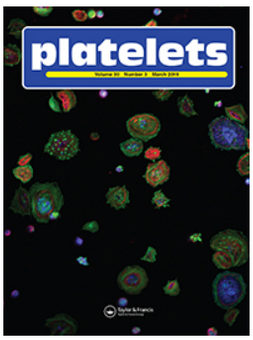

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PLENARY PAPER

Platelet Toll-like receptor expression and activation induced by lipopolysaccharide and sepsis

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Abstract

Platelets and Toll-like receptor (TLR) signalling play a role in the immune response during sepsis. Although preclinical knowledge about the role of platelet TLR signalling is increasing, data during human sepsis are less abundant. Moreover, controversy remains about the effect of the TLR4 agonist lipopolysaccharide (LPS) on platelet activation. We therefore assessed platelet TLR expression during human and murine sepsis. Moreover, we investigated the effect of TLR4 signalling on platelet activation and TLR expression. Platelets from healthy controls stimulated with LPS did not show classical platelet activation (P-selectin, CD63 and phosphatidylserine expression), potentiation of subthreshold agonist stimulation nor platelet-leukocyte complex formation. LPS stimulation however did increase maximal mitochondrial respiration in a TLR4-dependent manner. Platelet stimulation with LPS did not alter TLR expression. Platelet stimulation with thrombin receptor activating peptide increased TLR5 and TLR9, but not TLR2 or TLR4 expression. Platelets from patients with sepsis and mice with experimental sepsis showed platelet activation, but unaltered TLR expression. These results indicate that sepsis-induced platelet activation is not associated with altered platelet TLR expression and, although platelets are responsive to LPS, stimulation of platelet TLR4 does not result in classical platelet activation.

Keywords

Metabolism, platelet activation, platelets, sepsis, Toll-like receptors

History

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Introduction

Platelets are not only central for haemostasis, but also contribute to immune responses (1). Murine and human studies have established a role for platelets in the host response during sepsis (2,3). Toll-like receptors (TLRs) comprise a family of pattern recognition receptors that are crucial for innate immunity during sepsis (4). Platelets express functional TLRs (1,5), but their role during sepsis remains controversial. Some groups have shown platelet aggregation, granule release and P-selectin expression (hereunder referred to as 'classical platelet activation') by the TLR4 ligand lipopolysaccharide (LPS) (6,7), whereas others could not (2,8–10). Platelet TLR signalling can alter leukocyte activation (2,11), but did not impact host defense during gram-negative or gram-positive pneumosepsis (12,13).

Platelets from sepsis patients are commonly activated, with increased P-selectin expression, platelet-leukocyte-complex formation and thrombocytopenia (14,15). However, little is known about platelet TLR expression during sepsis. Platelet activation can upregulate platelet TLR2, TLR4 and TLR9 expression (5,16,17), and in mice TLR2 ligands can alter megakaryocyte TLR expression (18).

Here, we aimed to assess the effect of sepsis and *ex vivo* LPS stimulation on platelet activation and platelet TLR expression.

Methods

Patients

Citrated whole blood was drawn from healthy volunteers and from patients with sepsis or critically ill patients without infection, within 24 h after admission to the intensive care unit (ICU) (19). Patients were included if they were older than 18 years. Sepsis patients were included when they had a suspected infection for which the clinical team initiated therapeutic antibiotics and had at least two systemic inflammatory response syndrome criteria (body temperature ≤36°C or ≥38°C, tachycardia >90/min, tachypnoea >20/min or pCO2 < 4.3 kPA, leukocyte count $<4 \times 10^9$/L or $>12 \times 10^9$/L) (20). The medical ethical committee of the Academic Medical Center in Amsterdam approved the

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study (IRB No. NL 34294.018.10), and written informed consent was obtained from all patients (or legal representative).

Flow cytometry and assays

Human platelet activation was measured by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ) using mouse anti-CD61 monoclonal antibody (mAb; eBioscience, San Diego, CA), mouse anti-CD62 mAb (eBioscience), mouse anti-CD63 mAb (Biolegend, San Diego, CA) or lactadherin (MFG-E8, Haematologic Technologies, Essex Junction, VT; for phosphatidylserine (PS) expression) in combination with appropriate IgG control. TLR expression was determined on CD61+ platelets with mouse anti-TLR2 mAb (Clone 2.5), mouse anti-TLR4 mAb (HTA125, both eBioscience), mouse anti-TLR5 mAb (Abcam, Cambridge, UK) and mouse anti-TLR9 mAb (Novus, Abingdon, UK). Geometric mean fluorescence intensity (gMFI) was plotted. Expression murine platelet counts were measured using hamster anti-mouse-CD61 mAb (BioLegend) and platelet activation using rat anti-mouse CD62P (BD Biosciences, San Diego, CA). Murine TLR expression was determined with rat-anti-TLR2 (eBioscience), rat-anti-TLR4 (Clone MTS510, eBioscience) and mouse-anti-TLR5 mAbs (Abcam). Anti-human TLR4 antibody was validated on CD66b+ neutrophils (Figure S1). Chemokine (C-X-C motif) ligand 4 (CXCL4; R&D systems, Minneapolis, MI) and serotonin (Enzo Life Sciences, Bruxelles, Belgium) levels in plasma were determined by ELISA.

