Platelets: versatile effector cells in pneumonia and sepsis

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Platelet MyD88 does not contribute to the host response during gram-negative sepsis

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Submitted
Abstract

Beside their role in hemostasis, platelets serve as sentinel cells in host defense during infection. In sepsis, platelets have been implicated in both beneficial (antibacterial) and detrimental responses (thrombosis and organ damage). Toll-like receptors (TLRs) and their common adaptor MyD88 are essential for pathogen recognition and protective immunity. Platelets express functional TLRs and MyD88, which participate in platelet responsiveness to bacterial agonists. Considering the pivotal involvement of platelets and MyD88 in the host response to bacteria, we here studied the role of platelet MyD88 in gram-negative sepsis using intravenous and airway infections with the common human sepsis pathogen *Klebsiella pneumoniae*. Platelet specific *Myd88* mice were generated by crossing mice with a conditional *Myd88* flox allele with mice expressing Cre recombinase controlled by the platelet factor 4 promoter. In a reverse approach, full *Myd88* mice were transfused with wild-type platelets. In both settings platelet MyD88 did not impact on bacterial growth or dissemination. In addition, platelet MyD88 did not influence hallmark sepsis responses such as thrombocytopenia, coagulation or endothelial activation, or distant organ injury. Platelet MyD88 played no role in lung pathology during pneumonia derived sepsis. In conclusion, platelet MyD88 does not contribute to the host response during gram-negative sepsis.
Introduction

Sepsis is a syndrome that occurs when the host response to infection injures its own tissues. Sepsis is an important health burden, with an estimated incidence of over 19 million cases worldwide per year and high mortality rates even when appropriate antibiotic treatment is available. The most frequent cause of sepsis is pneumonia, responsible for up to 50% of all cases. *Klebsiella (K.) pneumoniae* is a common pathogen in human sepsis, especially in the context of pneumonia.

Platelets, renowned for their role in hemostasis, are increasingly recognized as essential players in host defense against infection. We recently revealed a strongly protective role for platelets in *Klebsiella* pneumonia derived sepsis, in which thrombocytopenic mice demonstrated platelet count dependent impaired survival together with enhanced bacterial growth and dissemination; severe thrombocytopenia in addition resulted in hemorrhage at the primary site of infection. Thrombocytopenic mice also displayed enhanced bacterial growth in respiratory tract infection caused by *Streptococcus (S.) pneumoniae* and a reduced clearance of *Escherichia (E.) coli* and *Bacillus cereus* after high dose systemic challenges, further supporting an antibacterial effect of platelets in vivo.

Toll-like receptors (TLRs) comprise a family of pattern recognition receptors that are critical for microbial surveillance and protective immunity during infection, including *Klebsiella* pneumonia derived sepsis. Human and mouse platelets express several TLRs, a relatively recent observation now documented by many laboratories. Myeloid differentiation factor 88 (MyD88) is the downstream adaptor protein of all TLRs except TLR3. We hypothesized that platelets exert their protective role in bacterial infection at least in part via signaling through MyD88. Considering the pivotal involvement of platelets and MyD88 in protective immunity against *Klebsiella* we here studied the role of platelet specific MyD88 signaling in the host response during pneumonia and sepsis caused by this common human pathogen.

Materials and Methods

Animals

Mice homozygous for the conditional *Myd88* flox allele (*Myd88<sup>lox/lox</sup>*) were kindly provided by Anthony DeFranco (University of California, San Francisco, CA). Platelet specific *Myd88* knock out (Plt-*Myd88<sup>-/-</sup>*) mice were generated by crossing *Myd88<sup>lox/lox</sup>* mice with mice expressing Cre recombinase under the control of the platelet factor 4 (PF4) promoter (Jackson Laboratory, Bar Harbor, Maine); Cre-negative *Myd88<sup>lox/lox</sup>* littermates were used as controls for Plt-*Myd88<sup>-/-</sup>* mice. Full *Myd88<sup>-/-</sup>* mice were kindly provided by Dr. S. Akira (Research Institute for Microbial Diseases, Osaka, Japan). All genetically modified mice were backcrossed >6 times to a C57Bl/6 genetic background. Wild-type (WT) C57Bl/6 mice were purchased from Harlan.
Sprague-Dawley (Horst, the Netherlands). Age (10-14 weeks) and sex-matched mice were used in all experiments. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

