Platelets: versatile effector cells in pneumonia and sepsis

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Platelet *and* endothelial cell P-selectin are required for host defense against gram-negative pneumosepsis

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Submitted
Abstract

**Background:** Sepsis is associated with activation of platelets and endothelial cells accompanied by enhanced P-selectin surface expression. Both platelet- and endothelial P-selectin have been associated with leukocyte recruitment and induction of inflammatory alterations. *Klebsiella (K.) pneumoniae* is a common human sepsis pathogen, particularly in the context of pneumonia.

**Methods:** Wild-type (WT) and P-selectin deficient (*Selp−/−*) mice, or bone marrow chimeric mice. Mice were infected with *K. pneumoniae* via the airways to induce pneumosepsis. Mice were sacrificed during early (12 hours after infection) or late stage (44 hours) sepsis for analyses, or followed in a survival study.

**Results:** *Selp−/−* mice displayed 10-1000-fold higher bacterial burdens in lungs, blood and distant organs during late stage sepsis. P-selectin deficiency did not influence leukocyte recruitment to the lungs, but was associated with decreased platelet-monocyte complexes and increased cytokine release. *Selp−/−* mice showed increased distant organ injury, while endothelial cell activation was reduced. Activation of coagulation was not impaired in *Selp−/−* mice. Bone marrow transfer studies revealed a role for both platelet and endothelial cell P-selectin in bacterial control and survival.

**Conclusion:** Both platelet and endothelial cell P-selectin contribute to host defense during *Klebsiella* pneumosepsis.
Introduction

Sepsis is a syndrome caused by a deregulated host response to infection resulting in organ injury. Sepsis has an estimated incidence of over 19 million cases worldwide per year and high mortality rates even when appropriate antibiotic treatment is available. *Klebsiella (K.) pneumoniae* is one of the most common causative agents of gram-negative sepsis, especially in the context of pneumonia.

The inflammatory response during sepsis involves both platelet and endothelial cell activation. P-selectin is a 140 kDa glycoprotein that is stored in Weibel-Palade bodies in endothelial cells or α-granulae in platelets. These storage vesicles rapidly fuse with the cell surface upon stimulation resulting in increased expression; surface P-selectin can subsequently be downregulated by internalization, degradation or by shedding into plasma. Both platelet and endothelial P-selectin expression have been associated with leukocyte recruitment. Infection and pro-inflammatory mediators induce upregulation of endothelial adhesion molecules including P-selectin in inflamed tissue, which results in a targeted accumulation of leukocytes. Platelets are additionally involved in leukocyte recruitment, contributing to an effective immune response at the primary site of infection.

We here sought to determine the role of P-selectin in *K. pneumoniae* induced pneumonia-derived sepsis. For this we infected P-selectin knock out (*Selp−/−*) mice with viable *K. pneumoniae* via the airways and compared them to wild-type (WT) mice. In order to dissect the roles of platelet and endothelial P-selectin, bone marrow chimeras were generated and challenged with *Klebsiella*.

Material and Methods

Experimental study design

The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments. Age (~10 weeks) and sex matched C57Bl/6 WT mice (Harlan Sprague-Dawley, Horst, the Netherlands) and C57Bl/6 Selp−/− mice (Jackson Laboratories, Bar Harbor, ME) were used. Pneumonia was induced by intranasal inoculation with log-phase *K. pneumoniae* serotype 2 (ATCC 43816 Rockville, MD, USA; 10,000 colony forming units (CFU) in 50 μL isotonic saline) as described. Mice were sacrificed 12 or 44 hours after induction of pneumonia or observed for 13 days (N = 15-18 per group). Bacterial quantification and storage of lungs, blood, liver and spleen from mice sacrificed at predefined time points was performed as described. In some experiments, bronchoalveolar lavage fluid (BALF) was collected by flushing the right lung with phosphate buffered saline (PBS). Total cell numbers in BALF were determined by Coulter Counter (Coulter Electronics, Hialeah, FL). Differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).
**Histopathology**

