Platelets: versatile effector cells in pneumonia and sepsis

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Thrombocytopenia impairs host defense in gram-negative pneumonia derived sepsis

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Abstract

Thrombocytopenia is a common finding in sepsis and associated with a worse outcome. We used a mouse model of pneumonia-derived sepsis caused by the human pathogen *Klebsiella pneumoniae* to study the role of platelets in host response to sepsis. Platelet counts (PC) were reduced to a median of <5x10^9/L or to 5-13x10^9/L by administration of a depleting antibody in mice infected with *Klebsiella* via the airways. Thrombocytopenia was associated with a strongly impaired survival during pneumonia-derived sepsis proportional to the extent of platelet depletion. Thrombocytopenic mice demonstrated PC-dependent enhanced bacterial growth in lungs, blood and distant organs. Severe thrombocytopenia resulted in hemorrhage at the primary site of infection, but not in distant organs. PC of 5-13x10^9/L were sufficient to largely maintain hemostasis in infected lungs. Thrombocytopenia did not influence lung inflammation or neutrophil recruitment, and did not attenuate local or systemic activation of coagulation or the vascular endothelium. PC below <5x10^9/L even resulted in enhanced coagulation and endothelial cell activation, which coincided with increased pro-inflammatory cytokine levels. In accordance, low PC in whole blood enhanced *Klebsiella* induced cytokine release. These data suggest that platelets play an important role in host defense to *Klebsiella* pneumosepsis.
Introduction

Sepsis is one of the most elusive syndromes in medicine, with an estimated incidence of over 19 million cases per year worldwide\(^1,2\) and high mortality rates despite appropriate antibiotic treatment\(^2\). The most common infection causing sepsis is pneumonia\(^3\). *Klebsiella* (*K.*) *pneumoniae* is a frequent causative agent in gram-negative sepsis and a common respiratory pathogen\(^2,4,5\).

Thrombocytopenia is a common finding in patients admitted to the Intensive Care Unit (ICU) and associated with a worse outcome\(^6-9\). Platelets are small anucleate cells widely renowned for their role in hemostasis. Notably, however, low platelet counts (PC) result in hemorrhage in only some patients. In accordance, thrombocytopenia *per se* does not induce bleeding in mice; in inflamed organs, however, platelets have a crucial role in preventing hemorrhage\(^10\). Next to hemostasis, platelets contribute to host defense against bacteria and may mediate a variety of proinflammatory effects\(^11,12\). On the other hand, platelets may inhibit macrophage-dependent inflammation during infectious and non-infectious systemic inflammation\(^13,14\). These properties implicate platelets as essential players in the pathogenesis of sepsis, involved in both protective and potentially injurious host responses\(^15\).

We here sought to determine the impact of thrombocytopenia on host defense during pneumonia derived sepsis caused by *Klebsiella*. For this we administered a platelet depleting antibody to mice at two different doses, causing thrombocytopenia of different severities, and studied bacterial growth and dissemination, hemostasis, and local and systemic inflammation after infection with a virulent *K. pneumoniae* strain via the airways.

Methods

**Animals**

Female C57Bl/6 mice (Harlan, Horst, the Netherlands) were used between 10 and 12 weeks of age. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

**Experimental study design**

Pneumonia was induced by intranasal inoculation with *K. pneumoniae* serotype 2 (ATCC 43816 Rockville, MD; \(10^4\) colony forming units (CFU) in 50 μL isotonic saline) as described\(^16,17\). Two hours before infection, mice were intravenously infused with 2 μg/g or 0.4 μg/g anti(α)-gycoprotein(GP)Iβα (Emfret analytics, Eibelstadt, Germany) or 2 μg/g control immunoglobulin (Ig)G (Emfret analytics)\(^10,18\). Mice were euthanized 12 or 44 hours after induction of pneumonia (N = 8 mice per group at each time point) or observed for 7 days (N = 20 per group). During the observation study, clinical signs were scored by an independent
animal biotechnician as follows: During the observation study, clinical signs were scored by an independent animal biotechnician as follows: 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 apathic, 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 seconds, 2 >5 seconds, 3 unresponsive); resulting in a maximum score of 16. Dead mice were scored with the highest clinical score. Lung, spleen and liver were harvested, individually weighed, and homogenized in sterile isotonic saline (4 mL per gram tissue). Bacterial quantification and storage of organs were performed as described 16,17.