**Ex vivo stimulations**

Heparinized whole blood from uninfected Myd88lox/lox, Plt-Myd88-/− and full Myd88−/− was incubated with 5x10^6 CFU growth arrested *K. pneumoniae* (UV-irradiation for 20 minutes; serotype 2, ATCC 43816, Rockville, MD) or medium (IMDM; Lonza, Basel, Switzerland); after 6 hours, blood was spun at 3000 rpm 10 min, and plasma supernatants were stored at -20°C until analysis. In separate experiments blood from naive or *Klebsiella* infected mice was incubated with or without protease activated receptor 4 activating peptide (PAR4AP; American Peptide Company, Sunnyvale, CA; 180 µg/ml) or control for 30 minutes at room temperature.

**Experimental study design**

Pneumonia was induced by intranasal inoculation with *K. pneumoniae* (ATCC 43816; 10.000 colony forming units (CFU) in 50 µL isotonic saline) as described 7,23. Sepsis was induced by intravenous infusion of 50.000 CFU *K. pneumoniae* 23. Mice were euthanized 2, 24 or 44 hours after induction of pneumonia or sepsis (N = 8 or 12 mice per group, as indicated in Figure legends). Bacterial quantification and storage of organs were performed as described 7,23.

**Measurements and assays**

To check the efficiency of the cre-recombinase process, platelets from Plt-Myd88−/− and control mice were isolated as described below and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (GE Healthcare, Eindhoven, the Netherlands) according to manufacturer’s instructions. MyD88 Western blotting was done using rabbit-anti-MyD88 (Cell Signaling Technology, Denvers, MA) and rat-anti-rabbit IgG-HRP secondary antibody (Cell Signaling Technology). β-actin was blotted as loading control (using rabbit-anti-β-actin (Cell Signaling Technology) and rat-anti-rabbit IgG-HRP secondary antibody). Platelet counts and platelet activation were determined in citrated whole blood by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ) using hamster anti-CD61-APC monoclonal antibody (BioLegend, San Diego, CA) and anti-CD62p-FITC (BD Biosciences, San Jose, CA) in accordance with manufacturers’ instructions. Interleukin (IL)-6, tumor necrosis factor (TNF-)α and monocyte chemotactic protein (MCP-)1 were determined using a cytometric beads array multiplex assay (BD Biosciences, San Jose, CA). Keratinocyte chemoattractant (KC), IL-1β, myeloperoxidase (MPO), P-selectin, E-selectin (R&D Systems, Abingdon, United Kingdom) and thrombin-antithrombin complexes (TATc; Bio-connect, Huissen, the Netherlands) were measured by ELISA. Matrix metalloproteinase (MMP)-9 was measured by Luminex assay (R&D Systems). 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 24.

**Histopathology**

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 23. H&E stained slides were coded and scored by a pathologist blinded for group identity for the following parameters: infiltrative surface (expressed as the percentage of total lung surface), bleeding, infiltration, 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. Ly-6G 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 positivity was expressed as a percentage of the total surface area 7.

