Platelets: versatile effector cells in pneumonia and sepsis

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Toll-like receptor signalling is not involved in platelet response to *Streptococcus pneumoniae in vitro or in vivo*

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Abstract

Background: *Streptococcus (S.) pneumoniae* strains vary considerably in their ability to cause invasive disease in humans, at least in part determined by the capsular serotype. Platelets have been implicated as sentinel cells in the circulation for host defence. One of their utensils for this function is the expression of Toll-like receptors (TLRs).

Objective: To determine the role of TLRs in platelet responses to *S. pneumoniae*.

Methods: Stimulation was performed with *S. pneumoniae* serotype 2, 3, 4 and mutated unencapsulated serotype 2 (ΔcpsD39). Platelet aggregation was measured in platelet-rich plasma; platelet granular release and platelet-neutrophil complexes were measured in human or mouse whole blood using flow cytometry. Platelet specific Myd88^−/− mice (Plt-Myd88^−/−) were generated by crossing Myd88lox/lox mice with mice expressing Cre under the platelet factor 4 promoter; mice were infected with ΔcpsD39 via the airways.

Results: Only ΔcpsD39 induced platelet aggregation. Whole blood incubation with all *S. pneumoniae* serotypes tested resulted in platelet degranulation and platelet-leukocyte complex formation. Platelet activation was TLR independent, as responses were not inhibited by TLR blocking antibodies, not induced by TLR agonists and were equally induced in wild-type and tlr2^−/−, tlr4^−/−, tlr2/4^−/−, tlr9^−/− and Myd88^−/− mice. Plt-Myd88^−/− and control mice displayed no differences in bacterial clearance or immune response to ΔcpsD39 pneumonia.

Conclusions: *S. pneumoniae* activates platelets activation through a TLR-independent mechanism that is impeded by the bacterial capsule. In addition, platelet MyD88-dependent TLR signalling is not involved in host defence to ΔcpsD39 in vivo.
Introduction

Streptococcus (S.) pneumoniae is a frequent inhabitant of the upper airways in healthy individuals, but also the most common cause of community-acquired pneumonia and a main cause of sepsis. Sepsis is a life-threatening condition, where the host response to infection is injurious to tissues and organs. During sepsis, activation of the coagulation cascade, together with endothelial damage, leads to platelet activation. Platelets can additionally be activated by pathogens and components thereof during bacterial dissemination. Sepsis patients indeed show an increase in platelet activation markers and a decrease in platelet counts, and the extent of these responses is associated with mortality.

Platelets are widely renowned for their role in haemostasis. More recently, platelets have been implicated as major players in host defence. The platelet releasate contains a number of pro-inflammatory proteins and antimicrobial peptides. Platelet activation and P-selectin expression lead to platelet-neutrophil interaction, which recruit neutrophils to an inflammatory site and stimulate the release of neutrophil extracellular traps. Platelet depletion in vivo leads to enhanced bacterial growth and increased mortality during murine S. pneumoniae and Klebsiella pneumoniae induced pneumosepsis.

Platelets express several immune related receptors such as Toll-like receptor (TLR) 1, 2, 4-7 and 9, Receptors for Fc domain of IgG FcRII and FcεRI, complement receptors, and cyto- and chemokine receptors; additionally, platelet protease activated receptor (PAR)1, PAR4, glycoprotein (GP)IV, GPIIbIIIa and GPIbα can play a role in inflammatory reactions. TLRs are a family of pattern recognition receptors that are critical for microbial surveillance and regulation of inflammatory and immune responses. Functional roles for some platelet TLRs have been described, indicating that they are not residual receptors conserved from their bone marrow precursors.

Considering the important role for platelets in host defense to S. pneumoniae, we here aimed to investigate whether and how S. pneumoniae can directly activate platelets. For this, we measured S. pneumoniae induced platelet activation in a variety of assays in human and mouse blood, investigated a possible role for TLR signalling herein and performed in vivo pneumonia experiments with platelet specific MyD88 depleted (Plt-Myd88−/) and control mice.