Stimulations

Citrated platelet rich plasma of healthy volunteers was stimulated with thrombin receptor activating peptide (TRAP; 15 μM; Sigma-Aldrich, St. Louis, MO) or *Klebsiella (K.) pneumoniae* LPS (100 ng/mL and 5 μg/mL, Sigma), *Pseudomonas (P.) aeruginosa* LPS (5 μg/mL, Sigma) or *Escherichia (E.) coli* LPS (5 μg/mL, Invivogen) for 20 min at room temperature. Kinetics were also tested by stimulating 10, 20, 60 and 90 min. Two different concentrations of *Klebsiella* LPS were chosen; 100 ng/mL as this gives a robust response in leukocytes (21,22) and 5 ng/mL as non-classical platelet activation has been described only with very high doses of LPS (2).

Alternatively, citrated blood was spun at 180 g to obtain platelet rich plasma, subsequently mixed with acid citrate dextrose (Sigma-Aldrich) and spun at 800 g. Washed platelets were resuspended in DMEM medium (Sigma) and stimulated with stimuli as mentioned earlier. Potentiation experiments were performed with washed platelets, stimulated with adenosine diphosphate (ADP, 2 μmol, Bio/Data Corporation Horsham, PA) or Collagen-related peptide (CRP-XL, 0.01 μg/mL, University of Cambridge, UK) with or without LPS (100 ng/mL and 5 μg/mL, Sigma). Citrated whole blood was also stimulated with TRAP, LPS or vehicle control in above-mentioned concentrations and platelet-leukocyte complex formation was assessed after 45 min using anti-human CD66b, anti-human CD14 (both BD Biosciences) and anti-human CD61 (eBioscience).

Mitochondrial respiration measurements

Platelets were isolated by centrifugation at 180 g for 15 min and 800 g for 15 min and resuspended in DMEM medium (Sigma) containing 25 mM glucose, 1 mM natrium-pyruvate and 2 mM glutamine. The plate was incubated in Seahorse XF Calibrant (Seahorse Bioscience, Agilent Technologies, Santa Clara, CA) for 30 min in a non-CO2 incubator at 37°C before measuring. Approximately, 30 million platelets per well were measured by XFe 96 extracellular flux analyzer (Seahorse Bioscience). Oxygen consumption rate (OCR) was measured over 2-min periods with a mixing of 2 min in each cycle, with 3 cycles in total. Inhibitors and activators were used at the following final concentrations: oligomycin (1,5 μM), carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μM), antimycin A (2,5 μM) and rotenone (1,25 μM; all Sigma), TRAP (15 μM, Sigma), LPS (100 ng/mL and 5 μg/mL, Sigma) or UV radiated *Klebsiella* ($5*10^6$ CFU) with or without anti-human TLR 2 (TLR2.5, 5 μg/mL, Hycult Biotech, Uden, the Netherlands) and anti-human TLR 4 (18H10, 5 μg/mL, Hycult Biotech) after 15 min incubation. Data are represented as OCR per million platelets, corrected for the OCR after antimycin A and rotenone injection.

Mice

Pneumonia was induced in female C57BL6J mice by intranasal inoculation with *K. pneumoniae* serotype 2 (ATCC 43816 Rockville, MD; 10^4 colony forming units (CFU) in 50 μL isotonic saline), as described (23). Both *K. pneumoniae* ATCC 43816 and LPS from *K. pneumoniae* (Sigma) are of the serotype O1, as determined by inhibition ELISA (A.F. de Vos, personal communication).

Mice were euthanized 42 h after infection ($n = 4$), non-infected mice were sacrificed simultaneously ($n = 4$). Mouse studies were approved by the Institutional Animal Care and Welfare Committee.

Statistical analysis

Data are expressed as box and whisker plots. Comparisons between groups were tested using the Mann-Whitney *U* test (when data were not normally distributed) or paired *t* test (when samples from the same donor when analysed in multiple conditions). Analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P values < 0.05 were considered statistically significant.

Results

Platelet stimulation by LPS does not lead to granule release or PS exposure

LPS is an important pro-inflammatory molecule during sepsis and signals via TLR4 (24). To assess the direct effect of LPS on platelet activation, we measured P-selectin and CD63 expression (reflecting alpha and dense granules release, respectively (1)) and PS positivity on platelets after stimulation with LPS. TRAP (used as positive control) induced platelet activation in platelet rich plasma (Figure1A-C, $P < 0.05$ vs. unstimulated); however, stimulation with LPS in a low or high dose did not alter activation (Figure 1A-C). Stimulations with washed platelets showed similar results (Figure 1D-E, $P < 0.05$ vs. unstimulated) with representative flow charts shown in Figure S2. Also stimulations of shorter (10 min) and longer (60 and 90 min) duration showed no effect of LPS on platelets (Figure S3). LPS derived from other gram-negative bacteria, *P. aeruginosa* and *E. coli* also did not induce platelet activation, expect for slightly higher PS positivity after stimulation with *E. coli* LPS (Figure S4). Release of CXCL4 and serotonin was also determined, but showed great inter-donor variation hampering interpretation of the effect of platelet activation (Figure S5). It has been shown that platelet TLR expression associated with different cardiovascular risk factors in woman and men (25). To exclude that gender effects might conceal any effect of LPS on platelet activation, we also separately assessed males and females. Both in males and females however, LPS did not influence platelet activation (Figure S6). Some reports have suggested that platelets can potentiate subthreshold concentrations of platelet stimuli such as ADP (26). However in our hands, subthreshold concentrations of ADP or CRP-XL in combination with LPS did not induce platelet activation (Figure 2A-F), whereas positive control TRAP did induce platelet activation (Figure 2A-F). Moreover, in human whole blood, no significant effect of LPS was seen on platelet-leukocyte complex

