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DOI:

[10.1016/j.pupt.2017.05.005](https://doi.org/10.1016/j.pupt.2017.05.005)

[10.1016/j.pupt.2017.05.005](https://doi.org/10.1016/j.pupt.2017.05.005)

Document Version

Early version, also known as pre-print

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Amison, R. T., Arnold, S., O'Shaughnessy, B. G., Cleary, S. J., Ofoedu, J., Idzko, M., Page, C. P., & Pitchford, S. C. (2017). Lipopolysaccharide (LPS) induced pulmonary neutrophil recruitment and platelet activation is mediated via the P2Y1 and P2Y14 receptors in mice. *PULMONARY PHARMACOLOGY AND THERAPEUTICS*, 45, 62-68. <https://doi.org/10.1016/j.pupt.2017.05.005>, <https://doi.org/10.1016/j.pupt.2017.05.005>

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Lipopolysaccharide (LPS) induced pulmonary neutrophil recruitment and platelet activation is mediated via the P2Y₁ and P2Y₁₄ receptors in mice.

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Abstract

Platelet activation occurs during host defence and in various inflammatory disorders. In animal models of infection and inflammation, experimental depletion of platelets leads to significantly reduced leukocyte recruitment and impaired clearance of pathogens from the lung. It is now appreciated that purinergic receptor activation is required for leukocyte activation, motility and adhesion, and platelet interactions with leukocytes can be modulated by purinergic stimulation of platelets. Here, we have investigated the role of platelet P2Y₁, P2Y₁₂, P2Y₁₄, and P2X₁ receptors on leukocyte recruitment and chemotaxis.

Mice were administered either vehicle controls or selective P2Y₁, P2Y₁₂, P2Y₁₄, or P2X₁ antagonists intravenously before intranasal administration of lipopolysaccharide (LPS) to investigate the effect of these drugs on pulmonary leukocyte recruitment, peripheral platelet counts, bleeding times, and *ex vivo* platelet aggregation. Separately, platelets were incubated with P2Y₁, P2Y₁₂, P2X₁ antagonists, or P2Y₁₄ agonists to assess effects on platelet-induced neutrophil chemotaxis *in vitro*.

Pulmonary neutrophil recruitment induced by intranasal LPS administration was inhibited in mice administered either with P2Y₁ or P2Y₁₄ antagonists, but not with P2Y₁₂ or P2X₁ antagonists. Furthermore, the administration of either a P2Y₁ or a P2Y₁₄ antagonist reversed the incidence of peripheral thrombocytopenia associated with LPS exposure. Bleeding times were significantly increased in mice administered P2Y₁, P2Y₁₂, or P2X₁ antagonists, whilst *ex vivo* platelet aggregation to ADP was significantly reduced. These haemostatic responses remained unaltered following antagonism of P2Y₁₄. *In vitro* chemotaxis assays revealed direct antagonism of platelet P2Y₁, but not P2Y₁₂ or P2X₁ receptors suppressed platelet-dependent neutrophil motility towards MDC. Furthermore, the stimulation of platelets with selective

P2Y₁₄ agonists (UDP-glucose, MRS2690) resulted in significant platelet-dependent neutrophil chemotaxis.

These results reveal a role for P2Y₁ and P2Y₁₄ activation of platelets following exposure to LPS, whilst haemostatic indices were unaffected by inhibition of platelet function with the P2Y₁₄ antagonist in response to LPS.

Introduction

Platelet activation has been described in patients with chronic obstructive pulmonary disease (COPD), acute lung injury (ALI), Inflammatory bowel disease (IBS), rheumatoid arthritis (RA), asthma and allergies, and in patients suffering from infectious diseases [1–10]. A number *in vivo* studies have previously demonstrated the presence of platelet activation through the upregulation of P-selectin expression, the formation of platelet-leukocyte conjugates and the release of pro-inflammatory mediators in septic, allergic, and sterile inflammation. These studies also demonstrate the importance of platelets for leukocyte recruitment [3,11–14].

The role of platelet purinergic receptors in the context of platelet aggregation has been well documented with co-stimulation of the P2Y₁ and P2Y₁₂ receptors by ADP, and following ATP stimulation of the P2X₁ receptor [15–22]. Furthermore, a number of studies have detailed the ability of purinergic receptor stimulation to induce increases in P-selectin expression and the incidence of platelet-leukocyte conjugates which are considered to be a prerequisite for leukocyte recruitment [23–25]. Using a murine model of allergic airway inflammation we have previously demonstrated that pulmonary leukocyte recruitment was mediated via RhoA signalling downstream of the platelet P2Y₁ receptor, whilst the P2Y₁₂ and P2X₁ receptors showed no involvement in this process [26]. More recently, platelets have been shown to express the P2Y₁₄ receptor on their surface [27]. However, the P2Y₁₄ receptor appears to be redundant in platelet aggregation, whilst demonstrating an ability to mediate cell migration in other cell types [27–30]. A physiological role for platelet P2Y₁₄ receptor activation therefore remains unknown in the context of host defence and inflammation. The aim of the present study was therefore to characterise the role of the P2Y₁, P2Y₁₂, P2Y₁₄ and P2X₁ receptors in a

murine model of LPS induced pulmonary inflammation which we and others have reported to be dependent on platelet activation.