Flow cytometry

Platelet counts (PC) were determined in citrated whole blood by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ) using hamster anti-CD61 monoclonal antibody (BioLegend, San Diego, CA) in accordance with manufacturers’ instructions.

Histology and assays

Four-micrometer sections of the left lung lobe, spleen and liver were stained with hematoxylin and eosin (H&E) or Ly-6G (BD PharMingen, San Diego, CA) and scored as described 16. Bleeding was scored on histology slides and by estimation of Hemoglobin (Hb) levels by evaluation of the absorbance of light with a wavelength of 410 nm by spectrophotometry (Nanodrop 2000, Thermo Scientific, Waltham, MA) of 50-fold diluted homogenate samples. Neutrophil extracellular traps (NETs) were stained in lung and liver sections by anti-citrullinated histone H3 (H3Cit, Abcam, Cambridge, UK) according to manufacturer’s instructions 19,20. IL-6 and TNF-α were determined using a cytometric beads array multiplex assay (BD Biosciences). Platelet Factor 4 (PF4; R&D Systems, Abingdon, United Kingdom), Myeloperoxidase (MPO; R&D Systems), E-selectin (R&D Systems) and thrombin-antithrombin complexes (TATc; Bioconnect, Huissen, the Netherlands) were measured by ELISA. Fibrinogen Western blotting was done as previously described 21, using polyclonal goat-anti-mouse-fibrinogen (Kordia, Leiden, the Netherlands) and donkey-anti-goat IgG-HRP secondary antibody (Abcam). Imaging was done on a LAS4000 dark box (Fujifilm, Tokyo, Japan). Anti-fibrin(ogen) reactive bands of fibrinogen, fibrin multimers and fibrin degradation products (Fragment X, D-dimer, D-monomer) were analyzed using ImageJ (version 2006.02.01, US National Institutes of Health, Bethesda, MD). Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured using a c702 Roche Diagnostics (Roche Diagnostics BV, Almere, the Netherlands).
In vitro experiments

Mice were intravenously infused with 2 μg/g α-GPlba or 2 μg/g control IgG and heparinized blood was collected from the inferior vena cava two hours thereafter. 90 μL whole blood was incubated with an equal volume of vehicle medium (IMDM; Lonza, Basel, Switzerland) with or without 10^6 CFU K. pneumoniae. After 6 hours, part of the blood was serially diluted in normal saline and CFUs were counted following 16 hours of incubation at 37°C. Remaining blood was spun at 3000 rpm 10 minutes and plasma supernatants were stored at -20°C until analysis.

Statistical analysis

Data are expressed as scatter dot plots or as box and whisker plots. Comparisons between groups were first performed using Kruskal-Wallis one-way analysis of variance test, followed Mann-Whitney U test where appropriate. Survival was compared by Kaplan-Meier analyses followed by log rank test. In order to compare clinical observation scores, areas under the curve were compared using analysis of variance with rank transformation. P-values < 0.05 were considered statistically significant.