**Platelet isolation and transfusion**

In order to obtain unperturbed platelets for transfusion, blood was collected with a 21-G needle from the inferior vena cava and diluted 4:1 with citrate. Blood was centrifuged at room temperature for 10 minutes at 180 g in order to obtain platelet rich plasma (PRP). PRP was re-centrifuged at 800 g for 10 minutes in the presence of prostaglandin E1 (PGE-1; Sigma Aldrich, St. Louis, MO, USA), acid citrate dextrose (ACD, 85 mM Na$_3$C$_6$H$_5$O(COO)$_3$, 110 mM Glucose, 65 mM C$_6$H$_8$O$_7$); platelet pellets were washed in 6 mL Buffer A (152 mM NaCl, 13.2 mM NaHCO$_3$, 6.16 mM Glucose, 1.1 mM MgCl$_2$6H$_2$O, 2.9 mM KCl, 1mM EDTA, pH6.5). Platelets were re-pelleted and resuspended in 1 mL SSP+ (Storage solution for platelets, Sanquin Blood Supply, Amsterdam, the Netherlands). Myd88$^{-/-}$ mice transfused with WT platelets (Myd88$^{-/-}$+WTplt) were compared to WT mice transfused with WT platelets (WT+WTplt) and Myd88$^{-/-}$ mice transfused with Myd88$^{-/-}$ platelets (Myd88$^{-/-}$+Myd88$^{-/-}$plt). Platelet transfusates (~ 4x 10$^8$ platelets per mouse) were administered directly after isolation via the tail vein, one hour before infection with Klebsiella.

**Statistical analysis**

Data are expressed as scatter dot plots or box and whisker plots. Comparisons between groups were performed using the Mann-Whitney U test. To correct for multiple testing, Kruskal-Wallis one-way analysis of variance test was used to compare groups in the transfusion experiments. In case of significant differences, differences between groups were additionally tested using the Mann-Whitney U test. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P-values < 0.05 were considered statistically significant.
Results

Blood from Plt-Myd88⁻/⁻ mice displays impaired TNF-α and KC production in vitro

Previous studies documented platelet specific deletion of floxed alleles by Cre recombinase driven by the PF4 promoter. To determine the efficiency and specificity of Cre-induced Myd88 deletion, we determined MyD88 expression in platelets and PBMCs from control and Plt-Myd88⁻/⁻ mice by Western blot. Plt-Myd88⁻/⁻ mice did not express MyD88 in platelets (confirming successful deletion), while MyD88 expression was unaffected in PBMCs (Figure 1A,B). In order to investigate whether platelet specific MyD88 deletion impacts on inflammatory responses in vitro, we incubated whole blood of control, Plt-Myd88⁻/⁻ and full Myd88⁻/⁻ mice with K. pneumoniae for 6 hours. Blood from Plt-Myd88⁻/⁻ mice produced significantly less TNF-α and KC when compared with blood from control mice (P<0.05, Figure 1C,E); as expected, blood from full Myd88⁻/⁻ mice hardly produced any TNF-α or KC (P<0.005 versus Plt-Myd88⁻/⁻; P=0.0005 versus control). Interestingly, IL-6 or MCP-1 release was only modestly reduced in blood from full Myd88⁻/⁻ mice (P<0.05 versus control) and not affected in blood from Plt-Myd88⁻/⁻ mice (Figure 1D,F). IL-1β remained undetectable in all incubations.

![Figure 1: Plt-Myd88⁻/⁻ mice display impaired TNF-α and KC production in vitro.](image-url) Naive Plt-Myd88⁻/⁻ (light gray bars) and control mice (dark gray bars) were sacrificed and platelets and PBMC's were isolated as described in the methods section. Western blot for MyD88 and β-actin are depicted in (A), quantitative analysis using ImageJ is depicted in (B). Whole blood from naive full Myd88⁻/⁻ (white bars), Plt-Myd88⁻/⁻ and control mice was stimulated with IMDM control or live K. pneumoniae for 6 hours, TNF-α (C), IL-6 (D), KC (E), and MCP-1 (F) were measured in plasma supernatants. Data are representative of two independent experiments. Data are depicted as bars or box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 4 mice per group. * P<0.05 versus control, ** P<0.005 versus control, *** P<0.0005 versus control, ## P<0.005 versus Myd88⁻/⁻ mice.
Platelet specific MyD88 deletion does not influence bacterial growth or platelet responses after intravenous infection with Klebsiella

