Canine platelets express functional Toll-like receptor-4: lipopolysaccharide-triggered platelet activation is dependent on adenosine diphosphate and thromboxane A2 in dogs.

Title
Canine platelets express functional Toll-like receptor-4: lipopolysaccharide-triggered platelet activation is dependent on adenosine diphosphate and thromboxane A2 in dogs.

Permalink
https://escholarship.org/uc/item/9pr75226

Journal
BMC veterinary research, 15(1)

ISSN
1746-6148

Authors
Li, Ronald HL
Nguyen, Nghi
Tablin, Fern

Publication Date
2019-07-15

DOI
10.1186/s12917-019-1997-3

Peer reviewed
Canine platelets express functional Toll-like receptor-4: lipopolysaccharide-triggered platelet activation is dependent on adenosine diphosphate and thromboxane A2 in dogs

Ronald H. L. Li¹*, Nghi Nguyen¹ and Fern Tablin²

Abstract

**Background:** Functional Toll-like receptor 4 (TLR4) has been characterized in human and murine platelets indicating that platelets play a role in inflammation and hemostasis during sepsis. It is unclear whether canine platelets could express functional TLR4 by responding to its ligand, lipopolysaccharide (LPS). We sought to determine if dogs express functional TLR4 and if LPS-induced platelet activation requires co-stimulation with ADP or thromboxane A₂ (TxA₂). Canine platelets were unstimulated (resting) or activated with thrombin or ADP prior to flow cytometric or microscopic analyses for TLR4 expression. We treated resting or ADP-primed platelets with LPS in the absence or presence of acetylsalicylic acid (ASA) and inhibited TLR4 with function blocking antibody or LPS from Rhodobacter sphaeroides (LPS-RS).

**Results:** We discovered that dog platelets have variable TLR4 expression, which was upregulated following thrombin or ADP activation. LPS augmented P-selectin expression and thromboxane B₂ secretion in ADP-primed platelets via TLR4. Inhibition of cyclooxygenase by ASA attenuated LPS-mediated P-selectin expression demonstrating that TLR4 signaling in platelets is partially dependent on TxA₂ pathway.

**Conclusion:** Expression of functional TLR4 on canine platelets may contribute to hypercoagulability in clinical septic dogs. Cyclooxygenase and TxA₂ pathways in TLR4-mediated platelet activation may present novel therapeutic targets in dogs with sepsis.

**Keywords:** Sepsis, Thrombosis, Platelet-priming, Inflammation, Aspirin, Pattern recognition receptor

Background

Despite recent advances in veterinary medicine, mortality remains high in dogs with sepsis secondary to Gram negative bacterial infections [1–3]. One of the major components of the outer membrane of Gram negative bacteria is the endotoxin, lipopolysaccharide (LPS), whose receptor, Toll-like receptor 4 (TLR4), is present on the surface of a wide variety of immune cells such as dendritic cells, epithelial cells, polymorphonuclear cells and macrophages. Formation of the TLR4 receptor complex in response to LPS initiates signalling pathways leading to proinflammatory cytokine production and inflammatory response. In addition to being the primary effector cell in hemostasis, there is growing evidence demonstrating that platelets function as innate immune cells [4]. Bacteria like E.coli and Streptococcus directly interact with platelets leading to platelet activation and aggregation [5]. Murine and human platelets also express several Toll-like receptors (TLRs), suggesting that platelets can act as sentinel cells in detecting pathogen-associated molecular patterns (PAMPs) like LPS.
Thrombocytopenia, a common finding in septic dogs, is associated with mortality, though the exact mechanism of this hematologic abnormality is poorly understood [6–8]. Proposed mechanisms of sepsis-associated thrombocytopenia include decreased thrombopoiesis and increased platelet consumption and sequestration. Systemic platelet activation, which precedes platelet accumulation in organs and microvasculature in human septic patients, suggests that platelets may be the key effector for systemic coagulation during bacterial infection [9]. Systemic hypercoagulability could progress to disseminated intravascular coagulation, further impeding blood flow to tissues causing organ dysfunction. Andonogui et al. showed that platelet TLR4 is an important regulator of endotoxin-mediated thrombocytopenia in mice [10]. In another in vivo sepsis model, transfection of TLR4 deficient platelets in platelet-depleted mice attenuated microvascular thrombosis [11]. These studies suggest that platelet TLR4 also may play a role in facilitating platelet activation in sepsis leading to microvascular thrombosis, and organ dysfunction in septic dogs. [10, 11] This, however, has never been demonstrated in this species. In one of the few canine studies, Yilmaz et al., demonstrated increased platelet aggregation in a lethal endotoxin shock model [8]. Another study, however, found that circulating platelets in dogs with septic peritonitis have decreased aggregation in response to multiple agonists [12].