Four-micrometer sections of the left lung lobe were stained with hematoxylin and eosin (H&E). Slides were coded and scored by a pathologist blinded for group identity for the following parameters as described 15: interstitial inflammation, endothelialitis, bronchitis, oedema, pleuritis and presence of thrombi. All parameters were rated separately from 0 (condition absent) to 4 (most severe condition). The total histopathological score was expressed as the sum of the scores of the individual parameters, with a maximum of 24. Granulocytes were stained with anti-Ly-6G (FITC-labeled; BD PharMingen, San Diego, CA) as previously described 16; monocytes were stained using rat-anti-F4/80 antibody (MCA4997Gal AbD Serotec, Oxford, UK), (FAC2)-anti-rat IgG (#613001; ITK Diagnostics, Uithoorn, the Netherlands and Powrecision Poly-HRP-anti-rabbit IgG (DPVM-5SHRP; Immunologic, Duiven, the Netherlands) and counterstained with methyl green (Sigma-Aldrich, Zwijndrecht, the Netherlands). 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 17,18 and counterstained with hematoxylin (Sigma-Aldrich). Ly-6G and F4/80 expression in the lung tissue sections were quantified by digital image analysis as previously described 16.

**Assays**

Tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β P-selectin, E-selectin and thrombin anti-thrombin complexes (TATc) were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA and Kordia, Leiden, the Netherlands for TATc). Fibrinogen Western blotting was done as previously described 19, using polyclonal goat-anti-mouse-fibrinogen (Kordia, Leiden, the Netherlands) and donkey-anti-goat IgG-HRP secondary antibody (Abcam, Cambridge, UK). Imaging was done on a LAS4000 dark box (Fujifilm, Tokyo, Japan). Anti-fibrin(ogen) reactive bands of fibrin degradation product D-dimer was analyzed using by densitometric analysis 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). Nucleosomes were measured by ELISA as described 20.

**Flow cytometry**

Platelet counts 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). Leukocytes were stained using anti-CD45 (eBioscience, San Diego, CA), anti-Ly-6G (BD Biosciences) and CD115 (eBioscience). Monocytes were defined as CD45+CD115+Ly6-G-, neutrophils as CD45+CD115-,Ly-6G+. Within these neutrophil and monocyte populations CD61 was used to delineate platelet complex formation. Bone
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Bone Marrow Transplantation (BMT)

BM chimeric mice were generated as described 21, with the exception that recipient groups received total body irradiation of 2x 6.5 Gy with a 24-hour interval for optimal megakaryocyte depletion, using a 137Cs irradiator (CIS Bio International, Gif-sur-Yvette, France). We transfused WT BM into WT recipient mice (producing mice with P-selectin positive endothelial cells (E) and platelets (P), Selp E+/P+ mice), WT BM into Selp -/- recipients (Selp E-/P+ mice), Selp -/- BM into WT recipients (Selp E+/P- mice) and Selp -/- BM into Selp -/- recipients (Selp E-/P- mice), thereby generating two chimeric and two control strains. Pneumonia was induced 6 weeks after transplantation (in mice aged 16 weeks).

Clinical scores

During the observation study, each individual mouse was scored by an independent research analyst for 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 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. Mice with a score of 12 or more were eliminated from the experiment for ethical reasons and were noted as if they had been found dead at the following scoring time. Dead mice were scored with the highest clinical score for the remaining observation period.

Statistical analysis

Data are expressed as indicated in the figure legends. Comparisons between groups were performed with Mann-Whitney U test. For more than two groups Kruskal-Wallis one-way analysis of variance test was used, followed by Mann-Whitney U test in case of significant differences. Survival was compared using Kaplan-Meier analyses followed by Gehan-Breslow-Wilcoxon test. Clinical observation scores between the groups were compared using a non-linear mixed effect model, assuming exponential growth ($y = b_0 + e^{(bx)}$). P-values < 0.05 were considered statistically significant.
Results

 SelP<sup>−/−</sup> mice demonstrate enhanced bacterial growth in lungs, blood and distant organs during late stage Klebsiella induced pneumonia

We quantified bacterial loads in lungs, blood, spleen and liver in WT and SelP<sup>−/−</sup> mice at predefined end points after infection with *K. pneumoniae* via the airways (Figure 1). At 12 hours after infection bacterial loads were similar in both mouse strains. 44 hours after induction of pneumonia however, 10 to 1000-fold higher bacterial loads were found in lungs, blood and distant organs of SelP<sup>−/−</sup> mice (P<0.05 to P<0.0005 versus WT mice).