Methods

Aggregation assay

Optical platelet aggregation was assayed on the aggregometer PAP-8E (Bio/data corporation, Horsham, PA) at 900 rpm and 37°C according to manufacturer’s instruction. Citrate-anticoagulated whole blood was collected from healthy volunteers. Platelet-rich plasma (PRP) was obtained by centrifugation at 180 g for 15 minutes at room temperature (RT). Blood was recentrifuged at 1500 g for 10 minutes to obtain platelet-poor plasma.
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Stimuli used were: *S. pneumoniae* serotype 2 (D39), *S. pneumoniae* serotype 3 (6303), *S. pneumoniae* serotype 4 (TIGR4), unencapsulated *S. pneumoniae* D39 (∆cpsD39 28), lipoteichoic acid (LTA; 5 μg/mL; *S. aureus*, Invivogen, San Diego, CA), Pam3CSK4 (5 μg/mL; Invivogen), lipopolysaccharide (LPS; ultrapure 5 μg/mL; *E. coli*, Invivogen) and recombinant *S. pneumoniae* serotype 2 capsule (rCPS2, 10 μg/mL; ATCC, Manassas, VA). Maximum platelet aggregation was determined in the presence of thrombin receptor activating peptide (TRAP, 15 μM; Sigma-Aldrich, St. Louis, MO). Indicated inhibitors were added 15 minutes prior to stimulation: Abciximab (glycoprotein IIbIIIa inhibitor Reopro, 10 μg/mL; Eli Lilly, Houten, the Netherlands), prostaglandin E1 (PGE1, 100 nM; Sigma-Aldrich), anti (α)-TLR2 (5 μg/mL, clone T2.5; HBT, Uden, the Netherlands), α-TLR4 (5 μg/mL, clone 18H10; HBT) and α-FcγRII (25 μg/mL, clone AT10; Abcam, Cambridge, UK). To evaluate platelet priming, PRP was stimulated for 5 minutes under stirring conditions at 37°C, before adding subthreshold concentration of TRAP. For each experiment, the TRAP concentration inducing the minimal measurable aggregation (hereby defined threshold concentration) was determined; usually 234 nM. Peripheral blood mononuclear cells (PBMCs) were isolated using Polymorphprep™ (Frensenius Kabi, Oslo, Norway) according to manufacturer’s instructions. Recordings were stopped after 10 or 15 minutes.

**HEK293-TLR2 cell stimulation**

HEK293 cells, stably transfected with TLR2 and CD14 29,30 were stimulated overnight with LTA (5 μg/mL), Pam3CSK4 (300 ng/mL) or 10^6 CFU ∆cpsD39, after a 30 minutes pre-incubation with 5 or 15 μg/mL α-TLR2 or medium control. Following 16 hours of stimulation at 37°C, supernatant was collected and IL-8 was determined using ELISA (R&D Systems, Abingdon, UK).

**Flow cytometry**

45 μL of citrated whole blood was stimulated with 5 x 10⁷ viable CFUs *S. pneumoniae* D39, ∆cpsD39, 6303 and TIGR4 in 45 μL PBS. Maximum platelet activation was determined in the presence of 15 μM TRAP. Where indicated, inhibitors were added 15 minutes prior to stimulation. Inhibitors were diluted in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 20 mM HEPES, 3.3 mM NaH₂PO₄, 1 g/l bovine serum albumin, 5.6 mM D-glucose, pH 7.4) and added in 5 μL to a final concentration of 5 μg/mL for α-TLR2, 10 μg/mL for Abciximab and 25 μg/mL for α-FcγRII AT10. Following 30 minutes incubation at RT, 5 μL stimulated blood was added to a mixture of antibodies in HEPES buffer, i.e., anti-CD61-APC (Dako, Heverlee, Belgium), anti-CD62p-PE (Beckman Coulter, Woerden, the Netherlands), anti-CD63-FITC (Beckman Coulter), anti-CD45-APC (BD biosciences, San Jose, CA), anti-CD14-FITC (BD biosciences) or isotype controls for human studies and anti-CD61-APC (BioLegend, San Diego, CA), anti-CD62p-FITC (BD biosciences) and isotype controls for mice and incubated at RT for 30 minutes. For platelet analysis, samples were fixed by addition of 2.5 mL 0.3%
paraformaldehyde-containing HEPES-buffer. For platelet-leukocyte complex analysis, samples were fixed by addition of 0.5 mL 0.3% paraformaldehyde-containing HEPES-buffer and erythrocytes were subsequently lysed by addition of 1.8 mL aquadest following centrifugation for 10 minutes at 400g, after which pellets were resuspended in 200 µL HEPES buffer. Flow cytometry was performed on a Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analysed using FlowJo (V10.0.4).

