

Platelets Play a Central Role in Sensitization to Allergen

Richard T. Amison, Simon J. Cleary, Yanira Riffo-Vasquez, Maida Bajwa, Clive P. Page, and Simon C. Pitchford

Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, London, United Kingdom

ORCID IDs: 0000-0002-2137-9430 (Y.R.-V.); 0000-0002-9358-3799 (C.P.P.); 0000-0003-3961-0407 (S.C.P.).

Abstract

Platelet activation occurs in patients with allergic inflammation, and platelets can be activated directly by allergen via an IgE-dependent process. Platelets have been shown to activate APCs such as CD11c⁺ dendritic cells *in vitro*. Although CD11c⁺ dendritic cells are a requisite for allergen sensitization, the role of platelets in this process is unknown. In this study, we investigated whether platelets were necessary for allergen sensitization. Balb/c mice sensitized to ovalbumin were exposed to subsequent aerosolized allergen (ovalbumin challenge). We analyzed lung CD11c⁺ cell activation, colocalization with platelets, and some other indices of inflammation. The role of platelets at the time of allergen sensitization was assessed through platelet depletion experiments restricted to the period of sensitization. Platelets colocalized with airway CD11c⁺ cells, and this association increased after allergen sensitization as well as after subsequent allergen exposure. Temporary platelet depletion (>95%) at the time of allergen sensitization led to a suppression of IgE and IL-4 synthesis and to a decrease in the pulmonary recruitment of eosinophils, macrophages, and lymphocytes after subsequent allergen exposure. Furthermore, in mice previously depleted of platelets at the

time of sensitization, the recovered platelet population was shown to have reduced expression of FcεRI. Pulmonary CD11c⁺ cell recruitment was suppressed in these mice after allergen challenge, suggesting that the migration of CD11c⁺ cells *in vivo* may be dependent on direct platelet recognition of allergen. We conclude that platelets are necessary for efficient host sensitization to allergen. This propagates the subsequent inflammatory response during secondary allergen exposure and increases platelet association with airway CD11c⁺ cells.

Keywords: platelets; allergen sensitization; FcεRI; dendritic cells; asthma

Clinical Relevance

This research will impact clinicians' research on platelet activation in patients with asthma and on cellular mechanisms involved in allergen sensitization. It adds to current knowledge of platelet activation by allergen during initial sensitization.

Platelet activation in allergic diseases such as asthma has been reported extensively over the last 30 years, with evidence of changes in platelet volume, presence of platelet microparticles, release of platelet-specific inflammatory mediators, and an increased incidence of circulating platelet-leukocyte (including eosinophil) complexes, both in patients undergoing spontaneous asthma

attacks and in patients after allergen challenge (1–4). Furthermore, platelet activation correlates strongly to changes in lung function (4–9). Various *in vivo* studies confirm that the activation of platelets during allergy and asthma plays a central role in the inflammatory response (3, 10–13). Of significance, pulmonary platelet recruitment and accumulation occur in the

lungs, initially via an IgE-dependent process whereby platelets migrate and enter the extravascular space (7, 14–17). The significance and temporal nature of allergen-induced activation of platelets via FcεRI has not been established; yet, of pertinence to allergy, platelets from patients allergic to *Dermatophagoides pteronyssinus* have been shown to be activated directly

(Received in original form November 14, 2017; accepted in final form January 23, 2018)

Supported by the Sackler Foundation.

Author Contributions: R.T.A., M.B., and S.J.C.: designed and performed experiments; Y.R.-V.: was involved with discussion and implications of data; and C.P.P. and S.C.P.: designed the project, analyzed data, and wrote the manuscript.

Correspondence and requests for reprints should be addressed to Simon C. Pitchford, Ph.D., Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, Room 5.43 Franklin Wilkins Building, Waterloo Campus, London SE9 1NH, UK. E-mail: simon.pitchford@kcl.ac.uk.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 59, Iss 1, pp 96–103, Jul 2018

Copyright © 2018 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2017-0401OC on January 24, 2018

Internet address: www.atsjournals.org

by the specific allergen, or by synthetic peptides derived from the allergen, through an IgE-dependent process (16, 18). Combined, these studies argue for a causal role of platelet activation in asthma and allergy and that such activation is not a consequence of the inflammatory milieu *per se*. In addition, it has been shown that platelet-derived serotonin is important for dendritic cell (DC) activation during allergic airway inflammation, further supporting a role for platelets and suggesting that the activation and migration of platelets induced by allergen might be of significance or causal to the sensitization process (19).

Myeloid dendritic cells are believed to be involved very early in the development of asthma by inducing sensitization to inhaled antigen, and they are increasingly viewed as essential proinflammatory cells that induce effector cytokine production in primed T-helper cell type 2 (Th2) cells during airway inflammation (20–23). It is therefore of interest that platelet CD40L has recently been reported to be involved in the promotion of allergic airway inflammation by polarizing Th2 responses after allergen exposure (24). We have previously demonstrated a presence of platelets extravascularly in the lungs of mice before subsequent allergen exposure in allergen-sensitized mice, but the vast majority of these platelets were attached to a resident undefined mononuclear cell population, and the relevance of this occurrence has yet to be established (16). We hypothesized that these platelets are a distinct population that had migrated to the lungs as a result of exposure to allergen (i.p. injection of ovalbumin [OVA]) and that a population of platelets might therefore be involved in initial sensitization to allergen. Temporally, the occurrence was clearly unrelated to subsequent aerosolized exposures to allergen (OVA), which drives platelet migration into the lungs together with recruited leukocytes (16). We report that CD11c⁺ cells (as a marker for DCs) are decorated with platelets during allergen sensitization, and we investigate the importance of these platelets during the process of allergen sensitization and their interactions with CD11c⁺ cells before and during subsequent exposure to aerosolized allergen. These studies provide a possible role for the specific activation of platelets by allergen from patients with asthma and the occurrence of platelets in the lungs of patients with asthma.