*P-selectin is not required for leukocyte recruitment to the lungs during Klebsiella pneumosepsis*

To determine the impact of P-selectin on pulmonary inflammation, we semi-quantitatively scored histology slides prepared from lungs harvested 12 or 44 hours after infection. The extent of lung inflammation and the percentage of infiltrated lung surface did not differ between SelP<sup>−/−</sup> and WT mice (Figure 2A-C). To obtain more insight in leukocyte recruitment in our model we performed neutrophil specific Ly-6G and macrophage/monocyte F4/80 staining on lung tissue sections. No differences were observed in neutrophil or macrophage numbers in lungs of WT and SelP<sup>−/−</sup> mice at either 12 or 44 hours post infection (Figure 2D-G). As another approach in determining leukocyte influx into lungs, we repeated the infection experiments and collected BALF. Surprisingly, no differences in bacterial burdens between WT and SelP<sup>−/−</sup> mice were detected in the intra-alveolar compartment (Figure 2H). Total cell counts in BALF did not differ between mouse strains (Figure 2I) and no differences in

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Figure 1: *SelP<sup>−/−</sup> mice display enhanced bacterial growth and dissemination.* WT (closed dots) and SelP<sup>−/−</sup> (open dots) mice were infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. Bacterial counts were determined in lungs (A), blood (B), spleen (C) and liver (D). Data are expressed as scatter dot plots with the median. N = 8 or 12 mice per group. * P<0.05, ** P<0.005, *** P<0.0005 versus WT.
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Figure 2: P-selectin is not required for leukocyte recruitment to the lungs during *Klebsiella* pneumosepsis. WT (gray) and Selp−/− (white) mice were infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. (A) Representative microphotographs of H&E stained tissue sections of 44 hours infected lungs (4x original magnification). Lung histopathology (B) and lung infiltration (C) were scored on H&E tissue sections by a pathologist blinded for groups. (D) Representative microphotographs of Ly-6G stained tissue sections of 44 hours infected lungs (4x original magnification); quantification in (E). (F) Representative microphotographs of F4/80 stained tissue sections of 44 hours infected lungs (4x original magnification); quantification in (G). BALF was obtained 12 and 44 hours after inoculation with *K. pneumoniae*; in BALF CFU (H) and cell counts (I) were determined. Cell differentiation was determined on cytopsins (J). (K) Representative microphotographs of H3Cit stained tissue sections of 44 hours infected lungs (4x original magnification); quantification in (L). Ly-6G, F4/80 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, F4/80 or NET positivity were expressed as a percentage of the total surface area. Data are depicted as scatter dot plots with the median or as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group.
recruited cell types (neutrophils, macrophages and lymphocytes) were found (Figure 2J). Platelets have been suggested to be involved in formation of neutrophil extracellular traps (NETs), secreted by neutrophils as an antibacterial defense strategy. To assess whether P-selectin is involved herein, we stained lung tissue sections for NETs using anti-citrullinated histone H3 (H3cit). No significant difference was however observed between WT and Selp mice 12 or 44 hours after infection (Figure 2K,L).

Selp mice display reduced platelet-monocyte complex formation and elevated cytokine levels during Klebsiella sepsis

Since platelet-leukocyte interactions can influence leukocyte functions, we next determined platelet-neutrophil and platelet-monocyte complexes in naïve and Klebsiella

![Figure 3](image-url)