Methods

Materials

PGE₁, lipopolysaccharide (LPS, extracted from *Escherichia Coli* O55:B5 serotype), Ticagrelor (1*S*,2*S*,3*R*,5*S*)-3-[7-[[*(1R,2S)*-2-(3,4-Difluorophenyl)cyclopropyl]amino]-5-(propylsulfanyl)-3*H*-[1,2,3] triazolo[4,5-*d*]pyrimidin-3-yl]-5-(2-hydroxyethoxy)-1,2-cyclopentane-1,2-diol) and adenosine-diphosphate (ADP) were purchased from Sigma (Poole, UK); MRS2179 (*N*⁶-methyl-2'-deoxyadenosine-3',5'-biphosphate), MRS2500 (2-Iodo-*N*⁶-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-biphosphate), AR-C66096 (2-propylthio-D-β,γ-difluoromethylene ATP), NF-279 (8-8'-[Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonyliminio)]bis-1,3,5-naphthalenetrisulfonic acid), and MRS2690 (Diphosphoric acid 1-α-D-glucopyranosyl ester 2-[(4'-methylthio)uridin-5''-yl] ester disodium salt) and uridine-di-phosphate glucose (UDP-glucose) from Tocris Bioscience (Bristol, UK). Murine MDC from Peprotech (New Jersey, USA); Chemotaxis plates were obtained from Neuroprobe (Maryland, USA).

Mouse LPS challenge and bronchoalveolar lavage

A murine model of LPS induced lung inflammation was established to investigate the importance of each of the 4 purinergic receptors expressed on platelets (P2Y₁, P2Y₁₂, P2X₁ and P2Y₁₄) for pulmonary leukocyte recruitment. All studies were carried out under the Animals (Scientific Procedures) Act of 1986 (United Kingdom) with local ethical approval of King's College London. Selected groups of female Balb/c mice (8-10 weeks; Harlan UK, Bicester,

United Kingdom) were treated with MRS2500, AR-C66096, NF-279 (3mg/kg administered intravenously), Ticagrelor (3, 10, 30mg/kg administered intra-peritoneally) or PPTN Mesylate (10mg/kg administered intravenously), Saline was used as a vehicle for MRS2500, AR-C66096, NF-279 and PPTN Mesylate, 0.1% Dimethyl Sulfoxide was used for Ticagrelor. 20 minutes post drug administration, mice were challenged with 1.25mg/kg LPS via intranasal administration under isoflurane anaesthetic. 4 hours post LPS challenge, bronchoalveolar lavage fluid was collected and processed for total and differential cell counts as previously described[11,26,31]. Circulating platelet numbers were enumerated at 4 hours post LPS challenge after blood was taken via cardiac puncture, and quantified in stromatol (1:100 dilution) on an Improved Neubauer haemocytometer using an Axioskop Microscope under an x40 objective.

Platelet aggregation

Blood from mice was collected by means of cardiac puncture 1 hour post LPS challenge and washed platelets isolated to confirm biological activity and to compare the effects on haemostasis of P2Y₁, P2Y₁₂, P2Y₁₄ and P2X₁ antagonists described above. Platelet aggregation was measured after stimulation with 10µmol/L ADP as previously described [26]. Platelet aggregation towards UDP-glucose (agonist for P2Y₁₄) was also assessed at increasing concentrations to 1µmol/L.

Bleeding times

Animals were kept under continuous anaesthesia with inhaled isoflurane anaesthetic. Bleeding assays were performed 1 hour post LPS challenge in mice by tail tip amputation, immersing the tail in saline at 37°C and continuously monitoring bleeding patterns. Each

animal was monitored for up to 10 minutes and bleeding times determined using a stop clock. At the conclusion of the experiment, animals were killed with an overdose of anaesthetic.

