Digging for melioidosis

New insights into the epidemiology and pathophysiology

Birnie, E.

Link to publication

Creative Commons License (see https://creativecommons.org/use-remix/cc-licenses):
Other

Citation for published version (APA):
CHAPTER 8

THROMBOCYTOPENIA IMPAIRS HOST DEFENSE AGAINST BURKHOLDERIA PSEUDOMALLEI (MELIOIDOSIS)
CHAPTER 8
THROMBOCYTOPENIA IMPAIRS HOST DEFENSE AGAINST BURKHOLDERIA PSEUDOMALLEI (MELOIDOSIS)

Emma Birnie*, Theodora A.M. Claushuis*, Gavin C.K.W. Koh, Direk Limmathurotsakul, Nicholas P.J. Day, Joris J.T.H. Roelofs, Jerry Ware, Baidong Hou, Alex F. de Vos, Tom van der Poll, Cornelis van ’t Veer, and W. Joost Wiersinga


*These authors contributed equally
ABSTRACT

Background: Infection with the Gram-negative bacillus *Burkholderia pseudomallei* (melioidosis) is an important cause of pneumosepsis in Southeast Asia and has a mortality of up to 40%. We aimed to assess the role of platelets in the host response against *B. pseudomallei* infection.

Methods: Association between platelet counts and mortality was determined in 1,160 patients with culture-proven melioidosis. Mice treated with (low or high-dose) platelet-depleting antibody were inoculated intranasally with *B. pseudomallei* and sacrificed. Additional studies using functional glycoprotein (GP)Iba-deficient-mice were conducted.

Results: Thrombocytopenia was present in 31% of patients at admission and predicted mortality in melioidosis patients even after adjustment for confounders. In our murine-melioidosis model, platelet counts decreased, and mice treated with a platelet-depleting antibody showed enhanced mortality and higher bacterial loads compared to mice with normal platelet counts. Low platelet counts had a modest impact on early-pulmonary neutrophil-influx. Reminiscent of their role in hemostasis, platelet depletion impaired vascular integrity, resulting in early-lung bleeding. GPIba-deficient mice had reduced platelet counts during *B. pseudomallei* infection together with an impaired local host defense in the lung.

Conclusion: Thrombocytopenia predicts 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 etiological agent of melioidosis, a life-threatening infection that often presents with pneumonia and sepsis and mainly occurs in Southeast Asia and Northern Australia. Unpublished work shows us that about 5% of sepsis patients (using Sepsis-3 criteria) is diagnosed with culture confirmed melioidosis in Northeast Thailand. Despite appropriate antibiotic treatment, mortality rates remain high, ranging from 10-40%. Melioidosis has been proposed as a good model to study Gram-negative sepsis.

A 2016 epidemiological study estimated that there are 165,000 cases, and 89,000 deaths annually, which suggest that the global burden of melioidosis is much larger than previously thought. 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. It has become clear that platelets also play an important role in inflammation and immunity. Platelets have been described to express several immune related receptors such as Toll-like receptors (TLRs), which are of importance for microbial surveillance and regulation of inflammatory and immune responses. Indeed, platelets can aid in the host defense against infection and can influence inflammation both in the lung and the systemic compartment. In murine studies, platelets have been shown to alter immune responses by influencing leukocyte functions and recruitment. In patients with sepsis, low platelet counts may dysregulate immune responses by decreasing leukocyte adhesion signaling.

We recently demonstrated that thrombocytopenia is a key feature of melioidosis and is correlated with mortality. Von Willebrand factor (which can bind and activate platelets via platelet glycoprotein (GP)Ib) levels are elevated in patients with melioidosis. We therefore sought to further define the role of platelets in melioidosis, using observational data from a large cohort of melioidosis patients and from clinically relevant murine models of 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. This cohort has been previously described elsewhere. Patients were stratified into three groups according to platelet counts at presentation; low platelets count (<100x10⁹/L), intermediate low platelet counts (100-150x10⁹/L), or normal platelet counts (≥150x10⁹/L). Boundaries were based on previous studies. The primary study outcome was in-hospital mortality, but we also pre-defined three secondary outcomes: hypotension, acute kidney injury, and respiratory failure. For further information, see supplementary materials.

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. Human IL4R/GPIba mice, are knock out for mouse GPIba, without the associated macrothrombocytopenia. For further information, see supplementary materials.