**Figure 3:** Selp mice display reduced platelet-monocyte complex formation and elevated cytokine levels during Klebsiella sepsis. WT (gray) and Selp (white) mice were sacrificed naïve or infected with K. pneumoniae via the airways and euthanized at the indicated time points. Whole blood obtained from uninfected (naive) mice and 44 hours after induction of pneumonia was stained with CD45, Ly-6G, CD115 and CD61 and cell complexes were identified as described in the online supplement. (A) and (C) are representative histograms of CD61 positivity for monocyte and neutrophil identified populations; (B) and (D) depict the Geomean MFI for the whole group. TNF-α, IL-6 and IL-1β were measured in plasma (E,F and below detection – not shown) and in lung homogenates (G,H,I). Data are expressed as bars or 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, ** P<0.005, *** P<0.0005 versus WT.
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Infected WT and Selp\(^{-/-}\) mice using flow cytometry. 44 hours after induction of pneumonia, Selp\(^{-/-}\) mice had fewer monocyte-platelet complexes than WT mice (P<0.005, Figure 3A,B), whereas neutrophil-platelet complexes were similar in both mouse strains (Figure 3C,D). Selp\(^{-/-}\) mice showed higher TNF-α and IL-6 levels in plasma and higher IL-6 and IL-1β levels in lungs when compared with WT mice 44 hours after induction of pneumonia (P<0.05 – P<0.0005; Figure 3E-I); plasma IL-1β remained undetectable in all mice. During this later time-point, the increase in bacterial burdens could be an additional explanation for the increase in cytokine release. It is therefore of special interest that the increase in lung IL-6 and IL-1β was already detected early after infection, while bacterial burdens in WT and Selp\(^{-/-}\) mice were equal (P<0.05 versus WT mice; P = 0.06 for TNF-α).

Role of P-selectin in activation of the coagulation system and the vascular endothelium during Klebsiella pneumosepsis

Platelet counts, indicative of platelet activation and consumption during sepsis, decreased similarly in WT and Selp\(^{-/-}\) mice during the infection (Figure 4A). Activation of the coagulation system was assessed by measuring TATc in plasma; levels did not differ between groups at any time point (Figure 4B). Considering that this model of severe pneumonia is associated with pulmonary coagulopathy, we analyzed D-dimer levels by western blotting of weight normalized whole lung homogenates to obtain insight in local activation of the coagulation system (Figure 4C,D). These analyses confirmed that P-selectin is not required for coagulation activation; on the contrary, Selp\(^{-/-}\) mice demonstrated higher D-dimer levels in their lungs at 44 hours after infection relative to WT mice. As expected, P-selectin was not detected in plasma of Selp\(^{-/-}\) mice (Figure 4E). Plasma P-selectin increased during late stage Klebsiella pneumosepsis in WT mice (P<0.01 versus naive). We measured soluble E-selectin in plasma and total E-selectin in whole lung homogenates to obtain insight in the role of P-selectin in systemic and local endothelial cell activation respectively. Both plasma and lung E-selectin levels increased in WT mice especially during late stage Klebsiella sepsis (Figure 4F,G). Naive Selp\(^{-/-}\) mice had lower plasma and lung E-selectin levels relative to WT mice (both P<0.05), suggesting that P-selectin contributes to the constitutive activation state of the vascular endothelium. Plasma E-selectin remained lower in Selp\(^{-/-}\) than in WT mice after infection with Klebsiella, whereas in lungs E-selectin increased similarly in both mouse strains.

Selp\(^{-/-}\) mice have increased distant organ damage during Klebsiella pneumosepsis

We measured plasma AST and ALT as measures of hepatocellular injury, and LDH and nucleosomes as markers of cellular injury in general (Figure 5A-D). As expected, plasma AST, ALT and LDH increased in WT mice during the course of the infection, which was paralleled by a rise in plasma nucleosome levels (P<0.05 – P<0.005 versus naive); organ damage was more severe in Selp\(^{-/-}\) mice as they displayed augmented levels of transaminases, LDH and nucleosomes at 44 hours after infection (P<0.05 – P<0.0005 versus WT). The enhanced
hepatocellular injury in Selp\(^{-}\) mice was associated with increased influx of neutrophils in hepatic tissue (P<0.05 versus WT mice; Supporting Figure 1).