Results

Infusion of α-GPlba induces dose dependent platelet depletion

Thrombocytopenia was induced by injection of α-GPlba antibodies, which deplete platelets in mice without affecting the function of the remaining platelet population. Naive mice had a median PC of 521x10^9. Infusion with 2 μg/g and 0.4 μg/g α-GPlba caused platelet depletion to a median of 2x10^9 platelets/L and 6x10^9/L, respectively, after 14 hours; PC rose to a median of 5x10^9/L and 153x10^9/L respectively after 46 hours in naive mice (Figure 1A,B). At 12 hours after infection with Klebsiella (and 14 hours after α-GPlba infusion) the extent of α-GPlba induced platelet depletion was similar compared to naive mice. After 44 hours of infection however, PC in control mice lowered to a median of 117x10^9 platelets/L. Whereas mice treated with 2 μg/g α-GPlba still displayed PC <5x10^9/L, mice infused with 0.4 μg/g α-GPlba replenished platelets only to 13x10^9 platelets/L (Figure 1C). In order to confirm that the platelet depletion by α-GPlba does not cause platelet lysis or activation, plasma PF4 was measured (Figure 1D). While PF4 was upregulated during Klebsiella pneumosepsis in control mice (indicative of platelet activation during sepsis) no PF4 could be detected in α-GPlba treated mice at any time point. α-GPlba did not induce alterations in any of the inflammatory outcome measurements in uninfected mice. Mice treated with 0.4 μg/g or 2 μg/g α-GPlba are subsequently referred to as low-PC and very low-PC mice respectively.
Thrombocytopenia is associated with a PC-dependent impaired survival in Klebsiella induced pneumonia

To determine the impact of thrombocytopenia on mortality during *K. pneumoniae* induced pneumonia, we carried out an observational study in very low-PC, low-PC and control mice during 7 days after infection (Figure 2A,B). Very low-PC mice demonstrated a rapidly increasing symptom score shortly after infection and all very low-PC mice succumbed in the second night (P<0.0005 versus low-PC and control mice). Low-PC mice developed symptoms shortly after very low-PC mice and showed a relatively delayed mortality (P<0.0005 versus control and very low-PC mice). Symptoms developed much slower in control mice, and 30% of control mice survived the infection while none of the very low-PC and only one of low-PC mice survived.
Thrombocytopenia results in PC-dependent enhanced bacterial growth and dissemination

To obtain a first insight in the underlying mechanism by which thrombocytopenia impairs outcome in *Klebsiella* pneumosepsis, we determined bacterial loads in lungs, blood, liver and spleen at 12 and 44 hours after infection. At 12 hours after infection, low-PC mice displayed a slightly enhanced bacterial outgrowth in the lungs (P<0.05 versus control mice); very low-PC mice displayed more pronounced increased bacterial burdens in lungs and liver (Figure 2C,F; P<0.05 versus control mice). These differences increased after 44 hours of infection (Figure 2C-F) with median bacterial loads that were ≥ 100-fold higher in all organs of very low-PC mice (P<0.005 - P<0.0005 versus control mice), and ≥ 10-fold higher in lungs, blood and spleen of low-PC mice (P<0.05 - P<0.005 versus control mice). Notably, at this late time point bacterial burdens were significantly higher in very low-PC mice relative to low-PC mice in all body sites tested (P<0.05 - P<0.005). Blood PC negatively correlated with blood *Klebsiella* CFU at 44 hours (Figure 2G).

Thrombocytopenia results in PC-dependent lung hemorrhage upon infection, with intact hemostasis in distant organs

We examined the occurrence of hemorrhages at the primary site of infection (lungs) and two distant body sites (spleen and liver) in very low-PC, low-PC and control mice in the presence or absence of *Klebsiella* pneumosepsis (Figure 3). Very low-PC mice demonstrated massive bleeding in their lungs upon infection with *K. pneumoniae*, both macroscopically (Figure 3A) and microscopically (Figure 3B,C). While infected low-PC mice did not display macroscopic signs of lung bleeding (Figure 3A), microscopic examination of H&E stained lung tissue sections revealed sites of hemorrhage (Figure 3B,C; P<0.005 versus control mice at both 12 and 44 hours), albeit to a lesser extent than in very low-PC mice (P<0.005 versus control mice and P=0.06 for the difference between low-PC and very low-PC mice at 12 hours). Of interest, neither low-PC nor very low-PC mice showed hemorrhages in spleen or liver (Supplemental Figure 1A,B). Bleeding was not found in any organ of uninfected mice from any of the three experimental groups. In accordance with histology, the infected lungs of very low-PC mice contained higher Hb levels relative to low-PC and control mice (Figure 3D; P<0.005 and P<0.0005 respectively), while in distant organs Hb concentrations were similar in all groups (Supplemental Figure 1C,D); Hb content of organs from uninfected mice were also similar in all groups.

