

RhoA signaling through platelet P2Y₁ receptor controls leukocyte recruitment in allergic mice

Richard T. Amison, MSci,^a Stefania Momi, PhD,^b Abigail Morris, PhD,^a Giorgia Manni, BSc,^b Sandra Keir, PhD,^a Paolo Gresele, MD,^b Clive P. Page, PhD,^a and Simon C. Pitchford, PhD^a *London, United Kingdom, and Perugia, Italy*

Background: Clinical studies reveal platelet activation in patients with asthma, allergic rhinitis, and eczema. This is distinct from platelet aggregation, which is critical for the maintenance of hemostasis and in which a role for platelet purinergic receptors is well documented. However, purines are also essential for inflammatory cell trafficking in animal models of allergic lung inflammation, which are known to be platelet dependent, yet the role of purines in the platelet activation accompanying inflammation is unknown.

Objectives: We investigated whether the involvement of purine activation of platelets during allergic inflammation is distinct from purine involvement in platelet aggregation.

Methods: BALB/c mice were sensitized to ovalbumin and subsequent airway ovalbumin challenge. Bronchoalveolar lavage fluid was analyzed for inflammatory cells, and blood samples were assessed for platelet activation. The role of platelet purinergic receptors and associated signaling mechanisms (RhoA) were assessed.

Results: P2Y₁, but not P2Y₁₂ or P2X₁, antagonism inhibited pulmonary leukocyte recruitment. The formation of platelet-leukocyte complexes *in vivo* and platelet/P-selectin-dependent polymorphonuclear cell migration *in vitro* were exclusively platelet P2Y₁ receptor dependent. Furthermore, platelet P2Y₁ activation resulted in RhoA activity *in vivo* after allergen challenge, and RhoA signaling in platelets through P2Y₁ stimulation was required for platelet-dependent leukocyte chemotaxis *in vitro*. Leukocyte recruitment in thrombocytopenic mice remained suppressed after reinfusion of platelets pretreated with a P2Y₁ antagonist or a Rho-associated kinase 1 inhibitor, confirming the crucial role of platelet P2Y₁ receptor and subsequent activation of RhoA.

Conclusion: RhoA signaling downstream of platelet P2Y₁, but not P2Y₁₂, represents a clear dichotomy in platelet activation during allergic inflammation versus hemostasis. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Platelets, P2Y₁, P2Y₁₂, P2X₁, allergic inflammation, RhoA

Abbreviations used

ADP: Adenosine diphosphate
IC₅₀: Inhibitory concentration of 50%
K_d: Dissociation constant
MDC: Macrophage-derived chemokine
OVA: Ovalbumin
PGE₁: Prostaglandin E₁
PMN: Polymorphonuclear cell
ROCK: Rho-associated kinase 1

The phenomenon of platelet activation in allergic patients, especially those with asthma, has been reported extensively over the last 30 years.¹⁻¹⁹ Significantly, platelet activation, as measured by changes in platelet volume, presence of platelet microparticles, and release of platelet-specific inflammatory mediators³⁻¹³; pulmonary platelet recruitment and accumulation in the lungs^{6,14,15}; and an increased incidence of circulating platelet-leukocyte (including eosinophil) complexes,^{16,17} has been recorded in asthmatic patients after spontaneous asthma attacks or allergen challenge. Furthermore, platelet activation correlates strongly to changes in lung function.^{3,5,6,10,11,17} Of pertinence to allergy, platelets from patients allergic to *Dermatophagoides pteronyssinus* have been shown to be activated by the allergen or by synthetic peptides derived from the allergen through a process mediated by IgE.^{18,20} Therefore these studies argue that platelet activation in the context of asthma and allergy is not a consequence of the inflammatory mediator milieu *per se* but rather that platelets are integral to allergic and IgE-dependent processes. The significance of platelet activation in patients with asthma and allergy has been assessed *in vivo* by using animal models of pulmonary allergic inflammation, where it has been demonstrated that (1) platelets are activated and migrate to the lungs immediately after exposure to allergen,^{18,21} (2) platelets are required for initiation of bronchial hyperresponsiveness in 3 animal species,^{4,14,22-26} (3) the pulmonary recruitment of leukocytes is platelet expressed and platelet selectin (P-selectin) dependent,^{4,16,22-25,27,28} and (4) platelets modulate airway remodeling.^{29,30} Nevertheless, the mechanisms by which platelets become activated in the context of allergic inflammation are not known, and despite heightened platelet activation being a hallmark of asthma, such patients have a mild hemostatic defect rather than an increased risk of thrombosis, which might be expected as a result of heightened platelet activation.^{6,19,31}

The role of platelet purinergic receptors (P2X₁, P2Y₁, and P2Y₁₂) in platelet aggregation has been well documented.³²⁻³⁵ Significantly, several studies have also detailed that activation of both the P2Y₁ and P2Y₁₂ receptors on platelets triggers an increase in surface P-selectin expression,^{32,35,36} suggesting that purinergic activation of platelets might also be involved in P-selectin-dependent inflammatory responses.^{24,25,27} Indeed, a

From ^athe Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, and ^bthe Department of Medicine, Section of Internal and Cardiovascular Medicine, University of Perugia.

Supported by the BBSRC (grant no. BB/H015728/1) and the Sackler Foundation and a grant from the Italian Ministry of University and Research (MIUR prot. 2012773NE3; to P.G.).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication January 27, 2014; revised July 21, 2014; accepted for publication September 19, 2014.

Corresponding author: Simon C. Pitchford, PhD, Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, Rm 5.91, Franklin Wilkins Building, Waterloo Campus, King's College London, London SE9 1NH, United Kingdom. E-mail: simon.pitchford@kcl.ac.uk.

0091-6749/\$36.00

© 2014 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2014.09.032>

role for purines has recently been described in the context of allergic inflammation.^{28,37} However, the importance of purines in the context of platelet activation during allergic inflammatory events remains unclear given the apparent incongruence of an associated mild hemostatic defect rather than increased thrombosis in patients with asthma.^{6,19,31} Therefore the aim of the present study was to characterize the involvement of platelet P2Y₁, P2Y₁₂, and P2X₁ receptors in leukocyte recruitment in a murine model of allergic inflammation, which is known to be platelet P-selectin dependent.

METHODS

Reagents used and details of the receptor pharmacology for the purinergic antagonists used in this study can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org. The methods outlined below appear in the order in which the experiments are described in the Results section.

Mouse ovalbumin sensitization and bronchoalveolar lavage

A murine model of allergic lung inflammation was established to deduce the importance of purinergic receptors on pulmonary leukocyte recruitment. All studies were carried out under the Animals (Scientific Procedures) Act of 1986 (United Kingdom) or the Italian Ministry of Public Health (authorization no. 260/2011) with local ethical approval from King's College, London, or the University of Perugia. Female BALB/c mice (20–25 g; Harlan UK, Bicester, United Kingdom) were sensitized with chicken egg ovalbumin (OVA; 30 µg/0.4 mL administered intraperitoneally) on days 0, 4, and 10. On days 14, 15, and 16, selected groups of mice were dosed with apyrase (100 U/mL, 50 µL per mouse administered intranasally), MRS2179 (1, 10, and 30 mg/kg administered intravenously), NF-279, MRS2500, or AR-C66096 (all at 0.1, 1, and 3 mg/kg administered intravenously); or MRS2395 (1, 3, and 10 mg/kg administered intravenously) 20 minutes before the start of allergen challenge. Clopidogrel (75 mg/kg by mouth) was administered for 5 days in total before the first day of allergen challenge.³⁸ Saline was used as a vehicle for all drugs other than MRS2395 (0.1% dimethyl sulfoxide). The highest drug doses were selected in excess of the reported activity of doses in rodents. Mice were subsequently nebulized with OVA (30 mg/mL) for 30 minutes. Twenty-four hours after the final allergen challenge (day 17), bronchoalveolar lavage fluid was collected and processed for total and differential cell counts, as previously described.^{16,27}

Platelet aggregation

Blood from mice was collected by means of cardiac puncture 1 or 6 hours after allergen challenge and washed platelets were isolated to confirm the biological activity of P2Y₁₂ antagonists used in the “Mouse ovalbumin sensitization and bronchoalveolar lavage” section. Platelet aggregation was measured after stimulation with 2 to 10 µmol/L adenosine diphosphate (ADP).

Flow cytometric analysis of platelet-leukocyte conjugation

Citrated blood was obtained by means of saphenous vein bleeds at 6 and 24 hours after allergen challenge and was analyzed for the presence of platelet-leukocyte aggregates to detect the activation of platelets and the influence of P2Y₁ and P2Y₁₂ antagonists in the “Mouse ovalbumin sensitization and bronchoalveolar lavage” section. Blood was incubated with an anti-CD41–phycoerythrin mAb, and leukocytes were labeled with an anti-CD45–fluorescein isothiocyanate mAb in saturating concentrations for 30 minutes, followed by red blood cell lysis with 500 µL of Optilyse C (Beckman Coulter, Fullerton, Calif) for 1 hour, and samples were analyzed on a flow cytometer (Epics XL; Beckman Coulter), as previously described.^{16,27}

Preparation of cell extracts and Western blots for detection of RhoA activity

Washed platelets were isolated from citrated blood 6 hours after the first allergen exposure outlined in the “Mouse ovalbumin sensitization and bronchoalveolar lavage” section to detect RhoA activity and the influence of P2Y₁ and P2Y₁₂ on these signaling events. Platelets were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethanesulfonyl fluoride, and 1 mmol/L Na₂VO₄) for 30 minutes on ice. Equal amounts of protein (30 µg) were separated by using 12% SDS-PAGE and immunoblotting, followed by enhanced chemiluminescence detection with either an anti-total RhoA antibody or anti-phosphorylated RhoA antibody (Ser188) and anti-rabbit horseradish peroxidase, as previously described.³⁹ Activated RhoA (RhoA–guanosine triphosphate) was selectively assayed with the Abcam RhoA Activation Assay Kit (Abcam, Cambridge, United Kingdom), according to the manufacturer's instructions. Platelets were isolated 1 hour after the first allergen challenge. A lysate of platelets activated with ADP 20 µmol/L was run as a positive control.

Platelet-induced leukocyte chemotaxis assay

An *in vitro* assay of platelet-dependent leukocyte chemotaxis was used to elucidate the importance of purinergic receptors expressed on platelets on subsequent leukocyte activation. Chemotaxis plates (3-µm pore size) were blocked with Dulbecco modified Eagle medium plus 2% FBS for 1 hour before blocking buffer was removed, and 100 nmol/L macrophage-derived chemokine (MDC) was added to each well. Washed platelets (1×10^8 platelets/mL by using the above protocol) were incubated with either NF-279, MRS2179, MRS2395, MRS2500, or AR-C66096 for 10 minutes at room temperature. In some experiments platelets were incubated with the P2Y₁ receptor agonist MRS2365 (in saline) and the Rho-associated kinase 1 (ROCK) inhibitor GSK429286 (in 0.1% dimethyl sulfoxide) at 1 and 100 µmol/L, respectively. Platelets and drug were subsequently incubated with 100 nmol/L ADP for 5 minutes at 37°C and then washed. Leukocytes were flushed from bone marrow (femurs) of mice and resuspended in assay buffer (5×10^6 cells/mL). Leukocytes were added at a 1:1 volume ratio with platelets. The chemotaxis assay was then performed, as previously described.⁴⁰ Analysis revealed the population of migrated leukocytes to consist of greater than 95% polymorphonuclear cells (PMNs).

