Platelets: versatile effector cells in pneumonia and sepsis

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Summary and general discussion
The role of platelets in infection and immunity is an exciting new theme, which is rapidly evolving. In this thesis, we studied the involvement of platelets in the host response to pneumonia and sepsis, using two clinically relevant human pathogens, and a combination of in vitro systems, experimental mouse models and observational studies in patients with sepsis.

We made use of two well-established mouse models of pneumonia and sepsis. Mice were infected with *Klebsiella (K.) pneumoniae* or *Streptococcus (S.) pneumoniae* via the airways, after which either clinical symptoms and mortality were scored, or experiments were terminated at predefined time points during the course of infection in order to obtain insight in bacterial growth and dissemination, and in host responses. We used these two pathogens because *K. pneumoniae* is a common gram-negative causative agent in hospital-acquired pneumonia and sepsis, while *S. pneumoniae* is a gram-positive bacterium and the most common causative microorganism of community-acquired pneumonia. Both bacteria cause fulminant pneumonia in mice, with dissemination into the bloodstream and to distant organs during later stages, initiating systemic inflammation and organ injury. These kinetics allowed us to study distinct phases of the host response to infection: the early phase, wherein innate immunity serves a protective role, and a later stage, wherein abundant inflammation may result in tissue injury and death. We considered the lungs as primary source of infection most appropriate since pneumonia by far is the most common cause of sepsis 1,2, and since platelets, our cell type of interest, especially exert immune modulatory effects in the lungs 3. In some experiments, we in addition administered bacteria directly into the bloodstream in order to cause sepsis in the absence of a primary source of infection.

In order to investigate the role of platelets in pneumonia and sepsis, we first depleted these cells in mice, and challenged thrombocytopenic animals with *K. pneumoniae* (chapter 3) or *S. pneumoniae* (chapter 4) via the airways. While platelet depletion did not harm uninfected mice, infectious challenges with *K. pneumoniae* or *S. pneumoniae* induced severe bleeding in lungs of severely platelet-depleted animals. Only a small platelet proportion of 1 to 9% in low platelet count (low-PC) mice protected mice from bleeding during *K. pneumoniae* pneumonia. Other studies using different inflammation models confirm these observations 4,5. During sepsis, platelets contribute to distant organ damage by enhancing disseminated intravascular coagulation 6, induction of apoptosis 7, Granzyme-B mediated toxicity 8 and by shedding of platelet microparticles 9-11. During *Klebsiella* pneumonia (chapter 3), low-PC mice were protected from hepatocellular damage, as indicated by low levels of liver damage markers AST, ALT and LDH. In the very low-PC group (exhibiting PC < 1% of normal), liver injury was as severe as in control – not platelet depleted - mice, probably as a result of overwhelming bacterial burdens. Low-PC mice displayed reduced thrombin levels together with reduced myeloperoxidase concentrations in plasma 12 hours after infection with *Klebsiella*, hinting at a role for the coagulation system and neutrophil activation in the development of organ
damage during later stage sepsis. Although low-PC mice were protected against bleeding and organ damage during *Klebsiella* pneumosepsis, these mice were did have an increased mortality, most likely due to high bacterial burdens and increased inflammation compared to control mice.

The regulatory role of platelets in cytokine release is still elusive. Several cytokines have been identified in \( \alpha \)-granules and are released upon activation \(^1\). Systemic TNF-\( \alpha \) release in response to low-dose intravenous lipopolysaccharide (LPS) was impaired in thrombocytopenic mice and restored upon platelet transfusion \(^3\). To the contrary, platelets in complex with macrophages inhibited TNF-\( \alpha \) and IL-1\( \beta \) production after high-dose intravenous LPS or *E. coli* challenges\(^4\). In our studies with *K. pneumoniae* and *S. pneumoniae* in vivo, and *K. pneumoniae* whole blood stimulation *in vitro*, cytokine production was enhanced in thrombocytopenic mice (chapters 3 and 4). Although initial cytokine production is indispensable for an adequate host response to invading pathogens, excessive cytokine production has opposite effects during severe infection \(^5\). 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 \(^4\).