Ethics Statement
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 by a native Thai speaker. Parents/guardians also provided written informed consent on behalf of child participants. All procedures performed were in accordance with the Helsinki Declaration of 1975 (revised 1983). The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments (permit number DIX 21) and ethical approval was obtained to use B. pseudomallei strain 1026b for animal experiments (08-150). Experiments were carried out in accordance with the Dutch Experiments on Animals Act.
Experimental study design
Melioidosis was induced by intranasal inoculation with \textit{B. pseudomallei} 350-500 colony forming units (CFU) in 50 μL (isotonic saline), as previously described. Two 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). 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. For further information see supplementary materials.

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) (for further information see supplementary materials).

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 scored by a pathologist blinded for group identity as previously described. To determine neutrophil influx in the lung, sections were stained with anti-mouse Ly6-G mAb (BioLegend) and Ly-6h positivity was measured using Image J (for further information see supplementary materials).

Protein measurements
For further information, see supplementary materials.

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. Difference in age was analyzed by ANOVA, days to infective symptoms prior to presentation by Kruskal-Wallis, gender by chi-square test, risk factors, organ involvement and distribution of disease by Fisher's exact test. Time to death to 28 days was analyzed using the Kaplan-Meier method. Logistic regression models were used to adjust for confounders identified using a conceptu-
al hierarchical framework (S1 Figure). Parameters were chosen on the basis of whether they were possible confounders for the effect of thrombocytopenia on mortality. For murine studies, data are expressed as box and whisker plots or as bars (respectively median with range or mean with SD). 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 1,160 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 previously. The lungs were the most frequent organs infected (in approximately 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 $150 \times 10^9/L$. There were 199 patients (17%) with low platelet counts of $<100 \times 10^9/L$, 163 patients (14%) with intermediate low counts between $100-149 \times 10^9/L$, and 798 (69%) had normal platelet counts ($\geq 150 \times 10^9/L$). Baseline characteristics between groups were largely similar; in the groups with low and intermediate low platelet counts, patients presented more with bacteremia and multi-organ disease ($P<0.001$), and the duration of symptoms prior to presentation was shorter than in the group with a normal platelet count ($P<0.001$; Table 1).
Table 1  Patient demographics and outcome of 1160 patients with a first hospital admission for culture-positive melioidosis

<table>
<thead>
<tr>
<th>Patients, (n)</th>
<th>Platelets &lt;100x10^9/L</th>
<th>Platelets 100-149x10^9/L</th>
<th>Platelets ≥150x10^9/L</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age in years, (mean, SD)</td>
<td>51 [14]</td>
<td>53 [14]</td>
<td>50 [14]</td>
<td>0.20</td>
</tr>
<tr>
<td>Gender male (n, %)</td>
<td>126 (63%)</td>
<td>99 (61%)</td>
<td>437 (55%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Days of infective symptoms prior to presentation (median, IQR)</td>
<td>7 [5-7]</td>
<td>7 [5-7]</td>
<td>10 [10-14]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Risk factors melioidosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice farmer (n, %)</td>
<td>159 (80%)</td>
<td>130 (80%)</td>
<td>616 (77%)</td>
<td>0.64</td>
</tr>
<tr>
<td>Known diabetes (n, %)</td>
<td>62 (31%)</td>
<td>46 (28%)</td>
<td>302 (38%)</td>
<td>0.04</td>
</tr>
<tr>
<td>Chronic kidney disease (n, %)</td>
<td>24 (12%)</td>
<td>24 (15%)</td>
<td>51 (6%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nephrolithiasis (n, %)</td>
<td>8 (4%)</td>
<td>9 (6%)</td>
<td>39 (5%)</td>
<td>0.78</td>
</tr>
<tr>
<td>Corticosteroid use (n, %)</td>
<td>11 (6%)</td>
<td>6 (4%)</td>
<td>32 (4%)</td>
<td>0.57</td>
</tr>
<tr>
<td>Thalassemia (n, %)</td>
<td>3 (2%)</td>
<td>2 (1%)</td>
<td>12 (2%)</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>Malignancy (n, %)</td>
<td>0 (0%)</td>
<td>1 (1%)</td>
<td>2 (&lt;1%)</td>
<td>0.43</td>
</tr>
<tr>
<td>Chronic liver disease (n, %)</td>
<td>3 (2%)</td>
<td>3 (2%)</td>
<td>6 (1%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Organ involvement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia (n, %)</td>
<td>80 (40%)</td>
<td>70 (43%)</td>
<td>311 (39%)</td>
<td>0.63</td>
</tr>
<tr>
<td>Skin soft tissue (n, %)</td>
<td>18 (9%)</td>
<td>22 (14%)</td>
<td>182 (23%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary tract (n, %)</td>
<td>33 (17%)</td>
<td>23 (14%)</td>
<td>73 (9%)</td>
<td>0.007</td>
</tr>
<tr>
<td>Liver abscess (n, %)</td>
<td>8 (4%)</td>
<td>11 (7%)</td>
<td>89 (11%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Spleen abscess (n, %)</td>
<td>19 (10%)</td>
<td>14 (9%)</td>
<td>92 (12%)</td>
<td>0.50</td>
</tr>
<tr>
<td>Septic arthritis (n, %)</td>
<td>10 (5%)</td>
<td>9 (6%)</td>
<td>69 (9%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Distribution of disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteremia (n, %)</td>
<td>159 (80%)</td>
<td>120 (74%)</td>
<td>397 (50%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Single organ disease (n, %)</td>
<td>92 (46%)</td>
<td>84 (52%)</td>
<td>501 (63%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypotension (n, %)</td>
<td>126 (63%)</td>
<td>81 (50%)</td>
<td>214 (27%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Respiratory failure (n, %)</td>
<td>118 (59%)</td>
<td>83 (51%)</td>
<td>196 (25%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acute kidney injury (n, %)</td>
<td>104 (52%)</td>
<td>67 (41%)</td>
<td>182 (23%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Died in hospital (n, %)</td>
<td>155 (78%)</td>
<td>98 (60%)</td>
<td>246 (31%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Difference in age analyzed by ANOVA, days to infective symptoms prior to presentation by Kruskal-Wallis, gender by chi-square test, risk factors, organ involvement, and distribution of disease by Fisher’s exact test. Patient outcomes were calculated using chi-square test. Abbreviations: SD=standard deviation, CI=confidence interval.
Thrombocytopenia predicts mortality in melioidosis patients