Busulfan induced platelet depletion and *ex vivo* drug treatment of platelets for reinfusion

By using the sensitization procedure explained in the “Mouse ovalbumin sensitization and bronchoalveolar lavage” section, the importance of platelet P2Y₁ and P2Y₁₂ on pulmonary leukocyte recruitment was assessed. Some groups of mice received busulfan (20 mg/kg administered intraperitoneally on days –5, –3, and 0 of the immunization protocol), a bone marrow-specific toxin, at a dose titrated to selectively target megakaryocyte maturation, resulting in gradual and pronounced depletion of circulating platelets, as previously stated.^{16,18,27,29} In some experiments blood was taken from separate OVA-sensitized platelet donor mice to harvest platelet-rich plasma. The platelet-rich plasma was incubated with 100 µmol/L MRS2500, AR-C66096, GSK429286, or vehicle for 30 minutes. Platelet suspensions were then washed free of remaining drug by means of centrifugation at 800g for 7 minutes in the presence of prostaglandin E₁ (PGE₁), and the platelet pellet was resuspended in PBS before infusion into busulfan-treated mice (0.1 mL administered intravenously, 1.5×10^8 platelets per injection) 15 minutes before the first 2 days of allergen challenge.

Statistical analysis

Data from allergic animal studies are displayed as means ± SEMs, whereas *in vitro* chemotaxis data are displayed as a chemotactic index. Data were analyzed by means of 1-way ANOVA, followed by the Dunnett multiple comparison posttest. A *P* value of less than .05 was considered significant.

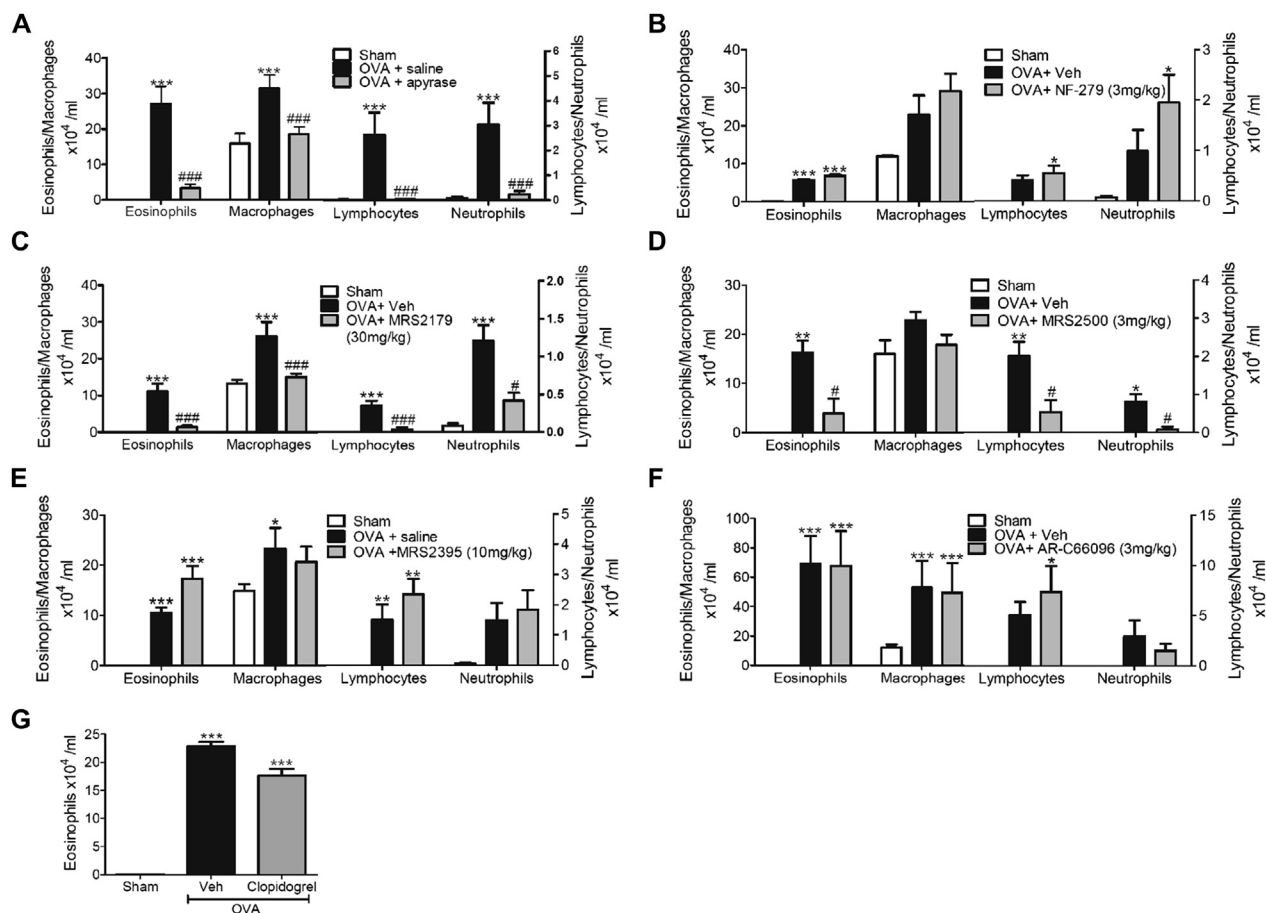


FIG 1. Effects of purinergic receptor antagonism on pulmonary leukocyte recruitment. Bronchoalveolar lavage fluid was analyzed from OVA-sensitized and challenged mice and pretreated with apyrase (**A**), P2X₁ antagonist (NF-279; **B**), P2Y₁ antagonist (MRS2179; **C**), P2Y₁ antagonist (MRS2500; **D**), P2Y₁₂ antagonist (MRS2395; **E**), P2Y₁₂ antagonist (AR-C66096; **F**), or P2Y₁₂ antagonist (clopidogrel; **G**) for differential cell counts ($n = 4-7$). Data are expressed as mean cell counts per milliliter of bronchoalveolar lavage fluid $\pm$ SEM. * $P < .05$, ** $P < .01$, and *** $P < .001$ versus the sham-sensitized control group. # $P < .05$ and ### $P < .001$ versus the OVA-sensitized control group.

RESULTS

ADP activation of P2Y₁, but not P2Y₁₂, is critical for pulmonary leukocyte recruitment in a murine model of allergic inflammation

Purines have been reported to induce the expression of selectins and integrins on the surfaces of platelets and leukocytes^{32,34,35} and are necessary for leukocyte chemotaxis.^{41,42} Therefore it is feasible that purines are involved in the pulmonary recruitment of leukocytes during allergic inflammation.^{28,37} OVA-sensitized mice were administered apyrase (100 U/mL) before each allergen challenge to test this hypothesis, which significantly attenuated pulmonary eosinophil, neutrophil, macrophage, and lymphocyte recruitment compared with that seen in vehicle-treated, OVA-sensitized and challenged mice ($P < .001$ for each leukocyte population; Fig 1, A, and see Fig E1, in this article's Online Repository at www.jacionline.org). Because apyrase hydrolyses both ATP and ADP into AMP, we specifically investigated the role of P2Y₁, P2Y₁₂, and P2X₁ receptors expressed on the surface of platelets, given the requirement for platelets in this murine model.^{16,27} The administration of the suramin analogue and highly selective P2X₁ channel antagonist NF-279 (0.1, 1, and

3 mg/kg administered intravenously) did not inhibit pulmonary leukocyte recruitment (Fig 1, B, and see Fig E1, B). However, the treatment of mice with the highly selective and competitive P2Y₁ antagonists MRS2179 (3, 10, and 30 mg/kg) or MRS2500 (0.1, 1, and 3 mg/kg) caused significant dose-dependent inhibition of leukocyte recruitment (see Fig E1, C). Specifically, the P2Y₁ antagonists inhibited eosinophil (MRS2179: 30 mg/kg, $P < .001$; MRS2500: 3 mg/kg, $P < .05$), macrophage (MRS2179: 30 mg/kg, $P < .05$), lymphocyte (MRS2179: 30 mg/kg, $P < .001$; MRS2500: 3 mg/kg, $P < .05$), and neutrophil (MRS2179: 30 mg/kg, $P < .05$; MRS2500: 3 mg/kg, $P < .05$) recruitment into the lungs compared with that seen in vehicle-treated, OVA-sensitized and challenged mice (Fig 1, C and D). In contrast, administration of selective P2Y₁₂ antagonists (the biphosphate acyclic analogue MRS2395, the adenosine triphosphate analogue AR-C66096, or the thienopyridine clopidogrel) was unable to inhibit pulmonary leukocyte recruitment at any dose tested (see Fig E1, E-G) or any particular leukocyte subpopulation (Fig 1, E-G). Given the inability of these P2Y₁₂ antagonists to modulate pulmonary leukocyte recruitment after allergen challenge, to check that these drugs were present at