Platelet-induced leukocyte chemotaxis

An *in vitro* assay of platelet-dependent leukocyte chemotaxis was used to elucidate the importance of each of the 4 purinergic receptors expressed on platelets on subsequent leukocyte activation as previously described [26]. Chemotaxis plates (3 μ m pore size) were blocked with Tyrode's buffer with 1% BSA for 1 hour. After blocking, the blocking buffer was removed and replaced with 100nmol/L macrophage-derived chemokine (MDC). Washed platelets at a concentration of 1×10^8 platelets/ml were prepared from whole blood as previously described [26] and were incubated with either the P2Y₁, P2Y₁₂ and P2X₁ antagonists MRS2500, AR-C66096 and NF-279 (1-1000nmol/L) respectively, or the P2Y₁₄ receptor agonists UDP-glucose (1-1000nmol/L) or MRS2690 (10-1000nmol/L) for 10 minutes at room temperature. Platelets treated with either MRS2500, AR-C66096 and NF-279 were stimulated with 100nmol/L ADP for 5 minutes, and then washed. Leukocytes were flushed from bone marrow (femurs) of mice and resuspended in a Dulbecco modified Eagle medium based assay buffer to a concentration of 5×10^6 leukocytes/ml. Leukocytes were then added to platelets at a 1:1 volumetric ratio and the chemotaxis assay performed as previously described[12].

Statistical Analysis

Data from *in vivo* animal studies are displayed as means \pm SEMs, whilst *in vitro* chemotaxis data are displayed as a chemotactic index. Data were analysed by means of 1-way ANOVA, followed by the Bonferroni multiple comparison post-test. A *P* value of less than 0.05 was considered significant.

Results

Activation of P2Y₁ and P2Y₁₄ but not P2Y₁₂ or P2X₁ receptors, is necessary for pulmonary leukocyte recruitment in a murine model of LPS induced inflammation

Exposure to 1.25mg/kg LPS induced a significant increase in total pulmonary leukocyte recruitment ($P < 0.001$, **Fig 1A**) which was accounted for by significant increases in neutrophil recruitment ($P < 0.001$, **Fig 1B**), and lymphocyte recruitment ($P < 0.05$, **Fig 1C**). The administration of the highly selective and competitive P2Y₁ receptor antagonist MRS2500 (3mg/kg) caused a significant attenuation of LPS induced leukocyte recruitment ($P < 0.05$, **Fig 1A**), specifically neutrophil recruitment ($P < 0.01$, **Fig 1B**), into the lungs when compared to vehicle controls. In contrast, neither administration of the specific P2Y₁₂ antagonist (the adenosine triphosphate analogue, AR-C66096) or a P2X₁ channel antagonist (the suramin analogue, NF-279) were unable to inhibit LPS induced leukocyte recruitment to the lungs (**Fig 1A-C**). Administration of the prodrug of a P2Y₁₄ antagonist (PPTN Mesylate)[33] suppressed LPS induced leukocyte recruitment ($P < 0.05$, **Fig 1A**), and specifically neutrophil recruitment ($P < 0.01$, **Fig 1B**). Lymphocyte recruitment was also reduced by 62%, although this did not reach statistical significance (**Fig 1C**).

Whilst these data demonstrating an involvement of P2Y₁ receptor activation in leukocyte recruitment towards LPS is in agreement with previous data we have published in allergic

inflammation[26], we further investigated P2Y₁₂ antagonism, since this receptor has been reported elsewhere to be involved in neutrophil inflammation [34–37]. Mice were administered a second P2Y₁₂ antagonist (Ticagrelor). However this did not lead to a suppression of pulmonary neutrophil recruitment induced by LPS at 3mg/kg or 10mg/kg (LPS+Veh: 30.9±9.8x10⁴ cells/ml, LPS+ 3mg/kg Ticagrelor: 44.9±16.7x10⁴ cells/ml, LPS+ 10mg/kg Ticagrelor: 35.8±16.7x10⁴ cells/ml). Ticagrelor administration at 30mg/kg however, did reduce neutrophil recruitment, but this did not reach statistical significance (30mg/kg: 12.4±16.7x10⁴ cells/ml).

Given that P2Y₁, P2Y₁₂, P2Y₁₄, and P2X₁ receptors are all expressed on platelets, and platelet activation is necessary for pulmonary leukocyte recruitment in this model of inflammation [12,22,27,38], we further examined the effects of these receptors on platelet dynamics (*in vivo*) and platelet-neutrophil interactions (*in vitro*).

Peripheral thrombocytopenia occurs after intranasal instillation with LPS, and this is reversed after antagonism of either the P2Y₁ or P2Y₁₄ receptors.

