Platelets: versatile effector cells in pneumonia and sepsis
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Thrombocytopenia impairs host defense during murine *Streptococcus pneumoniae* pneumonia

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Abstract

**Objective:** *Streptococcus (S.) pneumoniae* is the most common causative pathogen in community-acquired pneumonia. In patients, thrombocytopenia is correlated with an adverse outcome of pneumonia. Platelets can modulate the host response to infection in several ways, i.e. by facilitating clot formation, release and production of several inflammatory proteins and interaction with neutrophils. We studied the effect of thrombocytopenia during murine pneumococcal pneumonia.

**Design:** Animal study.

**Setting:** University research laboratory.

**Subjects:** Mice.

**Interventions:** Pneumonia was induced by intranasal inoculation of *S. pneumoniae*. Platelets were depleted by anti-mouse thrombocyte serum; controls received non-immunogenic serum. In separate studies mice were treated with the platelet P2Y₁₂ receptor inhibitor clopidogrel or placebo.

**Measurements and Main Results:** Thrombocytopenic mice (platelet counts <1% of uninfected controls) showed a reduced survival during pneumococcal pneumonia (27% versus 75% amongst controls; p=0.003), which was associated with higher bacterial loads in lungs, spleen, and blood. Thrombocytopenic mice showed enhanced coagulation activation (thrombin-antithrombin complexes) in plasma. Proinflammatory cytokine levels were higher in plasma but not in lungs of thrombocytopenic mice. Although clopidogrel treatment strongly prolonged the bleeding time, it did not impact on bacterial loads during pneumococcal pneumonia.

**Conclusions:** Platelets play a protective role during pneumococcal pneumonia independent of their aggregation.
**Introduction**

*Streptococcus (S.) pneumoniae* is the most prevalent microorganism in community-acquired pneumonia (CAP) and an important causative organism in sepsis in this context \(^1,2\). In the United States alone, the pneumococcus is responsible for more than half a million CAP cases each year and 50,000 episodes of bacteremia with a case fatality rate of 7 and 20\% respectively \(^3\). Similar figures have been reported for Europe \(^4\) and stress the importance of expanding our knowledge of the host defense mechanisms that influence the outcome of pneumococcal pneumonia.

Thrombocytopenia is a common finding in patients admitted to the Intensive Care Unit (ICU) and associated with a worse outcome \(^5-7\). Moreover, in a clinical study including over 800 ICU patients with CAP, thrombocytopenia was an independent predictor of mortality \(^8\). Platelets are well known chief cellular effectors of hemostasis, maintaining vascular integrity at sites of injury and inflammation. More recent investigations have revealed that platelets contribute to diverse processes that extend beyond hemostasis and thrombosis. A compelling body of evidence now exists indicating that platelets also contribute to inflammatory processes and defense against infection \(^9-11\).

Platelets act on the host innate and adaptive inflammatory response via release of a wide variety of preformed peptides stored in granules that mediate inflammation, chemotaxis and wound repair \(^9-11\). They are able to interact directly with polymorphonuclear leukocytes (PMNs, neutrophils) and this interaction can be induced by plasma from septic patients \(^9,12\). During systemic \(^13-15\) and lung inflammation \(^16,17\) platelets sequestrate in lungs, which in experimental models was neutrophil dependent \(^13,16,18\); conversely, platelet depletion inhibited neutrophil recruitment during acute lung injury in mice \(^18-20\). In addition, preventing neutrophil-platelet interaction protected from lung tissue damage during lung injury \(^16,18,20-22\). Through expression of pattern recognition receptors \(^13,23\) platelets also interact directly and indirectly with pathogens; they can bind, aggregate and internalize microorganisms \(^24,25\) and can augment antibacterial activities of other immune cells \(^26-28\). Additional antibacterial effects are achieved by production of antimicrobial mediators (e.g. cytotoxic oxygen metabolites) or platelet microbicidal proteins known as thrombicidins in humans, of which increased levels are found during infection \(^11,29,30\). Recent studies also demonstrated that platelets are able to induce neutrophil extracellular trap (NET) formation to entrap bacteria in septic blood \(^12\). Clearly platelets are equipped to play an important role in host defense against invading pathogens and this contribution may be especially important in the lung where they are found to accumulate during an inflammatory assault. We here aimed to study the role of platelets during CAP, for which we depleted mice of platelets or inhibited platelet aggregation and secondary activation in mice prior to intranasal infection with *S. pneumoniae*. 
Materials and Methods

Animals

Specific pathogen-free C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands). Experiments were conducted with age and gender-matched mice at 10–12 weeks of age. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