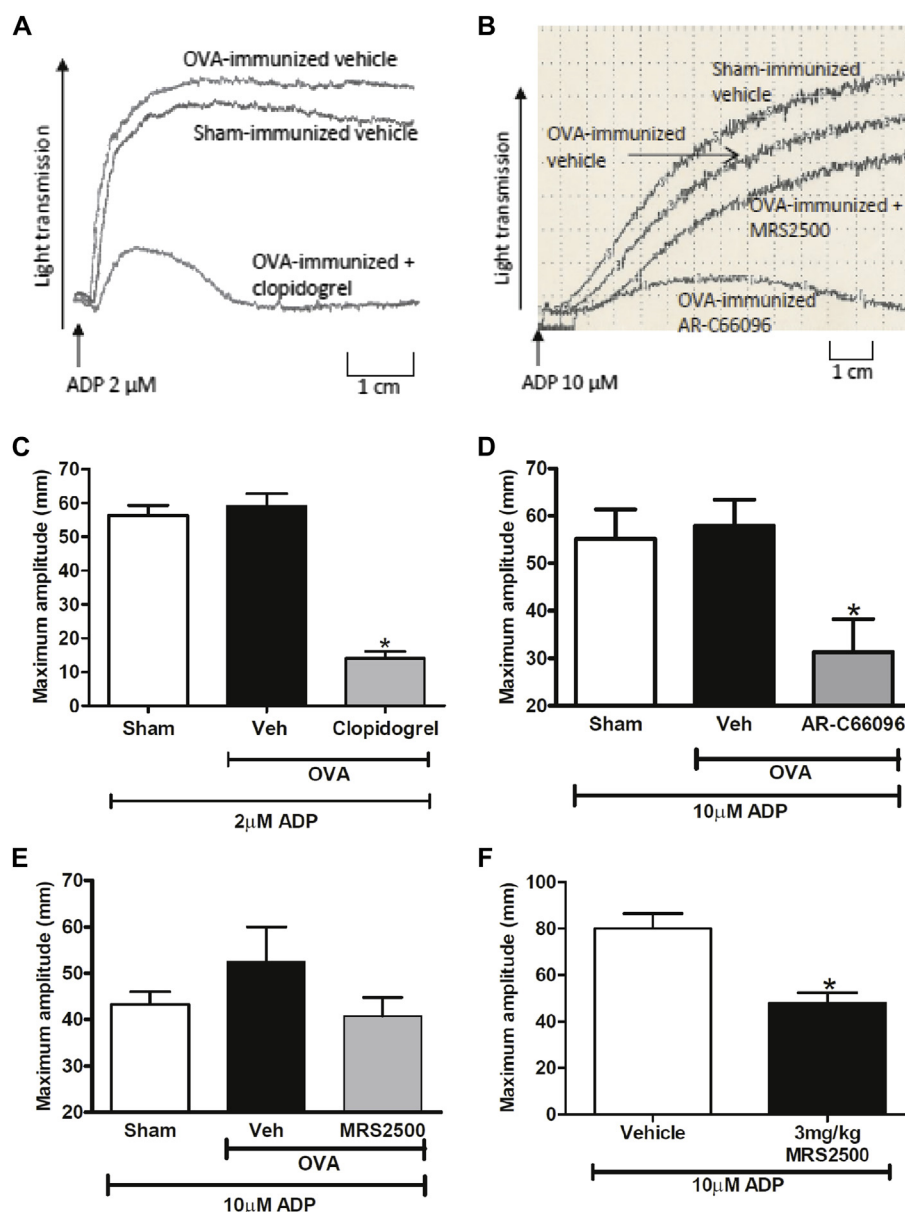


FIG 2. Effects of purinergic receptor antagonism on pulmonary platelet aggregation *ex vivo*. Platelets were isolated from OVA-sensitized mice pretreated with P2Y₁ antagonist (MRS2500), P2Y₁₂ antagonist (AR-C66096), or P2Y₁₂ antagonist (clopidogrel) 6 hours after allergen exposure, and *ex vivo* platelet aggregation to ADP was measured. Representative platelet aggregation traces to clopidogrel (A), AR-C66096 or MRS2500 (B), mean platelet aggregation to clopidogrel (C), AR-C66096 (D), MRS2500 (E), or MRS2500 (F) at 1 hour (n = 5-8). Data are expressed as means $\pm$ SEMs. **P* < .05 versus OVA-sensitized control groups.

effective doses, we tested the biological activity of AR-C66096 (3 mg/kg administered intravenously) and clopidogrel (75 mg/kg, 5 days by mouth) by using an *ex vivo* platelet aggregation assay and showed significant suppression of platelet aggregation to ADP 6 hours after allergen challenge (AR-C66096: *P* < .05 and clopidogrel: *P* < .05 vs the vehicle-treated control group; Fig 2, A-D). Inhibition of platelet aggregation induced by ADP *ex vivo* from mice administered the P2Y₁ antagonist MRS2500 (3 mg/kg administered intravenously) was modest at 6 hours (Fig 2, E) but more pronounced at an earlier time point (1 hour; Fig 2, F).^{43,44} Additionally, there was no evidence of increased platelet aggregation to ADP of platelets taken from OVA-sensitized mice compared with those from sham-sensitized

mice (Fig 2, A-E). Thus the biological actions of P2Y₁, P2Y₁₂, and P2Y₁ receptors differed remarkably in their ability to affect pulmonary leukocyte recruitment compared with platelet aggregation *ex vivo*. We next determined whether P2Y₁ receptors were involved in the process of platelet/leukocyte interactions observed after allergen exposure in allergen-challenged mice.

ADP activation of P2Y, but not P2Y₁₂, induces the formation of circulating platelet-leukocyte complexes after allergen challenge

Circulating platelet-leukocyte complexes that “prime” leukocytes for efficient adhesion to the vascular endothelium

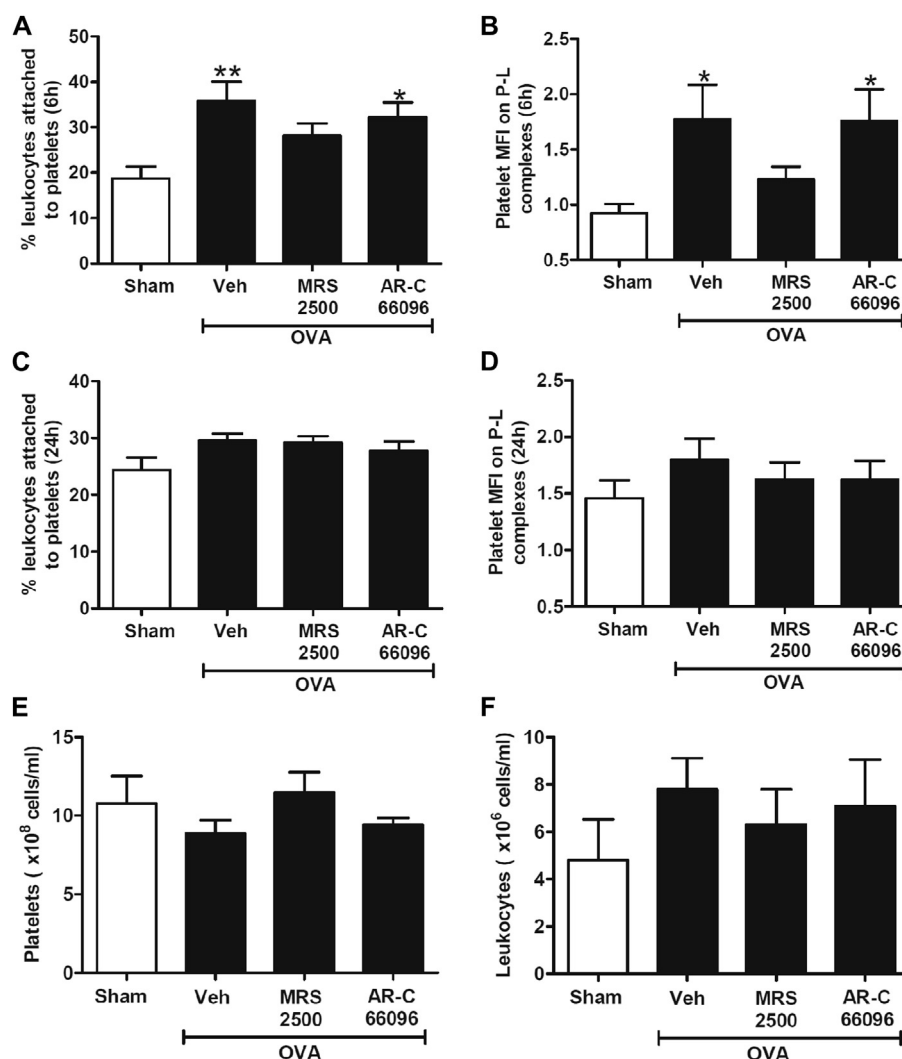


FIG 3. Effects of P2Y₁ and P2Y₁₂ receptor antagonism on the incidence of platelet-leukocyte complexes and circulating platelet and leukocyte numbers. **A-D**, Blood from sham- or OVA-sensitized mice treated with P2Y₁₂ antagonist (AR-C66096) or P2Y₁ antagonist (MRS2500) was quantified for the incidence (as a percentage) of platelet-leukocyte complexes (Fig 3, A and C) and the mean number of platelets attached per leukocyte (mean fluorescence intensity; Fig 3, B and D) at 6 (Fig 3, A and B) and 24 (Fig 3, C and D) hours after allergen challenge. **E** and **F**, Circulating platelet (Fig 3, E) and leukocyte (Fig 3, F) numbers (n = 6-11). Data are expressed as means $\pm$ SEMs. **P* < .05 and ***P* < .01 versus the sham-sensitized control group. P-L, Platelet-leukocyte.

are increased after allergen challenge in asthmatic patients and also in this platelet P-selectin-dependent model of allergic inflammation.^{16,27} Therefore we investigated the occurrence of these complexes as a measure of platelet activation in an inflammatory context after the administration of either P2Y₁ or P2Y₁₂ antagonists. Six hours after allergen challenge, there was an increase in circulating platelet-leukocyte complexes (*P* < .01) and the relative number of platelets attached to CD45⁺ leukocytes (CD41 mean fluorescence intensity on CD45⁺ events, *P* < .05) in blood taken from OVA-sensitized mice compared with that from sham-sensitized mice. The increase in circulating platelet-leukocyte complexes after allergen challenge was suppressed in OVA-sensitized mice administered a P2Y₁ antagonist (MRS2500: 3 mg/kg administered intravenously) but not a P2Y₁₂ antagonist (AR-C66096: 3 mg/kg administered intravenously; Fig 3, A and B), returning

to baseline 24 hours after allergen challenge (Fig 3, C and D). These data suggest that P2Y₁ activation of platelets occurred during the inflammatory response to allergen.

Because both P2Y₁ and P2Y₁₂ activation of platelets have been shown to be capable of inducing platelet P-selectin expression, the observation that only P2Y₁ antagonism reduced circulating platelet-leukocyte complexes after allergen challenge was unexpected. Therefore we assessed whether circulating platelet and leukocyte numbers in mice 6 hours after allergen challenge were affected by administration of either P2Y₁ or P2Y₁₂ antagonists. Neither administration of a P2Y₁ nor a P2Y₁₂ antagonist affected these hematologic values. Thus the suppression of platelet-leukocyte complex formation by the P2Y₁ antagonist (MRS2500) was not a result of changes to thrombopoiesis (Fig 3, E) or hematopoiesis (Fig 3, F).

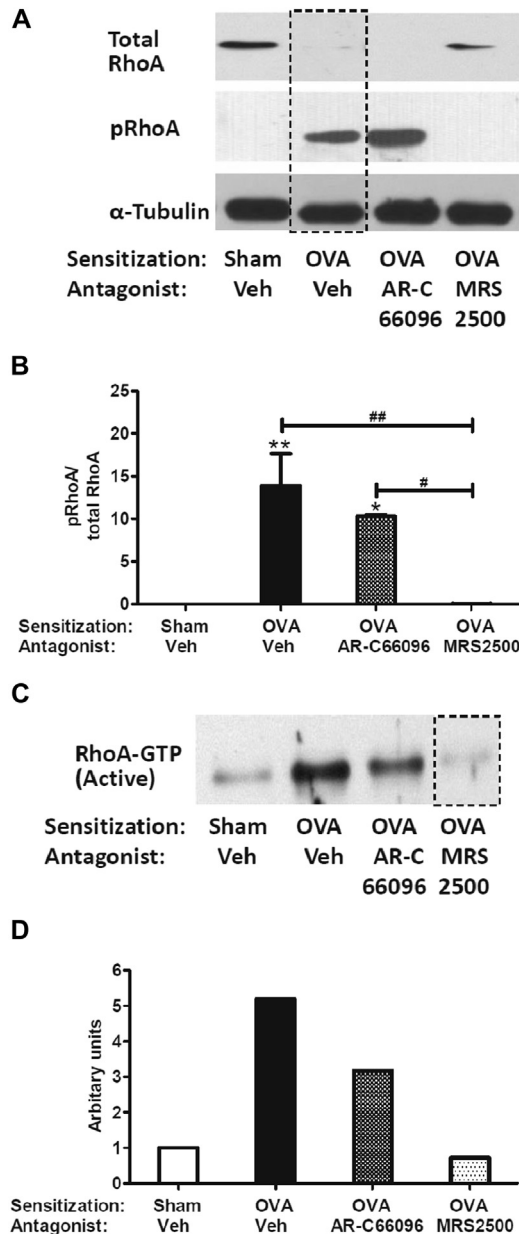


FIG 4. Allergen sensitization and challenge increase platelet RhoA activity *in vivo*. **A**, Washed platelets from OVA-sensitized and challenged mice administered P2Y₁ and P2Y₁₂ antagonists were analyzed for total RhoA, phosphorylated RhoA (pRhoA), and α-tubulin (as reference protein) 6 hours after allergen exposure and identified by means of Western blotting (representative shown), with densitometric analysis of the pRhoA/total RhoA ratio shown below (**B**). **C**, Western blot of active RhoA 1 hour after allergen exposure in washed platelets isolated from OVA-sensitized mice and with P2Y₁ or P2Y₁₂ antagonist administration, with densitometric analysis shown below (**D**). Representative columns from OVA-sensitized mice administered vehicle or P2Y₁ antagonist have been juxtaposed adjacent to the relevant left-hand columns from the same Western blot to remove repeated treatment columns or to reformat columns into a logical order and therefore to condense the image, as indicated by the dashed line, by using Microsoft Office and Powerpoint software (Fig 4, A and C). Data were produced from 4 mice per group (Fig 4, A and B), and pooled platelets were produced from 4 mice (Fig 4, C and D). Data are expressed as means ± SEMs. **P* < .05 and ***P* < .01 versus the sham-sensitized control group or #*P* < .05 and ##*P* < .01, where indicated.