The mechanism of platelet activation in sepsis has been extensively studied in mice and humans with conflicting results. While some investigators found that LPS stimulates human platelets to undergo activation and aggregation, others found that LPS does not directly stimulate platelets or that LPS-triggered activation requires synergistic stimulation by platelet agonists like ADP, collagen and thromboxane A2 (TxA2) [5, 13–16]. Because platelet activation mediated by TLR4 may account for the interplay between sepsis and thrombosis in dogs, a better understanding of platelet TLR4 expression and platelet response to LPS, is needed. We, therefore, aimed to examine platelet membrane TLR4 expression and determine if this expression is altered by the platelet agonists, ADP and thrombin. We also aimed to determine if LPS could activate platelets via TLR4. Specially, we sought to determine if LPS, in the absence or presence of ADP or TxA2, could stimulate platelet alpha-granule secretion. Lastly, we sought to determine if inhibition of platelet TLR4 could attenuate platelet response to LPS in the absence or presence of platelet priming by ADP.

Results
Out of the 30 dogs studied, 14 dogs were female and 16 dogs were male. Age ranged from 0.33 to 13 years of age (mean 4.92). Of the 30 dogs, 19 were mixed breed dogs; 11 were purebreeds including 2 Labrador Retrievers, 5 Golden Retrievers, 1 Cataoula Hog Dog, 1 Weimaraner, 1 Akbash Coban and 1 Bouvier des Flandres.

Canine platelets express surface TLR4 and its expression is upregulated by thrombin and ADP
Resting platelets had a low surface expression of TLR4 (9.50%; IQR = 0.70–16.88) and the expression was highly variable among subjects with a coefficient of variation (CV) of 135.54%. Thrombin or 10 μM ADP significantly increased the number of TLR4-positive platelets relative to resting platelets (20.80%, IQR = 5.39–43.43, p = 0.0078; 12.12%, IQR = 1.31–45, p = 0.016, respectively) (Fig. 1a to d). CVs of TLR4 expression in thrombin- and ADP-stimulated platelets were 85 and 97.62%, respectively. ADP increased TLR4 expression in a dose-dependent manner (p = 0.047) (Fig. 1d). Compared to ADP-stimulated platelets, thrombin stimulation resulted in higher TLR4 MFI fold change (0.065 ± 0.064 vs. 0.14 ± 0.12), but this difference did not reach statistical significance (p = 0.078). Flow cytometry findings were confirmed by directly visualizing surface TLR4 expression using confocal and STED immunofluorescence microscopy. The platelet membrane was identified by detecting the highly expressed platelet integrin subunit, β3 (Fig. 2). As expected, resting platelets have minimal exteriorization of P-selectin and TLR4 was either sparsely expressed or expressed in clusters on the cell membrane of unpermeabilized platelets (Fig. 2a, arrow). Following ADP or thrombin-induced activation, surface expression of TLR4 and P-selectin was upregulated on the platelet membrane. Compared to the clustered conformation seen on resting platelets, TLR4 was distributed evenly on the membrane surface (Fig. 2b, c). In addition, co-localization of TLR4 and P-selectin on the membrane surface was detected on either ADP or thrombin-stimulated platelets (Fig. 2b, c). We then determined the location of intracellular TLR4 by permeabilizing fixed resting platelets prior to immunostaining. We found that TLR4 (Fig. 2d, Merge, arrows) was concentrated within the platelet alpha-granules as outlined by P-selectin (Fig. 2d, P-selectin, asterisks).

LPS has a limited ability to stimulate alpha-granule secretion in canine platelets
To determine if LPS could activate canine platelets, we measured surface P-selectin expression, using flow cytometry, as a marker of alpha-granule secretion following treatment with 0, 1, 5 or 10 μg/ml LPS. P-selectin expression on resting platelets was highly variable among dogs (Range 2 to 34%, CV = 73.59%) despite a standardized protocol (Fig. 3a,b). Although we could not find a dose-dependent effect of LPS on platelet P-selectin expression (% P-selectin-positive platelets, p = 0.11; P-selectin MFI fold change, p = 0.38), 5 μg/ml LPS...
significantly elevated the number of P-selectin-positive platelets compared to resting platelets (26.23% ± 17.24 vs. 17.85% ± 10.53, \( p = 0.041 \)) (Fig. 4a). This elevation was similar to surface P-selectin expression in thrombin-stimulated platelets (36.14% ± 20.85, \( p = 0.066 \)). Treatment with either 1 or 10 \( \mu \)g/ml LPS did not significantly increase the number of P-selectin-positive platelets compared to resting platelets (16.82% ± 12.65, \( p = 0.74 \); 21.62% ± 15.4, \( p = 0.32 \), respectively) (Fig. 4a). No significant differences between P-selectin MFI fold change in platelets treated with 1, 5 or 10 \( \mu \)g/ml LPS (0.041 ± 0.11, 0.044 ± 0.047, 0.0064 ± 0.081, respectively, \( p > 0.05 \)) were found. Compared to thrombin as an agonist (0.5873 ± 0.38), LPS stimulation resulted in significantly lower P-selectin MFI fold change (1 \( \mu \)g/ml, \( p = 0.0057 \); 5 \( \mu \)g/ml, \( p = 0.0045 \); 10 \( \mu \)g/ml, \( p = 0.0018 \)) (Fig. 4c). Similarly, stimulation of platelets with all concentrations of LPS did not result in significant changes in the number of P-selectin-positive PDMV (\( p = 0.4833 \)) nor MFI fold change (\( p = 0.22 \)).