Experimental study design

*S. pneumoniae* serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD) was used to induce pneumococcal pneumonia. Bacteria were grown as described and ~5 x 10^4 colony-forming units (CFU) in 50 µL were inoculated intranasally. Mice were observed in a survival experiment or sacrificed at 24 or 48 hours after induction of pneumococcal pneumonia. Per group 8 animals were used, for the survival experiment 15 animals per group were used. In order to deplete platelets, mice were intravenously treated with rabbit anti-mouse platelet serum or normal rabbit serum as control (Accurate Chemical & Scientific Corporation, Westbury, NY) at -2 hours and at 24 and 48 hours after induction of pneumonia. Treatment effect was verified by determining platelet counts in a counting chamber. In one experiment, platelets were depleted using rat anti-mouse platelet glycoprotein Ib alpha chain (GP1b-alpha) 50 µg per mouse intravenously; controls received non-immune rat IgG (both antibodies from Emfret Analytics GmbH & Co, Würzburg, Germany). In separate experiments mice were treated with the platelet aggregation and activation inhibitor clopidogrel (30 mg kg⁻¹, Mylan, Hoeilaart, Belgium) or placebo by oral gavage at day -2, -1 and at the time of infection as used previously. Clopidogrel is an irreversible inhibitor of P2Y₃, the receptor for adenosine diphosphate on platelets. Blood diluted 4:1 with citrate, lungs, liver and spleen were harvested and processed using methods described previously. The left lung lobe was fixed in 10% buffered formalin and embedded in paraffin.

Bacterial quantification

Undiluted whole blood and serial ten-fold dilutions of organ homogenates and whole blood were made in sterile isotonic saline and plated onto sheep–blood agar plates. Colony forming units (CFU) were counted following 16 hours of incubation at 37°C.

Assays

Thrombin-antithrombin complexes (TATc) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (TATc: Behringwerke AG, Marburg, Germany, or Affinity Biologicals, Ancaster, Ontario, Canada), levels of macrophage–inflammatory protein (MIP)-2, keratinocyte-derived cytokine (KC), interleukin (IL)-1β (all R&D Systems, Abingdon,
UK) and myeloperoxidase (MPO; HyCult Biotechnology, Uden, The Netherlands) were measured using commercially available ELISA kits. Levels of tumor necrosis factor (TNF)-α, IL-6, monocyte chemotactic protein (MCP)-1 and interferon-γamma (IFN-γ) were determined using a commercially available cytometric beads array multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendations.

**Tail vein bleeding assay**

A mouse-tail vein bleeding assay was performed as described (27). In brief, at a standardized distance the tip of the mouse-tail was cut off (with a tail diameter approximately 1 mm). The tail was immediately placed in a 50-mL falcon tube filled with 37 °C saline and the bleeding time with a maximum of 5 minutes was recorded.

**Histopathology and immunohistochemistry**

Paraffin-embedded four-micrometer lung sections were stained with hematoxylin and eosin (H&E) and analyzed for hemorrhage, inflammation and tissue damage, as described previously (31,36). All slides were coded and scored by a pathologist who was blinded for group identity. Hemorrhage was scored separately and total pathology scores were determined as the sum of the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, pleuritis and presence of thrombi. Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of total lung surface. The remaining parameters were rated on a scale from 0 (condition absent) to 4 (most severe condition). Neutrophil stainings on mouse lung tissue were performed using an anti-mouse Ly-6G monoclonal antibody (BD Pharmingen, San Diego, CA). Slides were photographed with a microscope equipped with a digital camera (Olympus dotSlide, Tokyo, Japan) and stained areas were analyzed with ImageJ (version 2006.02.01; US National Institutes of Health) and expressed as percentage of the total lung surface area as described elsewhere (37).

**Statistical analysis**

Data are expressed as indicated. Differences between groups were analyzed using nonparametric analysis of variance (rank transformed variables) with modeled effects for strain and time, followed by post-hoc Mann-Whitney U tests at the individual time points. Survival curves and bleeding time were compared using log-rank test. All analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A p-value of < 0.05 was considered statistically significant.
Results

Thrombocytopenic mice show enhanced lethality in S. pneumoniae pneumonia

To study the effect of thrombocytopenia on mortality in an experimental model of CAP we depleted platelets in mice using anti-mouse platelet serum. Before infection with S. pneumoniae platelet counts were reduced to <1% (p=0.03) and after infection to <10% in mice treated with rabbit anti-mouse platelet serum compared to mice treated with control rabbit serum (p=0.002, Figure 1A). Thrombocytopenic mice showed a strongly enhanced mortality (73%) compared to control mice (25%, p=0.003, Figure 1B) after induction of S. pneumoniae pneumonia.