Methods

See the data supplement for methodology related to blood microsampling; immunohistochemistry of lungs; microscopic analysis and lung morphometry; flow cytometric analysis of CD11c⁺ cells and platelets; and quantification of IgE, IL-4, IL-5, and IFN- γ .

Sensitization of Mice to OVA and BAL

To understand the importance of platelets during allergen sensitization, as well as subsequent effects on the inflammatory response after secondary allergen exposure via an aerosol challenge, a murine model of allergic sensitization and lung inflammation was used in which the sensitization process can be delineated and confined by a defined route of administration (i.p. route), as compared with the more complex situation arising from continual exposure to allergen via the airways. All studies were performed according to the Animals (Scientific Procedures) Act of 1986 and 2012 amendments (United Kingdom), as well as with local ethical approval from King's College London. Our studies and reporting were undertaken according to the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

Female Balb/c mice (20–25 g; Harlan UK) were housed under standard husbandry conditions of 12-hour/12-hour light/dark cycles at 21–23°C in groups of four to six animals per cage with access to a pelleted chow diet and water *ad libitum*. The mice were randomly assigned to different treatment groups. They were sensitized with chicken egg OVA (grade V, 10 μ g/0.4 ml [Sigma-Aldrich], absorbed in a saturated solution of adjuvant aluminum hydroxide 0.1 M [Sanofi Winthrop]) or received adjuvant only (sham-sensitized mice) intraperitoneally on Days 0 and 7. On Days 15, 16, and 17, mice were subsequently nebulized with OVA (10 mg/ml) for 30 minutes, 24 hours after final allergen challenge (Day 18). BAL fluid was processed for total and differential cell counts as well as the enumeration of cytokines, and blood was processed for measurement of total IgE content as previously described (10, 25). Some groups of mice were killed 0, 24, and 48 hours after 1 day of allergen exposure on Day 15, and their lungs were taken for histological analysis. Groups of mice were also killed on Day 15 in the absence of allergen

exposure for measurement of platelet Fc ϵ RI expression from circulating blood and (Day 15, absence of allergen challenge; Day 16, 24 h after Day 15 allergen challenge) for enumeration of CD11c⁺ cells in lung homogenates.

Platelet Depletion

To produce profound but temporary platelet depletion for the selective period of allergen sensitization, groups of mice were administered intramuscularly (50- μ l i.m. injection into a hind limb) 25 μ g per mouse antiplatelet antibody (antiglycoprotein Ib α ; Emfret Technologies) or control IgG (monoclonal 2A3 antitrinitrophenol; Bio X Cell) 24 hours before allergen sensitizations on Days –1 and 6. Previous studies in our laboratory have demonstrated that mice receiving this antiplatelet antibody do not display pulmonary edema, hemorrhage, or weight loss beyond 10% body weight.

Statistical Analysis

The experimenters were blinded to the identity of experimental groups for the manual quantification of tissue samples/cells. Data from allergic animal studies are presented as mean $\pm$ SEM and were analyzed by one-way analysis of variance followed by Bonferroni's multiple comparisons posttest. A *P* value less than 0.05 was considered significant.

Results

CD11c⁺ Cell and Platelet Colocalization Occurs in the Airways during Sensitization to Allergen

Platelets, in low numbers, have previously been observed adjacent to mononuclear cells in lungs of mice before allergen exposure (16). To identify these cells and to quantify the extent of platelet decoration, lung sections were stained for CD11c using an antimouse anti-CD11c antibody (green, indicated by white arrow in Figure 1A) as a marker for DCs (26) and stained for the presence of platelets using an antimouse anti-CD41 antibody (red in Figures 1B–1D). CD11c⁺ cells were found in close proximity to the airway wall of sham-sensitized (Figure 1B) and allergen-sensitized mice on Day 15, before allergen exposure (Figure 1C). CD11c⁺ cells in tissue sections from allergen-sensitized mice showed evidence of positive CD41