Thrombocytopenic patients developed more respiratory failure, hypotension and acute kidney injury during admission (Table 1, P<0.001 vs. patients with normal platelet counts). Overall mortality was 43% and low (<100x10⁹/L) and intermediate low (100-149x10⁹/L) platelet counts on admission were associated with higher in-hospital mortality (Table 1, P<0.001). 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). Low platelet counts <100 x10⁹/L (OR 7.90, 95% CI 5.5-11.4) and intermediate low platelet counts 100-149x10⁹/L (OR 3.38, 95% CI 2.4-4.8) were associated with increased mortality compared to patients with normal platelet counts ≥150x10⁹/L. This association persisted after correcting for these confounders, with increased adjusted odds ratios (AOR) in patients with low platelet counts <100 x10⁹/L (AOR 7.98, 95% CI 5.5-11.6) and intermediate low platelet counts 100-149x10⁹/L (AOR 3.40, 95% CI 2.4-4.8) compared to normal platelet counts ≥150x10⁹/L. Important confounders were selected by hierarchical pathway analyses; gender, age, rice farming (malnutrition), liver cirrhosis, malignancy, and diabetes mellitus (S1 Figure). These results show that thrombocytopenia is associated with poor outcome and predicts mortality in melioidosis patients, even after correcting for important confounders. Disease severity scores of our patients were however not collected, hampering our ability to correct for this important confounder.
**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 counts (<100 x10^9/L) in light grey, intermediate low platelet counts (100-150x10^9/L) grey, or normal platelet counts in black (150≥x10^9/L). P<0.001 for comparison between three groups for survival analysis.](image)

*Platelet depletion impairs survival and host defense in experimental melioidosis*

As human studies are limited in their ability to investigate causality, we conducted murine studies to further assess the 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 dissemination to distant organs. Similar to patients with melioidosis, mice infected with *B. pseudomallei* showed a decline in platelet counts (median platelet counts at 72 hours after infection 152x10^9/L (IQR 112-260 x10^9/L)); non-infected mice had median platelet counts of 579x10^9/L (IQR 529-581x10^9/L) (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 we have described before. Mice remained thrombocytopenic during infection (Figure 2A-B).
Figure 2  Experimental melioidosis is associated with thrombocytopenia and effect of α-GPIbα on platelet counts

Mice were treated with anti-GPIbα (platelet depletion) or IgG control (both 0.4µg/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours or sacrificed uninfected (t= 0 hours).  