The results from these chapters clearly establish the hemostatic role that platelets fulfill in case of an infectious challenge. We additionally found an immunomodulatory role for platelets during gram-positive and gram-negative sepsis, resulting in altered cytokine levels during sepsis and in reduced organ damage (*Klebsiella* model).

To further dissect the mechanisms by which platelets influence host defense and disease outcome, we investigated the role of P-selectin (encoded by the *Selp* gene) during *Klebsiella* pneumonia (chapter 5). P-selectin is a cell adhesion protein stored in \( \alpha \)-granules in platelets and in Weibel-Palade bodies in endothelial cells. P-selectin is released to the surface of these cells upon activation \(^6\). Our main findings were that *Selp\(^{-/-}\)* mice displayed enhanced bacterial outgrowth in lungs and distant organs, which led to reduced survival compared to wild type (WT) mice. In order to dissect which of the P-selectin expressing cell type caused this effect, we carried out bone marrow transplantation experiments, creating mice with P-selectin expressed by platelets or endothelial cells only. Both platelet and endothelial cell P-selectin in contributed to protective immunity during *Klebsiella* sepsis, as P-selectin chimeric mice both displayed an intermediate phenotype compared to WT and full knock out controls. Both platelet- and endothelial P-selectin expression have been associated with leukocyte recruitment via interaction with P-selectin glycoprotein-1 (PSGL-1) \(^7\). We however found no influence of P-selectin deficiency on leukocyte recruitment to the lungs. We did find evidence for a platelet modulatory role when in complex with monocytes. During sepsis, there is an increase in circulating platelet-leukocyte complexes \(^8\) and P-selectin can contribute herein \(^2\). In our experiments, platelet-monocyte but not platelet-neutrophil complexes were diminished in *Selp\(^{-/-}\)* mice compared to WT mice after 44 hours of infection. Platelets
in complex with macrophages or monocytes have been shown to inhibit TNF-α and IL-1β production after intravenous LPS or E. coli challenges. We also found elevated levels of TNF-α, IL-6 and IL-1β upon infection with Klebsiella in Selp−/− mice as soon as 12 hours after induction of infection (when bacterial burdens in both mice strains were equal). Together these data suggest that P-selectin mediated complex formation between monocytes and platelets may inhibit proinflammatory cytokine release during Klebsiella sepsis.

Platelets need to become activated before they can exert their immunomodulatory tasks. We therefore investigated the way by which platelets are activated during pneumonia and sepsis in chapters 6 to 8. Platelets can be directly activated by several bacteria and chapter 7. Platelets express several toll like receptors (TLRs), a relatively recent finding now confirmed by many laboratories. TLRs are a family of pattern recognition receptors that are critical for microbial surveillance and regulation of inflammatory and immune responses. We investigated the role for platelet TLR signaling in host defense against Klebsiella pneumoniae (chapter 6) and mutated unencapsulated Streptococcus pneumoniae (ΔcpsD39) (chapter 7). MyD88 is the downstream adaptor protein of all TLRs except TLR3. Full Myd88 knock out (Myd88−/−) mice are dramatically susceptible during Klebsiella pneumonia, an effect that was dependent on both hematopoietic and resident cells. We have generated platelet specific Myd88−/− mice by crossing Myd88lox/lox mice with mice expressing Cre recombinase under the platelet factor (PF)4 promoter (Plt-Myd88−/−). Plt-Myd88−/− mice displayed reduced TNF-α and chemokine responses in whole blood stimulated with Klebsiella in vitro and during early sepsis in vivo. Despite these findings, and despite several reports stressing important roles for platelet-TLR signaling in several models of inflammation, the main outcomes for Plt-Myd88−/− (e.g., bacterial growth and dissemination, and organ damage) were equal to control mice during Klebsiella induced pneumonia and sepsis. Lack of platelet MyD88 signaling after K. pneumoniae infection might be insignificant in perspective of intact MyD88 signaling in other cell types involved (e.g., epithelial cells and leukocytes). Using a complementary approach, we transfused WT platelets into full Myd88−/− mice (generating Myd88−/−+WTplt mice) and challenged these mice with Klebsiella pneumonia. While median bacterial burdens were ≥2-log higher in Myd88−/− mice compared to WT mice in all body compartments, WT platelet transfusion did not improve outcomes for Myd88−/−+WTplt mice.