To investigate the impact of platelet specific MyD88 deletion on host responses during Klebsiella infection in vivo, we intravenously infused Plt-Myd88−/− and control mice with viable K. pneumoniae and sacrificed them 2 or 24 hours thereafter. Considering that platelets may contribute to an effective antibacterial defense, and that differences in bacterial burdens may drive differences in the extent of activation of host response systems, we first determined the impact of platelet specific MyD88 deficiency on bacterial loads in blood, spleen, liver and lungs. Bacterial loads were similar in Plt-Myd88−/− and control mice in all body compartments tested (shown for blood and spleen in Figure 2A and B). Plt-Myd88−/− and control mice showed a similar decline in platelet counts in Klebsiella induced sepsis (Figure 2C). Platelet TLR signaling has been associated with direct platelet activation and ‘priming’ for other platelet stimuli. We measured platelet P-selectin expression by flow cytometry as readout for platelet activation, which remained low and similar in both groups (Figure 2D). To determine a possible role for platelet MyD88 in priming of platelets for other stimuli, we obtained blood from Plt-Myd88−/− and control mice before and 2 or 24 hours after infection and incubated it with PAR4-AP (Figure 2E,F). Ex vivo PAR4-AP stimulation enhanced platelet P-selectin expression, most strongly at 2 hours after infection. Of interest, at 24 hours after infection the few remaining platelets from Plt-Myd88−/− mice displayed a reduced capacity to upregulate P-selectin upon exposure to PAR4-AP (P<0.05 versus control). Klebsiella sepsis elicited a strong rise in plasma P-selectin levels, which was not different between mouse strains (Figure 2G).

Platelet specific MyD88 deletion attenuates early TNF-α and MCP-1 release but does not impact leukocyte, coagulation or endothelial cell activation, or liver injury after intravenous infection with Klebsiella

Consistent with the in vitro results (Figure 1), TNF-α but not IL-6 release was significantly reduced in Plt-Myd88−/− mice at 2 hours after infection (P<0.05 versus control; Figure 3A,B). Additionally, MCP-1 but not KC was significantly decreased (P<0.05 versus control for MCP-1; Figure 3C,D). Plasma IL-1β remained undetectable throughout. Neutrophil activation markers MMP-9 and MPO did not differ between Plt-Myd88−/− and control mice (Figure 3E,F). Platelets have been implicated in activation of the coagulation system and the vascular endothelium during sepsis. To obtain insight in the role of platelet MyD88 herein, we measured the plasma concentrations of TATc and soluble E-selectin (Figure 3G,H). Plasma TATc and E-selectin were elevated 24 hours after infection, but not different between control and Plt-Myd88−/− mice. Platelets have been reported to contribute to liver injury after intravenous infection with E. coli. Therefore, we measured the plasma levels of AST, ALT and (as general cellular injury markers) LDH and nucleosomes in Plt-Myd88−/− and control mice 24 hours after infection (Figure 3I-L). Although Klebsiella sepsis clearly was associated with a rise in all injury markers, no differences between mouse strains were detected. Altogether these data suggest...
Figure 2: Platelet specific MyD88 deletion does not influence bacterial growth or platelet responses after intravenous infection with *Klebsiella*. Plt-Myd88\(^{-/-}\) (open dots / bars) and control mice (closed dots/ gray bars) were intravenously infected with *K. pneumoniae* and euthanized at the indicated time points. Bacterial burdens were quantified in blood (A) and spleen (B). Platelet counts (C) and *in vivo* platelet activation (D) were determined in citrated whole blood by flow cytometry using CD61 and CD62p. Blood from naive or infected mice was stimulated *ex vivo* with 181 µg/ml PAR4-AP after which platelet activation was determined using flow cytometry; representative histograms are depicted in (E), box- and whisker plots of all mice in (F). Soluble P-selectin concentrations were determined in plasma (G). Data are depicted as scatter dot plots or box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group. * P<0.05 versus control.
Figure 3: Platelet specific MyD88 deletion attenuates early TNF-α and MCP-1 release but does not impact leukocyte, coagulation or endothelial cell activation, or liver injury after intravenous infection with *Klebsiella*. Plt-Myd88−/− (open bars) and control mice (gray bars) were intravenously infected with *K. pneumoniae* and euthanized at the indicated time points. TNF-α (A), IL-6 (B), KC (C), MCP-1 (D), MMP-9 (E), MPO (F), TATc (G), E-selectin (H), AST (I), ALT (J), LDH (K) and nucleosomes (L) were measured in plasma. Data are depicted as scatter dot plots or box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group. * P<0.05 versus control.
that although platelet MyD88 enhances the release of some proinflammatory mediators during gram-negative sepsis, it has no major impact on other host responses implicated in sepsis pathogenesis.