To determine the impact of thrombocytopenia on lung inflammation and pathology, we semi-quantitatively scored lung histology slides obtained from naive and infected mice (Figure 3B,E). Despite increased bacterial loads in lungs of very low-PC and low-PC mice, no differences in histology scores or percentage infiltrated lung surface were found compared to control mice. Platelets have been reported to play a role in neutrophil recruitment to the lungs; we therefore measured neutrophil influx in lungs by determining the number of Ly-6G positive cells (Figure 4A,B) and by MPO in whole lung homogenates (Figure 4C); differences between groups were not significant. Platelet attachment to neutrophils has
Figure 2: Thrombocytopenia causes PC-dependent impaired survival and enhanced bacterial growth during *Klebsiella* induced pneumonia. Survival (A) and clinical observation score (B) of very low-PC (open dots), low-PC (half open dots) and control (closed dots) mice infected with *K. pneumoniae* via the airways. For bacterial quantification, very low-PC (open dots), low-PC (half open dots) and control (closed dots) mice were again infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. Bacterial counts were determined in lungs (C), blood (D), spleen (E) and liver (F). (G) Correlation of PC and *Klebsiella* CFU recovered from blood 44 hours after infection. Data are expressed as scatter dot plots with the median. N = 20 mice per group in the survival experiment and N = 8 mice per group for bacterial quantification. *** P<0.0005 for all comparisons in survival and clinical observation score graphs. * P<0.05, ** P<0.005, *** P<0.0005 versus control; # P<0.05, ## P<0.005 versus low-PC.
Figure 3: Thrombocytopenia results in PC-dependent lung hemorrhage upon infection, with intact hemostasis in distant organs. Very low-PC (white), low-PC (light gray) and control (dark grey) mice were infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. (A) Representative photographs of naive or infected lungs. (B) Representative microphotographs of H&E stained tissue sections of naive or 44 hours infected lungs (4x original magnification). Lung bleeding was scored on H&E tissue sections by a pathologist blinded for groups (C) and Hb was measured in 50-fold diluted lung homogenates by light density at 410 nm (D). Severity of inflammation (E) was scored on H&E sections. 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<0.005, *** P<0.0005 versus control; # P<0.05, ## P<0.005 versus low-PC. OD = optical density.
Figure 4: Platelets are not required for lung neutrophil accumulation or NETs formation in the lungs. Neutrophil accumulation in lung tissue was measured by quantification of Ly-6G stainings (4x original magnification microphotographs in A; quantification in B) and MPO measured in lung homogenates (C). NETs were stained using antibody to H3Cit (D 4x original magnification microphotographs for lung, E for liver; quantification in F,G). Ly-6G and NET positivity and total lung surface area were measured using Image J (U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij); the amount of Ly-6G or NET positivity was expressed as a percentage of the total surface area. 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.
been reported to be a threshold switch for release of neutrophil extracellular traps (NETs)\textsuperscript{24}. Whereas NETs were not visible in lungs of uninfected mice, \textit{Klebsiella} pneumonia resulted in enhanced NETs formation. Low-PC mice showed increased NETs in their lungs at 44 hours after infection relative to infected control mice (P<0.05, Figure 4D,F). NETs were not detected in the liver (Figure 4E,G).

\textit{Platelets are not required for coagulation or endothelial cell activation during \textit{Klebsiella} pneumonia}

To obtain insight in the role of platelets in systemic coagulation activation during \textit{Klebsiella} pneumonia derived sepsis, we measured TAT\textsubscript{c} levels in plasma of very low-PC, low-PC and control mice. At 12 hours post infection, plasma TAT\textsubscript{c} levels were not different between groups; remarkably, 44 hours after induction of pneumonia, very low-PC mice demonstrated increased plasma TAT\textsubscript{c} concentrations compared to control (P<0.005) and low-PC mice (P<0.05; Figure 5A). In order to measure local coagulation in the lungs, we performed fibrin(ogen) Western blotting on whole lung homogenates, and quantified fibrinogen multimers and degradation products (Fragment X and D-dimer; Figure 5B-E). \textit{Klebsiella} pneumonia resulted in enhanced pulmonary coagulation in control mice relative to uninfected mice. Thrombocytopenia was associated with increased pulmonary deposition of fibrinogen multimers and split products in mice infected with \textit{Klebsiella} via the airways, an effect that was PC-dependent. We measured (soluble) E-selectin in plasma and whole lung homogenates as parameters for systemic and local endothelial cell activation respectively\textsuperscript{25,26}. In line with their exaggerated procoagulant response, especially very low-PC mice showed strongly increased plasma and lung E-selectin levels (Figure 5F,G).