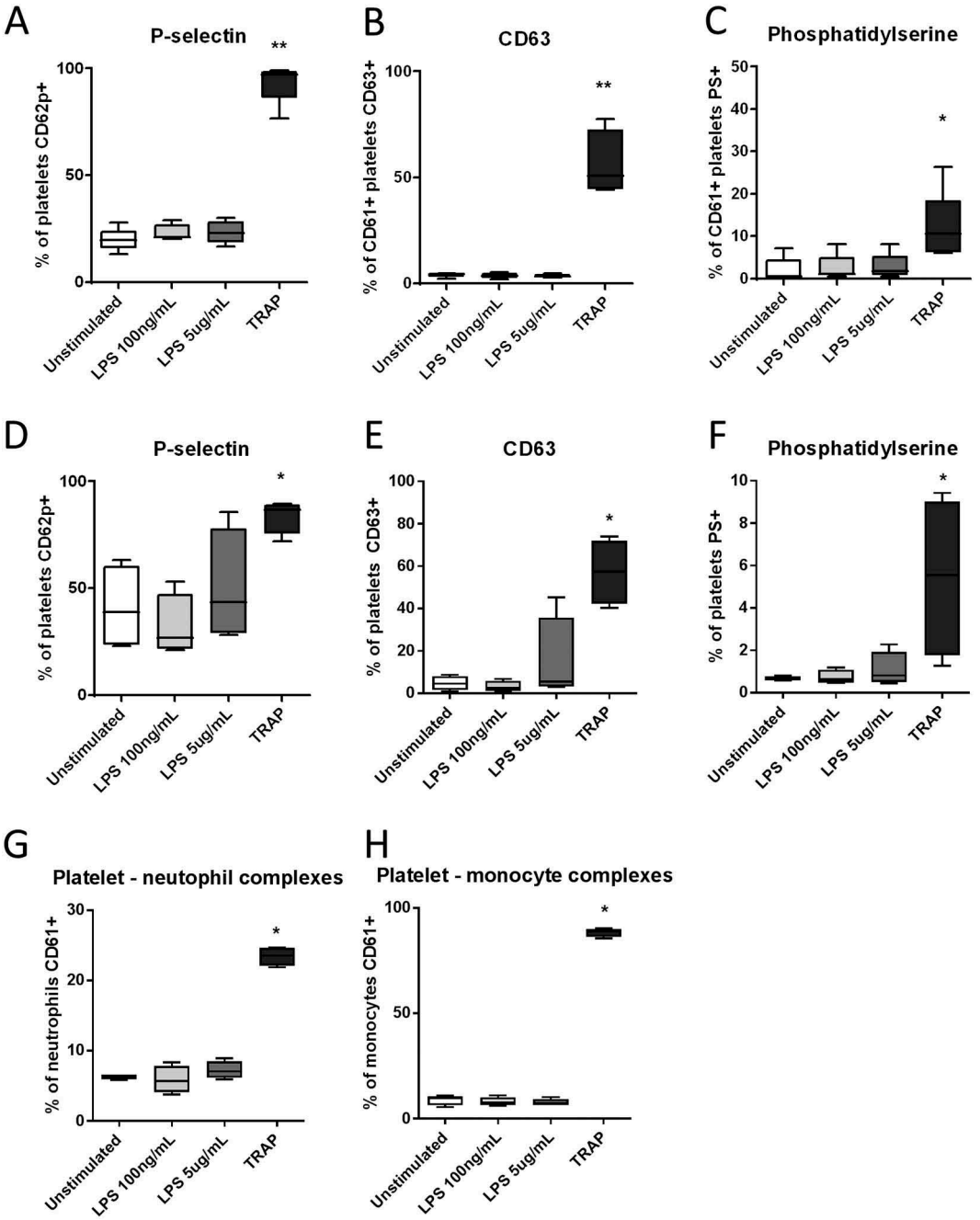


Figure 1. Effect of lipopolysaccharide (LPS) on classical platelet activation in platelet rich plasma and washed platelets.(A-C) Human platelet rich plasma was stimulated with thrombin receptor-activating peptide (TRAP), LPS or medium control for 20 min. (A) P-selectin expression, (B) CD63 expression and (C) phosphatidylserine (PS) positivity. (D-F) Washed human platelets were stimulated with TRAP, LPS or medium control. (D) P-selectin expression, (E) CD63 expression and (F) PS positivity. Data are represented as box and whisker plots. (G-H) Citrated whole blood was stimulated with TRAP, LPS or medium control for 45 min and platelet-neutrophil (G) and platelet-monocyte (H) complex formation was assessed. (A-F) Results are from 4–5 subjects. (G-H) Four replicated per stimulus, experiments were performed with at least two independent donors. $^{**}P < 0.005$, $^{*}P < 0.05$ vs. unstimulated.

formation, whereas TRAP did increase complex formation (Figure 1G,H, $P < 0.05$ vs. unstimulated).