Streptococcus pneumoniae has been reported to activate platelets in a TLR2 dependent manner. We confirmed platelet activation by S. pneumoniae in chapter 7, however, the activation mechanism was found to be TLR independent in our hands. Platelet activation was not inhibited by TLR2 or TLR4 blocking antibodies, activation patterns in tlr2−/−, tlr4−/−, tlr2/4−/−, tlr9−/− and Myd88−/− mice were equal to WT controls, and no platelet activation could be induced by direct TLR2 or TLR4 stimulation even when using supra-physiological dosages. S. pneumoniae strains vary substantially in the ability to cause invasive disease (sepsis) in humans, which is associated with the capsular serotype. The pneumococcal capsule inhibits mucosal clearance, facilitates binding to the epithelial surface and inhibits complement-
and phagocyte-mediated immunity. Besides reduction of exposure to several antibodies, capsular polysaccharide prevents interaction between Fc\(\gamma\) receptors to the Fc component of IgG fixed to pneumococci as it is highly negatively charged. AcpsD39 was the strongest inducer of platelet activation and platelet-neutrophil and platelet-monocyte complex formation. We therefore conducted pneumonia experiments with this pneumococcus using Plt-Myd88\(^{-/-}\) mice in order to determine the impact of platelet specific TLR signaling in vivo. No differences were however found in bacterial growth or inflammation between Plt-Myd88\(^{-/-}\) and control mice, extending the results described in chapter 6 for Klebsiella.

Research on platelet TLR activation, signaling and its influence on immune response is a challenging field with conflicting results, presumably caused by focus on different TLRs in a diversity of in vitro and in vivo models. We here attempted to study all platelet TLRs by targeting MyD88 using a relevant and virulent bacterial challenge. We were however unable to show a physiologic role for MyD88 dependent TLR signaling on platelets in host defense against K. pneumoniae or S. pneumoniae induced pneumonia.

Another important mechanism of platelet activation during pneumonia and sepsis is via thrombin. Our laboratory has previously documented local activation of coagulation with generation of thrombin during human and murine pneumococcal pneumonia by a mechanism that was dependent on the generation of tissue factor-factor VIIa-factor Xa. Thrombin is one of the major proteases capable of activating protease activated receptor (PAR) 1, 3 and 4. Human platelets express PAR1 and PAR4, in mice however, PAR4 is the only thrombin receptor present on platelets. PAR4 is additionally expressed by alveolar epithelial cells, neutrophils, alveolar macrophages and endothelium. We characterized the role of mouse (m)PAR4 signaling during S. pneumoniae pneumosepsis in chapter 8. mPAR4 deficiency resulted in enhanced bacterial outgrowth and enhanced inflammation during late infection. Whole blood stimulations in vitro showed that mPAR4 stimulation catalyzed cytokine response to S. pneumoniae. Considering the interspecies variety in PAR1 and 4 expression between mice and humans, we cannot extrapolate these mouse results to the human situation. Comparisons however could be made using thrombin, since this serine protease is the most potent activator of both PAR1 and 4. In line with our mouse whole blood experiments, where mPAR4 activation resulted in an enhanced cytokine response to S. pneumoniae, thrombin inhibition by lepirudin reduced cytokine levels after S. pneumoniae stimulation in human whole blood. These data suggest that in humans stimulation of human (h)PAR1 and hPAR4 enhances cytokine release elicited by S. pneumoniae. Further studies are however warranted to confirm that these observations are platelet-mediated.