**Platelet priming by ADP potentiates LPS-mediated alpha-granule secretion**

To determine if ADP could augment platelet response to LPS, we first primed platelets with 10 \( \mu \)M ADP, followed by 1, 5 or 10 \( \mu \)g/ml LPS. Compared to platelets treated with 5 \( \mu \)g/ml LPS alone, ADP priming prior to treatment with LPS of the same concentration resulted in significant elevation in P-selectin (20.51% ± 17.44 vs. 33.20% ± 19.83, \( p = 0.0032 \); MFI fold change 0.044 ± 0.047 vs. 0.18 ± 0.13, \( p = 0.0092 \)) (Fig. 4b, c). ADP-priming prior to LPS stimulation also led to significant increase in P-selectin expression compared to platelets activated with ADP alone (MFI fold change: 0.18 ± 0.13 vs. 0.13 ± 0.14,
When ADP-primed platelets were treated with 10 μg/ml LPS, P-selectin MFI fold was significantly higher compared to unprimed platelets treated with the same concentration of LPS (MFI fold change 0.11 ± 0.070 vs. 0.0064 ± 0.08, \( p = 0.00020 \)) but this elevation did not differ from that in ADP-treated platelets (\( p = 0.059 \)) (Fig. 4c). ADP-priming followed by LPS stimulation significantly increased the number of P-selectin-positive platelets relative to ADP-activated platelets (35.01% ± 20.48 vs 29.88% ± 19.70, \( p = 0.035 \)) and unprimed platelets treated with 1 μg/ml LPS (35.01% ± 20.48 vs 16.82% ± 12.65, \( p = 0.013 \)) (Fig. 4b).

TLR4 expression (MFI fold change) in resting platelets did not correlate with LPS-induced P-selectin expression (MFI fold change) in unprimed platelets (\( r = 0.25, r^2 = 0.065, p = 0.51 \)). Following ADP priming, however, TLR4
expression was positively and moderately correlated with LPS-mediated P-selectin expression ($r = 0.70$, $r^2 = 0.49$, $p = 0.036$).

**ADP priming augments LPS-induced TxA₂ synthesis**

To examine if LPS augments platelet TxA₂ synthesis in the presence or absence of ADP, we measured TxB₂ concentrations in platelet supernatant using ELISA. Using ASA-treated platelets as negative controls, we found that LPS did not significantly elevate TxB₂ secretion relative to ASA-treated platelets (1460 pg/ml, IQR: 695.4–3048 vs. 952 pg/ml, IQR: 495.3–2062, $p = 0.06$). TxB₂ concentration in LPS-treated platelets also was similar to that in resting platelets (1460 pg/ml, IQR: 695.4–3048 vs. 1839 pg/ml, IQR: 887.1–2788, $p > 0.99$). Following priming with ADP, LPS resulted in significant elevation in TxB₂ secretion (3988 pg/ml, IQR: 1042–6459) compared to resting platelets ($p = 0.0020$) and AA-reated platelets ($p = 0.005$). ADP-priming prior to LPS treatment also significantly increased TxB₂ secretion compared to stimulation with LPS ($p = 0.0039$) or ADP (1637 pg/ml, IQR: 921.3–5530, $p = 0.0039$) alone (Fig. 5a).