Both platelet and endothelial P-selectin contribute to protective immunity during Klebsiella pneumoniae

P-selectin is expressed upon activation by platelets and endothelial cells. To dissect the roles of endothelial cell and platelet P-selectin in host defense during Klebsiella sepsis, we created P-selectin BM chimeric mice. In order to determine the transplantation efficiency for megakaryocytes, platelet P-selectin expression was determined using flow cytometry in citrated whole blood after stimulation with PAR4AP (Figure 6A-E). Selp E+/P+ control mice and Selp E-/P+ chimeric mice displayed robust P-selectin expression on their platelets upon
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**Figure 5:** Selp^+/+ mice have increased distant organ damage during Klebsiella pneumosepsis. WT (gray) and Selp^−/− (white) mice were sacrificed naive or infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. AST (A) and ALT (B) were measured in plasma as markers for hepatocellular injury and LDH (C) and nucleosomes (D) as more general cell injury markers. 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, **P<0.005, ***P<0.0005 versus WT.

**Figure 6:** Successful generation of P-selectin BM chimeric mice. BM chimeric mice were produced as described in Methods, generating Selp^E+/P+ (A; closed dots) and Selp^E−/P− (D; open dots) control mice and Selp^E+/P− (B; right open dots) and Selp^E−/P+ (C; left open dots) chimeric mice. Citrated whole blood of naïve mice was stimulated with 330 μg/ml PAR4AP and platelet P-selectin expression was determined using flow cytometry. Histograms are depicted in (A-D) and percentage P-selectin positive platelets in (E). Soluble P-selectin was measured in plasma in of mice 44 hours after infection with *K. pneumoniae* (F). N = 12 mice per group. Data are expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 12 mice per group. ***P<0.0005 versus Selp^E+/P+; ###P<0.0005 versus Selp^E−/P−; °°°P<0.0005 versus Selp^E−/P+.
activation by PAR4AP, whereas platelets from Selp E+/P- and Selp E-/P- mice failed to express P-selectin, confirming successful BMT. These BMT studies revealed the cellular source of soluble P-selectin in mice with *Klebsiella* sepsis: while as expected Selp E-/P- mice had undetectable soluble P-selectin levels in plasma, both Selp E-/P+ and Selp E+/P- chimeric mice had detectable soluble P-selectin levels, albeit lower than Selp E+/P+ mice, indicating that both endothelial cells and platelets contribute to circulating P-selectin in sepsis (Figure 6F).

Figure 7: Both platelet and endothelial P-selectin contribute to protective immunity during *Klebsiella pneumosepsis*. BM chimeric mice were produced as described in Methods, generating Selp E+/P+ (closed dots) and Selp E-/P- (open dots) control mice and Selp E+/P- (right open dots) and Selp E-/P+ (left open dots) chimeric mice. Mice were infected with *K. pneumoniae* via the airways and euthanized 44 hours thereafter or observed for 13 days. Following 44 hours of infection, bacterial counts were determined in lungs (A), blood (B), spleen (C) and liver (D). The mean of clinical scores during the observation and survival curves are depicted in (E,F). Data are expressed as scatter dot plots with the median, curves depicting the mean or as Kaplan-Meier survival curve. N = 12 mice per group for the 44 hours and 15-18 mice per group during the observation study. * P<0.05, ** P<0.005, *** P<0.0005 versus Selp E+/P+ or for the indicated comparisons. # P<0.05, ## P<0.005, versus Selp E-/P-.
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*Selp* E-/P- mice demonstrated 10-1000-fold higher bacterial burdens when compared with *Selp* E+/P+ mice 44 hours after infection with *Klebsiella* (P<0.05 – P<0.0005; Figure 7A-D), replicating the impaired antibacterial defense of *Selp*+/ mice (Figure 1). *Selp* E+/P- and *Selp* E-/P+ mice displayed an intermediate phenotype with respect to bacterial outgrowth (P<0.05 – P<0.005 for comparisons indicated in Figure 7A-D). We next carried out an observational study to determine the impact of P-selectin on mortality during *K. pneumoniae* induced pneumonia. *Selp* E-/P- mice developed symptoms one day after induction of pneumonia, after which most of these mice died from 48 to 72 hours following the infection (Figure 7E,F). *Selp* E+/P+ mice developed symptoms later (P<0.005 versus *Selp* E-/P-), and deceased more slowly over the next 10 days (P<0.005 versus *Selp* E-/P- mice). Surprisingly, while *Selp* E+/P+ mice did not show symptoms during the first 48 hours after infection, in the days thereafter all but one *Selp* E+/P+ mice died. *Selp* E+/P- and *Selp* E-/P+ chimeric mice showed an intermediate phenotype. The onset of clinical scores was delayed for *Selp* E+/P- (P<0.05) and *Selp* E-/P+ (P<0.005) mice compared to *Selp* E-/P- mice (Figure 7E); there were no significant differences in survival of the P-selectin chimeric mice compared to *Selp* E+/P+ and *Selp* E-/P- controls (Figure 7F).