\textit{Thrombocytopenia has a bimodal PC-dependent effect on the occurrence of distant organ injury during \textit{Klebsiella} induced pneumosepsis}

This model of pneumosepsis is associated with distant organ damage during late stage infection\textsuperscript{16,17}. We measured plasma AST and ALT as measures of hepatocellular injury and LDH as a marker of cellular injury in general (Figure 6A-C). As expected\textsuperscript{16,17} plasma AST, ALT and LDH increased significantly in control mice during the course of the infection. Interestingly, distant organ injury was reduced in low-PC mice (significant for AST and LDH), while very low-PC mice were not different from control animals. Recently, NETs formation in the liver was shown to contribute to liver damage after intraperitoneal infection with high dose \textit{Eschericia (E.) coli}\textsuperscript{24}. However, we were unable to visualize NETs in liver tissue of infected mice in any of the experimental groups (Figure 4E,G). We speculated that platelets might influence systemic neutrophil activation\textsuperscript{27}, and therefore measured plasma MPO levels (Figure 6D). While very low-PC mice had elevated plasma MPO levels relative to infected control mice, low PC-mice displayed an attenuated increase in plasma MPO at 12 hours post infection.
Figure 5: Platelets are not required for coagulation or endothelial cell activation during *Klebsiella pneumoniae* pneumonia. Very low-PC (white), low-PC (light gray) and control (dark grey) mice were infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. Plasma TATc levels were measured as a marker for systemic coagulation activation (A). Fibrinogen was detected by Western blotting on lung homogenate samples (B). Semi-quantification of fibrinogen blot (C-E); quantification scores of naive control mice, very low-PC and low-PC mice were normalized to infected controls. E-selectin was measured in plasma (F) and lung homogenates (G) as marker for endothelial cell activation. Data are expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation or as bars depicting mean and SEM. N = 8 mice per group. * P<0.05, ** P<0.005 versus control; # P<0.05 versus low-PC. Fibr. = fibrinogen
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After infection with *Klebsiella*, thrombocytopenia was associated with elevated plasma TNF-α and IL-6 relative to control mice, an effect that was clear at 44 hours and most pronounced in very low-PC mice (Figure 7A, B). Considering that this effect could be the consequence of the much higher bacterial loads in thrombocytopenic mice, we next incubated whole blood from uninfected very low-PC and control mice with viable *Klebsiella* and measured pro-inflammatory cytokine levels in supernatants after 6 hours. *Klebsiella* CFU increased almost $10^5$-fold during the 6-hour incubation period in blood of both very low-PC and control mice, illustrating the high virulence of this bacterium (Supplemental Figure 2). Whole blood from very low-PC mice produced more TNFα ($P<0.05$) and IL-6 ($P<0.05$) than whole blood from control mice (Figure 7C, D). Of note, thrombocytopenic mice also displayed higher cytokine levels in lungs after infection, in particular during late stage infection in very low-PC mice (Supplemental Figure 3).
Chapter 3

Discussion

We investigated the role of platelets during gram-negative pneumonia induced sepsis by infecting thrombocytopenic and control mice with the common human sepsis pathogen *K. pneumoniae* via the airways. Our main findings were that thrombocytopenia enhanced mortality during *Klebsiella* induced pneumosepsis, and that even in mice with severe platelet depletion a modest rise in PC resulted in prolonged survival. In accordance, we found a PC-dependent enhanced bacterial growth in the lungs, blood and distant organs in thrombocytopenic mice during late stage sepsis. Very low-PC (<5x10⁹/L) resulted in hemorrhage at the primary site of infection but not in distant organs in septic mice; PC of 5-13x10⁹/L during the course of the infection were sufficient to largely maintain hemostasis. Low-PC additionally resulted in attenuated distant organ injury during gram-negative sepsis.