**Platelet specific Myd88 deletion does not influence bacterial burdens or platelet responses during Klebsiella induced pneumonia derived sepsis**

Pneumonia is the most common source of sepsis and platelets especially exert proinflammatory and immune modulatory effects in the lungs. In order to determine the impact of platelet specific MyD88 deletion in pneumonia, Plt-Myd88/− and control mice were infected with *K. pneumoniae* via the airways. This model is associated with a gradually growing bacterial load at the primary site of infection with subsequent dissemination of the infection and distant organ injury. Considering this more slowly progressive course, mice were sacrificed 24 or 48 hours after infection to study the impact of platelet specific MyD88 deletion on pneumonia-derived sepsis. Plt-Myd88/− and control mice showed no differences in bacterial burdens in lungs (Figure 4A) or distant organs (blood, spleen and liver; shown for spleen in Figure 4B). Platelet counts declined during Klebsiella pneumonia-derived sepsis in both Plt-Myd88/− and control mice to a similar extent (Figure 4C). Consistent with results obtained after intravenous infection, Plt-Myd88/− and control mice showed similar increases in soluble P-selectin levels throughout the infection (Figure 4D).

**Figure 4:** Platelet specific MyD88 deletion does not influence bacterial burdens or platelet responses during Klebsiella induced pneumonia derived sepsis. Plt-Myd88/− (open dots / bars) and control mice (closed dots/ gray bars) were infected with *K. pneumoniae* via the airways and euthanized at the indicated time points. Bacterial burdens were quantified in lung (A) and spleen (B). Platelet counts (C) were determined in citrated whole blood by flow cytometry using CD61. Soluble P-selectin concentrations were measured in plasma (D). Data are depicted as scatter dot plots or box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group.
Platelet specific MyD88 deletion does not modify lung inflammation or neutrophil recruitment during pneumonia induced Klebsiella sepsis

*Klebsiella* induced pneumonia is associated with clear histopathology of the lung compatible with lower respiratory tract infection. Platelets have been both associated with enhancement of histopathological damage during inflammatory challenges and the protection of vascular integrity during inflammation and *Klebsiella* and *S. pneumoniae* pneumosepsis. However, the extent and characteristics of lung inflammation did not differ between Plt-Myd88−/− and control mice, as reflected by the semi-quantitative scoring system described in the Methods section (Figure 5A,B). Hemorrhage was not observed in lungs of either Plt-Myd88−/− or control mice. Similar to the in vitro and early sepsis experiments, lung KC levels were diminished in Plt-Myd88−/− mice early after infection (P<0.05 versus control); other lung or plasma cytokine levels were comparable in both mouse strains (Table 1). However, neutrophil influx into lungs was similar in both mouse strains, as determined by Ly-6G staining of lung tissue slides (Figure 5C,D). As was found after intravenous infection, plasma levels of TAT, E-selectin, ALT, AST and LDH did not differ between Plt-Myd88−/− and control mice during pneumonia induced sepsis (Table 2).