**Animals**

Specific pathogen-free C57Bl/6 mice were purchased from Harlan Sprague-Dawley (Horst, the Netherlands). *Tlr2*−/−, *tlr4*−/−, *tlr9*−/− and *MyD88*−/− mice were generously provided by prof. Shizuo Akira (Research Institute for Microbial Disease, Osaka, Japan) 31-33. *Tlr2/4*−− double knock out mice were crossed from *tlr2*−/− and *tlr4*−/− as described 34. *MyD88* floxed mice (*Myd88*lox/lox) were kindly provided by prof. Anthony DeFranco 35. Platelet specific *MyD88* knock out (Plt-*Myd88*−/−) mice were generated by crossing these with mice expressing Cre recombinase under the platelet factor 4 (PF4) promoter (The Jackson Laboratory, Bar Harbor, Maine); littermates not expressing Cre were used as controls. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

**Experimental study design**

Pneumonia was induced by intranasal inoculation with ΔcpsD39 (2 x 10⁷ colony forming units (CFU) in 50 μL isotonic saline) using previously described methods 28,36. Mice were euthanized 16 hours after induction of pneumonia (N = 8 mice per group). Bacterial quantification and storage of organs were performed as described 28,36. Platelet counts and activation (by expression of P-selectin as described above) were determined in citrated whole blood by flow cytometry. Mouse tumour necrosis factor (TNF-)α, interleukin (IL-)6, IL-1β, keratinocyte chemoattractant (KC), PF4, soluble (s)P-selectin, E-selectin (R&D Systems) and thrombin-antithrombin complexes (TATc; Bio-connect, Huissen, the Netherlands) were measured by ELISA. Four-micrometer sections of the left lung lobe, spleen and liver were stained with hematoxylin and eosin (H&E). 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.

**Statistical analysis**

All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Comparisons between groups were tested using the Mann-Whitney U test. P-values < 0.05 were considered statistically significant.
Results

Unencapsulated, but not encapsulated, \textit{S. pneumoniae} induces platelet aggregation.

Whereas unencapsulated ΔcpsD39 induced platelet aggregation, \textit{S. pneumoniae} serotype 2, 3 and 4 (D39, 6303 and TIGR4 respectively) did not (Figure 1A). The finding that capsulated \textit{S. pneumoniae} failed to induce aggregation was confirmed by stimulation of recombinant \textit{S. pneumoniae} capsule (CPS2), which also was unable to induce platelet aggregation (Figure 1B). Platelet aggregation by ΔcpsD39 was activation dependent and required fibrinogen binding to GPIIb/IIIa, as it could be inhibited by PGE1 and the GPIIb/IIIa antagonist Abciximab. More specifically, we investigated whether platelet aggregation was induced via TLR2 signalling. Blocking of TLR2 however did not affect aggregation whereas inhibition of FcγRII completely

Figure 1: Unencapsulated, but not encapsulated, \textit{S. pneumoniae} induces platelet aggregation. Platelet aggregation was measured by light transmission as a percentage to the transmission through PPP in a stirring cuvette. TRAP was used as a positive control; PBS as a negative control. Aggregation curves are depicted for stimulation with \textit{S. pneumoniae} D39, ΔcpsD39, TIGR4 and 6303 (A) and rCPS2 (B). PRP was pre-incubated with α-TLR2, α-FcγRII, PGE1, abciximab or PBS prior to ΔcpsD39 stimulation in (C). Aggregation curves as a result of TLR agonist stimulation with LTA, Pam3CSK4 or LPS are shown in (D). All aggregation curves are representative of 3 independent experiments using different donors. HEK cells stably transfected with TLR2 and CD14 were pre-incubated with α-TLR2 and stimulated for 16 hours with LTA, Pam3-CSK4 and ΔcpsD39, IL-8 was measured in the supernatant (E). * P<0.05.
abolished aggregation (Figure 1C). These results are in contrast to a previous study in which TLR2-dependent *S. pneumoniae*-induced platelet activation was described (23). In a different attempt to induce platelet aggregation via TLR2, we stimulated platelets with the TLR2 agonists Pam3CSK4 and LTA. In addition, also the TLR4 agonist LPS was tested. However, all TLR agonists failed to induce any response even at high concentrations (5 μg/mL; Figure 1D).

We confirmed the capacity of the TLR2 antibody to inhibit TLR2 responses in another assay, in which HEK cells stably transfected with TLR2 and CD14 were stimulated with Pam3CSK4, LTA or ΔcpsD39. α-TLR2 reduced IL-8 to baseline levels for the direct agonists (P<0.05 versus no blocking antibody); ΔcpsD39-induced IL-8 was partially TLR2 dependent; some residual IL-8 production remained after TLR2 blockade, indicating induction of other signalling pathways besides TLR2 by this bacterium (P<0.05 versus no blocking antibody; Figure 1E).