Allergen sensitization and exposure induces RhoA activity in platelets through P2Y₁-dependent mechanisms

A downstream signaling pathway of the platelet P2Y₁ receptor is the GTPase RhoA. RhoA induces platelet shape change, filopodia formation, cytoskeletal changes, and granule centralization (in preparation for secretion), but RhoA activation does not lead to full platelet aggregation in response to ADP or other primary agonists.^{45,46} Similar processes in leukocytes are necessary for cell adhesion, and in conjunction with other GTPases (Rac1 and cdc42), RhoA coordinates cell movement.^{47–49} Here we show that total RhoA levels are significantly reduced in platelets taken from allergen-sensitized mice 6 hours after allergen challenge, a phenomenon replicated in allergen-sensitized mice administered a P2Y₁₂, but not a P2Y₁, antagonist (Fig 4, A). In contrast, phosphorylation at the serine residue 188 (Ser188) of RhoA in platelets is induced after allergen challenge and was selectively suppressed in mice administered a P2Y₁, but not a P2Y₁₂, antagonist (Fig 4, A). These data suggested that initial activation of the RhoA signaling pathway had occurred in allergen-challenged mice and that it was suppressed in mice administered a P2Y₁ antagonist, leading to subsequent inactivation (phosphorylation) 6 hours after allergen exposure.

We then measured active RhoA levels at earlier time point after allergen exposure (1 hour), confirming an active RhoA presence in platelets taken from allergen-sensitized and challenged mice and from P2Y₁₂ antagonist-treated mice but not in allergen-sensitized and challenged mice administered a P2Y₁ antagonist, in which active RhoA levels were similar to that found in sham-sensitized mice (Fig 4, B). Therefore we next investigated the importance of RhoA signaling downstream of platelet P2Y₁ receptor activation by ADP on leukocyte responses *in vitro*.

RhoA signaling downstream of P2Y₁, but not P2Y₁₂, receptor activation on platelets is critical in platelet-induced leukocyte chemotaxis toward MDC, a P-selectin-dependent process

The formation of platelet-leukocyte complexes after allergen challenge is P-selectin dependent and affected by P2Y₁ signaling (Fig 3). We hypothesized that this engagement of platelets with leukocytes *in vivo* and the dependency on P2Y₁ receptor activation might stimulate leukocyte adhesion molecule expression and increase motility to enhance their recruitment into tissues. We have previously reported that PMNs require the presence of platelets to undergo chemotaxis in response to the chemokines MDC or thymus and activation-regulated chemokine, which are known to be highly expressed in the lungs of asthmatic patients.^{40,50} Because MDC activates platelets through an ADP-dependent process,⁵¹ the ability of RhoA signaling downstream of platelet P2Y₁ receptor activation to induce the activation and motility of PMNs and the dependency of this chemotactic process on platelet P-selectin expression were also investigated.

Platelets and PMNs were isolated from the blood and bone marrow of allergen-sensitized mice. Platelets were incubated with either P2Y₁ antagonists (MRS2179 or MRS2500) or P2Y₁₂ antagonists (MRS2395 or AR-C66096) or a P2X₁ antagonist

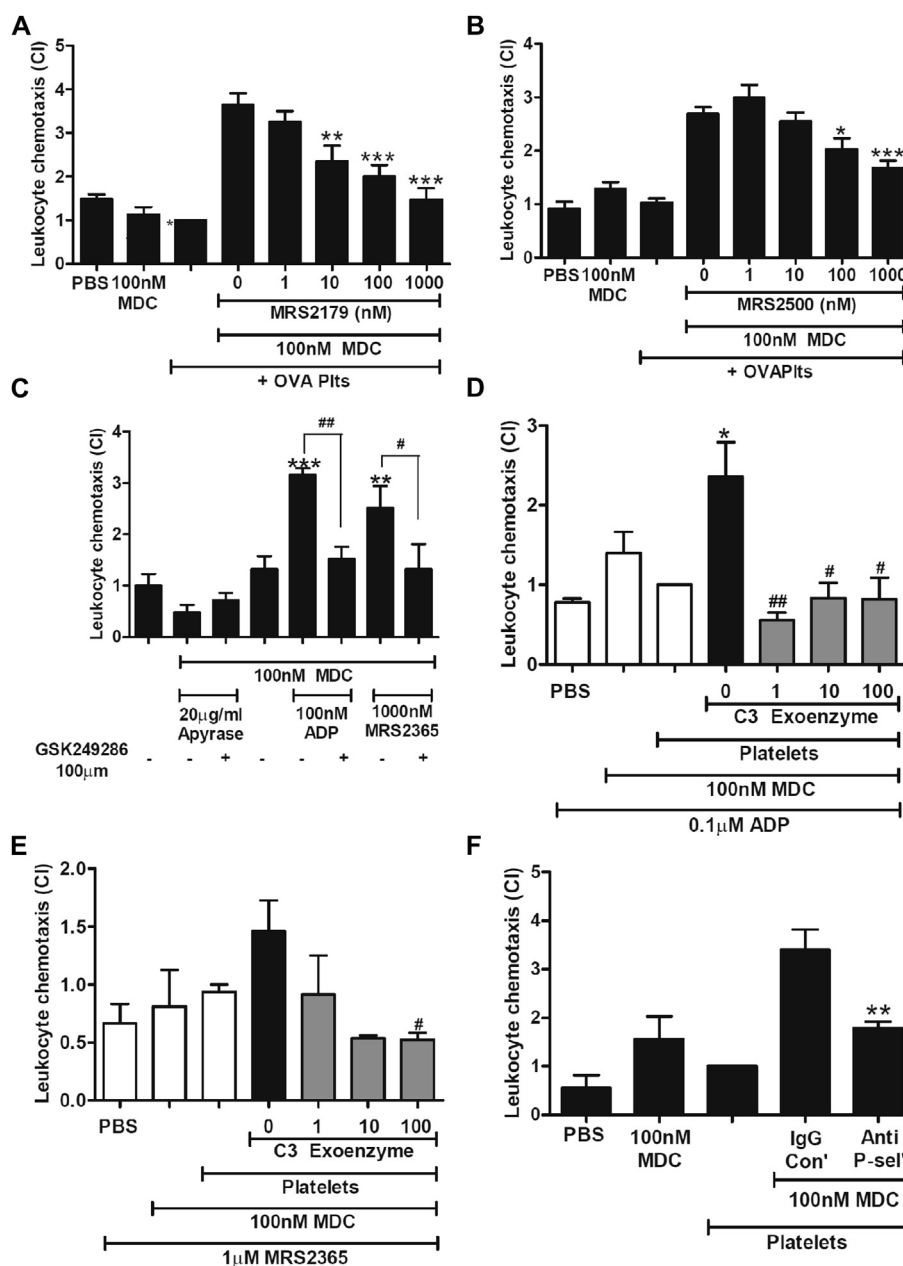


FIG 5. P2Y₁ receptor antagonism, RhoA inhibition, and P-selectin blockade suppress platelet-induced PMN chemotaxis *in vitro*. Platelets were treated with P2Y₁ antagonist (MRS2179; **A**), P2Y₁ antagonist (MRS2500; **B**), ROCK inhibitor (GSK249286; **C**), RhoA inhibitor ADP-ribosyltransferase C3 exoenzyme (**D** and **E**), or anti-P-selectin blocking antibody (**F**) and stimulated with 100 nmol/L ADP or 1000 nmol/L MRS2365 (P2Y₁ agonist; Fig 5, **C** and **E** only) and washed before mixture with PMNs. PMN chemotaxis toward MDC (100 nmol/L) was analyzed as a chemotactic index ($n = 3-8$). Data are expressed as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$ versus column 4 or # $P < .05$ and ## $P < .01$, where indicated.

(NF-279) before stimulation with ADP (100 nmol/L). Platelets were subsequently added to PMNs to stimulate their chemotaxis in response to MDC. As demonstrated, only the incubation of platelets with PMNs induced significant PMN chemotaxis toward MDC ($P < .001$; Fig 5, **A** and **B**) compared with that seen in negative controls (PMNs alone; PMNs plus MDC). Preincubation of platelets with the P2X₁ antagonist NF-279 or the P2Y₁₂ receptor antagonists MRS2395 and AR-C60996 did not affect the chemotaxis of PMNs to MDC, even at drug concentrations that exceeded those necessary to inhibit platelet aggregation

induced by ADP *in vitro* (see Fig E2, A-C, in this article's Online Repository at www.jacionline.org), thus supporting our *in vivo* observations (Fig 1). However, preincubation of platelets with the P2Y₁ antagonists MRS2179 or MRS2500 significantly inhibited PMN chemotaxis in a concentration-dependent manner (Fig 5, **A** and **B**). The preincubation of platelets with the ROCK inhibitor GSK429286, which inhibits the downstream signaling of RhoA in the presence of 100 nmol/L ADP or the P2Y₁ receptor agonist MRS2365, also significantly suppressed platelet-induced leukocyte chemotaxis toward MDC (Fig 5, **C**), whereas no