In both our *in vivo* and *in vitro* models, thrombocytopenia increased the release of pro-inflammatory cytokines to *Klebsiella*.

We induced thrombocytopenia of two distinct severities to investigate the hemostatic and immunomodulatory role of platelets during infection. PC of 5-13x10⁹/L would be sufficient...
to maintain hemostasis under inflammatory conditions \(^1\); however, PC in that range would hypothetically be insufficient to perform platelets’ immune functions. Notably, the thrombocytopenia that occurs in critically ill patients usually is less severe than induced here and in previous mouse studies examining the role of platelets in hemostasis and inflammation \(^10,28-30\). Hence, our results should be viewed upon in the context of an established and controlled mouse sepsis model, while sepsis patients represent a much more heterogeneous group with many more variables, as amongst other illustrated by differential genomic responses in blood leukocytes in experimental mouse sepsis and clinical human sepsis \(^31\).

We used a clinically relevant model of sepsis, induced by a low inoculum (10^4 CFU) of a virulent pathogen, associated with a gradually growing bacterial load at the primary site of infection and resulting in dissemination of the infection to distant organs during later stages, together with systemic inflammation and injury. This model, which is associated with clear sickness behavior such as detailed in the clinical scoring method, allowed us to study the role of platelets in distinct phases of the host response to infection, i.e., in the early phase, wherein innate immunity serves a protective role, and in a later stage, wherein abundant inflammation may result in tissue injury. We considered the lungs as primary source of infection most appropriate since pneumonia by far is the most common cause of sepsis \(^2,3\), and since platelets especially exert proinflammatory and immunomodulatory effects in the lungs \(^32\). In addition, this model permitted careful analyses of the role of platelets in vascular integrity at various body sites during sepsis. Previous investigations that addressed the role of platelets in the host response to sepsis used very high infectious doses of \(E.\ coli\) (10^7 CFU) administered via the intraperitoneal route, resulting in an immediate fulminant septic shock syndrome, resembling endotoxic shock \(^13,24\). One of these studies examined the role of platelets in antibacterial defense, reporting elevated bacterial loads in blood of thrombocytopenic mice but not at the primary site of infection (the peritoneal cavity) \(^24\). Similarly, thrombocytopenic mice had higher bacterial loads in their blood 1 and 4 hours after intravenous injection of high dose \(Bacillus (B.)\ cerus\) (5 x 10^7 CFU), a bacterial challenge that was rapidly cleared by mice with normal PC \(^33\). The \(Klebsiella\) strain used here is much more virulent, as illustrated by the high mortality rates after infection with a relatively low bacterial dose (Figure 2) and the logarithmic growth of this pathogen in whole blood (supplemental Figure 2). We showed that the presence of platelets in blood did not inhibit bacterial growth \textit{in vitro}. Notably, \textit{in vivo}, PC of only 5-13x10^9/L conferred significant improvement of antibacterial defense, as illustrated by the different bacterial loads between low-PC and very low-PC mice. While aspirin treatment increased mortality and inflammation in high dose LPS- or \(E.\ coli\) induced inflammation \(^13\), secondary platelet activation played no role in our \(Klebsiella\) pneumosepsis model. Treatment with clopidogrel, an irreversible inhibitor ADP platelet activation \(^34\), reduced rather than enhanced bacterial loads in distant organs in this model of gram-negative sepsis (our own unpublished data). Others have found that clopidogrel pre-treatment attenuated the drop in PC and improved end organ damage
in polymicrobial sepsis in mice. In contrast, clopidogrel treatment did impact on outcome parameters in *E. coli* endotoxin infused pigs. The discrepancy between the aforementioned studies are likely due to differences between the models or other anti-inflammatory effects of aspirin or clopidogrel.