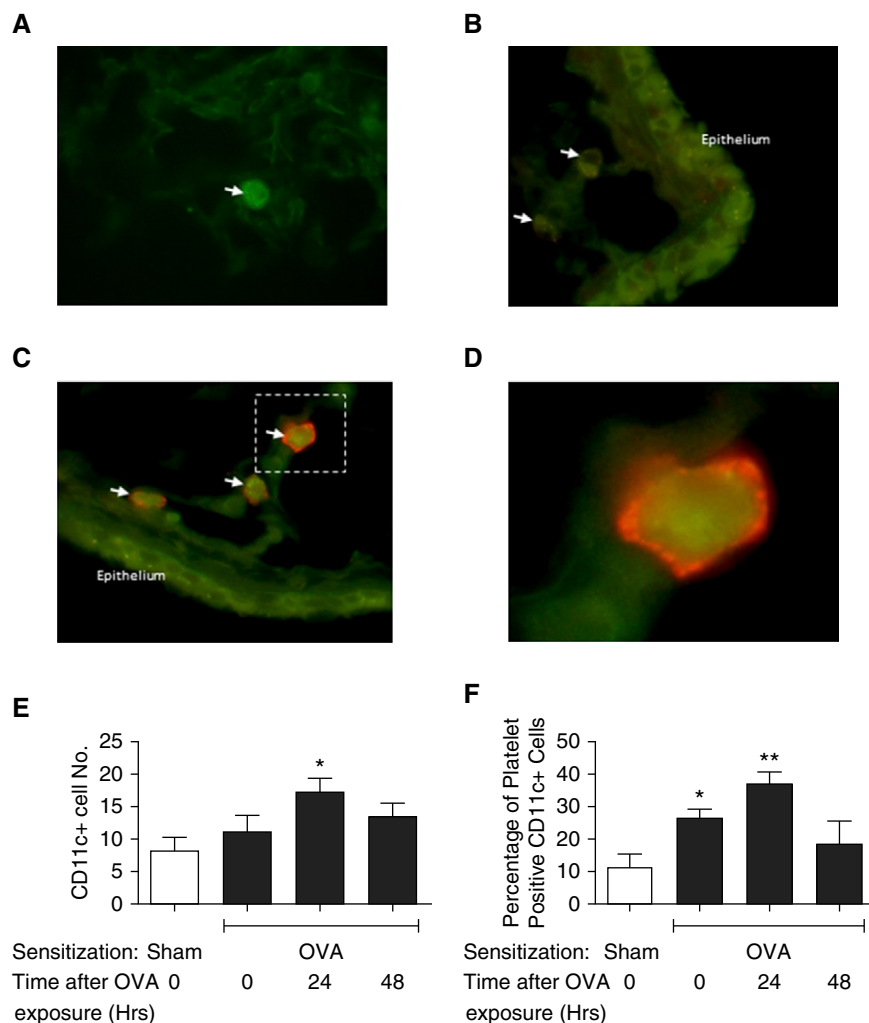


Figure 1. Association of platelets with CD11c⁺ cells in lungs of allergen-sensitized mice. Lungs from either sham-sensitized or allergen-sensitized (ovalbumin [OVA]) mice were collected at 0, 24, or 48 hours after exposure to aerosolized OVA from Day 15. Lung sections were stained for the presence of CD41⁺ (platelets, red fluorescence) and CD11c⁺ cells (green fluorescence). (A) Representation of CD11c⁺ cell (white arrow). (B) Platelets and CD11c⁺ cells localized to airways in sham-sensitized mice. (C) Platelets and CD11c⁺ cells localized to airways in allergen-sensitized mice. Arrows indicate the presence of CD11c⁺ cells. The dashed box inset is a zoomed image (created using Microsoft Office software). Individual platelets (red in C) are attached to the CD11c⁺ cell (D). Images were photographed using a $\times 40$ objective under a Zeiss Axiovert microscope. Images in A–D were enhanced to improve brightness/contrast using Microsoft PowerPoint software. (E) The presence of CD11c⁺ cells was quantified around airway walls. (F) The percentage of CD11c⁺ cells colocalized with CD41⁺ platelets was determined within the airway walls. $n = 4$ per group. * $P < 0.05$ and ** $P < 0.01$ compared with sham control group.

staining (Figure 1C); furthermore, individual platelets could be distinguished surrounding CD11c⁺ cells (Figure 1D). No difference in the number of CD11c⁺ cells was apparent between sham-sensitized and allergen-sensitized mice before allergen exposure (0 h) on Day 15 around the airway walls (Figure 1E). Twenty-four hours after allergen exposure (on Day 16), a significant increase in CD11c⁺ cell

localization was observed localized to the airway wall of allergen-sensitized mice ($P < 0.05$) (Figure 1E). Interestingly, there was also a significant increase in the incidence of platelets colocalized to CD11c⁺ cells around the airway walls in allergen-sensitized mice when compared with sham-sensitized mice before allergen exposure (0 h) on Day 15 (sham-sensitized vs. OVA-sensitized; $P < 0.05$)

(Figure 1F). This colocalization around the airway wall was further increased 24 hours after allergen exposure on Day 16 (sham-sensitized vs. OVA-sensitized; $P < 0.01$) (Figure 1F). Thus, the observation that platelets colocalized with tissue CD11c⁺ cells in response to allergen sensitization suggests that platelet activation (e.g., migration, intercellular communication) occurs and may therefore be relevant during allergen recognition by APCs.

Induction of Temporary Thrombocytopenia at Time of Allergen Sensitization

To evaluate whether platelets are involved in allergen sensitization, we developed a novel protocol to make mice temporarily thrombocytopenic during, but importantly restricted to, the distinct period of allergen sensitization rather than during allergen exposure, as previously reported (3, 10–12, 24, 25, 27). Platelet depletion using an antiglycoprotein Ib α antibody induced a 95% reduction of circulating platelets when administered 24 hours before each intraperitoneal administration of OVA (10 μ g/mouse on Days 0 and 7). Platelet counts were measured on Day –1, Day 0 (30 min before allergen sensitization), Day 9, and Day 14 (Figure 2A). Platelet counts had recovered by Day 14, 24 hours before the start of exposure to aerosolized allergen on Day 15 (Figure 2A). Importantly, temporary platelet depletion did not affect circulating leukocyte counts (Figure 2A).