**LPS-mediated alpha granule secretion requires TxA₂**

To determine whether TxA₂ was required for LPS-mediated alpha granule secretion, we first treated platelets with ASA prior to LPS stimulation in the absence or presence of ADP. We found that ASA did not significantly affect the number of P-selectin-positive platelets in resting (ASA: 8.06%, IQR: 6.38–16.12 vs. Resting: 7.87%, IQR: 6.94–17.54, $p = 0.84$) (Fig. 5b) or ADP-stimulated platelets (ASA + ADP: 23.85%, IQR: 16.53–36.18 vs. ADP: 32.25%, IQR: 18.00–44.50, $p = 0.10$). ASA also did not affect P-selectin MFI fold change in platelets activated with LPS (ASA + LPS: 0.014, IQR: −0.043–0.045 vs. LPS:0.015, IQR: −0.042–0.14, $p = 0.37$) or ADP (ASA + ADP: 0.15, IQR: 0.083–0.25 vs. ADP: 0.13, IQR: 0.097–0.29, $p = 0.74$) (Fig. 5c).

In unprimed platelets treated with LPS, number of P-selectin positive platelets was significantly lower in those with ASA treatment compared to those without (10.54%,...
Inhibition of TxA₂ synthesis by ASA also significantly attenuated the number of P-selectin positive platelets in ADP-primed platelets treated with LPS (16.35%, IQR: 11.03–31.63 vs. 28.15%, IQR: 15.33–31.68, p = 0.05) but not in P-selectin MFI fold change (0.13 ± 0.086 vs. 0.16 ± 0.071, p = 0.83) (Fig. 5b,c).

LPS-mediated alpha-granule secretion is dependent on TLR4

To investigate if LPS-mediated alpha-granule secretion in platelets is dependent on TLR4, platelets were pre-treated with TLR4 function blocking antibodies. We confirmed that TLR4 inhibition using a function blocking antibody did not interfere with detection of P-selectin expression in either resting or ADP-activated platelets (Fig. 6a, b). However, TLR4 inhibition significantly attenuated P-selectin expression in LPS-treated platelets. P-selectin MFI fold change in LPS-treated platelets was significantly decreased compared to those without TLR4 inhibition (−0.032 ± 0.023 vs. 0.0017 ± 0.031, p = 0.021) or those treated with the isotype control (−0.032 ± 0.023 vs 0.076 ± 0.031, p = 0.004) (Fig. 6a). TLR4 inhibition, however, did not significantly alter the percentage of P-selectin positive platelets in LPS-stimulated platelets (5.75 ± 1.32 vs 6.25 ± 3.93, p = 0.66).

Inhibition of TLR4 with LPS-RS, a TLR4 antagonist, had no attenuating effect on LPS-mediated P-selectin expression in unprimed platelets. However, in the presence of ADP, LPS-RS significantly lowered LPS-mediated P-selectin expression. Compared to vehicle
control, TLR4 inhibition by LPS-RS significantly decreased the percentage of P-selectin positive platelets (19.71% ± 10.86 vs 10.19% ± 4.51, \( p = 0.0003 \)) and MFI fold change (0.20 ± 0.17 vs 0.099 ± 0.13, \( p = 0.0049 \)). (Fig. 6c).

**Discussion**

To the authors’ knowledge, this is the first study documenting the expression of functional TLR4 in canine platelets. The present study indicates that platelet response to *E. coli* LPS via TLR4 is amplified by the agonists ADP and TxA2 in dogs.

The expression of functional TLR4 on canine platelets highlights a highly conserved mechanism of pathogen recognition utilized by many other cell types in mammals [17]. But unlike other immune cells, thrombin and ADP, which are platelet agonists that stimulate platelets in extending platelet plug formation, upregulate the surface expression of platelet TLR4 [18]. Here, we found that TLR4 resides within the cytoplasm and alpha granules in unstimulated platelets, similar to the findings in human platelets. The upregulation of surface TLR4 in thrombin-activated human platelets is secondary to the activation of calpain with subsequent cleavage of myosin-9 resulting in TLR4 trafficking from the alpha-granules to the platelet plasma membrane [19]. The co-expression of TLR4 and P-selectin on the surface of activated canine platelets suggests that TLR4 trafficking to the platelet surface also could be mediated by alpha-granule secretion. This unique mechanism of TLR4 up-regulation in platelets highlights the interplay between hemostasis and innate immunity.

Our results indicate a highly variable expression of surface TLR4 among dogs which is augmented once
platelets were activated by thrombin or ADP. The significant correlation between TLR4 expression and the degree of LPS-mediated alpha-granule release in the presence of ADP suggests that the upregulation of TLR4 augments platelets’ sensitivity to LPS. We further confirmed this finding by inhibiting TLR4 in ADP-primed platelets. While TLR4 inhibition did not interfere with ADP-induced activation, it abolished the stimulatory effects of LPS indicating that ADP potentiates LPS-mediated platelet activation by upregulating surface TLR4 expression. Dogs with naturally occurring sepsis are found to be in a hypercoagulable state with elevated thrombin generation and overconsumption of endogenous anticoagulants like antithrombin III [20]. It is unknown at this stage if elevated levels of thrombin in sepsis could trigger upregulation of surface TLR4 expression. Dogs with naturally occurring sepsis are found to be in a hypercoagulable state with elevated thrombin generation and overconsumption of endogenous anticoagulants like antithrombin III [20]. It is unknown at this stage if elevated levels of thrombin in sepsis could trigger upregulation of surface TLR4 expression. Dogs that were easily excitable, thrombocytopenia [21]. The prognostic and diagnostic significance of platelet TLR4 expression in dogs requires further investigations.