Platelets play a crucial role in protection of the vascular integrity during inflammation. While thrombocytopenia per se does not induce bleeding, locally instilled inflammatory stimuli caused hemorrhages in skin, brain and lungs in thrombocytopenic mice. The present study is the first to investigate the role of platelets in hemostasis in different organs during sepsis. Our results indicate that platelets are important in maintenance of hemostasis at the primary site of infection only, in spite of bacterial dissemination and evidence for distant inflammatory organ injury. Indeed, even in mice with very low PC (<5x10^9/L), liver and spleen did not show any sign of hemorrhage. Possibly, the high bacterial loads in distant organs would have resulted in inflammation-induced hemorrhage in thrombocytopenic mice after longer infection durations; earlier deaths precluded such analyses. Hemostasis in the lungs was largely preserved in low-PC mice, with median PC of 5 to 13x10^9/L at 12 and 44 hours after infection respectively (which is 1 to 2% and 9% of normal PC). In a previous study, transfusion of platelets into thrombocytopenic mice to PC of 4-8% of normal significantly reduced skin bleeding in a model of the reverse passive Arthus reaction, while platelet transfusion to PC of 10-15% of normal were required to completely prevent bleeding. Together these data suggest that the absolute threshold at which thrombocytopenia results in bleeding may differ dependent on body site and type of inflammatory challenge. In this respect it is important to note that previous studies examining the impact of thrombocytopenia on inflammation-induced hemorrhage all used non-infectious stimuli causing acute inflammation, whereas we studied thrombocytopenia in the setting of a gradually evolving inflammatory response elicited by a progressively expanding bacterial load.

Platelets provide a phospholipid surface for catalysis of tissue factor – factor VIIa mediated coagulation. The coagulation cascade was, however, not hindered by low or very low-PC in our model of pneumonia-derived sepsis. On the contrary, especially very low-PC mice displayed enhanced coagulation activation, both in plasma and lungs. Moreover, platelets can contribute to endothelial cell activation and vascular inflammation, which are hallmark features of sepsis. Using E-selectin as a marker for endothelial cell activation, we here show that platelets are not essential for this response during gram-negative sepsis. Very low-PC mice even demonstrated evidence for increased endothelial cell activation. Together these results suggest that coagulation and endothelial cell activation are driven by the systemic inflammatory response during sepsis with a limited role of platelets. It is likely that thrombocytopenia induced hemorrhage promotes coagulation activation, resulting into thrombin generation, which secondarily contributes to endothelial cell activation via protease activated receptors 1 and 2. Additionally, the exaggerated systemic inflammation in
severe thrombocytopenic mice provides an explanation for the enhanced procoagulant and endothelial cell responses in these animals.

Platelet activation is known to augment inflammatory responses and platelet depletion has been described to be protective in sterile LPS induced lung inflammation. Despite ≥100-fold higher bacterial burdens and macroscopic bleeding in the lungs of very low-PC mice 44 hours after *Klebsiella* infection, the extent of lung damage and inflammation were similar between groups and pulmonary neutrophil numbers were similar in thrombocytopenic and control mice. Previous studies on acute lung inflammation did reveal a role for platelets in neutrophil recruitment. Our data suggest that the influence of platelets on neutrophil influx to sites of lung inflammation likely depends on the stimulus and the time during which this inflammatory stimulus emerges in the airways. Although platelets have been implicated in NET formation, we did not find firm evidence for such a role in the lungs of mice infected with *Klebsiella*. One might argue however that in very low-PC mice NET formation was relatively impaired at 44 hours post infection when compared with low-PC and control mice considering the very high bacterial burdens in very low-PC mice.