(A) Platelet counts before (t= 0 hours) and after infection (t= 24, 48, or 72 hours).  

(B–C) Representative log scale scatter plots of CD61 positive platelets in blood of uninfected control and anti-GPIbα treated mice. Data are represented as bars (median with IQR). N=8 mice per group. *** P<0.001, * P<0.05 versus IgG control or versus uninfected mice.
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 numbers in lung, bronchoalveolar lavage fluid (BALF), and liver (Figure 3C, $P<0.05$-$P<0.01$-$P<0.001$ vs. controls), but not blood (Figure S2). These data indicate that during murine melioidosis, platelets are important for outcome and host defense.

**Figure 3**  Thrombocytopenia impairs survival and enhances bacterial growth during *B. pseudomallei pneumonia* derived sepsis

Mice were treated with anti-GPIbα (platelet depletion) or IgG control (both $0.4\mu g/g$) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours or were observed in a survival experiment. (A) Survival and (B) clinical observation score. (C) Bacterial quantification of indicated organs. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N=20 per group for survival experiment and n=8 mice per group for bacterial quantification. *** $P<0.001$, ** $P<0.01$, * $P<0.05$ versus IgG control. Abbreviations: CFU = colony forming units and BALF= bronchoalveolar lavage fluid.
Platelets do not exert direct anti-bacterial effect on
*B. pseudomallei* growth

To assess if thrombocytopenia 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 (S3 Figure A). Likewise, no differences were found in *B. pseudomallei* growth rate between human platelet rich and platelet poor plasma (S3 Figure B). Together, these data suggest that platelets do not directly influence *B. pseudomallei* growth.

Modest impact of platelets on early pulmonary neutrophil influx

As neutrophils influence antibacterial defense during melioidosis, we assessed if platelets mediate their protective effects via neutrophil recruitment. By quantification of Ly-6G-positive cells in lung tissue (Figure 4A-B) and by measuring myeloperoxidase (MPO) (Figure 4B) in whole lung homogenates we determined lung neutrophil influx for both platelet depleted and control mice at set time-points post-infection. 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 in thrombocytopenic mice at 72 hours post-infection (Figure 4B, P<0.05 vs. controls); however, neutrophil counts in BALF and lung Ly6G staining were no different between groups at this late time point (Figure 4B and S4 Figure). Platelets are potent inducers of neutrophil extracellular traps (NETs) which are used by neutrophils to ensnare and kill *B. pseudomallei*. To assess the role of platelets in NET formation during melioidosis *in vivo*, we determined cell free DNA (CfDNA) and citrullinated histone 3 (CitH3) levels in BALF (Figure 4C). *B. pseudomallei* showed to be a potent inducer of NET formation, which was, however, similar between groups (Figure 4C). We, therefore, conclude that platelet depletion has a modest impact on early neutrophil recruitment, but not on NET formation during murine melioidosis.
Mice were treated with anti-GPIbα (platelet depletion) or IgG control (both 0.4 µg/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours or sacrificed uninfected. (A) Representative images of Ly6G staining lung sections. (B) Neutrophil influx; measured by Ly6G staining quantification, MPO levels in lung and neutrophil counts in BALF. (C) Cell free DNA and Citrinullated histon 3 levels in BALF. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. N=8 mice per group. *P<0.05 versus IgG control. Abbreviations: MPO=myeloperoxidase and BALF=bronchoalveolar lavage fluid.
Platelet depletion increases local and systemic inflammatory responses

Platelets can influence inflammatory responses of other cells, e.g. monocytes. 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 cytokine and chemokines levels in both lung and plasma (most notably tumor necrosis factor (TNF)-α and C-X-C motif ligand (CXCL)-2; S1 Table), in part likely driven by higher bacterial loads. This aggravated cytokine response in platelet depleted mice was not reflected in altered systemic organ damage, which is a hallmark feature of sepsis. There were no differences in liver damage (as scored by a blinded pathologist) or plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels between groups (S5 Figure A-D).