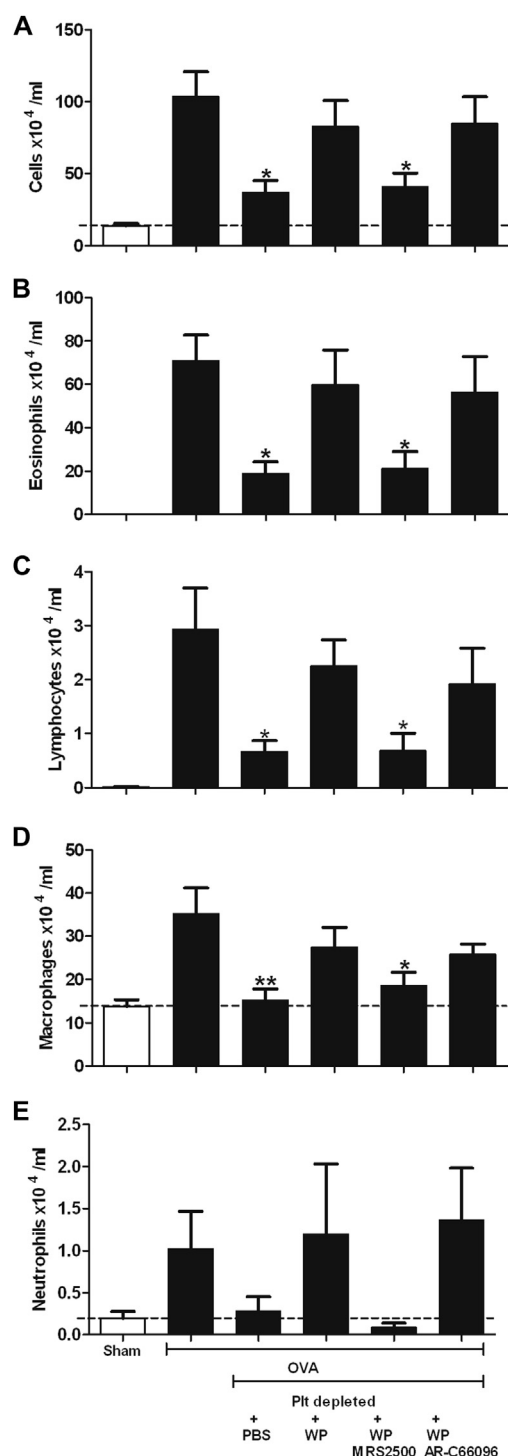


FIG 6. The requirement of P2Y₁ for pulmonary leukocyte recruitment is platelet specific. Bronchoalveolar lavage fluid was analyzed from OVA-sensitized and challenged mice for total cell counts (A), eosinophils (B), lymphocytes (C), macrophages (D), and neutrophils (E). Selected groups were rendered thrombocytopenic and then transfused with washed platelets (WP) or platelets incubated with either MRS2500 or AR-C66096 (100 μ mol/L; $n = 8-10$). Data are expressed as means $\pm$ SEMs. * $P < .05$ and ** $P < .01$ versus OVA-sensitized control group.

chemotaxis occurred in the absence of ADP (apyrase-treated groups), suggesting that RhoA signaling downstream of P2Y₁ receptor stimulation was necessary (Fig 5, C). We then further

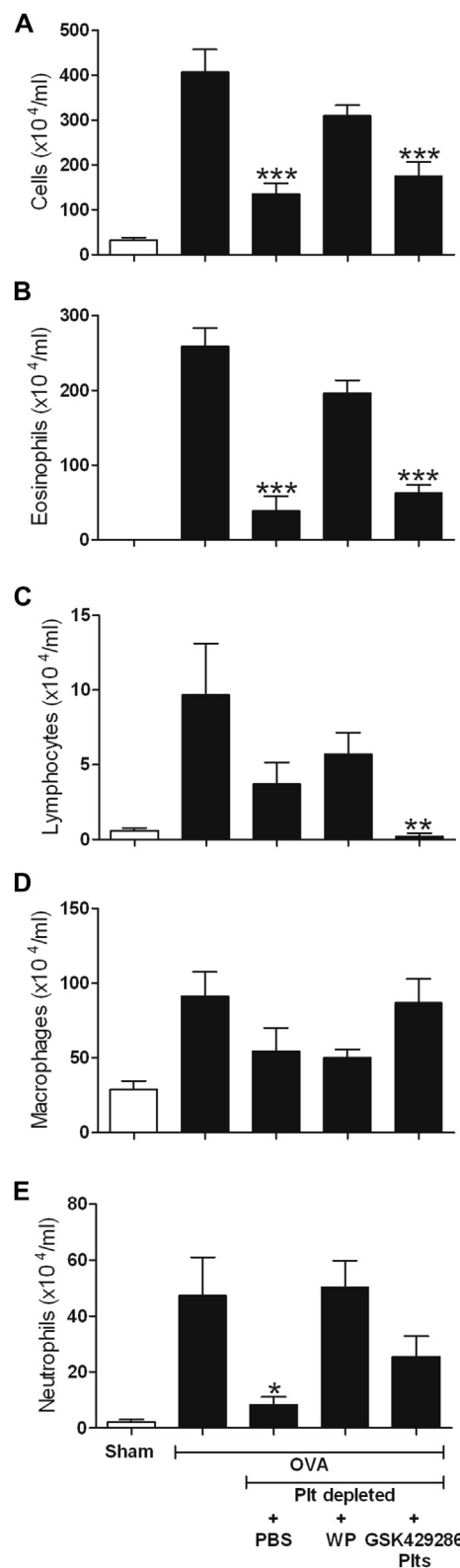


FIG 7. Platelet RhoA activation is necessary for pulmonary leukocyte recruitment. Bronchoalveolar lavage fluid was analyzed from OVA-sensitized and challenged mice for total cell counts (A), eosinophils (B), lymphocytes (C), macrophages (D), and neutrophils (E). Selected groups were rendered thrombocytopenic and then transfused with washed platelets (WP) or platelets incubated with the ROCK inhibitor GSK429286 (100 μ mol/L; $n = 5$). Data are expressed as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$ versus the OVA-sensitized control group.

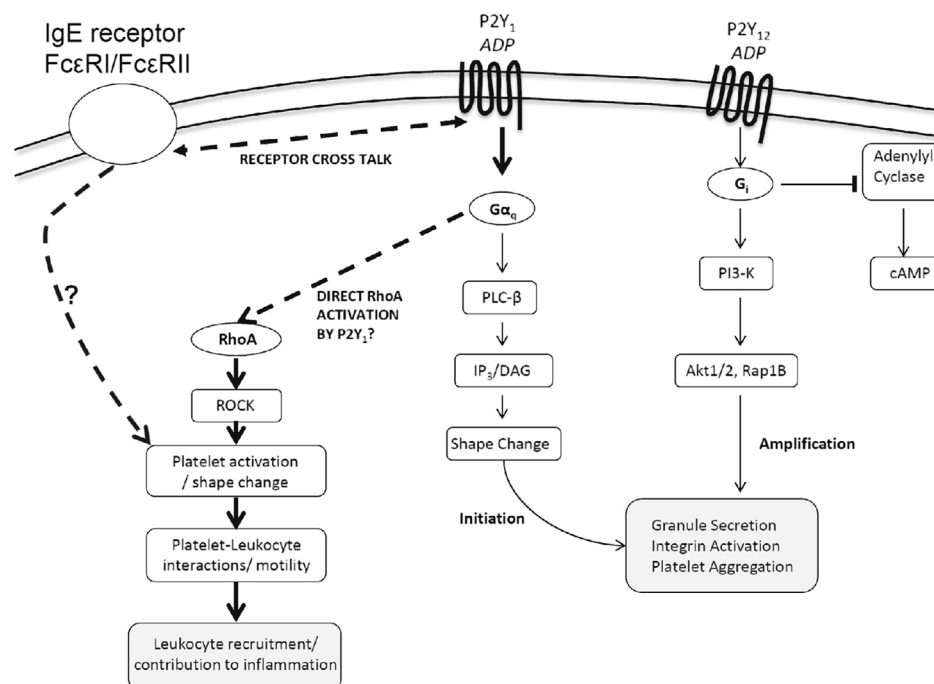


FIG 8. The role for platelet P2Y₁ receptor activation in allergic inflammation. The putative signaling pathways involved in P2Y₁ activation during inflammation compared with the role of platelet P2Y₁ and P2Y₁₂ receptors in hemostasis. Platelet RhoA activity through P2Y₁ stimulation is augmented after allergen sensitization. DAG, Diacylglycerol; IP₃, inositol trisphosphate; PI3-K, phosphatidylinositol 3-kinase; PLC-β, phospholipase C β.

confirmed that platelet RhoA activity downstream of P2Y₁ receptor stimulation was necessary by incubating platelets with ADP-ribosyltransferase (C3 exoenzyme), which significantly inhibited leukocyte chemotaxis after platelets were stimulated with ADP (Fig 5, D) or the P2Y₁ agonist MRS2365 (Fig 5, E). Lastly, preincubation of platelets with an anti-P-selectin blocking antibody significantly inhibited platelet-dependent PMN chemotaxis induced by MDC, suggesting that P-selectin-dependent platelet-PMN complexes were necessary for PMN chemotaxis in this assay, which was also dependent on RhoA signaling in platelets downstream of P2Y₁ receptor stimulation ($P < .01$; Fig 5, F).

Requirement for P2Y₁ and RhoA on pulmonary leukocyte recruitment is platelet specific

Given that the activation of platelets through P2Y₁ receptors was critical for platelet P-selectin-dependent migration of PMNs *in vitro* and for the formation of platelet-leukocyte complexes after allergen challenge *in vivo*, we determined whether the requirement for P2Y₁ receptor activation on pulmonary leukocyte recruitment was indeed platelet specific because it was conceivable that the phenomenon of leukocyte recruitment inhibited by P2Y₁ antagonism was the result of leukocyte activation by ADP and not solely platelets, given the expression of P2Y₁ receptors by different leukocyte subtypes and endothelium.⁵² Therefore we used a method to induce thrombocytopenia that would allow subsequent platelet restoration with administration of platelets that could be manipulated *in vitro* before transfusion, as reported previously.^{16,18,27,29} Busulfan (a bone marrow-specific toxin) was administered at a dose (20 mg/kg) that affects almost exclusively thrombopoiesis (targeting megakaryocyte

maturation) rather than hematopoiesis in mice (see Fig E3 in this article's Online Repository at www.jacionline.org).

Thrombocytopenia significantly reduced eosinophil ($P < .05$), lymphocyte ($P < .05$), macrophage ($P < .01$), and neutrophil (70% reduction, not significant) recruitment to the lungs 24 hours after final allergen challenge compared with OVA-sensitized and challenged vehicle-treated mice (Fig 6). However, pulmonary leukocyte recruitment was restored in thrombocytopenic mice reinfused with washed platelets, whereas leukocyte recruitment remained significantly suppressed with the infusion of platelets preincubated with a P2Y₁ antagonist (MRS2500, Fig 6). In contrast, the infusion of platelets preincubated with a P2Y₁₂ antagonist restored pulmonary leukocyte recruitment (AR-C66096, Fig 6). Thus selective inhibition of platelet P2Y₁ receptors led to a profound suppression of pulmonary leukocyte recruitment after allergen challenge.

Given that administration of a P2Y₁ antagonist inhibited platelet RhoA activity after allergen challenge (Fig 4, B) and that inhibition of RhoA suppressed P2Y₁-dependent platelet-induced leukocyte chemotaxis (Fig 5, C), we evaluated the importance of platelet RhoA on pulmonary leukocyte recruitment. Platelets preincubated with the ROCK inhibitor (GSK429286) and reinfused into thrombocytopenic mice before allergen challenge were unable to restore leukocyte recruitment compared with the reinfusion of washed platelets incubated with vehicle (Fig 7), demonstrating the importance of platelet RhoA signaling on leukocyte recruitment in a model in which platelet stimulation by P2Y₁ is necessary.