Platelet stimulation by LPS increases maximal mitochondrial respiration in a TLR4-dependent manner

Although LPS did not lead to classical platelet activation, several reports have shown that LPS stimulated platelets can have other effects such as increased leukocyte activation (2,11). To assess if platelets were responsive to LPS stimulation, we measured the OCR in platelets, which predominantly reflects mitochondrial respiration through oxidative phosphorylation (27). Addition of the chemical uncoupler FCCP during measurement of respiration induces maximal oxidative phosphorylation and can be used as a readout of oxidative phosphorylation capacity (27). Stimulation with TRAP led to increased maximal (FFCP induced) respiration in platelets (Figure 3A, $P < 0.0005$ vs. unstimulated). Incubation with different doses of LPS also increased maximal respiration in platelets (Figure 3A, $P < 0.005$ vs. unstimulated). *K. pneumoniae*, the source of LPS, also increased maximal respiration rates (Figure 3A, $P < 0.005$ vs. unstimulated). In line with a

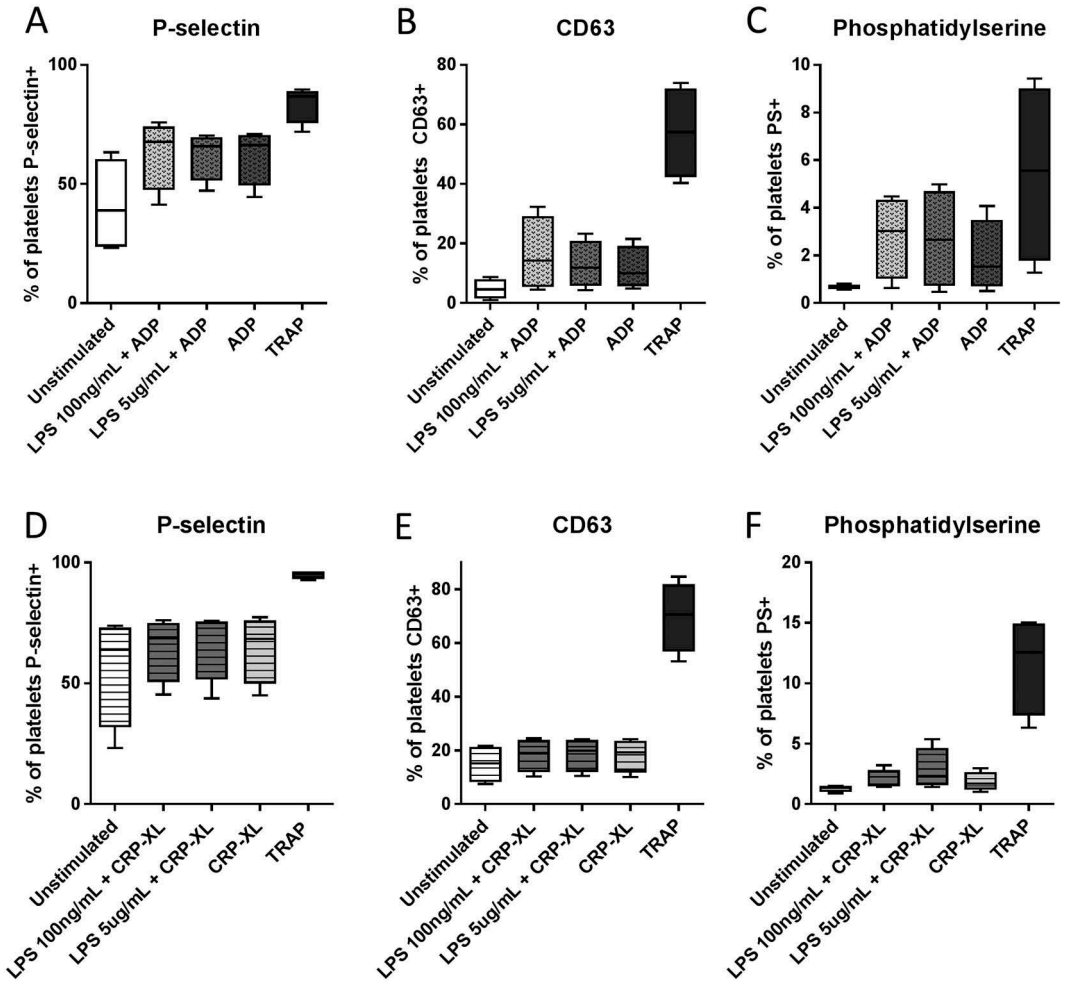
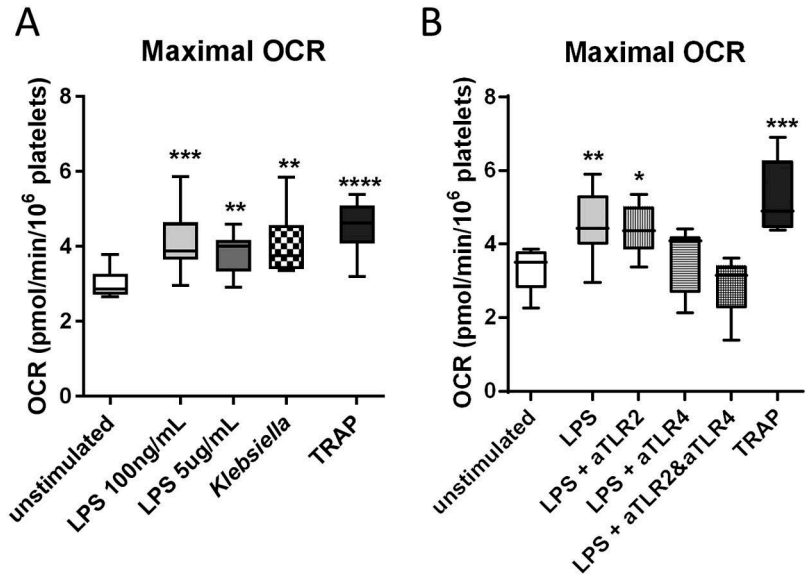