Figure 5: Platelet specific MyD88 deletion does modify lung inflammation or neutrophil recruitment during pneumonia induced *Klebsiella* sepsis. Plt-Myd88−/− (open bars) and control mice (gray bars) 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 24 hours infected lungs (10x original magnification). Lung histopathology (B) was scored on H&E tissue sections by a pathologist blinded for groups as described in the methods section. (C) Representative microphotographs of Ly-6G stained sections of 24 hours infected lungs (10x original magnification); quantification in (D). Ly-6G 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 was expressed as a percentage of the total surface area. Data are depicted as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group.
WT platelets do not improve host response in full Myd88 deficient mice

Lack of platelet MyD88 signaling after *K. pneumoniae* infection might be insignificant in perspective of intact MyD88 signaling in other cell types involved in host defense, especially myeloid cells 16. We therefore performed platelet transfusion experiments in order to generate Myd88−/− mice with intact platelet MyD88 signaling and infected these mice with *K. pneumoniae* via the airways. We compared these Myd88−/−+WTplt mice with WT mice transfused with WT platelets (WT+WTplt) and Myd88−/− mice transfused with Myd88−/− platelets (Myd88−/−+Myd88−/− plt). As expected 13,15, median bacterial burdens were ≥2-log higher in Myd88−/−+Myd88−/− plt mice compared to WT+WTplt mice in all body compartments after 24 hours of infection (P<0.0005, shown for lung and spleen in Figure 6A,B). Transfusion of WT platelets did not lower bacterial burdens in Myd88−/−+WTplt mice. In addition, platelet counts and soluble P-selectin did not differ between groups (Figure 6C,D). In fact, transfusion of WT platelets did not alter any of
**Figure 6:** WT platelets do not improve host response in full Myd88 deficient mice. *Myd88*−/− mice were transfused with WT platelets (*Myd88*−/−+WTplt, light gray bars), *Myd88*−/− platelets (*Myd88*−/−+Myd88−/−plt, white bars) and WT mice were transfused with WT platelets (WT+WTplt, dark gray bars), after which mice were infected with *K. pneumoniae* via the airways. Mice were euthanized after 24 hours of infection. Bacterial burdens were quantified in lung (A) and spleen (B). Platelet counts (C) were determined in citrated whole blood by flow cytometry using CD61. Soluble P-selectin concentrations were measured in plasma (D). Data are depicted as scatter dot plots or box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. Figures represent combined data from three independent experiments with 4 mice per group. ***P<0.0005 versus WT+WTplt mice.

**Figure 7:** WT platelets do not alter inflammatory cytokines in full MyD88 deficient mice. *Myd88*−/− mice were transfused with WT platelets (*Myd88*−/−+WTplt, light gray bars), *Myd88*−/− platelets (*Myd88*−/−+Myd88−/−plt, white bars) and WT mice were transfused with WT platelets (WT+WTplt, dark gray bars), after which the mice were infected with *K. pneumoniae* via the airways. Mice were euthanized after 24 hours of infection. TNF-α (A), IL-6 (B), IL-1β (C) and KC (D) were determined in lung homogenates. Data are depicted as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. Figures represent combined data from three independent experiments with 4 mice per group. *P<0.05 versus WT+WTplt mice, **P<0.005 versus WT+WTplt mice.
the local or systemic host responses measured in Myd88⁻/⁻ recipient mice, when compared with transfusion of Myd88⁻/⁻ platelets in Myd88⁻/⁻ mice (Figure 7 and Table 3). Notably, lung cytokine levels were altered in Myd88⁻/⁻ recipient mice when compared with WT recipient mice irrespective of the genotype of the transfused platelets (P<0.05-0.005), reflecting the role of MyD88 of the recipient herein. Finally, lung pathology did not differ between groups (Supplemental Figure 1).

Table 3:

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<td>622 (465-781)</td>
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Plasma cytokines, coagulation and endothelial cell activation and organ damage markers in Control and Myd88⁻/⁻ platelet transfused mice during Klebsiella pneumonia induced sepsis. TNF-α, IL-6, IL-1β and MCP-1 are in pg/mL, TATc and E-selectin are in ng/mL. Data are measured 24 hours post-infection and expressed as the median with 25th and 75th percentile.