Increased Platelet Expression of Fc ϵ RI Is Suppressed in the Recovered Platelet Population When Temporary Thrombocytopenia Occurs during the Period of Allergen Sensitization

Histological analysis revealed the colocalization of CD11c⁺ cells and platelets in lung tissue of allergen-sensitized mice before allergen exposure, and our previous data revealed that the accumulation of platelets into the lung after sensitization was dependent on platelet Fc ϵ RI (16). Furthermore, we have previously reported that the expression of Fc ϵ RI on platelets is increased in mice sensitized to allergen compared with sham-sensitized mice (16). We therefore examined the effect of platelet depletion at the time of sensitization on the expression of platelet Fc ϵ RI. Using a gating strategy to identify CD41⁺ platelet

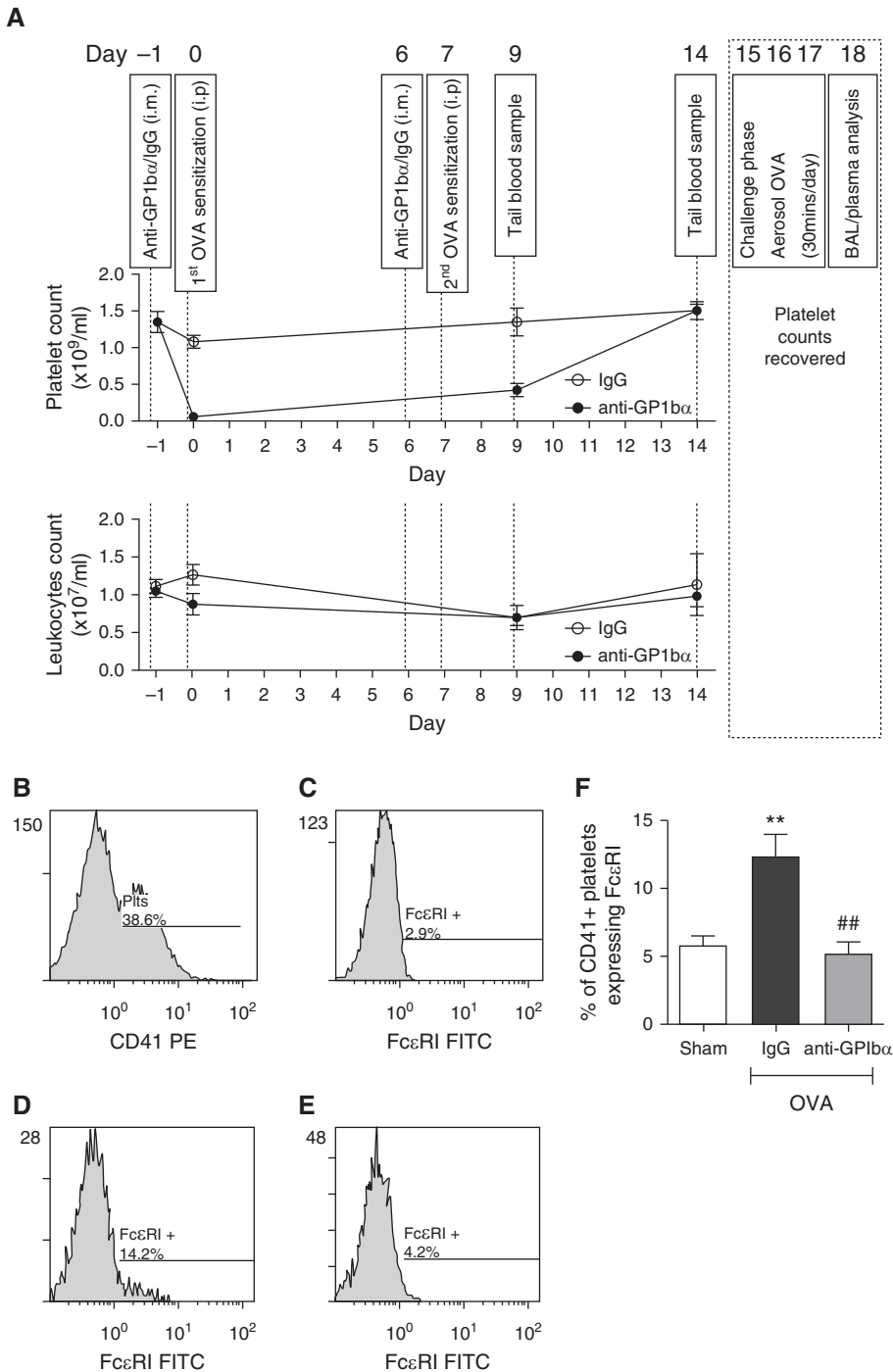


Figure 2. Induction of temporary thrombocytopenia during the period of allergen sensitization and effects on platelet FcεRI expression. (A) Mice were administered either a control IgG antibody or platelet-depleting antiglycoprotein 1bα (anti-GP1bα) antibody on Days -1 and 6 of the sensitization procedure to deplete circulating platelets within the time frame of allergen sensitization (OVA) on Days 0 and 7. Tail vein bleeds were conducted on Days -1, 0, 9, and 14 to enumerate circulating platelet and leukocyte numbers. Representative histograms show (B) CD41⁺ platelets from lysed whole blood and (C) FcεRI expression (using anti-FcεRI FITC conjugated antibody) in sham-sensitized mice, (D) OVA-sensitized + IgG mice, and (E) OVA-sensitized + anti-GP1bα mice. (F) FcεRI expression was recorded as the percentage of cells expressing antigen on Day 15 in the absence of secondary allergen exposure. *n* = 4–5 per group. Data are presented as mean ± SEM. ***P* < 0.01 versus sham-sensitized control group; ##*P* < 0.01 versus OVA-sensitized IgG group. PE = phycoerythrin.