**Prestimulation with *S. pneumoniae* fails to modulate platelet aggregation to subthreshold concentrations of TRAP**

Previous studies have described a role for LPS in platelet ‘priming’, where LPS pretreatment induced platelet hypersensitivity to subthreshold concentrations of classical platelet agonists (24;25). However, we failed to observe any priming effect of pre-incubation of platelets with either *S. pneumoniae* or TLR agonists before stimulation with subthreshold concentration TRAP (Figure 2A,B). The priming effect of LPS described by Montrucchio (24) was monocyte-dependent; we therefore repeated these experiments in the presence of isolated PBMC’s. Still, no platelet hypersensitivity to subthreshold TRAP was found (not shown).
Platelet activation by different agonists can induce a variety of responses. We therefore focused on platelet granule release. Alpha granule degranulation was detected by CD62p (P-selectin) surface expression and dense granule release was detected based on surface expression of CD63. Whole blood stimulation by *S. pneumoniae* D39, 6303, TIGR4 and ∆cpsD39 all resulted in platelet CD62p and CD63 exposure, ∆cpsD39 being the most potent activator (Figure 3). *S. pneumoniae* did not activate platelets via TLR2 or 4, as pre-incubation with α-TLR2 and α-TLR4 did not inhibit CD62p expression by *S. pneumoniae* (Figure 4A). Opposed to aggregation, FcγRII and GPIIbIIIa inhibition did not block CD62p expression by *S. pneumoniae*.

**Figure 3: S. pneumoniae D39, ∆cpsD39, TIGR4 and 6303 all induce platelet degranulation.** Whole blood was stimulated with *S. pneumoniae* D39, ∆cpsD39, TIGR4 or 6303. Following 30 minutes of incubation platelets were stained and analysed by flow cytometry for surface expression of CD62p (A) and CD63 (B). Percentages were determined using isotype control antibodies to set the gate. TRAP was used as a positive control and induced CD62p - and CD63 expression on 87% and 56% of platelets respectively; PBS induced CD62p - and CD63 expression on 10% and 2% of platelets. Histograms are representative of 2 independent experiments using different donors.
pneumoniae D39 or ΔcpsD39; PGE1 did (Figure 4A). In line with these observations, platelet surface expression of CD62p or CD63 was not induced by direct TLR agonists LTA, Pam3CSK4 or LPS (Figure 4B,C).

**Whole blood S. pneumoniae incubation results in platelet-leukocyte complex formation**

To determine whether S. pneumoniae whole blood stimulation results in formation of platelet-leukocyte complexes, platelet markers CD61 and CD62p were measured on neutrophils, monocytes and lymphocytes (shown for CD61 in Figure 5). All S. pneumoniae strains tested induced some platelet-neutrophil complexes; ΔcpsD39 being the most potent (Figure 5A). Platelet-monocyte complex formation occurred readily upon stimulation with all S. pneumoniae serotypes tested (Figure 5B), platelet-lymphocyte complexes were not induced (Figure 5C). TLR2 and TLR4 were not directly involved in platelet-leukocyte complex formation as it was not induced by the TLR agonists LTA, Pam3CSK4 or LPS (shown for neutrophils and monocytes in Figure 5D,E).

**Wild-type mouse platelets respond to S. pneumoniae D39 and ΔcpsD39 in a similar manner as platelets from Tlr2−/−, Tlr4−/−, Tlr2/4−/−, Tlr9−/− and Myd88−/− mice.**

In order to confirm that TLR2 and 4 signalling are not directly involved in platelet responses to S. pneumoniae without the use of antibodies or synthetic agonists, we conducted similar whole blood stimulation experiments in mouse blood comparing wild-type platelets with platelets of Tlr2−/−, Tlr4−/− and Tlr2/4−/− strains using CD62p expression as readout for platelet activation. Recently, a functional role for platelet TLR9 was described 25. We therefore