Previous studies showed increased organ failure during endotoxin shock or after *B. cereus* infusion in thrombocytopenic mice. In accordance, very low-PC mice displayed increased LDH plasma levels during *Klebsiella* pneumosepsis. Interestingly, low-PC mice were protected from distant organ injury with a significant reduction in transaminases and LDH released in plasma. While in fulminant acute *E. coli* sepsis platelets were shown to contribute to liver injury at least in part through enhancing NETs formation, we did not detect NETs in liver tissue in our more gradually evolving sepsis model. Interestingly, low-PC but not very low-PC mice had attenuated systemic neutrophil activation 12 hours after infection, suggesting that platelets may inhibit neutrophil degranulation, an effect that is overruled by the high bacterial burdens in very low-PC mice. Possibly, the reduced systemic neutrophil degranulation in low PC-mice might explain the diminished hepatocellular injury in this group. Other platelet mediated mechanisms for organ injury during sepsis could be via microthrombosis and ischemia or via induction of apoptosis, Granzyme-B mediated toxicity and by shedding of platelet microparticles. The protective role of platelets may not be restricted to gram-negative pneumonia-derived sepsis. Our own preliminary data suggest that severe thrombocytopenia results in enhanced bacterial dissemination during pneumonia caused by *Streptococcus pneumoniae* (data not shown); moreover, in lymphocytic choriomeningitis virus infection, profound platelet depletion caused systemic bleeding and death, while nonhemorrhagic and partially platelet depleted mice were unable to control viral replication.

The regulatory role of platelets in cytokine response is still elusive. Systemic TNF-α release in response to low-dose intravenous LPS was impaired in thrombocytopenic mice and restored upon platelet transfusion. To the contrary, platelets in complex with macrophages
inhibited TNF-α and IL-1β production after high-dose intravenous LPS or *E. coli* challenges 13. Additionally, platelet GPIb-IX had suppressive effects on inflammation in a cecal ligation and puncture model 50. While platelets potentiated macrophages to produce TNF-α and IL-6 after low dose (100 pg/mL) LPS stimulation *in vitro* 51, high dose LPS stimulation (50-10,000 ng/mL) induced platelet-macrophage interaction that inhibited TNF-α production 13,14. In our studies, in which high levels of LPS-expressing *K. pneumoniae* were present, cytokine production *in vivo* and after whole blood stimulation *in vitro* was enhanced. Although initial cytokine production is indispensable for an adequate host response to invading pathogens, excessive cytokine production might have opposite effects during severe infection 52. Platelets might serve as a switch for the inflammatory response during sepsis, i.e., by promoting inflammation at an early stage of infection when the inflammatory stimulus is low, and by inhibiting cytokine production when inflammatory stimuli become high 13.

In conclusion, severe thrombocytopenia is associated with a strongly impaired host defense during pneumonia derived gram-negative sepsis, which is proportional to the extent of platelet depletion. While in sepsis patients low PC could merely be a marker of disease severity, our current results suggest that thrombocytopenia plays a causative role in increased sepsis mortality.

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References


Supplemental figures

**Supplemental Figure 1: Thrombocytopenia does not result in hemorrhage in distant organs.** Very low-PC (white), low-PC (light gray) and control (dark grey) mice were infected with *K. pneumoniae* via the airways and euthanized 44 hours later. Liver and spleen bleeding were scored on H&E tissue sections by a pathologist blinded for groups (C) and liver and spleen Hb were measured in 50-fold diluted organ homogenates by light density at 410 nm (D). 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.

**Supplemental Figure 2: Thrombocytopenia does not impact on *Klebsiella* outgrowth in vitro.** Heparinized whole blood obtained from naive very low-PC and control mice was 1:1 diluted in IMDM and incubated with *Klebsiella* or IMDM control. After 6 hours, part of the blood was serially diluted in normal saline and CFUs were counted. Data are expressed as scatter dot plots with the median; *Klebsiella* CFU at t = 0 (gray dots) and *Klebsiella* growth in very low-PC (open dots) or control (closed dots).
Supplemental Figure 3: Thrombocytopenia increases lung TNF-α and IL-6 levels during *Klebsiella* sepsis.

Very low-PC (white), low-PC (light gray) and control (dark grey) mice were infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. TNF-α (A) and IL-6 (B) were measured in lung homogenates. Data are expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation or as bars depicting mean and SEM. N = 8 mice per group. * P<0.05, ** P<0.005 versus control.