Figure 2. LPS does not potentiate platelet activation elicited by low dose adenosine diphosphate (ADP) or collagen related peptide (CRP-XL).Washed human platelets were stimulated with subthreshold levels of (A-C) ADP (2 μmol), or (D-F) CRP-XL with or without LPS for 20 min, as well as positive control TRAP. (A,D) P-selectin expression, (B,E) CD63 expression and (C,F) phosphatidylserine positivity. Data are represented as box and whisker plots. Results are from four subjects.

Figure 3. Effect of LPS on maximal mitochondrial respiration in platelets.(A) FCCP-induced maximal oxygen consumption rate (OCR) after stimulation with TRAP (15 μM), LPS (100 ng/mL and 5 μg/mL) or UV radiated *Klebsiella* ($5*10^6$ CFU). (B) FCCP-induced maximal OCR after stimulation with TRAP (15 μM), LPS (100 ng/mL) and LPS (100 ng/mL) with or without inhibition of TLR2 and TLR4. Experiments were performed at least twice with different donors. Data are represented as box and whisker plots. $*P < 0.05$, $**P < 0.005$, $***P < 0.005$, $****P < 0.0005$ vs. unstimulated.



TLR4-dependent role for LPS-induced effects in platelets, inhibition of TLR4 (with or without TLR2 inhibition) abolished the rise in maximal respiration after LPS stimulation (Figure 3B). These results show that platelets are responsive to LPS in a TLR4-dependent manner, but that does not lead to classical platelet activation.

Platelet stimulation by LPS does not alter TLR expression

Platelets express multiple TLRs (5,11,16,17) and previous studies have shown that TLR expression can be modulated by platelet activation (5,16,17). As LPS induces changes in platelet metabolism, we assessed if LPS stimulation can influence platelet TLR surface expression. LPS stimulated platelets however had unaltered TLR2, TLR4, TLR5 or TLR9 expression (Figure 4A-D). TRAP stimulation did increase platelet TLR5 and TLR9 expression as expressed by gMFI (Figure 4A-D, $P < 0.05$ vs. unstimulated).

Sepsis induces platelet activation but does not influence platelet TLR expression

Sepsis is characterized by sustained hyperinflammation and signalling by TLRs is a crucial feature herein (4). We first determined the extent of platelet activation in sepsis patients (Table I). Sepsis patients showed increased platelet activation compared to healthy volunteers, with increased expression of P-selectin, CD63 and PS (Figure 5A-C, $P < 0.05$ vs. healthy volunteers).

We also compared sepsis patients to non-infectious ICU controls, which showed similar trends for platelet activation as sepsis patients and also increased CD63 expression compared to healthy volunteers (Figure 5A-C, $P < 0.05$ vs. healthy volunteers). These data suggest that platelet activation occurs in critically ill patients both with and without infection.

As platelets are activated *in vivo* and TLR signalling is important during sepsis, we assessed if sepsis is associated with altered TLR expression on platelets. These effects could be mediated by sepsis-induced platelet activation (5,16) or alternatively by changed megakaryopoiesis resulting in altered platelets (18). TLR expression was however similar between healthy volunteers and sepsis patients for TLR2, TLR4, TLR5 and TLR9 (Figure 5D-G). Also in non-infectious ICU controls, TLR expression was not different compared to healthy volunteers or sepsis patients (Figure 5D-G).

We moreover assessed platelet TLR expression in mice infected with *K. pneumoniae*, who develop severe pneumosepsis with associated platelet activation and thrombocytopenia (Figure 6A-B, $P < 0.05$ vs. uninfected). Also in mice, sepsis did not influence platelet TLR expression, as septic and uninfected mice showed similar expression levels of TLR2, TLR4 and TLR5 (Figure 6C-E). These results show that although platelets are activated during sepsis, their TLR expression is unaltered.