Discussion

We here studied the role of P-selectin during murine gram-negative pneumonia-derived sepsis. Our main findings were that *Selp*+/ mice displayed enhanced bacterial outgrowth in their lungs and distant organs. P-selectin deficiency was associated with reduced platelet-monocyte complex formation together with an increased release of proinflammatory cytokines and enhanced distant organ injury. BMT experiments revealed a role for both platelet and endothelial cell P-selectin in protective immunity during *Klebsiella* sepsis, as indicated by enhanced bacterial growth and dissemination together with a reduced survival in *Selp* E-P- relative to *Selp* E+P+ mice, with intermediate phenotypes for P-selectin chimeric mice.

Our study is the first to show a role for P-selectin in the control of bacterial growth in pneumonia induced sepsis. We considered the lungs as primary source of infection most appropriate since pneumonia is by far the most common cause of sepsis 1. P- and E-selectin double knock-out mice have been challenged in a pneumonia model with high dose (10^7 CFU) *Streptococcus (S.) pneumoniae*; this study reported no differences in bacterial burdens or survival 30. Impaired host defense has been shown in *Selp*+/ mice in a model of pneumococcal peritonitis, induced by injecting 10^7 CFU *S. pneumoniae* into the abdominal cavity 31, an unusual route of infection by this pathogen. Experimental *K. pneumoniae* pneumonia represents a clinically more relevant model, induced by a low inoculum of a virulent pathogen, associated with a gradually growing bacterial load at the primary site of infection.
with subsequent dissemination to distant organs and tissue injury. Our results indicate that P-selectin limits invasive growth of *Klebsiella* during advanced pneumonia. Indeed, at 44 hours after infection Selp<sup>−/−</sup> mice had higher bacterial loads in all body compartments except for BALF, suggesting that P-selectin inhibits bacterial translocation from the bronchoaveolar space.

Most studies describing a role for P-selectin in inflammation used non-infectious models. Infusion of activated platelets led to the formation of platelet-leukocyte aggregates and increased leukocyte rolling, which was both platelet and endothelial cell P-selectin dependent<sup>10</sup>. Endothelial cell P-selectin also mediated platelet and leukocyte rolling and adherence to microvascular endothelial cells in a model of intestinal ischemia and reperfusion<sup>8</sup>. Conversely, platelet P-selectin mediated neutrophil recruitment in models of acid-induced acute lung injury<sup>7</sup> and post-ischemic renal failure<sup>11</sup>. In our *Klebsiella* pneumosepsis model, P-selectin did not contribute to leukocyte recruitment to either BALF or lung tissue, which is in accordance with earlier studies examining leukocyte influx in response to *S. pneumoniae* infection in mice with either isolated P-selectin deficiency or P-selectin deficiency combined with absence of intercellular adhesion molecule-1<sup>30-32</sup>. While platelets have been found to be threshold switch cells for neutrophil NET release during sterile LPS and *Escherichia (E.) coli* infectious challenges<sup>22,23</sup>, P-selectin mediated platelet-neutrophil interaction was not involved in NET formation during *Klebsiella* induced pneumosepsis.