Peripheral thrombocytopenia occurs during inflammation, perhaps because of localised recruitment [38]. Here, the intranasal administration of LPS lead to a significant decrease in circulating platelets at 4 hours compared to sham treated control mice (P<0.001, **Fig 2**). This thrombocytopenia induced by LPS administration was significantly reversed in mice administered either the P2Y₁ antagonist (P<0.001, **Fig 2**), or the P2Y₁₄ antagonist (P<0.01, **Fig 2**) compared to mice receiving LPS + vehicle. In contrast, the thrombocytopenia induced by LPS remained in mice administered either the P2Y₁₂ antagonist (P<0.001, **Fig 2**), or the P2X₁ antagonist (P<0.01, **Fig 2**) compared to sham treated control mice. These data suggested

platelet activation was modulated by P2Y₁ or P2Y₁₄ receptor activation in this model, whereas pulmonary neutrophil recruitment is dependent on platelet activation.

Platelet induced-leukocyte migration is mediated by the P2Y₁ and P2Y₁₄ receptors *in vitro*

P2Y₁ and P2Y₁₄ receptors are expressed systemically and are not restricted solely to the platelets surface [29,30,39]. We have previously used an *in vitro* model of platelet-induced leukocyte chemotaxis to better understand how platelets taken from allergen-sensitized mice and treated with P2Y₁ antagonists can modulate leukocyte motility and chemotaxis [26]. In non-allergic inflammation, we have used the same assay to assess how platelet P2Y₁ or P2Y₁₄ receptors can mediate leukocyte chemotaxis and correlate with P2Y₁ or P2Y₁₄ dependent neutrophil recruitment into tissue in response to LPS observed *in vivo*. Platelets and PMNs were isolated from the blood and bone marrow of mice. Platelets were subsequently incubated with either the P2Y₁ antagonist (MRS2500), P2Y₁₂ antagonist (AR-C66096) or the P2Y₁ antagonist (NF-279) prior to stimulation with 100nM ADP. Following incubation and stimulation with drugs or ADP, platelets were subsequently added to PMNs to stimulate their chemotaxis with 100nmol/L macrophage derived chemokine (MDC). In agreement with previous work [26], only the incubation of platelets with PMNs induced significant chemotaxis towards MDC (P < 0.001; **Fig 3A, B and C**; P < 0.001) compared to negative controls (PMNs alone, PMNs with MDC). Pre-incubation with the P2Y₁₂ antagonist AR-C66096 or the P2Y₁ antagonist NF-279 failed to modulate platelet induced chemotaxis of PMNs towards MDC (**Fig 3B and 3C**), which was in agreement with work previously published by the authors [26]. However, pre-incubation of platelets with the P2Y₁ antagonist MRS2500 induced a significant concentration-dependent inhibition of PMN chemotaxis towards MDC (P < 0.001, **Fig 3A**). As the P2Y₁₄ receptor antagonist (PPTN) is a prodrug and requires metabolism within the liver to

produce its active form, we investigated whether stimulation of platelets via P2Y₁₄ receptors could mimic the ADP (P2Y₁) –dependent platelet activation in this assay. Thus, the P2Y₁₄ agonist UDP-glucose, and a highly specific P2Y₁₄ agonist (MRS2690) were used to stimulate the platelet expressed P2Y₁₄. Following incubation and stimulation of platelets with these agonists, platelet-induced PMN chemotaxis was induced to MDC (100nmol/L) in a concentration dependent manner with 1000nmol/L UDP-glucose (P<0.05; **Fig 3D**) or 1000nM MRS2690 (P<0.05, **Fig 3E**). Levels of PMN chemotaxis were similar in these conditions to when platelets are stimulated with 100nmol/L ADP (P<0.05, **Fig 3D & 3E**).

Effects of P2Y₁, P2Y₁₂, P2Y₁₄ and P2X₁ antagonist administration on haemostatic measurements *in vivo* and *in vitro*.

Given the inability of either the P2Y₁₂ or P2X₁ antagonists to effect leukocyte recruitment in response to exposure to LPS, the biological activities of AR-C66096 (P2Y₁₂ antagonist) and NF-279 (P2X₁ antagonist) were assessed by *ex vivo* platelet aggregation assays, and *in vivo* bleeding time assays. This was done to confirm that the drugs were present at effective doses, and to compare with the P2Y₁ and P2Y₁₄ antagonists that did suppress LPS-induced pulmonary leukocyte recruitment. *Ex vivo* aggregation of platelets from mice administered LPS revealed that the possible activation of platelets by LPS had no effect on platelet aggregation towards ADP (10µmol/L) *per se* (**Fig 4A & 4B**), yet mice administered the P2Y₁₂ antagonist (AR-C66096) had a similarly significant reduction in platelet aggregation to those administered the P2Y₁ antagonist (MRS2500) (P<0.05, **Fig 4A & 4B**). However, we failed to demonstrate any effect of the P2X₁ antagonist or the P2Y₁₄ antagonist on platelet aggregation towards ADP *ex vivo*, as expected given the stimulus (**Fig 4A & 4B**). We therefore also investigated the effect of these antagonists on *in vivo* bleeding times. We demonstrated that LPS challenge had no