Thrombocytopenia is associated with higher systemic bacterial loads during pneumococcal pneumonia

To evaluate the effect of thrombocytopenia on bacterial loads during pneumococcal pneumonia, bacteria were quantified locally (lung homogenates) and systemically (whole blood and spleen homogenates). Thrombocytopenic mice demonstrated a trend towards higher bacterial counts in lungs at 24 hours (p=0.065, Figure 2A) together with increased dissemination, as reflected by higher bacterial loads in blood at both time points (p<0.05, Figure 2B) and spleen at 24 hours (p<0.001, Figure 2C). To confirm that thrombocytopenia facilitates bacterial dissemination, we depleted mice of platelets by a different approach, using an antibody directed against GP1b-alpha. In accordance with previous studies, anti-GP1b-alpha treatment reduced platelet counts to 7 ± 2% relative to controls (p=0.03). Similar to mice made thrombocytopenic with anti-platelet serum, anti-GP1b-alpha treated...
mice displayed increased bacterial loads in blood and spleen (Fig 2E, F) at 24 hours after infection with *S. pneumoniae* via the airways (both p<0.05).

**Thrombocytopenia does not impact on lung inflammation during pneumococcal pneumonia**

Platelets have been found to contribute to several forms of sterile lung inflammation at least in part by facilitating neutrophil recruitment. We were therefore interested in the impact of thrombocytopenia on the pulmonary inflammatory response to infection with *S. pneumoniae*. The extent of lung pathology, as determined by the semi-quantitative histology scores described in the Methods section, did not differ between thrombocytopenic and control mice (Figure 3G, with representative slides for control A-C and thrombocytopenic mice D-F 0, 24 and 48 hours after infection). Notably, signs of pulmonary hemorrhage were almost exclusively found in thrombocytopenic mice in the setting of infection (Figure 3H); thrombocytopenia did not result in hemorrhage in uninfected mice. Thrombocytopenia did not influence neutrophil influx during pneumonia, as indicated by the number of Ly6+ cells in lung tissue slides and concentrations of MPO in whole lung homogenates (Figure 4). Similarly, thrombocytopenia did not affect cytokine or chemokine levels in lung homogenates, with the exception of MCP-1 (48h) concentrations, which were higher in lungs of thrombocytopenic...
mice (Table 1). Altogether these data indicate that thrombocytopenia did not have a major impact on the local inflammatory response during pneumococcal pneumonia.

**Thrombocytopenia enhances the systemic cytokine response during pneumococcal pneumonia**

To obtain insight in the systemic inflammatory response, we measured cytokine and chemokine levels in plasma harvested 24 or 48 hours after infection. At 24 hours after infection plasma levels of these mediators did not differ between groups; at 48 hours after infection, however, thrombocytopenic mice showed strongly increased plasma concentrations of TNF-α, IL-6, IFN-γ and MCP-1 (Table 2).
Thrombocytopenia impairs host defense during murine Streptococcus pneumoniae pneumonia.

Figure 4. Thrombocytopenia does not influence neutrophil accumulation in lung tissue during pneumococcal pneumonia. Mice were treated with rabbit anti-platelet (P) serum or control serum and infected intranasally with *Streptococcus pneumoniae*; samples were obtained at 0, 24 or 48 hours post-infection. Concentrations of myeloperoxidase (MPO) per milliliter whole lung homogenate (A) and neutrophil accumulation in lung tissue expressed as total Ly-6G positivity as percentage of lung tissue surface (B) in control (grey boxes) and thrombocytopenic (open boxes) mice with representative slides of Ly-6G staining of control (C) and thrombocytopenic (D) mice at 48 hours. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (N = 8 per group). Scale bar indicates 200 µm.