P-selectin can contribute to the formation of platelet-leukocyte complexes<sup>4</sup>. Platelet-monocyte complexes were diminished in Selp<sup>−/−</sup> mice compared to WT after 44 hours of infection, signifying a role for platelet P-selectin herein. Platelets in complex with macrophages or monocytes have been shown to inhibit TNF-α and IL-1β production after high-dose intravenous LPS or *E. coli* challenges; the same authors additionally found that platelet-macrophage interaction inhibited TNF-α production upon high-dose LPS stimulation *in vitro*<sup>26,27</sup>. During *in vitro* platelet stimulation experiments using thrombin however, monocyte interaction with activated platelets resulted in increased cytokine production<sup>33</sup>. Induction of proinflammatory signaling in monocytes by platelets was dependent on secreted platelet products, not by direct platelet-monocyte interaction in another study<sup>34</sup>. Although cytokine production is indispensable for an adequate host response to invading pathogens, excessive cytokine production might have detrimental effects during severe infection<sup>35</sup>. Indeed, decreased cytokine levels conferred protection during LPS and *E. coli* sepsis<sup>26</sup>. Selp<sup>−/−</sup> mice displayed elevated levels of TNF-α, IL-6 and IL-1β upon infection with *Klebsiella*. While higher proinflammatory cytokine concentrations during the later stage of infection could be due to increased bacterial burdens in Selp<sup>−/−</sup> mice, increased levels were found in the lungs as soon as 12 hours after induction of infection, when bacterial burdens were equal in both genotypes. Together these data suggest that P-selectin mediated complex formation...
between monocytes and platelets may inhibit proinflammatory cytokine release during *Klebsiella sepsis in vivo.*

Activation of platelets can lead to P-selectin mediated systemic inflammation, resulting in leukocyte activation and microvesicular tissue factor generation, thus eliciting inflammation-induced procoagulant activity. *Selp*−/− mice were protected in a deep venous thrombosis model, which was endothelial cell P-selectin dependent. During fecal sepsis, blockade of P-selectin significantly reduced septic blood flow stoppage by platelet deposition in organ capillaries. Moreover, activated platelets can stimulate endothelial cells to secrete von Willebrand Factor in a P-selectin manner. We here used E-selectin levels as a readout for endothelial cell activation and found that naïve (uninfected) *Selp*−/− mice had lower concentrations of soluble E-selectin in plasma and total E-selectin in whole lung homogenates. In plasma, soluble E-selectin levels remained lower in *Selp*−/− mice when compared with WT mice, while in whole lungs E-selectin increased to a similar extent in both mouse strains. Together these results point to involvement of P-selectin in systemic endothelial cell activation during sepsis, while such a role is not detectable in lungs in the presence of a strong local inflammatory response. P-selectin deficiency did not impair activation of coagulation in our model, which is in accordance with results obtained in non-infectious systemic inflammation model, in which P-selectin deficiency did not impact on the development of microvascular thrombosis. D-dimer levels were even higher in lungs of *Selp*−/− mice - likely caused by the higher bacterial burdens in these animals, providing a stronger procoagulant stimulus.

*Klebsiella pneumosepsis* is associated with distant organ damage during late stage infection. In addition to elevated transaminases and LDH, we now shown increased systemic release of nucleosomes, thereby mirroring elevated nucleosome levels in patients with severe sepsis. Previously, interaction between platelets and neutrophils was found to be essential for the induction of liver injury after administration of a high infectious dose of *E. coli*, with a pivotal role for lymphocyte function-associated antigen 1, an adhesion molecule expressed by multiple cell types including neutrophils. The present data indicate that P-selectin played no role herein. On the contrary, *Selp*−/− mice displayed higher plasma levels of all organ injury markers measured, likely due to the higher bacterial burdens. Like in lungs, P-selectin was not important for neutrophil recruitment in liver during *Klebsiella* sepsis, as reflected by higher neutrophil numbers in liver tissue sections of *Selp*−/− mice at 44 hours after infection.

Our results clearly establish for the first time that P-selectin is involved in host defense against gram-negative pneumosepsis, with protective roles for both platelet and endothelial cell P-selectin.
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References


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Figure S1: Increased neutrophil recruitment to the liver in Selp^−/− mice. WT (gray) and Selp^−/− (white) mice were infected with K. pneumoniae via the airways and euthanized at the indicated time points. (A) Representative liver microphotographs of Ly-6G stained tissue sections after 44 hours of infection (4x original magnification). Neutrophil accumulation in liver tissue is expressed as a quantification of Ly-6G staining; the amount of Ly-6G positivity was expressed as a percentage of the total surface area (E). N = 8 mice per group. * P<0.05.