events by flow cytometry (Figure 2B), we identified FcεRI on the surface of these cells (Figures 2C–2E). On Day 15, a significant increase in the proportion of platelets expressing FcεRI was observed in allergen-sensitized mice compared with sham-sensitized mice in the absence of allergen exposure (*P* < 0.01) (Figure 2F). This distinct population of platelets expressing FcεRI was significantly suppressed in allergen-sensitized mice depleted of platelets during the sensitization period compared with allergen-sensitized mice administered control IgG antibody (*P* < 0.01) (Figure 2F). Thus, given that only a distinct population of platelets express FcεRI, and given that the murine platelet life-span is 3–4 days, the process of sensitization to allergen continues to directly affect the manifestation of this platelet population or bone marrow megakaryocytes sometime after the platelet population temporally associated with the administration of allergen would have been consumed. These data suggest that the sensitization process toward allergen directly affects the phenotype of circulating platelets.

Temporary Thrombocytopenia at Time of Allergen Sensitization Inhibits Pulmonary Leukocyte Recruitment in Response to Subsequent Allergen Exposure

On Days 15, 16, and 17, mice were exposed to aerosolized allergen (OVA). Twenty-four hours after final allergen exposure (Day 18), BAL was performed to enumerate pulmonary leukocyte recruitment. Allergen exposure induced significant pulmonary recruitment of inflammatory cells in allergen-sensitized mice compared with sham-sensitized mice (eosinophils, *P* < 0.001 [Figure 3A]; macrophages, *P* < 0.05 [Figure 3B]; neutrophils, *P* < 0.05 [Figure 3C]; and lymphocytes, *P* < 0.01 [Figure 3D]). The presence of thrombocytopenia at the time of allergen sensitization in mice suppressed leukocyte recruitment compared with allergen-sensitized mice administered the nondepleting control IgG antibody (eosinophils, *P* < 0.01 [Figure 3A]; macrophages, *P* < 0.05 [Figure 3B]). Lymphocyte recruitment was also suppressed, but not to a significant degree (Figure 3D), whereas neutrophil recruitment was not altered in platelet-depleted animals (Figure 3C).

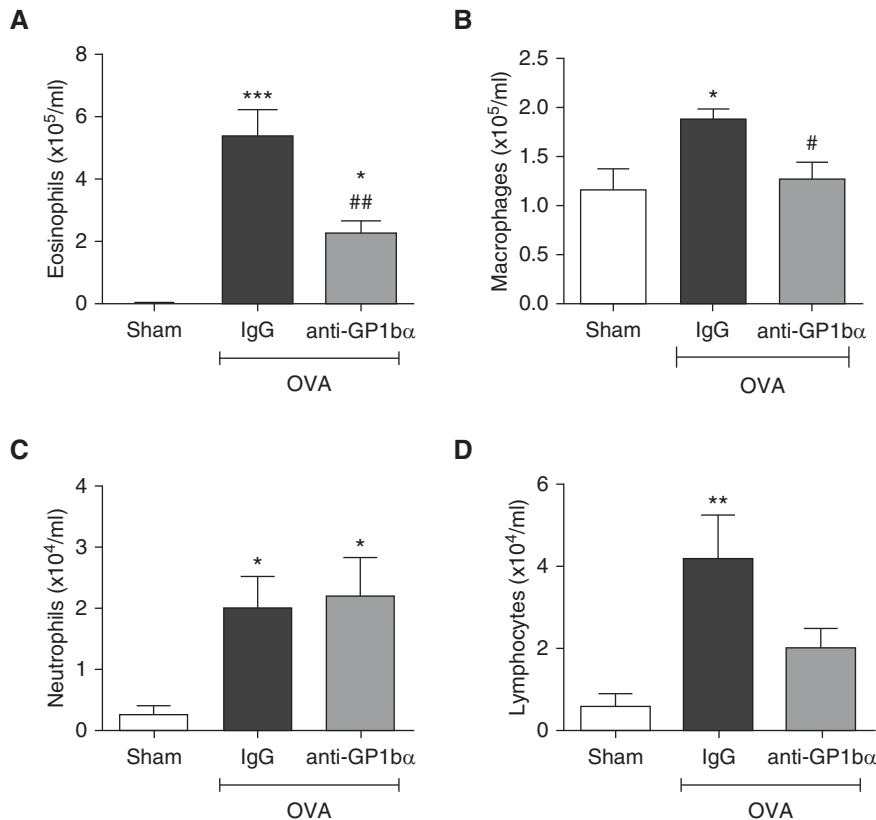


Figure 3. Effects of temporary thrombocytopenia during the period of allergen sensitization on the inflammatory response after subsequent allergen exposure. Mice were administered either a control IgG antibody or platelet-depleting anti-GP1b α antibody on Days -1 and 6 of the sensitization procedure to deplete circulating platelets within the time frame of allergen sensitization (OVA) on Days 0 and 7 . On Days 15 , 16 , and 17 , mice were subsequently exposed to aerosolized allergen (OVA) for 30 min/d before BAL was performed on Day 18 and enumerated for (A) eosinophils, (B) macrophages, (C) neutrophils, and (D) lymphocytes. $n = 8-12$ per group. Data are presented as mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus sham-sensitized control group. # $P < 0.05$; ## $P < 0.01$ versus OVA-sensitized group (OVA+ IgG).