Discussion

Platelets have been shown to play an important role in the regulation of the host response during severe infection. We here hypothesized that platelets at least in part exert their function in bacterial sepsis via MyD88 dependent signaling. Considering the established role of MyD88 in protective immunity against Klebsiella, we utilized two different infection routes with this clinically relevant human sepsis pathogen (intravenous and via the airways) and two opposite approaches (mice with platelet specific MyD88 deficiency and transfusion of WT platelets into Myd88⁻/⁻ recipients) to determine the impact of platelet MyD88 in sepsis. Although Plt-Myd88⁻/⁻ mice displayed impaired TNF-α and KC production during whole blood stimulation in vitro and during early Klebsiella sepsis in vivo, our data clearly show that platelet MyD88 does not contribute to either control of bacterial growth or to the subsequent collateral tissue damage. These results argue against an important functional role for platelet TLRs in the host response during severe sepsis.

MyD88 dependent TLR signaling is critical for microbial surveillance and regulation of inflammatory and immune responses. Several research groups have confirmed expression of TLR1-7 and 9, and MyD88 human and mouse platelets and functional roles for some of these TLRs have been described. For example, the TLR4 agonist LPS and the...
Platelet MyD88 does not contribute to host defense during gram-negative sepsis

TLR9 agonist carboxyalkylpyrrole protein adducts have been shown to stimulate platelet granule secretion in a MyD88 dependent manner. MyD88 is the only TLR adaptor present in platelets, since Tir-domain containing adaptor-inducing interferon-β (TRIF), which mediates signaling via TLR3 and 4, has not been demonstrated in this cell type. Previous studies have indicated that platelets may either inhibit or enhance cytokine production by mononuclear cells depending on the experimental conditions and the stimuli used. We here found that platelet MyD88 contributed to Klebsiella-induced TNFα release in whole blood in vitro and during sepsis in vivo. In accordance, LPS prestimulated platelets increased the secretion of TNF-α by human PBMCs. The role of platelet MyD88 in cytokine release was modest and of insignificant biological relevance, considering that none of the more downstream sepsis responses were altered in Plt-Myd88−/− mice. Nonetheless, studies that address the effect of platelet MyD88 on cytokine production by leukocytes and the cellular mechanisms involved are of interest.

LPS infusion has been shown to cause profound thrombocytopenia in WT, but not in Tlr4−/− mice. Klebsiella sepsis, however, caused thrombocytopenia in Plt-Myd88−/− and control mice to a similar extent, indicating comparable platelet activation and consumption. Although Klebsiella sepsis did not directly enhance P-selectin expression on circulating platelets, the infection did increase the responsiveness of platelets to ex vivo stimulation with PAR4-AP that triggers the sole thrombin receptor on mouse platelets. In accordance, platelet ‘priming’ has been described for LPS, as pretreatment of LPS induced platelet hypersensitivity to subthreshold concentrations of classical platelet agonists in a manner that was either dependent on platelet cyclic guanosine monophosphate (cGMP) upregulation or externally via monocytes. Notably, while platelet reactivity to PAR4-AP similarly increased during early sepsis for Plt-Myd88−/− and control mice, 24 hours after infection remaining Plt-Myd88−/− platelets displayed reduced capacity to upregulate P-selectin. Plasma soluble P-selectin, indicative of platelet degranulation but also secreted by endothelial cells, increased equally in Plt-Myd88−/− and control mice. Taken together with the similar rise in plasma soluble E-selectin, indicative of endothelial cell activation in both mouse strains, these results suggest that platelet MyD88 signaling does not contribute to sepsis induced platelet or endothelial cell activation.