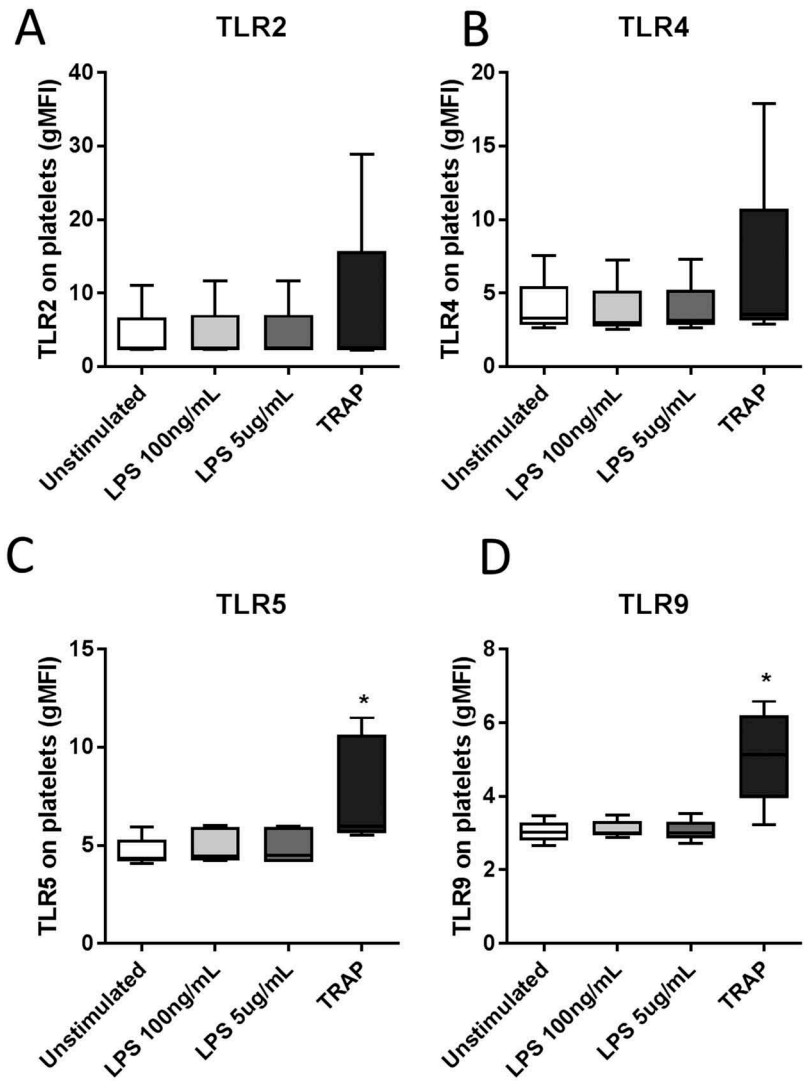


Figure 4. Effect of LPS on platelet TLR expression.Human platelet rich plasma was stimulated with TRAP, LPS or medium control for 20 min. (A-D) Expression of platelet TLRs; (A) TLR2, (B) TLR4, (C) TLR5 and (D) TLR9. Data are represented as box and whisker plots. Results are from five subjects. *$P < 0.05$, **$P < 0.05$ vs. unstimulated.

Table I. **B**aseline characteristics healthy volunteers and sepsis patients and ICU controls.

	Healthy volunteers ($n = 8$)	Sepsis ($n = 7$)	ICU controls ($n = 4$)
Demographics			
Age (years), mean (range)	39 (29–57)	60 (33–83)	64 (24–87)
Gender male, *n* (%)	6 (75%)	6 (86%)	4 (100%)
Primary source of infection, *n* (%).			
Respiratory tract		3 (43%)	
Abdomen		2 (29%)	
Brain		1 (14%)	
Urinary		1 (14%)	
Causative pathogen	–	*Pseudomonas aeruginosa* (29%), *Klebsiella pneumoniae* (14%), *Cryptococcus neoformans* (14%), Unknown (43%)	–
Mechanical ventilation	–	4 (57%)	2 (50%)
30-day mortality	–	3 (43%)	1 (25%)