We demonstrated that E.coli LPS, to a limited extent, activates canine platelets to undergo alpha-granule secretion required for normal thrombus formation. P-selectin, a marker of alpha-granule secretion, is an integral protein of the alpha granule membrane. In accordance with previous studies, we were unable to detect significant elevation in P-selectin expression, and TxA2 generation elicited by LPS [16]. Despite finding a significant increase in the numbers of platelets expressing P-selectin, the lack of MFI fold change suggests that LPS had minimal effect on augmenting P-selectin density, a marker of substantial alpha-granule secretion. Another explanation is the variable P-selectin expression on unstimulated platelets potentially due to stress from handling, excitement or in vitro platelet activation from PRP generation (Fig. 3) [22]. This likely could decrease platelet sensitivity to LPS. Dogs that were easily excitable,
stressed and difficult to restrain for blood draws were later on excluded from the TLR4 blocking experiments, as evidenced by the lower mean resting P-selectin expression (Fig. 5a, c).

Once platelets are primed with ADP, treatment of platelets with LPS potentiates alpha-granule secretion and TxA2 generation, suggesting that LPS synergizes with ADP in amplifying platelet response to LPS. This observation might be due to several underlying mechanisms. First, surface P-selectin on human platelets has been shown to enhance E.coli LPS binding to platelet TLR4 by forming a heterodimeric complex with TLR4 and myeloid differentiation protein-2 (MD-2). Downstream signaling pathway of TLR4 leads to α-granule secretion, which is amplified by ADP and thromboxane A2 (TxA2). Activation of G-protein coupled receptors, P2Y1/P2Y12 and thromboxane receptor (TP), leads to phospholipase C (PLC) activation and, subsequently, 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) for intracellular calcium release and alpha-granule secretion. LPS acts synergistically with ADP to increase generation of TxA2, serving a positive feedback mediator. TLR4 and ADP signaling activates cyclooxygenase-1 (COX-1), which converts arachidonic acid (AA) to TxA2, likely by the Akt/p38 MAPK pathway.

Fig. 7 Schematic diagram of LPS-mediated platelet activation and TLR4 expression in canine platelets. ADP activation via P2Y1 or P2Y12 receptor upregulates surface TLR4 expression. TLR4 trafficking to cell membrane from granules may be mediated by alpha-granule secretion. LPS binding protein (LBP) presents LPS to CD14 forming a heterodimeric complex with TLR4 and myeloid differentiation protein 2 (MD-2). Downstream signaling pathway of TLR4 leads to α-granule secretion, which is amplified by ADP and thromboxane A2 (TxA2). Activation of G-protein coupled receptors, P2Y1/P2Y12 and thromboxane receptor (TP), leads to phospholipase C (PLC) activation and, subsequently, 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) for intracellular calcium release and alpha-granule secretion. LPS acts synergistically with ADP to increase generation of TxA2, serving a positive feedback mediator. TLR4 and ADP signaling activates cyclooxygenase-1 (COX-1), which converts arachidonic acid (AA) to TxA2, likely by the Akt/p38 MAPK pathway.
p38MAP kinase axis leading to phospholipase A2 phosphorylation and subsequent generation of TxA2 in human platelets [16]. Further studies are needed to characterize the non-genomic pathway of TLR4 in dog platelets (Fig. 7).

By directly inhibiting the enzyme, cyclo-oxygenase (COX-1) with ASA, we showed that platelet activation mediated by TLR4 and ADP signaling is partially dependent on TxA2. Given its short half-life (~ 30 s), TxA2 acts as an autocrine or paracrine to nearby platelets amplifying platelet activation via platelet TxA2 receptors. As expected, ASA did not completely attenuate LPS-mediated alpha granule secretion and, in some dogs, had no effect on P-selectin expression. This may be due to the variable thromboxane responsiveness found in some dogs, in which inhibition of TxA2 production may not negatively impact LPS-mediated platelet activation [25]. This also may be due to the presence of other mediators likely involved in the positive feedback mechanism of TLR4 signaling. Antiplatelet therapy is the cornerstone of antithrombotic therapy in dogs. In addition to their antithrombotic properties, antiplatelet drugs have been shown to modulate inflammation in people and dogs by reducing acute phase response and proinflammatory biomarkers like C-reactive protein [26]. Since TxA2 serves as a positive feedback in amplifying LPS-mediated platelet activation, antiplatelet therapy may have potential benefits in clinical septic dogs [26]. In observational studies, prehospital administration of ASA has been shown to be associated with reduced mortality and lower prevalence of acute respiratory distress syndrome in critically ill humans with sepsis [27, 28]. Prospective clinical trials are needed to identify the clinical benefits of ASA therapy in at-risk dogs.