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

We have recently shown that during K. pneumoniae induced pneumosepsis, platelet depletion towards a levels of <1% of normal has a more pronounced phenotype compared to platelet depletion towards a levels of <5% of normal. 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% also increased bacterial loads in lung and liver (S6 Figure A). Additional, lung MPO levels were decreased, however Ly6G staining was similar between groups (S6 Figure B). Despite an increased local and systemic inflammatory response (S2 Table), distant organ damage was similar (S6 Figure B-C). 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. 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 started at 24 hours after infection as measured by increased lung and BALF hemoglobin levels and lung pathology bleeding scores (Figure 5A-5C, P<0.05 vs. control).
Mice were treated with anti-GPIbα (platelet depletion) or IgG control (both 0.4µg/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours or sacrificed uninfected. (A) Representative photographs of naïve or infected lungs and BALF. (B) Representative microphotographs of H&E stained tissue sections (original magnification 40x), bleeding indicated by arrow. (C) Quantification of lung bleeding; both scored on H&E stained tissue sections by a pathologist blinded for groups and hemoglobin measurement in 50-fold diluted lung homogenates or BALF. Data are represented as bars (mean with SEM). N=8 mice per group. Abbreviations: BALF=bronchoalveolar lavage fluid and OD=optical density. *** P<0.001, * P<0.05 versus IgG control.
Furthermore, we 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. A decrease in platelet counts may also indicate pathologic coagulation activation, which can contribute to complications in melioidosis such as disseminated intravascular coagulation and multiple organ failure. 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 (S7 Figure A-D). This is in line with findings in patients with melioidosis. Platelet depletion (<5%) further increased lung D-dimer and TATc levels in lung and plasma (S7 Figure A-D) when compared to controls. The increased accumulation of fibrin products in the lung in the thrombocytopenic mice can be due to extravascular formation of fibrin as a result of the bleeding or may be due to increased coagulation as a result of the increased bacterial burden and inflammatory response (Figure 3 and S1 Table). Platelet depletion <1% also induced lung bleeding during melioidosis (S6 Figure C). Taken together, these results show that platelet depletion impairs vascular integrity in the lung during melioidosis.

**Mice lacking platelet GPIba, but not platelet TLR signaling, display impaired local host defense during melioidosis**

To assess which platelet receptors were involved in the protective effect of platelets during melioidosis, we investigated mice lacking platelet TLR signaling or GPIba. Platelet TLRs and GPIba can both influence platelet leukocyte interactions and cytokine release. Moreover, it was recently shown that platelet GPIba can influence host defense during *Klebsiella pneumoniae* induced pneumosepsis. To investigate platelet-specific TLR signaling, we used platelet-specific MyD88 KO mice (Plt-MyD88-/-), which lack the crucial TLR signaling protein MyD88 only in platelets and megakaryocytes. Total MyD88-/- mice have impaired host defense during *B. pseudomallei* infection. However, infected Plt-MyD88-/- and littermates had similar bacterial loads in all organs during *B. pseudomallei* infection (S8 Figure A). Moreover, platelet activation, platelet-leukocyte interactions and thrombocytopenia were similar between both groups during experimental melioidosis (S8 Figure B). To investigate the role of platelet GPIba, IL4R/GPIba mice (that lack GPIba, but without the associated macro-thrombocytopenia) were infected with *B. pseudomallei*. IL4R/GPIba mice had no GPIba expression (Figure 6A, P<0.001 vs. con-
trols) and reduced platelet counts during infection (Figure 6B, P<0.01 vs. controls). Platelet activation as measured by P-selectin on platelets was similar between mice strains during infection (Figure 6B), platelet neutrophil-complexes were reduced in IL4R/GPIbα compared to controls (Figure 6B, P<0.01 vs controls). IL4R/GPIbα mice showed increased lung bacterial loads compared to controls in our experimental melioidosis model. This was not to a similar extent as observed in the experiments in which platelets were depleted with anti-GPIbα antibody (Figure 6C, P<0.05 vs. controls) as bacterial loads in distant organs were unaffected (Figure 6C). Both Plt-MyD88−/− mice and IL4R/GPIbα mice did not show increased lung bleeding during melioidosis (Figure S9). These results show that GPIbα, but not platelet TLR signaling, contributes to the local host defense against B. pseudomallei.
**Figure 6** Platelet GPIb deficiency decreases platelet counts and leads to 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) GPIb expression on platelets; (B) Platelet counts, P-selectin expression, and platelet-neutrophil complex formation. (C) Bacterial quantification in indicated organs. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N=8 mice per group. ***P<0.001, ** P<0.01, * P<0.05 versus IgG control.
Here, we show that during clinical melioidosis thrombocytopenia is an independent predictor of 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\textsuperscript{20} as well as other studies looking at sepsis.\textsuperscript{19,41,42} 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.\textsuperscript{19} Disease severity scores of our patients were, however, not collected, 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 towards levels <5% of normal was associated with increased mortality. Moreover, platelets directly contributed to host defense against \textit{B. pseudomallei}, both at the local site of infection as well as distant organs, such as the liver. Of interest, platelet depletion increases local and systemic inflammatory responses during experimental melioidosis. These findings are in line with other murine studies of Gram-negative sepsis using \textit{E. coli}\textsuperscript{15,16} and \textit{K. pneumoniae},\textsuperscript{14} as well as our previous findings in patients with sepsis.\textsuperscript{19}