Temporary Thrombocytopenia at the Time of Allergen Sensitization Suppresses IgE and IL-4 Production

We next investigated whether platelet depletion during the phase of allergen sensitization might affect the immune phenotype of the mice. IL-4, IL-5, and IFN- γ concentrations in BAL fluid, as well as IgE in serum, were measured from samples taken on Day 18 , 24 hours after the last day of allergen exposure on Day 17 . Significantly increased concentrations of IgE, IL-4, and IL-5, but not IFN- γ , were observed in allergen-sensitized mice compared with sham-sensitized mice (IgE, $P < 0.05$ [Figure 4A]; IL-4, $P < 0.05$ [Figure 4B]; IL-5, $P < 0.05$ [Figure 4C]). Temporary thrombocytopenia during the period of allergen sensitization resulted in suppression of IgE production ($P < 0.05$ [Figure 4A])

and IL-4 production ($P < 0.05$ [Figure 4B]), whereas IL-5 production (Figure 4C) and IFN- γ were not affected (Figure 4D). These data nonetheless suggest that platelets directly affect the Th2 sensitization process during this period.

Temporary Thrombocytopenia during the Sensitization Process Inhibits Pulmonary CD11c⁺ Cell Recruitment

We have previously reported that in noninflamed tissue, a small number of platelets reside in lung tissue, and 90% of these are attached to mononuclear cells (16). Indeed, there is a 29.7% increase in these events in OVA-sensitized mice compared with sham-sensitized mice as measured by immunohistochemistry of lung tissue sections for CD41⁺ events (sham, 84.1 ± 20.6 platelets/mm²; OVA, 109.1 ± 31.9

platelets/mm² [16]). Furthermore, this increased accumulation of platelets extravascularly and their association with mononuclear cells were suppressed in mice deficient in functional Fc ϵ RI, suggesting that platelet localization to lung-resident mononuclear cells in response to allergen sensitization was dependent on platelet activation via Fc ϵ RI (16). Given that platelet Fc ϵ RI expression is significantly decreased in animals made temporarily thrombocytopenic at the time of allergen sensitization (Figure 2F), we assessed whether the increase in CD11c⁺ cell numbers in the lungs after allergen exposure (Figure 1F) was platelet dependent. Lungs were taken from either sham-sensitized or allergen-sensitized mice at either 0 (Day 15) or 24 hours (Day 16) after allergen exposure, and they were processed into a single-cell suspension to allow quantitative flow cytometry of CD11c⁺ events with the use of a fixed number of fluorescent beads to assist with accurate enumeration (Figure 5A). In accordance with immunohistochemistry of mouse lungs, a significant increase in the number of CD11c⁺ cells was observed 24 hours after allergen exposure on Day 16 in allergen-sensitized mice compared with sham-sensitized mice ($P < 0.001$) (Figure 5C). This was a result of secondary allergen exposure rather than the sensitization process, because no increase in CD11c⁺ cells was observed before allergen exposure (0 h) on Day 15 (Figure 5B). This accumulation was reduced in allergen-sensitized mice rendered temporarily thrombocytopenic during the period of allergen sensitization ($P < 0.05$) (Figure 5C), suggesting that allergen-exposed platelets are necessary for pulmonary CD11c⁺ cell recruitment.

Discussion

We have shown, for the first time to our knowledge, that platelets are causal in the initial processing of allergen *in vivo*. The temporary depletion of platelets during the initial sensitization period suppressed inflammatory cell recruitment upon subsequent allergen exposure (aerosol allergen challenge) (Figure 3), and this correlated with an inhibition of IL-4 synthesis and IgE production (Figure 4). We have previously reported that allergen can induce platelet motility and migration