Table 1

<table>
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<th></th>
<th>control (N=8)</th>
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<th>control (N=8)</th>
<th>anti-platelet (N=8)</th>
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<td>TNFα (pg/ml)</td>
<td>114 (19-432)</td>
<td>714 (194-1094)</td>
<td>380 (74-441)</td>
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<td>IL-6 (pg/ml)</td>
<td>433 (71-2993)</td>
<td>1870 (173-2512)</td>
<td>2994 (941-3459)</td>
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<td>1753 (720-4534)</td>
<td>5329 (1706-6490)</td>
<td>5931 (3401-8034)</td>
<td>9789 (8136-10240)*</td>
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<td>IFNγ (pg/ml)</td>
<td>11 (3-50)</td>
<td>19 (5-42)</td>
<td>59 (17-84)</td>
<td>62 (31-149)</td>
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<td>IL-10 (pg/ml)</td>
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<td>170 (106-331)</td>
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<td>KC (pg/ml)</td>
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<td>39806 (26774-52093)</td>
<td>40814 (25092-44103)</td>
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<td>19276 (9490-39503)</td>
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<td>568 (72-1393)</td>
<td>1666 (402-3919)</td>
<td>870 (452-979)</td>
<td>439 (294-617)</td>
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Cytokine and chemokine levels in whole lung homogenates. Levels of cytokines and chemokines in lung homogenates 24 and 48 h after induction of pneumococcal pneumonia in mice treated with anti-mouse platelet serum (am-platelet) or control serum. Data are expressed as median (interquartile ranges). IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; KC, keratinocyte-derived cytokine; MCP-1, monocyte chemotactic protein-1; MIP-2, Macrophage–inflammatory protein–2. * P < 0.05 compared to control.
Thrombocytopenia is associated with an enhanced procoagulant response during pneumococcal pneumonia

Platelets are important for hemostasis and amplification of thrombin generation. To evaluate the effect of thrombocytopenia on coagulation in mice, we measured TATc levels in plasma before and 24 and 48 hours after infection (Figure 5). Directly before infection, TATc levels were similar in thrombocytopenic and control mice. At 24 and 48 hours post infection, thrombocytopenia was associated with increased plasma levels of TATc (p=0.06 and p<0.001 respectively).

Clopidogrel does not influence bacterial loads or the inflammatory response during pneumococcal pneumonia

To investigate whether the protective role of platelets in pneumococcal pneumonia depends on the aggregation and secondary activation of platelets, we inhibited P2Y12 receptor mediated aggregation and activation of platelets using clopidogrel. Although this treatment was effective as reflected by a profoundly increased tail vein bleeding time (Figure 6A, p=0.007), it did not affect bacterial counts in any of the organs tested at either 24 or 48 hours post infection (Figure 6B-E). Of note, no macroscopic or intrapulmonary signs of bleeding were found in mice treated with clopidogrel. In addition, clopidogrel did not influence lung pathology or the systemic cytokine response (data not shown).

Table 2

<table>
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<th>Cytokine and chemokine levels in plasma.</th>
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<th>t=48</th>
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<td>anti-platelet (N=7)</td>
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<td>TNFα (pg/ml)</td>
<td>12 (9-24)</td>
<td>28 (11-32)</td>
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<td>IL-6 (pg/ml)</td>
<td>67 (10-238)</td>
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<td>131 (67-522)</td>
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<td>IFNγ (pg/ml)</td>
<td>7 (2-47)</td>
<td>32 (3-80)</td>
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<tr>
<td>IL-12 (pg/ml)</td>
<td>28 (5-39)</td>
<td>34 (5-64)</td>
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Cytokine and chemokine levels in plasma. Levels of cytokines and chemokines in plasma 24 and 48 h after induction of pneumococcal pneumonia in mice treated with anti-mouse platelet serum or control serum. Data are expressed as median (interquartile range). IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic protein-1; *, ** and *** indicate P < 0.05, P < 0.01 and P < 0.001 versus control.

Figure 5. Thrombocytopenia is associated with an enhanced procoagulant response during pneumococcal pneumonia.

Mice were treated with anti-platelet (P) serum or control serum and infected intranasally with Streptococcus pneumoniae. Thrombin-antithrombin complex (TATc) levels in plasma of uninfected or infected control (grey boxes) and thrombocytopenic (open boxes) mice at 24 and 48 hours post-infection. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (N = 8 per group). ***p<0.001, #p<0.1 compared with controls.
Discussion

Besides their well-known hemostatic function, platelets are increasingly appreciated as key effectors in inflammatory responses. Thrombocytopenia frequently occurs in critically ill patients and lower platelet counts are an independent risk factor of mortality in patients admitted to the ICU for severe CAP. We here investigated the role of platelets during CAP using thrombocytopenic mice infected with S. pneumoniae via the airways and show that thrombocytopenia facilitates bacterial dissemination, resulting in enhanced systemic inflammation and increased mortality.

The absence of platelets promoted bacterial dissemination, indicating that platelets contribute to local containment of pneumococcal infection in the lung. Platelets promote endothelial barrier integrity and in experimental animals thrombocytopenia increased pulmonary vascular permeability, however, to date it is not clear whether platelets also influence the lung epithelial barrier. In contrast with our results, in a recent study platelets facilitated dissemination of S. pyogenes in murine sepsis. This implies that distinct streptococcal species vary in their interaction with platelets. Indeed, S. pyogenes can rapidly adhere to and promote aggregation of platelets, while S. pneumoniae appears to optimally induce aggregation by an antibody-dependent mechanism.