Neutrophils recruited to the site of infection can influence outcome during melioidosis,\textsuperscript{31} mostly during late-stage infection. Additionally, interactions between platelets and neutrophils can influence bacterial killing.\textsuperscript{11} 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 during \textit{Pseudomonas} infection and during a (polymicrobial) cecal-ligation and puncture model.\textsuperscript{43,44} However, during \textit{Klebsiella infection}, platelets did not influence neutrophil recruitment.\textsuperscript{14} Interestingly, we observed an effect of platelets on antibacterial defense 48 hours after infection, whereas previous studies showed an effect of neu-
trophils 72 hours after B. pseudomallei infection.\textsuperscript{31} We made use of a platelet depletion antibody and depleted platelets to < 5% of normal. Although this mice model is used often to examine the effect of platelets on the host defense, it is important to note that the thrombocytopenia in those mice is more severe as compared to the much more modest levels of thrombocytopenia in patients with melioidosis. Previous studies have shown that platelets can induce NET formation and this can influence bacterial growth of E. coli and S. aureus.\textsuperscript{31,45} During melioidosis, however, platelet depletion did not impair NET formation, as assessed by cfDNA and CitH3 levels.

Earlier, we reported that compromised NETs (by DNase treatment) also did not affect outcome in murine melioidosis.\textsuperscript{34} Previous studies have shown that platelet TLR signaling was important for restriction of E. coli growth,\textsuperscript{11} but during infection with S. pneumoniae, K. pneumonia and B. pseudomallei\textsuperscript{23,46} platelet TLR signaling did not contribute to host defense. These differences might be explained by differences in bacteria, the intracellular nature of B. pseudomallei, or the model (acute high dose vs. slowly growing).

Mice lacking platelet GPIbα did show impaired host defense in the lung. Il4R/GPIbα mice showed increased bacterial burden in the lung after three days of infection, but not to the similar extent as platelet depleted mice. Il4R/GPIbα mice are GPIbα deficient mice without the associated macrothrombocytopenia,\textsuperscript{24} 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 caused by K. pneumoniae, using a GPIbα blocking antibody.\textsuperscript{17}

Platelets protect against bleeding, specifically at the site of infection and inflammation.\textsuperscript{14,47} Platelet depletion resulted in bleeding in the lung during B. pseudomallei infection, a finding consistent with previous reports in Klebsiella and Streptococcal pneumosepsis.\textsuperscript{14,48} However, in contrast to Klebsiella infection,\textsuperscript{14} melioidosis also induced severe bleeding when platelet counts were < 5%. Possibly, B. pseudomallei infection of cells causes more severe damage to tissue as well as 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 addition, increased bacterial growth and cytokine levels observed could be a directly consequence or cause of the bleeding seen in the thrombocytopenic mice, as the release of heme and transferrin free iron can activate and deteriorate the immune system. In human melioidosis, bleeding complications are rarely observed. The importance of the risk of bleeding in melioidosis patients remains to be elucidated.

In conclusion, we found that thrombocytopenia predicted mortality in melioidosis patients even after adjustment for confounders, and that in murine melioidosis, platelet depletion severely hampered survival, host defense as well as vascular integrity.

Acknowledgments

We thank Joost Daalhuisen, Marieke ten Brink, and Jasmin Ersoz for technical assistance during the animal experiments and Regina de Beer for preparations of lung tissue slides.
REFERENCES


SUPPLEMENTARY MATERIALS

METHODS

Cohort study
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. 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. The primary study outcome was in-hospital mortality, but we also pre-defined three secondary outcomes: hypotension (a systolic blood pressure of less than 90 mmHg at any point during admission), acute kidney injury (diagnosed by attending physicians), and respiratory failure (hypoxia judged clinically to require mechanical ventilation; arterial blood gases are not taken routinely in our setting).

