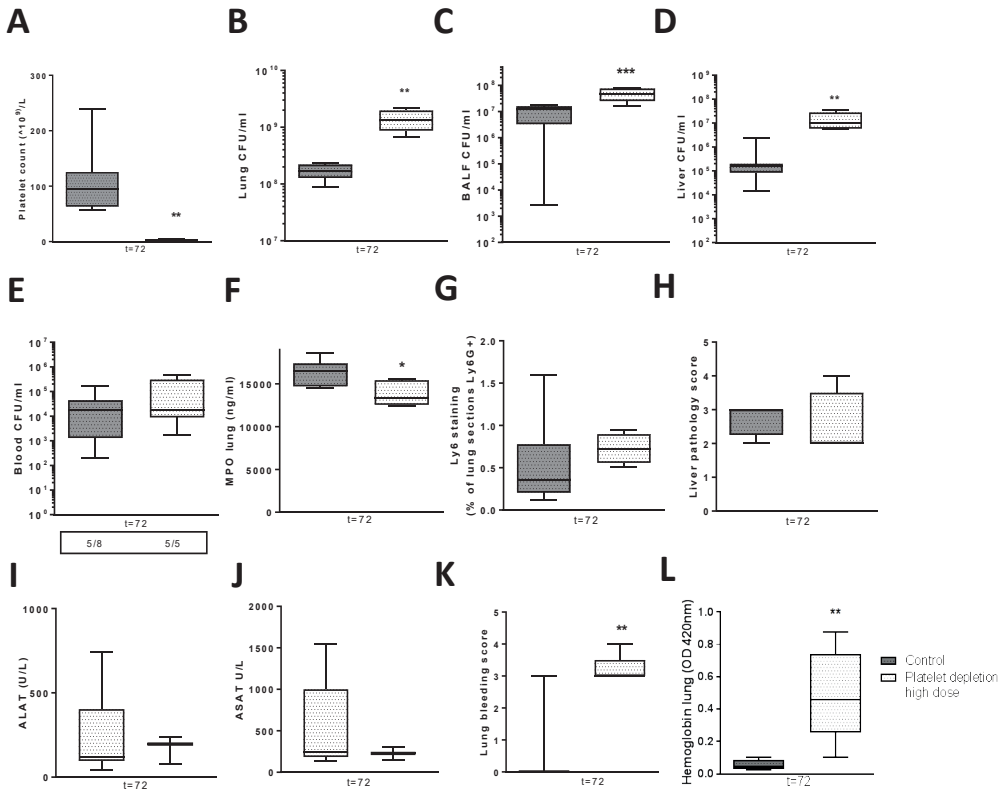
Figure S6. Platelet depletion <1% also impairs host defense and vascular integrity during melioidosis

Mice were treated with high dose a-GPIbα 2ug/g or IgG control and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48 or 72 hours.   (A) Blood platelet counts. (B-E) Bacterial loads in organs indicated. (F) MPO levels lung (G) Ly6G staining lung. (H) liver pathology. Plasma (I) ALAT (J) ASAT levels. (K) Lung bleedings score. (L) Lung hemoglobin levels. N=8 mice per group. ***P<0.005, **P<0.05, * P<0.05 versus IgG control.
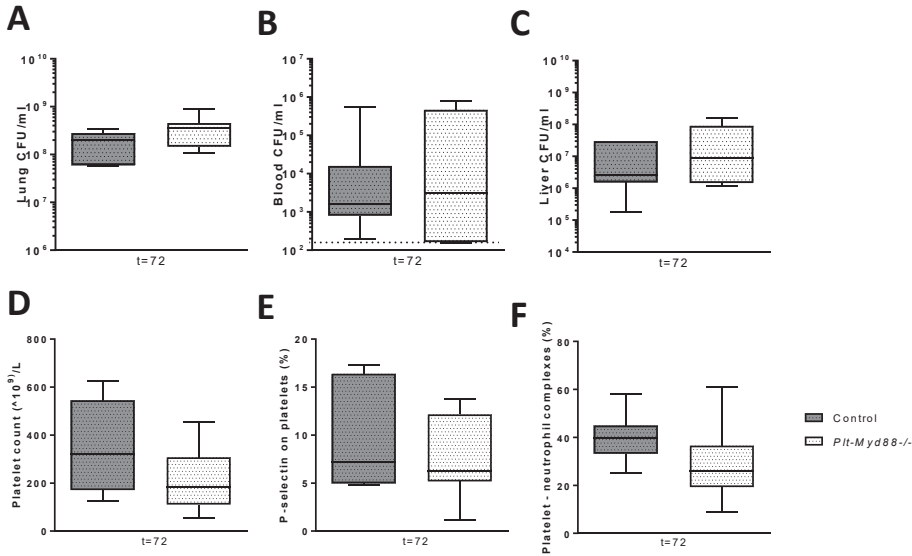
Figure S7. Platelet Toll-like receptor (TLR) signalling does not influence host defense during murine melioidosis
(A-E) Plt-Myd88^{-/-} (white boxes) or control mice (grey boxes) were infected with *B. pseudomallei* via the airway and sacrificed after 72 hours. (A-C) Bacterial quantification in indicated organs. (D) Platelet counts in blood, (E) Platelet P-selectin expression (F) Platelet-neutrophil complex formation in blood. n=8 mice per group.