effect on bleeding times *per se* (**Fig 4C**). Administration of the P2Y₁ antagonist (MRS2500), the P2Y₁₂ antagonist (AR-C66096) or the P2X₁ antagonist (NF-279) demonstrated significant prolongation in bleeding time when compared to vehicle controls (P2Y₁: P < 0.05, P2Y₁₂: P < 0.05, P2X₁: P < 0.05, **Fig 4C**) which in addition to the attenuation of ADP induced *ex vivo* platelet activation confirms their biological activity at the tested doses. In contrast, administration of 10mg/kg PPTN (P2Y₁₄ antagonist) had no impact on bleeding times when compared to vehicle controls (**Fig 4C**) despite demonstrating attenuation of leukocyte recruitment following LPS challenge. Furthermore, whilst the P2Y₁₄ agonist UDP-glucose was able to stimulate platelet-induced PMN chemotaxis towards MDC *in vitro* (1 µmol/L, **Fig 3D**), the same concentration of this drug did not induce platelet aggregation *in vitro* (**Fig 4D**), further suggesting that P2Y₁₄ receptor activation does not affect haemostatic functions of platelets.

Discussion.

In this present study, we have shown that LPS induced pulmonary leukocyte recruitment in mice is dependent on P2Y₁ receptor activation, in a distinct model to our previous studies where we used a murine model of allergic airways inflammation [26]. Whilst this present study has revealed that antagonism of P2Y₁ receptors led to a suppression of inflammation and separately, effects on haemostasis, we have reported an apparent dichotomy in platelet P2Y₁ signalling in the context of inflammation compared to haemostasis [26,38]. Within the context of this study, it is apparent that the intranasal administration of LPS, which induced robust platelet dependent pulmonary neutrophil recruitment [12,38], did not affect bleeding time, or the magnitude of platelet aggregation in response to ADP *ex vivo*. These observations

give further weight to the hypothesis that platelet activation involved in inflammation and haemostasis are not necessarily linked [40].

For the first time, we reveal that P2Y₁₄ receptor activation can affect platelet function by stimulating platelet-induced PMN chemotaxis (*in vitro*), and in the context of platelet-dependent pulmonary neutrophil recruitment *in vivo*. However, all measured haemostatic parameters were not affected by P2Y₁₄ *in vivo*. Whilst the role of platelet purinergic receptors (P2X₁, P2Y₁, P2Y₁₂) in platelet aggregation has been well documented, P2Y₁₄ has an unknown significance, with various studies suggesting that classical platelet activation is not altered [22–25,33]. P2Y₁₄ receptors are coupled to the G protein G_i, and are activated by uridine diphosphate (UDP) and UDP-glucose [33]. Currently, signalling downstream of the P2Y₁₄ receptor remains to be elucidated in platelets. With respect to host defence, activation of purinergic receptors also induces selectin and integrin adhesion molecule expression on the surface of leukocytes [23,24,32], receptors known to be necessary for leukocyte chemotaxis [32,41,42]. Whilst it is not yet known whether P2Y₁₄ has any consequence with regards to platelet function (during haemostasis, or inflammation), P2Y₁₄ does promote RhoA-mediated signalling and chemotaxis in neutrophils [42]. Platelet shape change, filopodia formation, cytoskeletal changes and granule centralization are presumably events required to elicit platelet function during inflammatory events; nevertheless a role for Rho-GTPases in purine-induced platelet activation in the context of host defence is not yet known.

With similarities to our previous work, we were unable to provide evidence for a role for P2Y₁₂ and P2X₁ receptors in the context of LPS-induced inflammation, or P2Y₁₂ and P2X₁ dependent platelet function with respect to enhancement of neutrophil chemotaxis. We recognise that

other groups have reported a role for P2Y₁₂ receptors during a model of allergic inflammation [34], but this is perhaps contrary to other reports stating that LPS-induced systemic inflammation is actually more severe in P2Y₁₂ deficient mice [35], whilst also mediating LPS induced inflammation [36]. It is also interesting to note that the P2Y₁₂ antagonist ticagrelor may have anti-inflammatory characteristics due to effects on adenosine receptors other than P2Y₁₂ [43], and recently this drug has been reported to exhibit antagonism of P2Y₁₃ [39]. Thus, the non significant trend depicting inhibition of neutrophil recruitment at the highest dose of ticagrelor tested here (30mg/kg) might involve secondary off-target effects on additional receptors involved in the inflammatory response, since in both a model of allergic airway inflammation and in this present study investigating LPS-induced lung inflammation, no other P2Y₁₂ antagonist has displayed any potency as an anti-inflammatory agent [22]. Finally, despite our inability to show a role for P2X₁ receptors in either allergic or LPS-induced inflammation, it should be noted that other groups have separately reported a role for P2X₁ receptor activation in acute inflammatory events [40].