The present study has several limitations. First, the concentration of LPS that consistently activated canine platelets in the absence or presence of ADP was 5 μg/ml, a higher concentration than expected in systemic circulation during sepsis. A plausible explanation for the need of this concentration is the low levels of plasma proteins in our washed platelet system. The binding of LPS to TLR4 is complex and requires 3 other extracellular proteins including LPS binding protein (LBP), CD14, and myeloid differentiation protein 2 (MD-2). LBP, a soluble acute-phase protein, binds to LPS and presents it to CD14 on platelets to form a heterodimeric complex with TLR4 and its accessory protein, MD2 (Fig. 6). Whether human platelets express CD14 is controversial [13, 29, 30]. Damien et al. found that the response to LPS in washed platelets is dependent on soluble CD14 suggesting that platelets may obtain CD14 from systemic circulation [30]. We supplemented canine platelets with a small concentration of canine serum in order to enhance platelet response to LPS. However, we did not investigate if canine platelets express the necessary TLR4 signaling complex including surface CD14 expression. Since LPS-RS antagonizes LPS by directly competing with LPS for the binding site on MD-2, our data suggests that dog platelets may express MD-2 on the cell surface. Further studies using platelet rich plasma to determine if concentrations of LPS found in septic dogs could activate platelets is needed. Another plausible explanation is that canine platelets may have variable responses to LPS from different strains of E. coli as the affinity for TLR4 on mouse platelets is dependent on the LPS serotypes [23]. Finally, we did not investigate the effects of LPS on other markers of platelet activation such as fibrinogen binding, dense granule secretion and CD40L expression, which have all been shown to increase by LPS stimulation in human and murine platelets [13, 23].

Conclusion
Our study demonstrated that canine platelets express functional TLR4, which can be upregulated by thrombin or ADP. Although E. coli LPS is a limited stimulus for platelet activation, in the presence of ADP or TxA2, LPS is a potential platelet activator in dogs. The findings of this study provide novel insights into the mechanisms of thrombosis and potential therapeutic targets in septic dogs.

Methods
Animals
The study protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Thirty clinically healthy staff- or student-owned dogs greater than 5 kg were used in this study and 8 dogs were enrolled for each experiment. Dogs were deemed to be clinically healthy by physical examination and a complete blood count using an automated hematology analyzer (Coulter ACT diff, Beckman-Coulter Inc., Miami, FL) and blood smear evaluation. Dogs were not enrolled in the study if they were vaccinated 30 days prior to enrollment, on any concurrent medications, or had any abnormalities on hematological examination. Eight dogs were enrolled.

Generation of gel-filtered platelets
Whole blood (4 to 6 ml) was drawn from either the jugular or cephalic vein using a 22 gauge needle connected to a 6 ml syringe before transferring into 3.2% sodium citrate tubes. Blood tubes were gently inverted 2 to 3 times and carefully inspected for clots. Citrated blood was transferred to polypropylene tubes and platelet rich plasma was generated by centrifugation (300 x g, 5 min, no brakes) at room temperature. Platelets were separated from plasma by gel-filtration over a Sephrose 2B column at 37 °C and eluted with filtered Tyrodes-HEPES buffer (pH 7.4, 5 mM dextrose,
0.5% canine serum, without divalent cations) [31]. Gel-filtered platelets were observed to determine if they exhibited the “swirling” characteristic found in truly discoid resting platelets [31, 32]. Isolated platelets that failed to display swirling movements were not included in the study. Platelet count was obtained using an automated analyzer (Coulter ACT diff, Beckman-Coulter Inc., Miami, FL) and confirmed by bloodsmear evaluation. All experiments were carried out in a sterile manner.