**Figure 4: TLR2, 4, FcyRII and Abciximab are not involved in S. pneumoniae induced platelet degranulation.**

Following pre-incubation with blocking antibodies to TLR2, TLR4, or FcyRII, or with PGE1, whole blood was stimulated with S. pneumoniae ΔcpsD39. Platelets were stained and analysed by flow cytometry for surface expression of CD62p (A). As an opposite approach, whole blood was incubated with TLR2 and 4 agonists LTA, Pam3CSK4 and LPS and analysed by flow cytometry for surface expression of CD62p (B) or CD63 (C). Histograms are representative of 2 independent experiments using different donors.
Tlr9−/− mouse blood to investigate a possible role for TLR9 in this model. As a final control, we performed the stimulation experiments with blood obtained from Myd88−/− mice, blocking downstream signalling of all TLR receptors except for TLR3. Tlr2−/−, Tlr4−/−, Tlr2/4−/−, Tlr9−/− and Myd88−/− platelets all showed enhanced CD62p expression to a similar extent as wild-type platelets upon stimulation with S. pneumoniae D39 or ΔcpsD39, implicating that there is no role for TLR signalling in direct platelet response to S. pneumoniae (Figure 6A,B).

Platelet MyD88 is not involved in host defence and response to ΔcpsD39 in vivo.

It is known that platelets especially exert proinflammatory and immune modulatory effects in the lungs. To determine the impact of platelet specific TLR signalling during pneumonia in vivo, Plt-Myd88−/− and littermate control mice were inoculated with 2 × 10⁷ CFU ΔcpsD39 via the airways. We chose to conduct these experiments with ΔcpsD39, the strain that was the most potent inducer of platelet activation and platelet-leukocyte formation in our in vitro experiments. As control mice clear this unencapsulated S. pneumoniae strain within 24 hours, we therefore sacrificed the mice after 16 hours when bacterial loads are still present. No differences were detected in bacterial burdens in the lungs, blood, spleen or liver between control and Plt-Myd88−/− mice (Figure 7A). Additionally, no differences were found in platelet counts (Figure 7B) or platelet activation measured by platelet surface CD62p (P-selectin) expression, PF4 and platelet and endothelial cell activation marker sP-selectin (Figure 7C-
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E). (Activated) platelets are considered to play an essential role in coagulation by providing a phospholipid surface for the assembly of activated clotting factors. To obtain insight in the role of MyD88 dependent platelet signalling in systemic coagulation activation during ΔcpsD39 pneumonia, we measured TATc levels in plasma of infected Plt-Myd88-/- and control mice. No differences were detected between the groups (Figure 7F). Lastly, platelet MyD88

Figure 6: Wild-type mouse platelets respond to S. pneumoniae D39 and ΔcpsD39 in a similar manner as platelets from Tlr2-/-, Tlr4-/-, Tlr2/4-/-, Tlr9-/- and Myd88-/- mice. Mouse wild-type, Tlr2-/-, Tlr4-/-, Tlr2/4-/-, Tlr9-/- and Myd88-/- whole blood was stimulated with S. pneumoniae D39 (A) or ΔcpsD39 (B). Following 30 minutes of incubation platelets were stained and analysed by flow cytometry for surface expression of CD62p. N = 2-3 mice per group; histograms are representatives for the mice genotypes.

Figure 7: Platelet MyD88 is not involved in host defence to ΔcpsD39 in vivo. Control (closed dots, grey bars) and Plt-Myd88-/- mice (open dots, white bars) were infected with S. pneumoniae ΔcpsD39 via the intranasal route and euthanized 16 hours thereafter. Bacterial counts were determined in lungs, blood, spleen and liver (A). Platelet counts (B) and platelet activation (CD62p; C) were determined by FACS analysis for CD61 and CD62p. PF4 (D), sP-selectin (E), TATc (F) and E-selectin (G) were measured in plasma using ELISA. Data are expressed 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.
signalling had no influence on endothelial cell activation during ΔcpsD39 pneumonia as E-selectin levels did not significantly differ (Figure 7G).

Platelets secrete cytokines upon activation and platelets in complex with leukocytes can influence leukocyte effector function. During ΔcpsD39 pneumonia however, cytokine production in the lungs did not differ between control and Plt-Myd88−/− mice (Figure 8A-D); plasma cytokine levels were below detection. Platelets have been both associated with enhanced histopathological damage during inflammatory challenges and the protection of vascular integrity during inflammation. However, no differences for inflammation parameters or infiltrated lung surface were found between Plt-Myd88−/− and control mice, as reflected by the sem-quantitative scores of lung histopathology slides (Figure 8E,F). Additionally, no bleeding was found in the lungs of either Plt-Myd88−/− or control mice.