In conclusion, we have demonstrated a role for P2Y₁ and P2Y₁₄ receptor activation in a platelet-dependent LPS induced model of neutrophil recruitment in the mouse, and that these two receptor types stimulate platelet-induced neutrophil chemotaxis *in vitro*. Furthermore, the P2Y₁₄ dependent events did not affect haemostatic measurements, whilst P2Y₁₂ and P2X₁ receptors expressed on platelets did not modulate LPS-induced inflammation.

Acknowledgements

This study was financially supported by grants from the Sackler Foundation and Abcam to Professor Clive Page.

Author Contributions

RA, CP, SP were involved with the conception, hypotheses delineation, and design of the study. RA, SA, BGO, SC, JO, MI, SP, CP were involved with the acquisition of the data, analysis and interpretation of the data. SP wrote the article. All authors were involved with manuscript revision prior to submission. There are no conflicts of interest to declare.

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Figure legends

Figure 1: Effect of antagonism of purinergic receptors that are characteristically expressed on platelets on LPS induced lung inflammation. Mice were administered either vehicle, P2Y₁, P2Y₁₂, P2Y₁₄, or P2X₁ antagonists intravenously 20 minutes prior to the instillation of

1.25mg/kg LPS. 4 hours post challenge, broncho-alveolar lavages (BAL) were performed for the enumeration of total leukocytes counts (**A**); neutrophils (**B**); and lymphocytes (**C**). Data: means \pm SEM. n= 8-15 per group. *** P <0.001 vs sham+ vehicle, # P <0.05 vs LPS + vehicle, ## P<0.01 vs LPS + vehicle.

Figure 2: Effect of LPS and purinergic receptor antagonists on circulating peripheral platelet numbers. Mice were administered either vehicle, P2Y₁, P2Y₁₂, P2Y₁₄, or P2X₁ antagonists intravenously 20 minutes prior to the instillation of 1.25mg/kg LPS. 4 hours later, blood samples were taken via cardiac puncture for the enumeration of circulating platelet counts. Data: means \pm SEM. n= 4-8 per group. ** P <0.01 vs sham+ vehicle, *** P <0.001 vs sham+ vehicle, ## P <0.01 vs LPS + vehicle, ### P<0.001 vs LPS + vehicle.

Figure 3: Effect of P2Y₁, P2Y₁₂, P2Y₁₄ and P2X₁ on platelet-induced PMN chemotaxis. Platelets were isolated from mouse blood and leukocytes collected from femoral bone marrow. Platelets were treated with the P2Y₁ antagonist (MRS2500) (**A**), the P2Y₁₂ antagonist (AR-C66096) (**B**), or the P2X₁ antagonist (NF-279) (**C**), or UDP-glucose (P2Y₁ agonist) (**D**), or MRS2690 (P2Y₁ agonist) (**E**); and washed before being mixed with leukocytes. This cell mixture was added to the top well of a chemotaxis chamber consisting of 3 μ m pores. PMN chemotaxis towards MDC (bottom well 100nmol/L) was then quantified after a 90 minute incubation and analysed as a chemotactic index. n=8 (**A**), 7 (**B**), 4 (**C**), 3 (**D**), 4 (**E**). Data are expressed as mean \pm SEM. * P < 0.05, *** P < 0.001 vs column 3 (or 1 for **D & E**), or # P < 0.05, ### P < 0.01 vs positive control (black column).

Figure 4: Effect of purinergic receptor antagonism on haemostatic responses and platelet aggregation. Mice were administered either vehicle, P2Y₁, P2Y₁₂, P2Y₁₄, or P2X₁ antagonists intravenously 20 minutes prior to the instillation of 1.25mg/kg LPS. 1hour post challenge, blood samples were collected via cardiac puncture to assess platelet aggregation towards 10µmol/L ADP *ex vivo*. An example aggregation trace is shown (**A**), and group % aggregation (**B**). In other experiments, bleeding time was assessed in anaesthetised mice at 1 hour post LPS challenge (**C**). In separate experiments, cardiac bleeds were undertaken from naive mice and platelets harvested for assessment of aggregation to ADP and UDP-glucose (**D**) Data: means ± SEM. n= 4 (**B**), 3-4 (**C**), 4 (**D**). * P <0.01 vs LPS+ vehicle (white column in **D**).