Detection of platelet TLR4 and P-selectin surface expression by flow cytometry
Platelet count was adjusted to a concentration of 1 × 10^7 cells/ml with Tyrodes-HEPES (pH 7.4, 5 mM dextrose, 0.5% canine serum, without divalent cations) and platelets were either unstimulated (resting) or stimulated with 0.1 unit/ml bovine-derived alpha-thrombin (Haematologic Technologies, Inc., Essex Junction, VT) or ADP (1 μM or 10 μM) for 15 min at 37 °C [33]. Surface TLR4 expression was assessed using a biotinylated mouse anti-human monoclonal antibody to TLR4 (1:400, Clone: HTA 125, BioRad Laboratoires, Hercules, CA), known to cross react with canine TLR4 [34]. Following 45 min of incubation at 37 °C, platelets were incubated with streptavidin conjugated to phycoerythrin cy7 (1:200, Invitrogen, Carlsbad, CA) for an additional 45 min at 37 °C. For the detection of P-selectin (CD62p), platelets were either resting or first primed with 10 μM ADP (15 min, 37 °C) prior to treatment with 1, 5, or 10 μg/ml LPS from Escherichia coli 0111:B4 (EMD Millipore, Temecula, CA) for 30 min at 37 °C. Unstimulated and thrombin-activated (0.1 U/ml) platelets served as negative and positive controls, respectively. Following activation, platelets were incubated with fluorescein isothiocyanate-conjugated rat anti-mouse monoclonal antibody to P-selectin (CD62p) (1:200, Clone:RB40.34, BD Biosciences, San Diego, CA) for 45 min at 37 °C. Platelets were identified by forward and side scatter properties as well as the platelet integrin, β3a (CD61), using a mouse anti-human monoclonal antibody conjugated to allophycocyanin (1:1000, Clone:VI-PL2, eBioscience, San Diego, CA) (45 min, 37 °C). Cells were fixed in 0.1% paraformaldehyde and analyzed using 5-color flow cytometer (Beckman-Coulter FC500, Beckman-Coulter Inc., Miami, FL) within 4 h. Gating boundaries were established by fluorescence-minus-one controls. Anti-mouse compensation beads (BD Biosciences, San Diego, CA) conjugated to matched experimental fluorochromes were used for compensation and compensation matrices were calculated using commercially available software (Flowjo, Tree Str Inc., Ashland, OR). TLR4 and P-selectin expression was measured as percent positive events or fold change in mean fluorescence intensity (MFI) between activated platelets and resting platelets using the following formula:

\[
\text{MFI fold change} = \left( \frac{\log_{10} \text{MFI}_{\text{Activated}}}{\log_{10} \text{MFI}_{\text{Resting}}} \right)
\]

The platelet and platelet-derived microvesicle (PDMV) gates were determined as previously described using 0.5 μM and 3 μM calibration beads [35, 36]. In brief, PDMV were quantified based on either the number of CD62P-positive events or MFI fold change in CD62P. Flow cytometry data were analyzed using commercially available software (Flowjo, Tree Str Inc., Ashland, OR).

Immunofluorescence microscopy of platelet TLR4
Gel-filtered platelets (1 × 10^5/ml) were either unstimulated or activated with 0.1 unit/ml thrombin or 10 μM ADP. Following activation, platelets were fixed in 1% paraformaldehyde for 15 min at room temperature and concentrated onto microscope slides using a cyt centrifuge (Cytospin 4, ThermoScientific Inc., Grand Island, NY) at 1500 rpm for 5 min. Platelets were then washed 3 times in phosphate buffered saline (pH 7.4) and were either unpermeabilized or permeabilized with 0.1% NP40 (Surfact-AMPS™ NP-40, Pierce, Rockford, IL) for 2 min at room temperature. After washing, cells were blocked with 10% bovine serum albumin (1 h, 37 °C), and subsequently incubated with biotinylated mouse anti-human monoclonal antibody to TLR4 (25 μg/ml, Clone: HTA125, BioRad Laboratories, Hercules, CA) (1 h, 37 °C), followed by streptavidin conjugated to Alexa Fluor 555 (1:200 in 2% BSA, S21381, ThermoFisher Scientific, Waltham, MA) (1 h, 37 °C). P-selectin (CD62P) and integrin β3a (CD61) were detected by incubating cells with rat anti-mouse CD62P monoclonal ab (10 μg/ml, Clone:RB40.34, BD Biosciences, San Diego, CA) and mouse anti-human monoclonal antibody (1 μg/ml, Clone:VI-PL2, eBioscience), respectively, followed by incubation with Alexa Fluor 488-conjugated goat anti-rat IgG and Alexa Fluor 405-conjugated goat anti-mouse IgG (eBioscience, ThermoFisher Scientific, Waltham, MA) overnight at 4 °C. The final dilution of all the secondary antibodies used was 1:50 diluted in 5% goat serum. After washing, a #1.5 glass coverslip was placed on the cells with an anti-fade mounting medium (Pro-Long Gold, ThermoFisher Scientific, Waltham, MA) and cured overnight at 4°C. Interference controls were prepared by excluding incubation with the primary antibodies in the second immuno-labelling step.