DISCUSSION

We provide evidence of a distinct mechanism of platelet activation in the context of allergic inflammation leading to

pulmonary leukocyte recruitment. Specifically, costimulation of the platelet P2Y₁ receptor by ADP is required, leading to downstream RhoA activation (Fig 8). This represents a divergence of platelet activation by inflammatory compared with aggregatory stimuli, in which ADP, acting to costimulate platelets, does not lead to platelet aggregation. Significantly, others have shown heightened release of purines in asthmatic patients that could account for activation of platelets after exposure to allergen.³⁷ We show no involvement of P2Y₁₂ receptors in this activity, even though administration of the P2Y₁₂ antagonists AR-C66096 and clopidogrel retained the ability to inhibit platelet aggregation *ex vivo* 6 hours after allergen challenge, suggesting that the drug was present in sufficient amounts to antagonize P2Y₁₂ receptors. Furthermore, we have previously reported that using the same dosing schedule, clopidogrel protects mice from collagen plus epinephrine-induced pulmonary embolism.³⁸ The P2Y₁₂ receptor is crucial for full platelet aggregation by all known platelet agonists, irrespective of the activating stimulus.³⁴ Therefore it is surprising that platelet P2Y₁₂ is not involved in activation of platelets in the inflammatory context presented here. Previous reports displaying a role for P2Y₁₂ receptor on allergic inflammation are in contrast to this present study and suggest P2Y₁₂ activation might be dependent on the provocation stimulus.²⁸ In our model platelets are activated by an IgE-dependent mechanism after antigen (OVA) challenge rather than the direct stimulation of platelets by leukotriene D₄/E₄ presented in other models.²⁸

It is not yet understood why platelet activation by inflammatory mediators would not ultimately lead to thrombosis and coagulation *in vivo*. Nor is it understood why ADP-induced activation of P2Y₁ receptors does not lead to coactivation of P2Y₁₂ and thus result in platelet aggregation. The P2Y₁₂ receptor signals through G protein G_i by means of either the α subunit or the β/γ subunits. Signaling through the α subunit inhibits cyclic AMP production through inhibition of adenylyl cyclase.³² On the other hand, signaling through the β/γ subunit activates phosphatidylinositol 3-kinase, leading to granule secretion.^{34,53} In contrast, the P2Y₁ receptor is coupled to the G protein G_{αq}, which, when stimulated, results in Ca²⁺ mobilization and protein kinase C activation, initiating platelet shape change, granule release, and thromboxane A₂ generation and aggregation (Fig 8).^{34,53} Interestingly, the P2Y₁ receptor can also activate the GTPase RhoA that in other cells is important for cell polarity and chemotaxis.⁴⁷⁻⁴⁹ We show here that platelet activation through P2Y₁ leads to downstream activation of RhoA after allergen challenge *in vivo* and that platelet RhoA is necessary for platelet-induced PMN motility *in vitro* and leukocyte recruitment *in vivo*. RhoA has previously been reported to induce platelet shape change, filopodia formation, cytoskeletal changes, and granule centralization (presumably events required to elicit platelet function during inflammatory events) through G_{αq}, but RhoA activity does not lead to full platelet aggregation in response to ADP or other primary agonists (Fig 8).^{45,46,54} Here, we postulate that platelet RhoA activity through P2Y₁ stimulation is augmented after allergen sensitization, inducing platelet function that is applicable to inflammatory rather than hemostatic events. We have previously shown that allergen stimulation through FcεRI modulates platelet activation and accumulation to the lungs, and it has been suggested that FcεRI can interact with G protein-coupled receptors, such as purinergic receptors (Fig 8).^{18,55}

The expression of platelet P-selectin and P-selectin glycoprotein ligand 1 is important in the conjugation of platelets with leukocytes, which increases their subsequent recruitment to the lungs.^{24,25,27} Several studies have revealed that both P2Y₁ and P2Y₁₂ receptor activation increase platelet P-selectin expression through granule secretion.^{32,36} Therefore it is surprising that P2Y₁₂ receptor activation was not involved in the platelet P-selectin-dependent mechanism of pulmonary leukocyte recruitment described here. This would suggest that the mechanism of platelet activation by inflammatory mediators, although requiring ADP costimulation of platelets through P2Y₁ receptor activation, is fundamentally different from the mechanism of platelet activation induced by “classical” platelet agonists, in which ADP costimulation of platelets through P2Y₁₂ activation is involved, even when the end result, such as P-selectin expression, is similar but ultimately produces a difference in platelet function. This scenario of platelet activation in the context of an inflammatory stimulus, without an alteration in hemostasis, reveals a fundamental dichotomy in platelet activation.⁵⁶ This has implications for a wide variety of diseases in which platelets are activated and known to be functionally important, including asthma, allergic rhinitis, and eczema.

Key messages

- ADP stimulation of platelets through P2Y₁, but not P2Y₁₂, receptors is necessary for leukocyte recruitment into the lungs of allergic mice.
- RhoA signaling downstream of platelet P2Y₁, but not P2Y₁₂, receptor activation affects platelet function in response to allergic inflammatory stimuli.
- A dichotomy in platelet activation exists and determines platelet function, which can be exploited for the development of new anti-inflammatory and antiallergic drugs.

REFERENCES

- Gresele P, Todisco T, Merante F, Nenci GG. Platelet activation and allergic asthma. *N Engl J Med* 1982;306:549.
- Morley J, Page CP, Sanjar S. Platelets in asthma. *Lancet* 1985;2:726-7.
- Gresele P, Dottorini M, Selli ML, Iannacci L, Canino S, Todisco T, et al. Altered platelet function associated with the bronchial hyperresponsiveness accompanying nocturnal asthma. *J Allergy Clin Immunol* 1993;91:894-902.
- Dürk T, Duerschmied D, Müller T, Grimm M, Reuter S, Vieira RP, et al. Production of serotonin by tryptophan hydroxylase 1 and release via platelets contribute to allergic airway inflammation. *Am J Respir Crit Care Med* 2013;187:476-85.
- Knaur KA, Lichtenstein LM, Adkinson NF, Fish JE. Platelet activation during antigen-induced airway reactions in asthmatic subjects. *N Engl J Med* 1981;304:1404-6.
- Kowal K, Pampuch A, Kowal-Bielecka O, DuBuske LM, Bodzenta-Lukaszyk A. Platelet activation in allergic asthma patients during allergen challenge with *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 2006;36:426-32.
- Yasuba H, Chihara J, Kino T, Satake N, Oshima S. Increased releasability of platelet products and reduced heparin-induced platelet factor 4 release from endothelial cells in bronchial asthma. *J Lipid Mediat* 1991;4:5-21.
- Yamamoto H, Nagata M, Tabe K, Kimura I, Kiuchi H, Sakamoto Y, et al. The evidence of platelet activation in bronchial asthma. *J Allergy Clin Immunol* 1993;91:79-87.
- Kasperska-Zajac A, Rogala B. Markers of platelet activation in plasma of patients suffering from persistent allergic rhinitis with or without asthma symptoms. *Clin Exp Allergy* 2005;35:1462-5.
- Averill FJ, Hubbard WC, Proud D, Gleich GJ, Liu MC. Platelet activation in the lung after antigen challenge in a model of allergic asthma. *Am Rev Respir Dis* 1992;145:571-6.