Figure 1

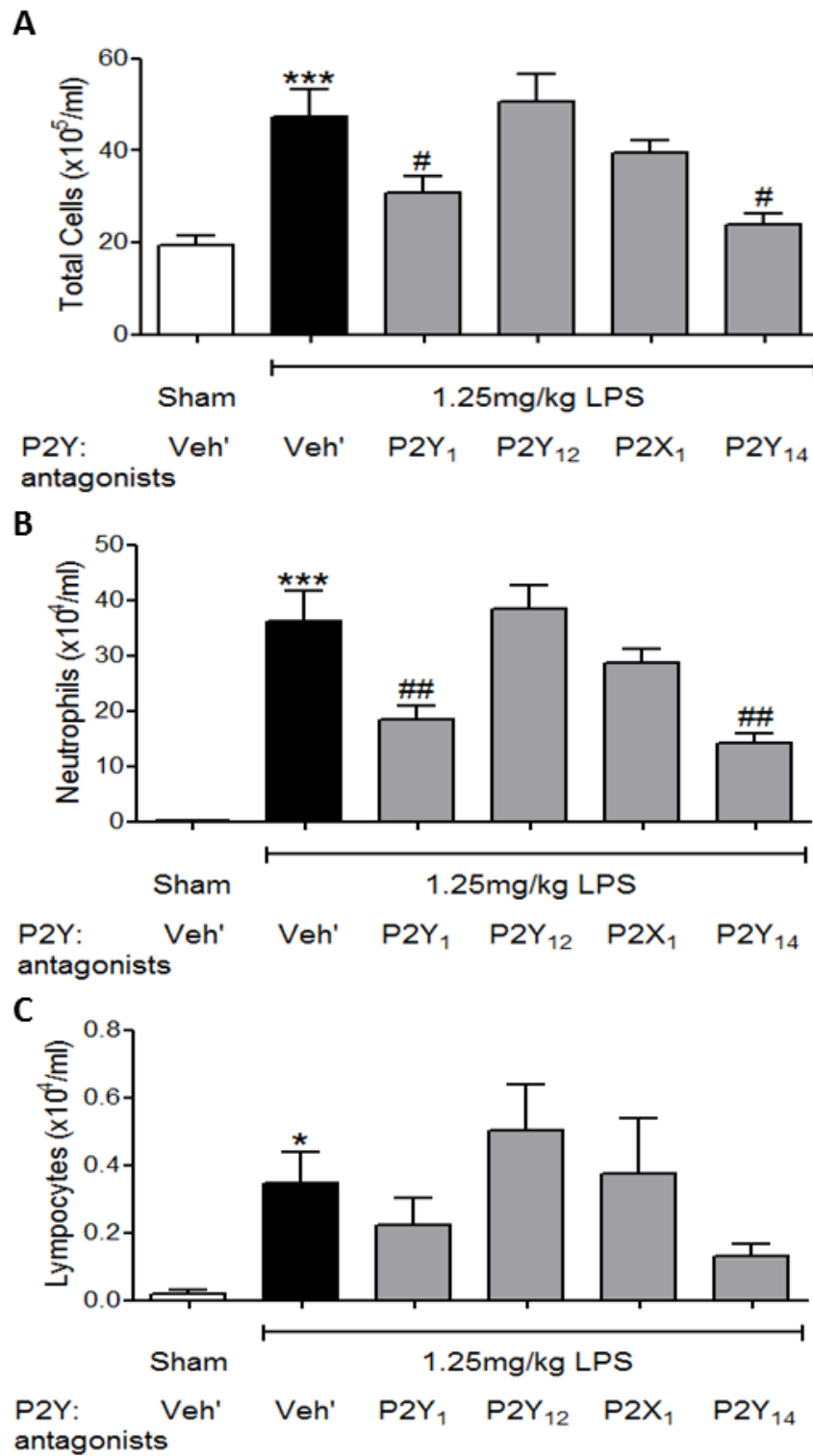


Figure 2

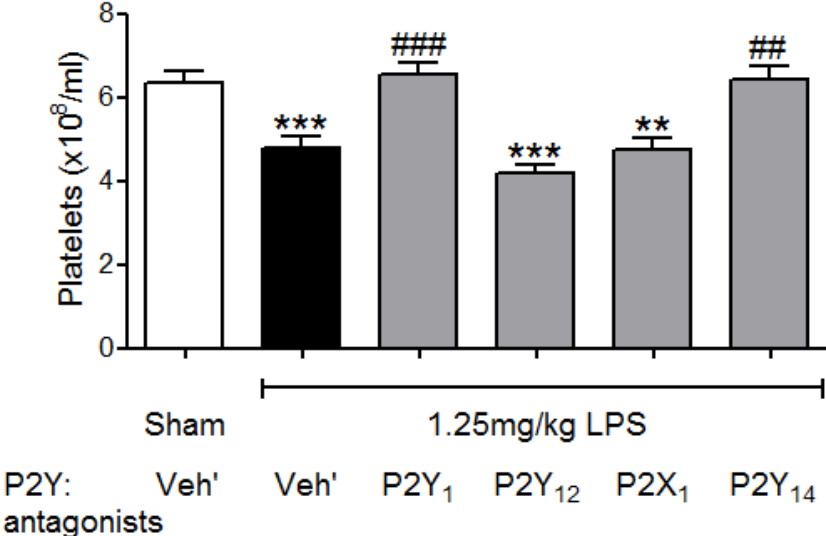