Platelets are considered to play an essential role in coagulation by providing a phospholipid surface for the assembly of activated clotting factors. During sepsis, increased cell death results in release of histones into the circulation. We here indeed demonstrated a rise in the plasma levels of nucleosomes (complexes of DNA and histones) during Klebsiella sepsis. Histones can promote thrombin generation via platelet TLR2 and TLR4, contributing to a pro-coagulant phenotype. In accordance, platelets from WT mice but not Tlr4−/− mice diminished microvascular flow in Tlr4−/− mice exposed to LPS. Additionally, carboxyalkylpyrrole protein adducts, released during infection and injury, have been shown to engage platelet TLR9 and
thereby promote platelet hyperreactivity and thrombosis. In our study, however, we did not find differences in thrombin generation between control and Plt-Myd88<sup>-/-</sup> mice. Hence, considering that TLRs 2, 4 and 9 are MyD88 dependent and *Klebsiella* responsive receptors, these data suggest that platelet TLR stimulation is not involved in activation or perpetuation of coagulation during *Klebsiella* sepsis.

Platelets have been implicated in the organ damage during severe infection. Indeed, platelets contributed to liver injury in systemic infection caused by either *E. coli*<sup>9</sup> or *B. cereus*<sup>10</sup>, and in the model of gram-negative pneumonia derived sepsis used here.<sup>7</sup> In the hyperacute challenge models produced by high dosages of *E. coli* or *B. cereus* injection platelets caused liver damage by facilitating the formation of neutrophil extracellular traps (NETs)<sup>9,10</sup>. Platelets assist in NETs formation by a mechanism that at least in part relies on platelet TLR4<sup>19</sup>. However, NETs formation unlikely plays a role in liver damage during the more gradually evolving infection caused by *Klebsiella*<sup>7</sup>. We did provide evidence that platelets enhance early neutrophil activation in *Klebsiella* sepsis, as reflected by reduced plasma MPO levels in mice with moderate thrombocytopenia<sup>7</sup>. In the present investigation we establish that platelet MyD88 dependent signaling is not involved herein: the increase in plasma transaminases was not altered by the presence or absence of platelet MyD88. Similarly, plasma MPO and MMP9 were not influenced by platelet MyD88. In addition, platelet MyD88 did not impact on cellular injury in general during *Klebsiella* sepsis, as indicated by similar rises in plasma LDH and nucleosome concentrations in all experimental groups. Altogether these results indicate that while platelets can contribute to organ damage during sepsis, platelet MyD88 signaling has no role herein.

Platelets exert potent proinflammatory and immune modulatory effects in the lungs.<sup>33</sup> Platelets have been shown to be recruited to the lungs after LPS administration by a TLR4 dependent mechanism<sup>18</sup>, and to enhance LPS-induced lung architectural damage<sup>34</sup>, at least partially through stimulating neutrophil influx<sup>5,6,52</sup>. Moreover, platelets are essential for vascular integrity during pulmonary inflammatory challenges, including *Klebsiella* pneumonia<sup>7,35</sup>. In the present series of experiments we found no evidence for a role of platelet MyD88 in either lung inflammation, neutrophil recruitment or prevention of hemorrhage during *Klebsiella* pneumonia.

Platelets can play a dual role in the pathogenesis of bacterial sepsis - on the one hand assisting in innate defense, while on the other hand contributing to later organ damage. Although platelet TLRs have been implicated in both beneficial and detrimental responses during infection, the present study is the first to show that platelet MyD88 does not impact on the host response during severe infection by a common human gram-negative sepsis pathogen.
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References


Supplemental figure

**Figure S1:** WT platelets do not alter histopathological changes in full MyD88 deficient mice. Myd88<sup>−/−</sup> mice were transfused with WT platelets (Myd88<sup>−/−</sup>+WTplt, light gray bars), Myd88<sup>−/−</sup> platelets (Myd88<sup>−/−</sup>+Myd88<sup>−/−</sup>plt, white bars) and WT mice were transfused with WT platelets (WT+WTplt, dark gray bars), after which the mice were infected with *K. pneumoniae* via the airways. Mice were euthanized after 24 hours of infection. (A) Representative microphotographs of H&E stained tissue sections of 24 hours infected lungs (10x original magnification). Lung histopathology (B) was scored on H&E tissue sections by a pathologist blinded for groups as described in the methods section. Data are depicted as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group.