11. Sullivan PJ, Jafar ZH, Harbinson PL, Restrik LJ, Costello JF, Page CP. Platelet dynamics following allergen challenge in allergic asthmatics. *Respiration* 2000;67:513-7.
12. Gallagher JS, Bernstein IL, Maccia CA, Splansky GL, Glueck HI. Cyclic platelet dysfunction in IgE-mediated allergy. *J Allergy Clin Immunol* 1978;62:229-35.
13. Duarte D, Taveira-Gomes T, Sokhatska O, Palmares C, Costa R, Negrão R, et al. Increased circulating platelet microparticles as a potential biomarker in asthma. *Allergy* 2013;68:1073-5.
14. Metzger WJ, Sjoerdsma K, Richerson HB, Moseley P, Zavala D, Monick M, et al. Platelets in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced late phase responses. *Agents Actions Suppl* 1987;21:151-9.
15. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 1989;140:1745-53.
16. Pitchford SC, Yano H, Lever R, Riffo-Vasquez Y, Ciferri S, Rose MJ, et al. Platelets are essential for leukocyte recruitment in allergic inflammation. *J Allergy Clin Immunol* 2003;112:109-18.
17. Johansson MW, Han ST, Gunderson KA, Busse WW, Jarjour NN, Mosher DF. Platelet activation, P-selectin, and eosinophil $\beta 1$ -integrin activation in asthma. *Am J Respir Crit Care Med* 2012;185:498-507.
18. Pitchford SC, Momi S, Baglioni S, Casali L, Giannini S, Rossi R, et al. Allergen induces the migration of platelets to lung tissue in allergic asthma. *Am J Respir Crit Care Med* 2008;177:604-12.
19. Szczeklik A, Milner PC, Birch J, Watkins J, Martin JF. Prolonged bleeding time, reduced platelet aggregation, altered PAF-acether sensitivity and increased platelet mass are a trait of asthma and hay fever. *Thromb Haemost* 1986;56:283-7.
20. Cardot E, Pestel J, Callebaut I, Lassalle P, Tsicopoulos A, Gras-Masse H, et al. Specific activation of platelets from patients allergic to *Dermatophagoides pteronyssinus* by specific peptides derived from the allergen Der p 1. *Int Arch Allergy Immunol* 1992;98:127-34.
21. Yoshida A, Ohba M, Wu X, Sasano T, Nakamura M, Endo Y. Accumulation of platelets in the lung and liver and their degranulation following antigen-challenge in sensitized mice. *Br J Pharmacol* 2002;137:146-52.
22. Lellouch-Tubiana A, Lefort J, Simon MT, Pfister A, Vargaftig BB. Eosinophil recruitment into guinea pig lungs after PAF-acether and allergen administration. Modulation by prostacyclin, platelet depletion, and selective antagonists. *Am Rev Respir Dis* 1988;137:948-54.
23. Coyle AJ, Page CP, Atkinson L, Flanagan R, Metzger WJ. The requirement for platelets in allergen-induced late asthmatic airway obstruction. Eosinophil infiltration and heightened airway responsiveness in allergic rabbits. *Am Rev Respir Dis* 1990;142:587-93.
24. Lukacs NW, John A, Berlin A, Bullard DC, Knibbs R, Stoolman LM. E- and P-selectins are essential for the development of cockroach allergen-induced airway responses. *J Immunol* 2002;169:2120-5.
25. De Sanctis GT, Wolyniec WW, Green FH, Qin S, Jiao A, Finn PW, et al. Reduction of allergic airway responses in P-selectin-deficient mice. *J Appl Physiol* 1997;83:681-7.
26. Robertson DN, Page CP. Effect of platelet agonists on airway reactivity and intrathoracic platelet accumulation. *Br J Pharmacol* 1987;92:105-11.
27. Pitchford SC, Momi S, Giannini S, Casali L, Spina D, Page CP, et al. Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation. *Blood* 2005;105:2074-81.
28. Paruchuri S, Tashimo H, Feng C, Maekawa A, Xing W, Jiang Y, et al. Leukotriene E₄-induced pulmonary inflammation is mediated by the P2Y₁₂ receptor. *J Exp Med* 2009;206:2543-55.
29. Pitchford SC, Riffo-Vasquez Y, Sousa A, Momi S, Gesele P, Spina D, et al. Platelets are necessary for airway wall remodeling in a murine model of chronic allergic inflammation. *Blood* 2004;103:639-47.
30. Svensson Holm AC, Bengtsson T, Grenegård M, Lindström EG. Platelet membranes induce airway smooth muscle cell proliferation. *Platelets* 2011;22:45-55.
31. Ind PW. Platelet and clotting abnormalities in asthma. *Clin Exp Allergy* 1991;21:395-8.
32. Leon C, Ravanat C, Freund M, Cazenave JP, Gachet C. Differential involvement of the P2Y₁ and P2Y₁₂ receptors in platelet procoagulant activity. *Atheroscler Thromb Vasc Biol* 2003;23:1941-7.
33. Sun B, Li J, Okahara K, Kambayashi J. P2X₁ purinoceptor in human platelets. Molecular cloning and functional characterization after heterologous expression. *J Biol Chem* 1998;273:11544-7.
34. Gachet C. Regulation of platelet functions by P2 receptors. *Annu Rev Pharmacol Toxicol* 2006;46:277-300.
35. Storey RF, Sanderson HM, White AE, May JA, Cameron KE, Heptinstall S. The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. *Br J Haematol* 2000;110:925-34.
36. Storey RF, Judge HM, Wilcox RG, Heptinstall S. Inhibition of ADP-induced P-selectin expression and platelet-leukocyte conjugate formation by clopidogrel and the P2Y₁₂ receptor antagonist AR-C69931MX but not aspirin. *Thromb Haemost* 2002;88:488-94.
37. Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, et al. Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* 2007;13:913-9.
38. Momi S, Pitchford SC, Alberti PF, Minuz P, Del Soldato P, Gesele P. Nitroaspirin plus clopidogrel versus aspirin plus clopidogrel against platelet thromboembolism and intimal thickening in mice. *Thromb Haemost* 2005;93:535-43.
39. Momi S, Falcinelli E, Giannini S, Ruggeri L, Cecchetti L, Corazzi T, et al. Loss of matrix metalloproteinase 2 in platelets reduces arterial thrombosis in vivo. *J Exp Med* 2009;206:2365-9.
40. Komerup KN, Salmon GP, Pitchford SC, Liu WL, Page CP. Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. *J Appl Phys* 2010;109:758-67.
41. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, et al. ATP release guides neutrophil chemotaxis via P2Y₂ and A₃ receptors. *Science* 2006;314:1792-5.
42. Corriden R, Insel PA. New insights regarding the regulation of chemotaxis by nucleotides, adenosine, and their receptors. *Purinergic Signal* 2012;8:587-98.
43. Hechler B, Nonne C, Roh EJ, Cattaneo M, Cazenave JP, Lanza F, et al. MRS2500 [2-Iodo-N⁶-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-biphosphate], a potent, selective, and stable antagonist of the platelet P2Y₁ receptor with strong antithrombotic activity in mice. *J Pharmacol Exp Ther* 2006;316:556-63.
44. Bird JE, Wang X, Smith PL, Barbera F, Huang C, Schumacher WA. A platelet target for venous thrombosis? P2Y₁ deletion or antagonism protects mice from vena cava thrombosis. *J Thromb Thrombolysis* 2012;34:199-207.
45. Pleines I, Hagedorn I, Gupta S, May F, Chakarova L, van Hengel J, et al. Megakaryocyte-specific RhoA deficiency causes macrothrombocytopenia and defective platelet activation in haemostasis and thrombosis. *Blood* 2012;119:1054-63.
46. Eckly A, Gendraul JL, Hechler B, Cazenave JP, Gachet C. Differential involvement of the P2Y₁ and P2Y₁₂ receptors in the morphological changes of platelet aggregation. *Thromb Haemost* 2001;85:694-701.
47. Aslan JE, McCarty OJT. Rho GTPases in platelet function. *J Thromb Haemost* 2013;11:35-46.
48. Laudanna C, Campbell JJ, Butcher EC. Role of Rho in chemoattractant activated leukocyte adhesion through integrins. *Science* 1996;271:981-3.
49. Sakumura Y, Tsukada Y, Yamamoto N, Ishii S. A molecular model for axon guidance based on cross talk between rho GTPases. *Biophys J* 2005;89:812-22.
50. Hirata H, Arima M, Cheng G, Honda K, Fukushima F, Yoshida N, et al. Production of TARC and MDC by naive T cells in asthmatic patients. *J Clin Immunol* 2003;23:34-45.
51. Gear AR, Suttitanamongkol S, Viisoreanu D, Polanowska-Grabowska RK, Raha S, Camerini D. Adenosine diphosphate strongly potentiates the ability of the chemokines MDC, TARC and SDF-1 to stimulate platelet function. *Blood* 2001;97:937-45.
52. Burnstock G. Purine and pyrimidine receptors. *Cell Mol Life Sci* 2007;64:1471-83.
53. Hardy AR, Jones ML, Mundell SJ, Poole AW. Reciprocal cross-talk between P2Y₁ and P2Y₁₂ receptors at the level of calcium signalling in human platelets. *Blood* 2004;104:1745-52.
54. Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circ Res* 2006;99:1293-304.
55. Kuehn HS, Gilfillan AM. G-protein-coupled receptors and the modification of FcεRI-mediated mast cell activation. *Immunol Lett* 2007;113:59-69.
56. Page CP. The involvement of platelets in non-thrombotic processes. *Trends Pharmacol Sci* 1988;9:66-71.

METHODS

Reagents

Apyrase, MRS2395, clopidogrel (Methyl 2-(4,5,6,7-tetrahydrothieno [3,2-*c*]pyridin-5-yl)-2-(2-chlorophenyl)acetate hydrochloride), PGE₁, OVA (grade V), busulfan, and ADP were obtained from Sigma-Aldrich (Poole, United Kingdom); MRS2179 (*N*⁶-methyl-2'-deoxyadenosine-3', 5'-biphosphate), MRS2500 (2-Iodo-*N*⁶-methyl-[*N*]-methanocarpa-2'-deoxyadenosine-3',5'-biphosphate), MRS2365 (2,2-dimethyl-propionic acid 3-[2-chloro-6-methylaminopurin-9-yl]-2-[2,2-d imethyl-propionyloxymethyl]-propyl ester), 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 GSK429286 (4-[4-(Trifluoromethyl)phenyl]-*N*-[6-Fluoro-1*H*-indazol-5-yl]-2-methyl-6-oxo-1,4,5,6-tetrahydro-3-pyridinecarboxamide) were from Tocris Bioscience (Bristol, United Kingdom); rabbit anti-RhoA (phosphoS188) polyclonal antibody and the RhoA activation assay kit were from Abcam (Cambridge, United Kingdom); the rabbit anti-total RhoA mAb was from Cell Signaling Technology (Danvers, Mass); goat anti-rabbit IgG-conjugated Alexa Fluor 488 was from Invitrogen (Carlsbad, Calif); rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, Calif); rat anti-mouse CD41-phycoerythrin-conjugated antibody, rat anti-mouse CD45-fluorescein isothiocyanate-conjugated antibody, rat anti-mouse P-selectin antibody (blocking, clone RB40.34), and control antibodies were from BD Biosciences (Oxford, United Kingdom); murine MDC was from PeptoTech (Rocky Hill, N); chemotaxis plates were from Neuro Probe (Gaithersburg, Md); and C3 exoenzyme (*Clostridium botulinum*) was from Enzo Life Sciences (Farmingdale, NY).

Receptor pharmacology of purinergic antagonists

MRS2179 and MRS2500 have high affinity for the P2Y₁ receptor (dissociation constant [*K*_d] of 109 and 0.78 nmol/L, respectively), with no affinity or functional effect on P2Y₁₂ receptors.^{E1-E7} The inhibitory concentration of 50% (IC₅₀) values for MRS2179 and MRS2500 at inhibiting ADP-induced platelet aggregation are less than 1 μmol/L and 0.95 nmol/L, respectively.^{E1,E3,E7} MRS2395 is a competitive P2Y₁₂ antagonist (*K*_d of 3.6 μmol/L) and inhibits ADP-induced platelet aggregation (IC₅₀ of 7 μmol/L) and antagonizes ADP-induced inhibition of cyclic AMP in rat and human platelets in the presence of PGE₁ without affecting P2Y₁ receptor-induced phospholipase C activity in transfected astrocytoma cells.^{E8} AR-C66096 is also a competitive P2Y₁₂ antagonist with high affinity (*K*_d of 2.5 nmol/L) and 9000-fold selectivity for P2Y₁₂ over P2Y₁ and potently inhibits ADP-induced platelet aggregation (IC₅₀ of 6 nmol/L).^{E9,E10} Clopidogrel is a prodrug, the metabolite of which is an irreversible P2Y₁₂

antagonist (*K*_d of 125 nmol/L) that inhibits ADP-induced platelet aggregation (IC₅₀ 1.6 μmol/L).^{E11} NF-279 and demonstrates high potency and selectivity for the P2X₁ receptor, as demonstrated by an IC₅₀ of 19 nmol/L.^{E12}

REFERENCES

- E1. Boyer AL, Mohanram A, Camaioni E, Jacobson KA, Harden TK. Competitive and selective antagonism of P2Y₁ receptors by N⁶-methyl 2'-deoxyadenosine 3',5'-biphosphate. *Br J Pharmacol* 1998;124:1-3.
- E2. Houston D, Costanzi S, Jacobson KA, Kendall HT. Development of selective high affinity antagonists, agonists and radioligands for the P2Y₁ receptor. *Comb Chem High Throughput Screen* 1998;11:410-9.
- E3. Baurand A, Raboisson P, Freund M, Leon C, Cazenave JP, Bourguignon J, et al. Inhibition of platelet function by administration of MRS2179, a P2Y₁ receptor antagonist. *Eur J Pharmacol* 2001;412:213-21.
- E4. Hechler B, Eckly A, Ohlmann P, Cazenave JP, Gachet C. The P2Y₁ receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol* 1998;103:858-66.
- E5. Hechler B, Nonne C, Roh EJ, Cattaneo M, Cazenave JP, Lanza F, et al. MRS2500 [2-iodo-N⁶-methyl-(*N*)-methanocarpa-2'-deoxyadenosine-3',5'-biphosphate], a potent, selective, and stable antagonist of the platelet P2Y₁ receptor with strong antithrombotic activity in mice. *J Pharmacol Exp Ther* 2006;316:556-63.
- E6. Kim HS, Ohno M, Xu B, Kim HO, Choi Y, Ji XD, et al. 2-substitution of adenine nucleotide analogues containing a bicycle[3.1.0]hexane ring system locked in a northern conformation: enhanced potency as P2Y₁ receptor antagonists. *J Med Chem* 2003;46:4974-87.
- E7. Cattaneo M, Lecchi A, Ohno M, Joshi BV, Besada P, Tchilibon S, et al. Antiaggregatory activity in human platelets of potent antagonists of the P2Y₁ receptor. *Biochem Pharmacol* 2004;68:1995-2002.
- E8. Xu B, Stephens A, Kirschenheuter G, Greslin AF, Cheng X, Sennelo J, et al. Acyclic analogues of adenosine bisphosphates as P2Y₁ antagonists: phosphate substitution leads to multiple pathways of inhibition of platelet aggregation. *J Med Chem* 2002;45:5694-709.
- E9. Humphries RG, Tomlinson W, Ingall AH, Cage PA, Leff P. FPL 66096: a novel, highly potent and selective antagonist at human platelet P2T- purinoceptors. *Br J Pharmacol* 1994;113:1057-63.
- E10. Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McNally JJ, et al. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. *J Med Chem* 1999;41:213-20.
- E11. Savi P, Labouret C, Delesque N, Guette F, Lupker J, Herbert JM. P2Y₁₂(12), a new platelet ADP receptor, target of clopidogrel. *Biochem Biophys Res Commun* 2001;283:379-83.
- E12. Damer S, Niebel B, Czeche S, Nickel P, Ardanuy U, Schmalzing G, et al. NF279: a novel potent and selective antagonist of P2X₁ receptor-mediated responses. *Eur J Pharmacol* 1998;350:R5-6.