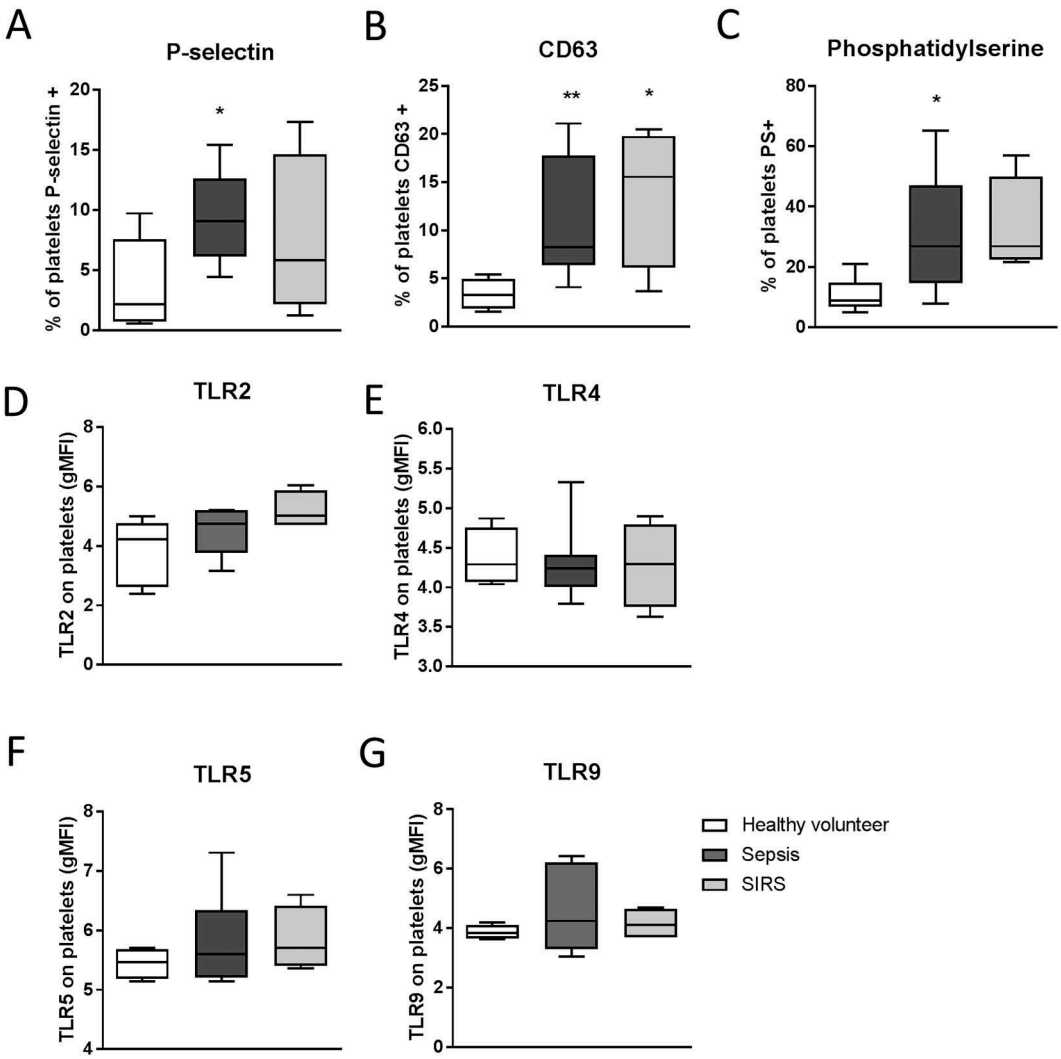


Figure 5. Platelet activation and TLR expression in human sepsis.(A-C) Expression of platelet activation markers in healthy controls ($n = 8$) and sepsis patients ($n = 7$). (A) P-selectin, (B) CD63 expression and (C) PS positivity. (D-G) Platelet TLR expression on platelets of healthy controls and sepsis patients. (D) TLR2, (E) TLR4, (F) TLR5 and (G) TLR9. Data are presented as box and whisker plots. $^*P < 0.05$, $^{**}P < 0.005$ vs. controls.

Discussion

The importance and effects of platelet TLR signalling during sepsis remain controversial. We here show that LPS induces a TLR4-dependent platelet response at a metabolic level, but does not lead to classical platelet activation or altered platelet TLR expression. Furthermore, although sepsis results in platelet activation, TLR expression is unaltered in this condition.

Platelet activation can result in 'classical' activation with aggregation and increased expression of markers such as P-selectin, which aid in haemostasis (28). Alternatively, some

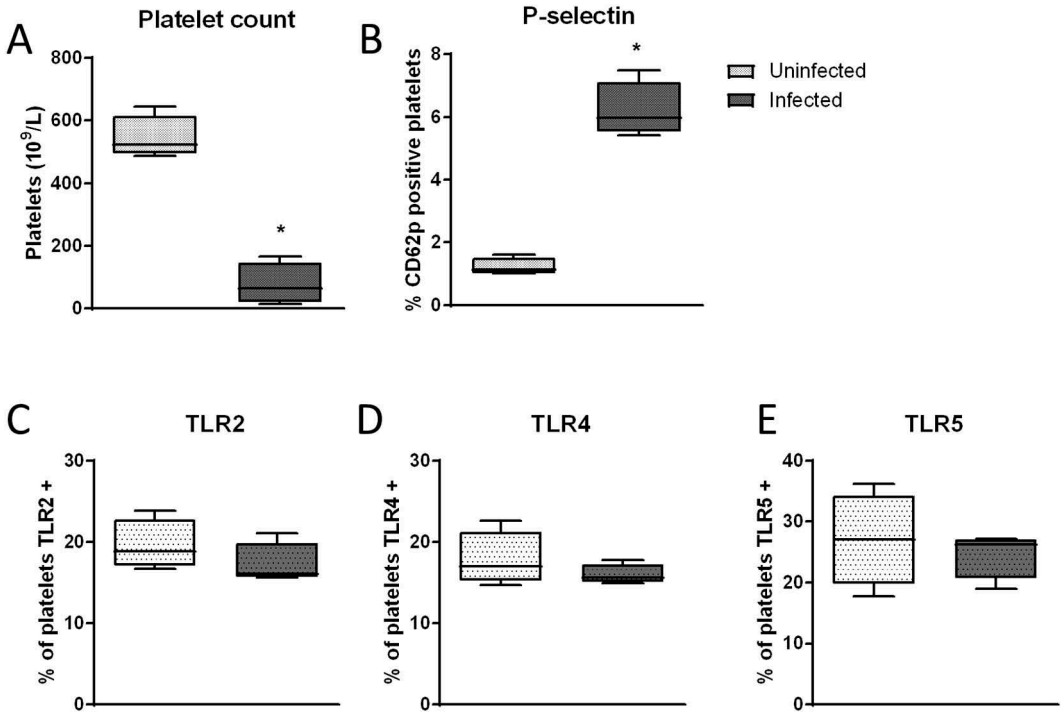


Figure 6. Platelet activation and TLR expression in murine sepsis.Murine platelet counts (A) and platelet P-selectin expression (B) in uninfected mice and mice with 42 h *Klebsiella* pneumosepsis. (C-E) Platelet TLR expression in uninfected control mice and mice with 42 h *Klebsiella* pneumosepsis. (C) TLR2, (D) TLR4 and (E) TLR5. $n = 4$ uninfected and four infected mice. Data are presented as box and whisker plots. $^*P < 0.05$ vs. uninfected mice.

stimuli, including LPS, have been reported to activate platelets in a different way that can, without any signs of classical platelet activation, lead to, e.g., increased platelet-leukocyte complex formation and neutrophil extracellular trap formation (2).