Animals
Human IL4R/GPIba mice (University of Arkansas for Medical Sciences, Little Rock, USA) are knock out for mouse GPIba, 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 GPIba. 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.

Experimental study design
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 bronchoalveolair 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 GPib (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-6G&C 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 scored by a pathologist blinded for group identity as previously described. 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. 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. To determine neutrophil influx in the lung, sections were stained with anti-mouse Ly6-G mAb (BioLegend). 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, TNF-α, CCL2 and interferon (IFN)-γ were determined with a commercially available cytometric beads array multiplex assay (BD Biosciences). Myeloperoxidase (MPO) and CXCL2 were measured by ELISA (all R&D systems, Minneapolis, MN) as well as TATc levels (Affinity Biologics 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. Hemoglobin concentrations were measured in 50-fold diluted lung homogenates by light density at 410 nm by NanoDrop spectrophotometer (Thermo Fisher Scientific). AST and ALT were measured using a c702 Roche Diagnostics (Roche Diagnostics BV, Almere, the Netherlands). cfDNA 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). 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).

Statistics of murine experiments
For murine studies, data are expressed as box and whisker plots or as bars (respectively median with range or mean with SD). 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.

REFERENCES
S1 Table  Cytokine and chemokine levels in lung homogenate and plasma during experimental melioidosis

Mice were treated with anti-GPIbα or IgG control (0.4µg/g), infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours. Cytokine and chemokine levels in lung homogenate and plasma. Values are in ng/mL and presented as median (interquartile range). N=8 mice per group. *P < .05, **P < .01 vs IgG control. Abbreviations: b.d.= below detection, n.d.= not determined.

<table>
<thead>
<tr>
<th></th>
<th>Lung homogenates</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Platelet depletion</td>
</tr>
<tr>
<td></td>
<td>ng/mL</td>
<td>24 h</td>
</tr>
<tr>
<td>TNF-α</td>
<td>3.8 (0.1-8.5)</td>
<td>3.3 (0.2-9.3)</td>
</tr>
<tr>
<td>IL-6</td>
<td>23.1 (6.6-36.9)</td>
<td>41.0 (30.6-43.5)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.2 (0.8-2.1)</td>
<td>2.3 (1.0-3.0)</td>
</tr>
<tr>
<td>CCL2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>20.6 (12.7-23.6)</td>
<td>23.8 (23.8-31.1)*</td>
</tr>
<tr>
<td>IL-6</td>
<td>82.0 (41.6-108.8)</td>
<td>113.3 (91.4-135.0)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.7 (2.5-2.9)</td>
<td>2.5 (2.1-3.1)</td>
</tr>
<tr>
<td>CXCL2</td>
<td>166.2 (112.6-194.5)</td>
<td>310.8 (215.6-343.1)**</td>
</tr>
<tr>
<td>CCL2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Mice were treated with anti-GPIbα or IgG control (0.4µg/g), infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours. Cytokine and chemokine levels in lung homogenate and plasma. Values are in ng/mL and presented as median (interquartile range). N=8 mice per group. *P < .05, **P < .01 vs IgG control. Abbreviations: b.d.= below detection, n.d.= not determined.
S2 Table  Cytokine and chemokine levels in lung homogenate and plasma during murine melioidosis in experiments in which the high dose of anti-GPIbα was used

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

Mice were treated with anti-GPIbα or IgG control (2.0µg/g), infected with B. pseudomallei via the airway and sacrificed after 72 hours. Cytokine and chemokine levels in lung homogenate and plasma. Values are in ng/mL and presented as median (interquartile range). N=8 mice per group. *P < .05, **P < .01 vs IgG control. Abbreviations: b.d.= below detection, n.d= not determined.

S1 Figure  Conceptual hierarchical framework for risk factors for thrombocytopenia and mortality

1. Gender
2. Age
3. Diabetes mellitus
4. Liver cirrhosis
5. Malignancy
6. Malnutrition*
7. Melioidosis
8. Thrombocytopenia
9. Bleeding
10. Immunothrombosis
11. Sepsis
12. Respiratory failure
13. Hypotension
14. Kidney failure
15. Mortality

Gender, age, and occupation 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; a conceptual hierarchical framework its built around social and biological plausibility of which came first in time.* Factors in level 4 are immediate proximate causes of death. *We used rice farming (occupation) as a proxy for malnutrition.