Figure 6. Inhibition of thrombocyte activation by clopidogrel increases bleeding time but does not influence bacterial loads during pneumococcal pneumonia. Mice were infected intranasally with Streptococcus pneumoniae and treated with the platelet activation inhibitor clopidogrel or placebo; samples were harvested 24 or 48 hours post-infection. Tail vein bleeding time (A) and number of colony forming units (CFU) per milliliter lung (B), liver (C), spleen (D) homogenates and whole blood (D) in control mice (grey boxes) and mice treated with clopidogrel (striped boxes). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (N = 8 per group). N.d, not determined; b.d, below detection. **p<0.01 compared with controls.
Platelets can modulate the immune response in several ways. In experimental models of sterile acute lung injury, thrombocytopenia protected against pulmonary injury, whereas it worsened lung injury in septic shock. We found little effect on lung damage caused by platelet depletion after induction of pneumococcal pneumonia, as reflected by similar lung histopathology scores and pulmonary cytokine and chemokine levels. Of note, pneumonia caused major lung hemorrhage during thrombocytopenia, in line with reports from previous studies using non-infectious challenges, again implicating platelets are required to maintain vascular barrier integrity in the setting of inflammation. In a recent study demonstrating a protective effect of platelets during septic shock, no lung hemorrhage was observed and it was suggested that this was due to a milder extent of platelet depletion, i.e. 90% compared to more than 97.5% in the aforementioned studies. In the studies here carried out, platelets were depleted for ~90% 48 hours after pneumococcal infection, still major hemorrhage was observed, which could be attributed to the fact that the site of primary infection was located in the lungs.

Platelet depletion inhibited neutrophil recruitment into lungs in several studies of acute lung injury in mice. However, in another study of murine endotoxemia, platelet depletion did not affect neutrophil accumulation. In line with the latter study, we did not observe any difference in neutrophil counts in lung tissue between control and thrombocytopenic mice, as reflected by similar numbers of Ly6-G positive cells and MPO levels in lungs. At the same time, we demonstrated higher cytokine levels in plasma of thrombocytopenic mice during pneumococcal pneumonia, which may at least in part be explained by the higher number of bacteria present in the circulation. Furthermore, it was recently shown that platelets inhibit IL-6 and TNF-α production in endotoxemia in a macrophage dependent manner. Therefore, a direct effect of platelet depletion on cytokine release may have contributed to the increased systemic cytokine response observed here. An overwhelming, ongoing systemic inflammatory response likely has added to tissue damage and mortality in thrombocytopenic mice.

Next to their central role in primary hemostasis, activated platelets are able to induce a coagulation response as they facilitate coagulation by providing a suitable phospholipid surface for the assembly of activated coagulation factors. Surprisingly, we found enhanced activation of coagulation in plasma of thrombocytopenic mice during infection, which together with enhanced systemic inflammation may have resulted in increased microthrombotic organ failure. This, combined with major bleeding complications associated with strongly reduced platelet counts, likely additionally contributed to enhanced lethality in thrombocytopenic mice. The increased coagulation response may in part be attributed to an augmented systemic pro-inflammatory response in thrombocytopenic mice. In addition, *S. pneumoniae* is able to activate coagulation and increased bacterial counts in plasma of thrombocytopenic mice may provide an alternative explanation for the enhanced
procoagulant state. Together, these results clearly show that platelets are not required for coagulation activation during pneumococcal pneumonia.

Activation of platelets is a typical feature during systemic inflammation and \textit{S. pneumoniae} can induce platelet aggregation. Retrospective data investigating the outcome of CAP patients suggested a benefit for those patients who had used anti-platelet drugs for over six months before hospital admission. We studied the effect of platelet aggregation and secondary activation inhibition with clopidogrel during pneumococcal pneumonia. Clopidogrel markedly increased the bleeding time in mice, but did not impact on bacterial loads or dissemination. Together these data indicate that the mere inhibition of the hemostatic properties of platelets does not impact on host defense in the setting of pneumonia caused by \textit{S. pneumoniae}, whereas decreasing platelet numbers does.

In conclusion, we here demonstrated that platelets, independent of aggregation and secondary activation, play a favorable role in the setting of experimental CAP caused by \textit{S. pneumoniae} by protecting from dissemination of bacteria and mortality.

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References


Chapter 4