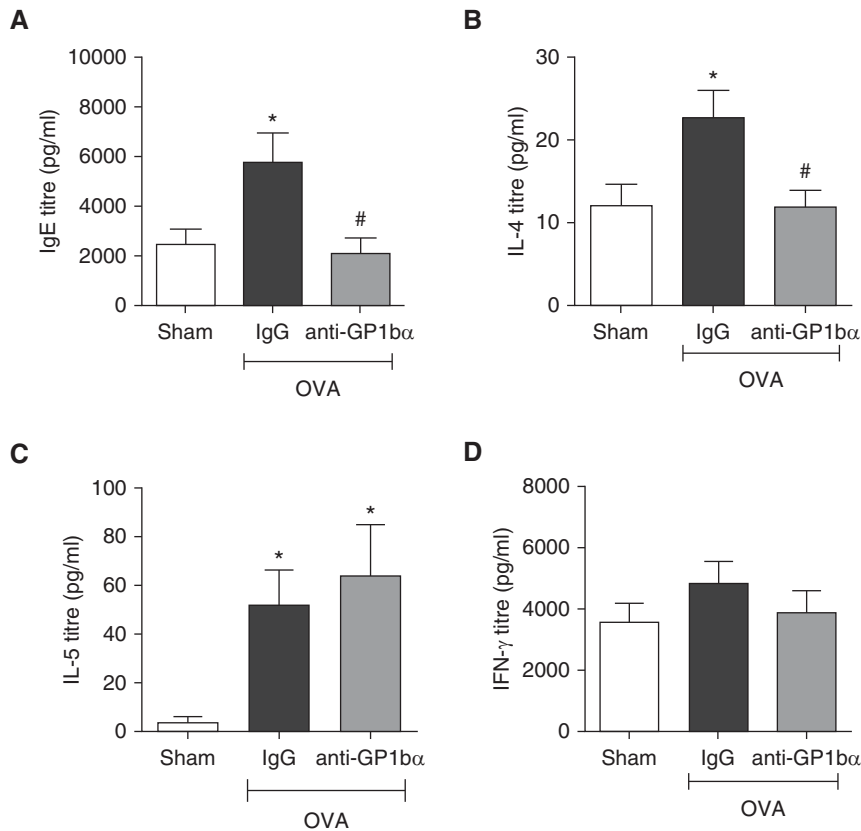


Figure 4. Effects of temporary thrombocytopenia during the period of allergen sensitization on the cytokine expression and IgE titer after subsequent allergen exposure. Mice were administered either a control IgG antibody or platelet-depleting anti-GP1b α antibody on Days -1 and 6 of the sensitization procedure to deplete circulating platelets within the time frame of allergen sensitization (OVA) on Days 0 and 7 . On Days 15 , 16 , and 17 , mice were subsequently exposed to aerosolized allergen (OVA) for 30 min/d before BAL. (A) Blood was harvested on Day 18 for plasma IgE analysis, and BAL was enumerated for (B) IL-4, (C) IL-5, and (D) IFN- γ titers. $n = 6-8$ per group. Data are presented as mean $\pm$ SEM. * $P < 0.05$ versus sham-sensitized control group; # $P < 0.05$ versus OVA-sensitized group (OVA+ IgG).

into lungs after allergen exposure (16), whereas others have also reported activation of platelets via IgE-dependent mechanisms (18). As shown in the present study, one consequence of platelet accumulation in tissue in response to allergen is platelet colocalization with CD11c $^{+}$ cells, a phenomenon that occurs before subsequent allergen exposure (Figure 1). Given the reduction in the Th2 response, we conclude that the actions of platelets during allergen sensitization are probably delivered via an IgE-dependent Fc ϵ RI process to interact with lung APCs, such as CD11c $^{+}$ DCs, to process allergen signaling with T cells in bronchial lymph nodes, although the data in the present study are restricted to CD11c $^{+}$ cells rather than specifically DCs that express the CD11c antigen. We note that in contrast to

other studies (24), we did not observe effects of platelet depletion on IgE and Th2 cytokine production after allergen exposure, either acutely (3 d of allergen exposure) or chronically (8 wk of allergen exposure), and the effects of platelets on Th2 cytokine production are constrained temporally to the time of allergen sensitization (3, 13, 25).

The phenomenon of allergen-exposed platelets being necessary for pulmonary CD11c $^{+}$ cell recruitment after secondary allergen exposure is unlikely to be due to an inability of the restored platelet population to efficiently recruit CD11c $^{+}$ cells from the circulation, because our previous studies have revealed that platelets from non-allergen-sensitized mice retain their ability to recruit leukocytes (3). Furthermore, neutrophil

recruitment to the lungs was not influenced by platelet depletion at the time of allergen sensitization, even though it is well understood that pulmonary neutrophil recruitment is dependent on intravascular interactions with platelets (10, 27).

It remains to be understood why professional APCs such as DCs might require platelet interactions for more efficient processing of allergen *in vivo*. DCs also express Fc ϵ RI in different conformations with cross-linked IgE; yet, it has been reported that IgE-mediated activation of DCs in allergic Th2-type inflammation might be immune-regulatory rather than proinflammatory (26, 28). Clearly, IgE/Fc ϵ RI-mediated antigen presentation by DCs is considered to be a critical event in the priming of Th2-type cells (29), but recent evidence suggests that this response is dependent on low antigen concentrations rather than on receptor signaling *per se* (30). T-cell activities induced after IgE/Fc ϵ RI-dependent processes by DCs have been reported not to develop as efficiently into Th2-type effector cells as those generated via an IgE/Fc ϵ RI-independent process (31). The process by which DCs process antigen and elicit a Th2-type response is complex and might therefore involve other cell types, including platelets that have been implicated in the innate response and express Fc ϵ RI, CD23, and other immunoglobulin receptors, as well as Toll-like receptors (2). Indeed, it has been reported that platelet communication with APCs is regulated by CD40/CD40L interactions, and this might be an important component of DC communication with T cells in the draining lymph nodes after platelet activation by allergen (26, 32). Currently, the understanding of why APCs might require platelets to effect an immune response is incomplete, but perhaps platelet activation by allergen or pathogens via IgE-dependent processes is a more direct innate response with less regulation and control. Two recent studies showed the ability of platelets to modulate the immune response in mouse models of eosinophilic and allergic airway inflammation (33, 34). The transfer of human platelets restored papain-induced airway eosinophilia on an IL-33-deficient background in mice, supporting a hypothesis that platelets are important in the IL-33-dependent provocation of Th2 immune cells (33). Furthermore, the platelet-derived Wnt