Figure 3

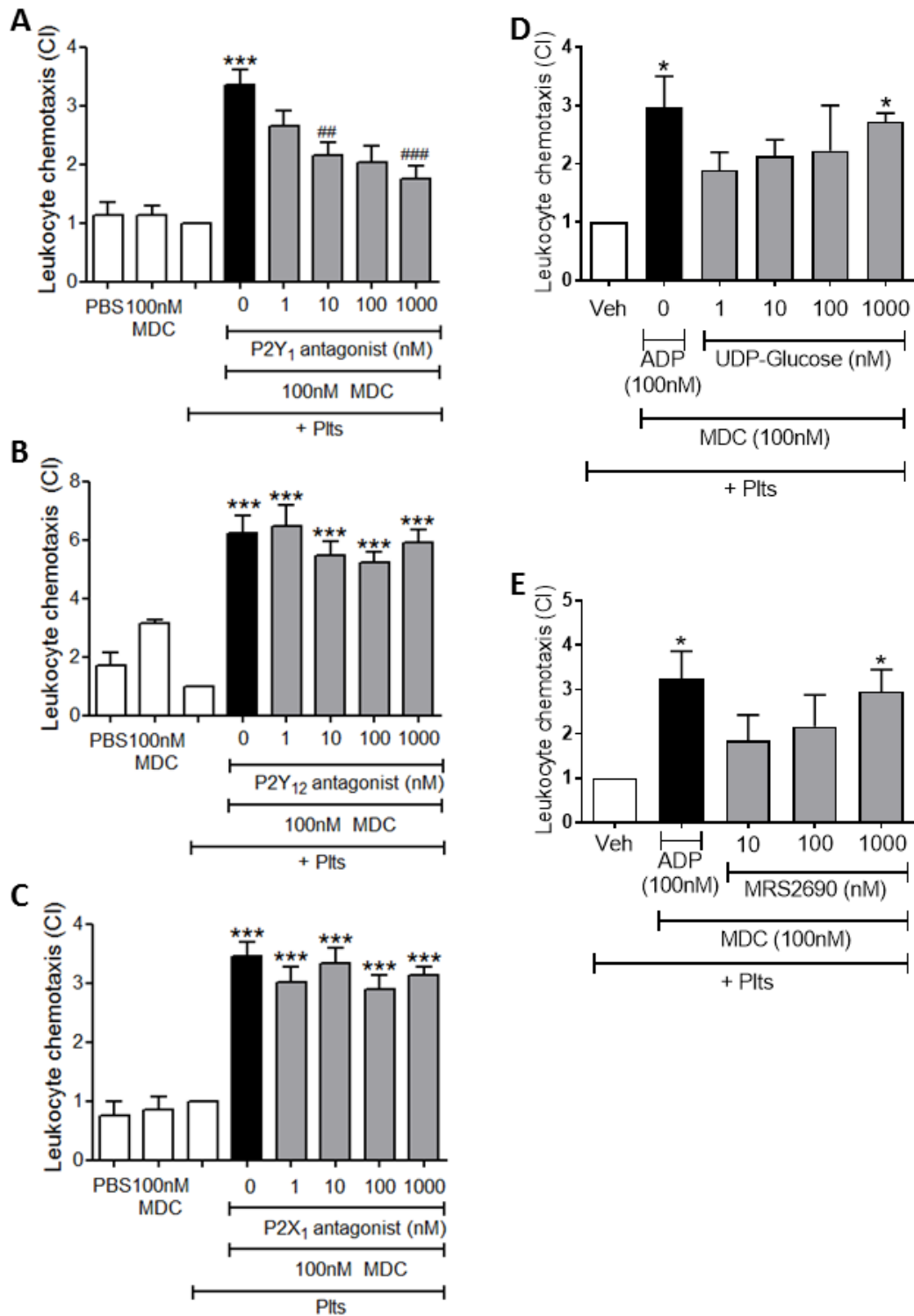


Figure 4