While platelets seem indispensable for host response at to an invading pathogen, platelet activation during sepsis contributes to sepsis complications such as disseminated intravascular coagulation (DIC), multiple organ failure, acute lung injury and acute kidney injury. Several experimental and retrospective clinical studies have shown beneficial
effects of antiplatelet therapy in sepsis models. We aimed to better understand the role of platelet activation in a clinical setting in Chapter 9, where the influence of pre-existing antiplatelet therapy on disease outcome was investigated in a prospectively followed sepsis cohort encompassing 1070 patients, 27.8% of whom used antiplatelet drugs before admission to the intensive care unit. To account for differential likelihoods of receiving antiplatelet therapy, a propensity score was constructed, including variables associated with use of antiplatelet therapy. Antiplatelet therapy did not influence sepsis severity at presentation, the primary source of infection, causative pathogens, the development of organ failure or shock during ICU stay, or mortality up to 90 days after admission, in either the unmatched or propensity matched analyses. In addition, antiplatelet therapy did not modify induction of the cytokine network or endothelial cell activation in these patients. Hence, this observational study strongly suggests that pre-existing antiplatelet therapy does not influence the presentation or outcome of, or the host response to sepsis. Our results differ from previous retrospective studies that studied the effect of antiplatelet therapy on sepsis outcome, which reported a reduced mortality of sepsis patients on platelet therapy. Our study is different from these previous reports in that we prospectively enrolled patients and classified patients as having sepsis based on strict diagnostic criteria and posthoc assessment of dedicated research physicians taking all available clinical and microbiological information into account.

Clinical impact

In the models used in this thesis (mostly pneumonia induced sepsis in mice), modulation of platelets or platelet pathways almost invariably caused a disadvantage to the host. Translation from pre-clinical animal research to a clinical setting however is difficult. Sepsis in humans is a highly heterogeneous syndrome, entailing different causes and sources of infection in usually elderly patients with different comorbidities, unlike experimental mouse research conducted in a controlled laboratory setting. Nonetheless, even when taking this limitation into account, the results presented in this thesis clearly suggest that platelets play a protective role in host defense against bacterial infection. An observational study in humans supports this notion: clopidogrel treatment (inhibiting secondary platelet activation) was associated with increased risk for the development of community-acquired pneumonia and in-hospital treatment; among pneumonia patients, those with active clopidogrel prescriptions had a higher incidence of sepsis than those with no clopidogrel prescriptions.

Should we aim for higher platelet counts or qualitatively better platelets during pneumonia or sepsis? Upon presentation, pneumonia patients typically do not have thrombocytopenia. During sepsis however thrombocytopenia is a predictor of mortality. Because of this and because of the risk of bleeding in sepsis patients caused by DIC, thrombocytopenia
and inflamed organs, one could argue that platelet transfusion could be a therapeutic strategy. Platelet substitution therapy should however not be instituted based on low platelet counts alone, but is advised only in patients with active bleeding or with an increased risk of bleeding complications. It has never been proven that the transfusion of blood components will ‘fuel the fire’ during DIC; however, nor have beneficial effects of platelet transfusions been established. Platelet transfusions are associated with risks such as the transmission of infectious agents, hemolysis (although both rare in developed countries where blood products are securely tested), and transfusion-related acute lung injury (TRALI). Additionally, there is a step-wise increase in complications with exposure to progressively older platelets. Nonetheless, platelet transfusion could be effective in critically ill patients with thrombocytopenia due to impaired platelet production. Randomized controlled trials are required to define the optimal use of platelet reconstitution therapy in sepsis patients.

On the other hand: if platelet activation contributes to sepsis pathogenesis during the inflammatory and coagulation dysbalance that characterize this syndrome, could platelet inhibitory therapy benefit sepsis patients? A concern when administering anti-platelet therapy to sepsis patients might be enlarging the risk of bleeding. Benefit of pre-existing therapy with antiplatelet drugs during severe infection was however also evident in patients with high bleeding risks such as in neurosurgery patients; thus, the contribution of antiplatelet therapy to unfavorable bleeding might be overestimated. While the results presented in chapter 9 argues against a role for antiplatelet therapy in sepsis management, the effect of such an intervention can only be adequately addressed in a randomized trial.

**Conclusion**

The work presented in this thesis provides evidence for an important role for platelets in the host response to bacterial infection. Low platelet counts render animals more susceptible to uncontrolled growth and dissemination of bacteria, resulting in enhanced mortality. On the other hand, platelets can contribute to the organ damage caused by severe infection, exemplifying the double-edged-sword character of the action of these cells. The time that platelets were merely viewed upon as cells important for primary hemostasis is way beyond us. Clearly, platelets play a multifaceted role in infection. Future research should focus on which granulae and constituents of platelets are the main drivers of their effects in sepsis.
References