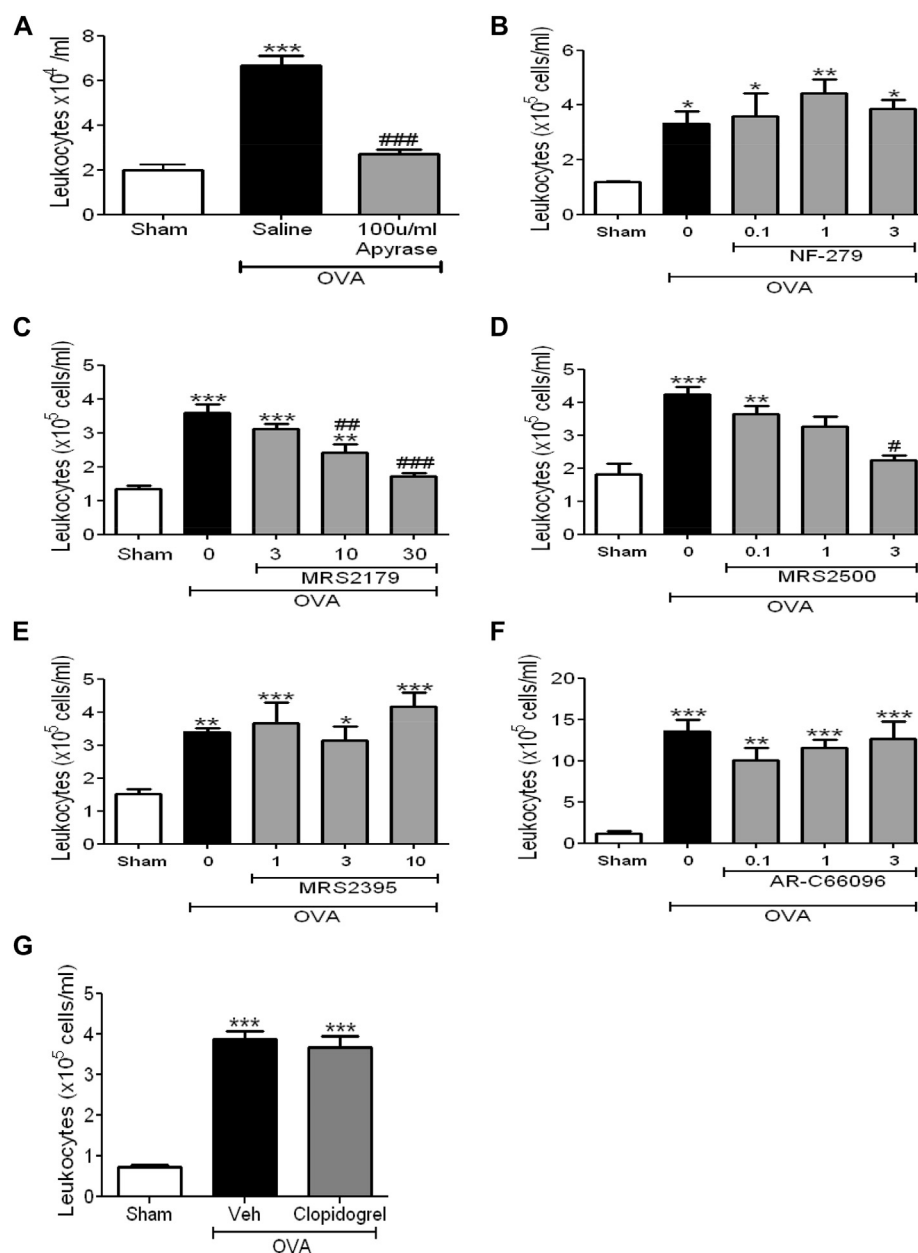


FIG E1. Effects of purinergic receptor antagonism on pulmonary leukocyte recruitment in a murine model of allergic inflammation. Mice sensitized to OVA were treated with apyrase (**A**), the P2X1 antagonist NF-279 (**B**), the P2Y1 antagonist MRS2179 (**C**), the P2Y1 antagonist MRS2500 (**D**), the P2Y12 antagonist MRS2395 (**E**), the P2Y12 antagonist AR-C66096 (**F**), or the P2Y12 antagonist clopidogrel (**G**) before exposure to 3% OVA through nebulization on days 14, 15, and 16. Twenty-four hours after the final challenge, animals were culled, bronchoalveolar lavage was performed, and total cell counts were determined at each drug dose ($n = 4-7$). Data are expressed as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with the sham-sensitized control group and # $P < .05$, ## $P < .01$, and ### $P < .001$ compared with the OVA-sensitized control group.

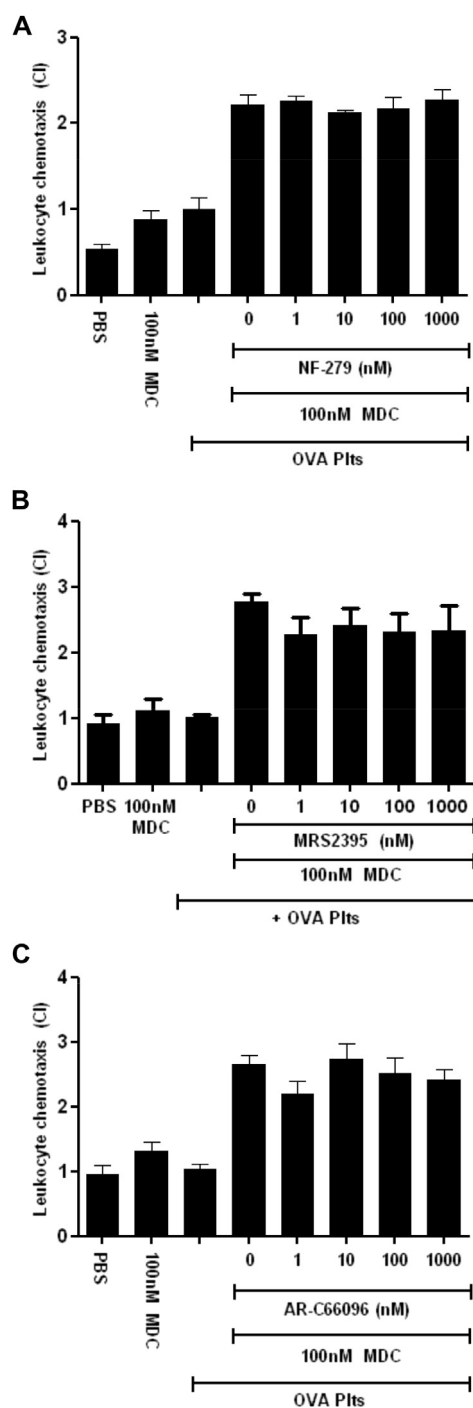


FIG E2. Effects of P2X1 or P2Y12 receptor antagonism on platelet-induced PMN chemotaxis *in vitro*. Platelets were isolated from mouse blood, and leukocytes were collected from femoral bone marrow. Platelets were treated with the P2X1 antagonist NF-279 (**A**) or the P2Y12 antagonists MRS2395 (**B**) or AR-C66096 (**C**) and stimulated with 100 nmol/L ADP and washed before being mixed with leukocytes. This cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis toward MDC (bottom well, 100 nmol/L) was then quantified after a 90-minute incubation and analyzed as a chemotactic index ($n = 5$). Data are expressed as means $\pm$ SEMs. CI, Chemotactic index.

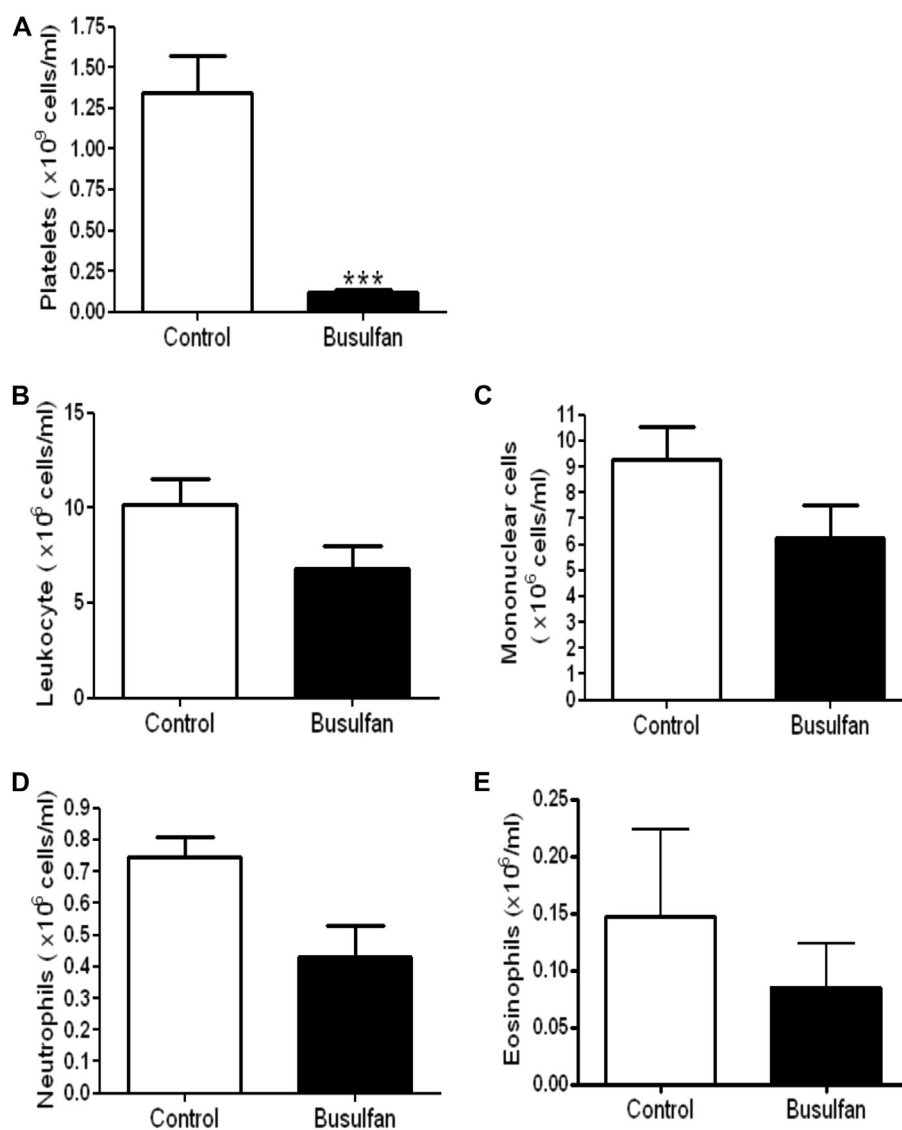


FIG E3. Effect of busulfan on circulating platelet and leukocyte numbers. Mice were treated with busulfan (20 mg/kg administered intraperitoneally) on days 0, 3, and 5, and blood was collected by means of cardiac bleed on day 22. Bronchoalveolar lavage fluid was analyzed for platelets (**A**), total cell counts (**B**), mononuclear cells (**C**), neutrophils (**D**), eosinophils (**E**). $n = 4$. Data are expressed as means $\pm$ SEM. *** $P < .001$.