**S2 Figure**  \( B.\) pseudomallei growth in blood during experimental melioidosis

Mice were treated with anti-GPIba or IgG control (0.4µg/g), infected with \( B.\) pseudomallei via the airway and sacrificed after 24, 48, or 72 hours. Bacterial quantification in blood. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. \( N=8\) mice per group.

**S3 Figure**  \( \text{Ex vivo } B.\) pseudomallei growth

(A) Mice were treated with anti-GPIba (0.4µg/g, platelet depletion) or IgG control (0.4µg/g) and sacrificed uninfected. Whole blood was incubated with viable \( B.\) pseudomallei (10^7 CFU/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 \( B.\) pseudomallei (5*10^5 CFU/mL) for 20 hours at 37 degrees after which bacterial growth was quantified. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. \( N=4\) replicates or mice per group. Human experiments were performed at least twice with 2 independent donors.
**S4 Figure**  Platelet depletion does not influence total cell influx in the bronchoalveolar space during experimental melioidosis

Mice were treated with anti-GPIbα (platelet depletion) or IgG control (0.4µg/g), infected with *B. pseudomallei* via the airway and sacrifice after 72 hours or uninfected. Total cell numbers in the BALF. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N=8 mice per group. Abbreviation: BALF= bronchoalveolar lavage fluid.
A D Mice were treated with anti-GPIba (platelet depletion) or IgG control (0.4µg/g), infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours. (A-C) Representative images, original magnification 40x and (D) quantification of liver damage. (D) ALT and AST plasma levels. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. N=8 mice per group. *P < .05 vs IgG control. Abbreviations: BPS = *Burkholderia pseudomallei*, ALT = alanine transaminase and AST = aspartate transaminase.
Platelet depletion <1% also impairs host defense and vascular integrity during melioidosis

(A-C) Mice were treated with high dose anti-GP Ibα 2 µg/g or IgG control and infected with B. pseudomallei via the airway and sacrificed after 24, 48, or 72 hours. (A) Bacterial loads in organs indicated. (B) Blood platelet counts, MPO levels lung, Ly6G staining lung, and liver pathology. (C) ALT, AST levels, lung bleedings score, and lung hemoglobin levels. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. N=8 mice per group. *P < .05, **P < .01, ***P < .001 vs IgG control.

Abbreviations: ALT= alanine transaminase and AST= aspartate transaminase.
**Figure**  Thrombocytopenia results in increased local and systemic coagulation

(A-D) Mice were treated with anti-GPIba (platelet depletion) or IgG control (both 0.4µg/g) and infected with *B. pseudomallei* via the airway and sacrificed after 24, 48, or 72 hours or sacrificed uninfected. (A) Plasma and lung thrombin-anti-thrombin complex (TATc) levels. (B) Lung fibrinogen western blot showing D-dimer, and semi quantification D-dimer in uninfected mice. Lung fibrinogen westerns blots for (C) 24 hours and (D) 72 hours. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile, and largest observation or as bars (mean and SEM). N=8 mice per group. *P < .05 and **P < .01 vs IgG control. Abbreviations: NP= naïve plasma, +=positive D-dimer control.
Platelet Toll-like receptor (TLR) signaling does not influence host defense during murine melioidosis

(A-B) Plt-Myd88-/- (white boxes) or control mice (grey boxes) were infected with *B. pseudomallei* via the airway and sacrificed after 72 hours. (A) Bacterial quantification in indicated organs. (B) Platelet counts in blood, platelet P-selectin expression, and platelet-neutrophil complex formation in blood. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. N=8 mice per group.
**Figure**  Lung bleeding in IL4R/GPIba, Plt-Myd88−/− and control mice

(A) IL4R/GPIba or control mice were infected with *B. pseudomallei* via the airway and sacrificed after 72 hours and hemoglobin levels were determined in lung homogenates. (A) Plt-Myd88−/− or control mice were infected with *B. pseudomallei* via the airway and sacrificed after 72 hours and hemoglobin levels were determined in lung homogenates. (B) Lung pathology quantification, lung bleeding score and (C) representative images original magnification 40x of IL4R/GPIba and control mice. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. N=8 mice per group. Abbreviations: OD= optical density and BPS= *Burkholderia pseudomallei*.