**Figure 8: Platelet MyD88 is not involved in the inflammatory response to ΔcpsD39 in vivo.** WT (gray bars) and Plt-Myd88−/− mice (open bars) were infected with S. pneumoniae ΔcpsD39 via the intranasal route and euthanized 16 hours thereafter. Lung cytokine levels of TNF-α (A), IL-1β (B), IL-6 (C), and KC (D) were measured by ELISA. Lung histopathology was scored by an independent pathologist; representative microphotographs are shown in (E; 10x magnification) and pathology scores in (F). Data are depicted as expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group.
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Discussion

*S. pneumoniae* represents a major health burden worldwide. Recently, platelets have been implicated as major players in infection and immunity. Platelets are activated during sepsis, directly by an invading pathogen or indirect via host coagulation activation. In this paper we demonstrate that *S. pneumoniae* directly activates platelets in a TLR independent fashion. Platelet activation by all serotypes tested resulted in surface expression of CD62p and CD63 and platelet-leukocyte complex formation; ΔcpsD39 additionally induced platelet aggregation. Plt-Myd88−/− mice were unaffected during ΔcpsD39 pneumonia.

The pneumococcal capsule inhibits mucosal clearance, facilitates binding to the epithelial surface and inhibits complement- and phagocyte-mediated immunity. Besides reduction of exposure to several antibodies, capsular polysaccharide was suggested to prevent interaction between Fcy receptors to the Fc component of IgG bound to pneumococci. This could be why the only pneumococcal strain capable of inducing (FcyRII dependent) platelet aggregation was unencapsulated DcpsD39. Our results are in conflict with an earlier report showing that both encapsulated and unencapsulated *S. pneumoniae* induced platelet aggregation, via TLR2 mediated signalling. The strains we tested however did not induce platelet aggregation unless in its mutated unencapsulated form (ΔcpsD39). ΔcpsD39 did not induce aggregation in a TLR2 dependent manner, as we could not inhibit the reaction by adding TLR2 blocking antibodies. In addition, direct TLR2 stimulation by TLR2 agonists LTA and Pam3CSK4 failed to induce platelet activation. Although several papers reported functional roles for platelet TLRs in vivo, controversy still surrounds the functionality of these receptors in *in vitro* assays. Stimulation of platelets with TLR2 or 4 ligands sometimes did or did not induce CD62p (P-selectin) expression, did or did not induce Ca2+ mobilisation or thrombin generation. We were unable to induce platelet activation by direct TLR2 agonists LTA and Pam3CSK4 or TLR4 agonist LPS, in a variety of functional assays. Opposed to direct activation two groups found a priming effect on platelets of LPS alone or in co-incubation with monocytes, whereafter platelets were ‘hyperexcitable’ and aggregated by addition of subthreshold levels of classic platelet agonists. Nevertheless, TLR agonists or encapsulated *S. pneumoniae* strains did not modulate the platelet response to subthreshold concentrations of TRAP in our hands. Lastly, platelets from WT and Tir2−/−, Tir4−/−, Tir2/4−/−, Tir9−/− and Myd88−/− mice showed similar surface CD62p expression after *S. pneumoniae* stimulation in whole blood stimulation assays.

All *S. pneumoniae* serotypes caused platelet α- and dense granule release, with release of inflammatory regulators stored in α-granules. Moreover, all serotypes induced platelet-leukocyte complex formation, which influences leukocyte function. In contrast to aggregation responses, the pneumococcal capsule can only partly reduce platelet degranulation and complex formation.
Thrombocytopenic mice have indeed been shown to be subjected to increased bacterial growth and mortality during *S. pneumoniae* pneumonia. MyD88 dependent TLR signalling is not involved herein, as bacterial clearance was similar in Plt-Myd88−/− and control mice during ΔcpsD39 pneumonia and platelet MyD88 deletion had no influence on platelet counts, platelet activation or coagulation activation. No bacteria were detected in blood and blood cytokine levels were below detection, indicating lack of significant bacterial stimulus in the blood compartment. It is probably therefore that in vivo platelet CD62p expression was only 2-3%.

Platelet TLR4 has been reported to modulate TNF-α production to bacterial lipopolysaccharide (LPS). Platelet activation during infection could additionally influence cytokine levels by release of cytokines from their own granules or by influencing leukocyte effector function. TNF-α and other cytokine levels were however similar in lungs of Plt-Myd88−/− and control mice in our experiments. While platelets are additionally known to regulate lung architectural changes and vascular integrity during inflammation, platelet activation via MyD88 dependent TLR signalling seems not involved as we found no histopathological differences between the groups in our pneumonia model.

In conclusion, we show that *S. pneumoniae* activates platelets by a TLR-independent mechanism. The described activation patterns provide additional evidence that platelets function as circulatory sentinel cells in our immune system.

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