Fluorescent images were acquired using a combination of confocal and super-resolution stimulated emission depletion (STED) microscopy (Leica TCS SP8 STED 3x, Leica Microsystems, Buffalo Grove, IL). Imaging powers of STED wavelengths were set to 20 to 50% of excitation wavelengths. The following imaging sequence was
performed to avoid photobleaching; 1. CD61 was first detected by confocal microscopy excited with 405 nm with 1–6 nsec HyD gating; 2. Alexa Fluor 555 (TLR4) was then excited with 555 nm and 660 nm STED depletion laser; 3. Alexa Fluor 488 (P-selectin) was excited with 488 nm and 592 nm STED depletion laser with 1.2–6 nsec HyD gating. All images were acquired at 100x magnification with pixel dwell time of 800 nsec; pixel size (20 to 25 nm) was optimized at four times the image format of 512 × 512 pixels. Deconvolution of microscope images was performed using commercially available software (Huygens Professional, 18.10, Scientific Folume Imaging) and analyzed using publically available software (FIJI, NIH).

**Thromboxane B₂ ELISA**

Resting or ADP-primed platelets (10 µM ADP, 15 min, 37 °C) were treated with 5 µg/ml *E.coli* 0111:B4 LPS (EMD Millipore, Temecula, CA) for 30 min at 37 °C. Platelets pre-treated with 100 µM acetylsalicylic acid (ASA) (Sigma-Aldrich, St. Louis, MO) (30 min, 37 °C) served as negative control. Immediately following stimulation, samples were centrifuged (1500 RPM, 18 °C, 10 min, no brakes) and platelet supernatant was collected, flash frozen in liquid nitrogen and stored at − 80 °C until analysis. Concentration of thromboxane B₂ (TxB₂) in the platelet supernatant was measured using a commercial ELISA kit (Enzo Life Sciences Inc., Farmingdale, NY).

**Platelet TLR4 inhibition**

Gel-filtered platelets, corrected to 1 × 10⁷ cells/ml with Tyrodes-HEPES, were either incubated with preservative-free function blocking antibody to TLR4 (50 µg/ml, Clone: HTA125, BioRad Laboratories, Hercules, CA) or equivalent concentration of mouse IgG2a (Clone: OX-34Mouse IgG2a, BioRad Laboratories, Hercules, CA) as isotype control for 20 min at room temperature. An additional population of platelets were incubated with 50 µg/ml ultrapure LPS from *Rhodobacter sphaeroides* (LPS-RS) (InvivoGen, San Diego, CA), a TLR4 antagonist, for 1 h at 37 °C. Following TLR4 blocking, platelets were either unstimulated (resting) or stimulated with 10 µM ADP (15 min, 37 °C) before treatment with 5 µg/ml LPS (30 min, 37 °C). CD62P and CD61 were labeled and detected by flow cytometry as described above.

**Statistical analysis**

Normality of data was tested using Shapiro-Wilk normality test or visual inspection of normal quartile plots. Non-parametric data were presented as median and interquartile range (IQR) and parametric data were presented as mean ± standard deviation. Normally distributed and paired data were analyzed using t-tests while nonparametric and paired data were analyzed using Wilcoxon signed-rank test. Unpaired data was measured using either Mann-Whitney U test or unpaired t test. One-way repeated measures ANOVA was used for comparing the means of dose-response studies followed by post-hoc analysis using Dunnet test. Data that violated the assumption of sphericity were analyzed using Greenhouse-Geisser correction. Correlation and correlation coefficient were calculated using Pearson correlation. An alpha-priori of < 0.05 was considered statistically significant. Interindividual variability was calculated as the ratio between the standard deviation of a group and its means and expressed as coefficient of variation (CV). Data were analyzed using commercially available software (Prism 7.0, GraphPad Software, La Jolla, CA).

**Abbreviations**

ASA: Acetylsalicylic acid; LPS-RS: LPS from *Rhodobacter sphaeroides*; MFI: Mean fluorescence intensity; TLR4: Toll-like receptor 4; TxA₂: Thromboxane A₂; TxB₂: Thromboxane B₂

**Acknowledgements**

The authors would like to thank Dr. Ingrid Brust-Mascher for assistance with immunofluorescence microscopy, the canine blood donors and their owners.

**Authors’ contributions**

RHLL and FT contributed to the experimental design, writing and editing of the manuscript. RHLL, and NN contributed to collection of samples, conduction of experiments, interpretation of data, and immunofluorescence microscopy. RHLL was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

**Funding**

This work was supported by the Center for Companion Animal Health, University of California, Davis [2017–8-F, 2017]. The corresponding author was funded by the Morris Animal Foundation [D15CA-907, 2015].

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

The study protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Davis. All samples were collected after informed written consent was obtained from the owners.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