Multiple reports have shown conflicting results regarding the effect of LPS on platelet activation. In line with our findings, some investigations have reported an inability of LPS to induce classical platelet activation (2,8–10). Others, however, found a direct (6,7) or potentiating (26) effect. Differences in (isolation) techniques or different sources of LPS may explain these discrepancies, although we did not find an effect of LPS derived from both *K. pneumoniae* and *P. aeruginosa. E. coli* LPS did result in increased PS positivity. Previous studies have also shown that platelets can react differently to various forms of LPS, and suggested that this might be due to differences in TLR4 and CD14 binding (29) or structure of the O-antigen region of LPS and activation of the lectin pathway of the complement system (30). Platelets do not express CD14, but can absorb large amounts from plasma (31), which might also play a role in our results – as stimulations were performed in platelet rich plasma. The difference between stimulating in platelet rich plasma or washed platelets is also mentioned as a possible explanation for the varying results of LPS on platelet activation (1). In our hands, however, LPS did not induce classical platelet activation in either platelet rich plasma or when using washed platelets.

Whereas P-selectin expression and CD63 expression showed relatively minor inter-donor variation, CXCL4 release was subject to greater inter-donor variation. Although all are readouts of platelet activation, previous studies have shown that not only alpha and dense granule secretion is differentially regulated upon platelet activation (32), but also granule cargo is differentially released (33) possibly by spatially segregation of certain cargo into subregions of the granule (34). Therefore, some granule cargo can be more rapidly released than others (35). Although we have not found previous studies describing this, our experiences are that CXCL4 release is occurring more quickly and to a greater extent than P-selectin expression after blood withdrawal, possibly explained by the differential cargo release.

LPS stimulation also did not increase platelet-leukocyte complex formation, which is in line with previous results by our group (12), however, in contrast to a previous paper (2). Differences in experimental setup again might explain these differences; in the paper by Clark et al. immobilized neutrophils were perfused with platelets in a flow chamber, whereas we used a (static) whole blood stimulation (2).

LPS stimulation did increase mitochondrial respiration. Like in other cell type, mitochondrial respiration is linked to apoptosis in platelets. A number of stimuli including thrombin (signalling (in part) via a similar receptor as our stimulus TRAP) can, at low concentrations, cause platelet activation, whereas higher concentrations can cause apoptosis (36). As we used stimulations with a short duration, (no prolonged exposure was present) the mitochondrial apoptotic pathway was however most likely not induced.

Although LPS activated platelets at a metabolic level, it did not alter platelet TLR expression. We aimed to study all TLRs expressed on the platelet surface and therefore assessed TLRs 2, 4, 5 and 9 (5,17). Although TLR1 is also reported to be expressed on the cell membrane of platelets, we could not detect this (data not shown). Platelet activation by TRAP did increase TLR5 and TLR9 expression. Previous studies have also found changes in TLR4 expression after platelet stimulation (5,16) and reported increased TLR2 and TLR9 expression after platelet stimulation with collagen, thrombin and ADP (5,16,17). The differences in stimuli, methods or antibodies as well as possibly the small effects previously reported (5,16,17) in combination with inter-donor variation, could explain differences in previous studies compared to our results.

When assessing TLR4 expression on both mice and human platelets, we found higher TLR4 expression on murine platelets. Previous reports have shown varying expression of TLR4 on human platelets, ranging from 5% to 50%(5,37). From these and our data, it remains hard to conclude if murine platelets have a

higher TLR4 expression. Differences in the ability of anti-human TLR4 antibodies to recognize platelet TLR4 most likely also influence the expression measured.

In patients and mice with sepsis, we found evidence for classical platelet activation. These findings are in accordance with previous reports of platelet activation during sepsis (14,15). Although platelet activation by TRAP or other stimuli can alter TLR expression (5,16,17), and platelets are activated *in vivo* during sepsis (14,15), human or murine sepsis did not influence platelet TLR expression. A recent study did find increased TLR4 protein levels in platelets of patients with pneumonia; however, these patients were not septic and TLR4 surface expression was not determined (26). Although platelet TLR expression was unaltered in sepsis patients, platelet TLR is expressed and TLR signalling can still play a role in sepsis patients.

In conclusion, although LPS induces TLR4-dependent platelet responses at a metabolic level, it does not lead to classical platelet activation or altered TLR expression. Also during human or murine sepsis, platelet TLR expression was unaltered. These data, taken together with our previous findings showing that absent TLR signalling in platelets does not modify the host response during experimental murine sepsis (12,13), argue against a major role for platelet TLRs in the pathogenesis of sepsis.

Declarations of interest

The authors report no conflicts of interest.

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Supplemental material

Supplemental data for this article can be accessed on the publisher's website

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