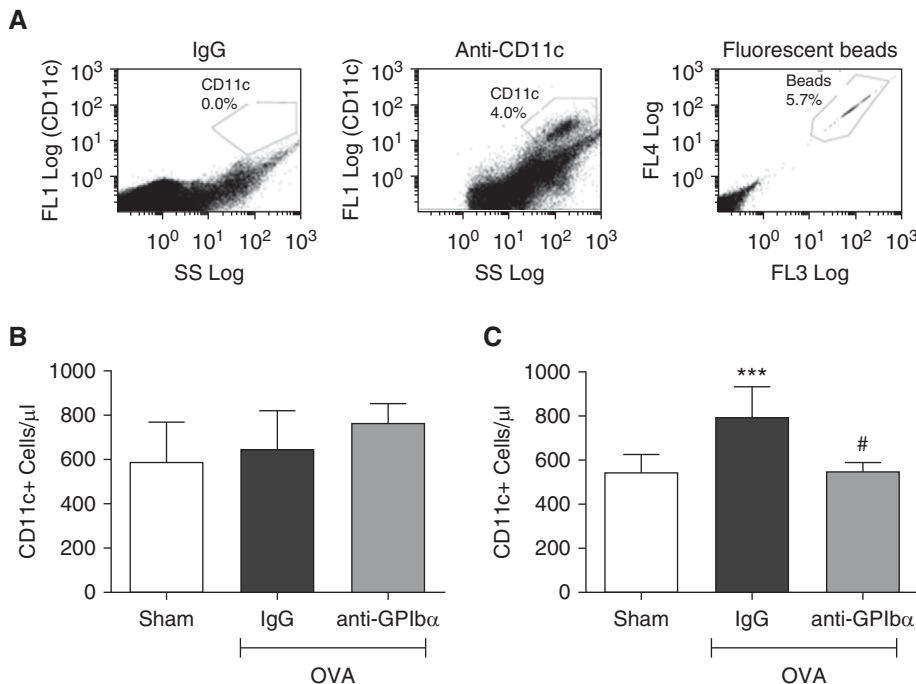


Figure 5. Effect of platelets and allergen on dendritic cell recruitment. Allergen-sensitized mice had previously been administered either control IgG or platelet-depleting anti-GPIIbα antibody during the allergen sensitization period, and lungs were harvested at 0 (Day 15) and 24 hours after (Day 16) exposure to aerosolized allergen (OVA) for histology or flow cytometry. (A) Lungs were processed into a single-cell suspension, and CD11c⁺ cells were quantified via flow cytometry using fluorospheres to obtain an accurate count at (B) 0 hours and (C) 24 hours after allergen challenge. $n = 3-9$. Data are presented as mean $\pm$ SEM. *** $P < 0.001$ versus sham-sensitized group; # $P < 0.05$ versus OVA-sensitized IgG group. FL = fluorescence.

antagonist Dickkopf-1 was reported to be important for Th2-cell cytokine production in a house dust mite model of asthma (34). The pathways by which platelets might affect sensitization to allergens in patients with asthma has not yet been described, but it is of interest that platelets from patients allergic to *D. pteronyssinus* have been shown to be

activated directly by the specific allergen and not to be activated by other known allergens to which the allergic blood donor was not sensitized (16, 18). Thus, clinical data are suggestive of an interaction between platelets and the sensitization process.

It is not understood how induced expression of FcεRI occurs on circulating

platelets (Figure 2). The percentage of platelets that express these receptors remains a small subset of the whole platelet population, which might suggest that megakaryocytes residing in the bone marrow are capable of differentiation to ultimately determine the phenotype of subsets of platelets. It is interesting to note, however, that this increase in platelet FcεRI expression is suppressed in mice subjected to temporary thrombocytopenia at the time of allergen sensitization (Figure 2). The life-span of murine platelets is between 3 and 4 days; yet, the recording in the present study was done when the platelet population had fully recovered, suggesting that there is establishment of immune memory in a subset of megakaryocytes to continue producing a population of platelets with increased expression of FcεRI.

To conclude, the initial processing of antigen in naive animals requires the involvement of platelets and leads to increased production of allergen-responsive platelets perhaps transmitted from megakaryocytes during thrombopoiesis. These findings support earlier clinical data regarding the ability of platelets to become activated by specific allergens to which individual patients with allergy become sensitized (16, 18, 35). These platelets respond by recruiting to organs that are exposed to the external environment and migrate to prime resident APCs for an efficient antigen-dependent immune response after subsequent exposure to allergen. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Page C, Pitchford S. Platelets and allergic inflammation. *Clin Exp Allergy* 2014;44:901-913.
- Idzko M, Pitchford S, Page C. Role of platelets in allergic airway inflammation. *J Allergy Clin Immunol* 2015;135:1416-1423.
- Pitchford SC, Yano H, Lever R, Rizzo-Vasquez Y, Ciferri S, Rose MJ, et al. Platelets are essential for leukocyte recruitment in allergic inflammation. *J Allergy Clin Immunol* 2003;112:109-118.
- Johansson MW, Han ST, Gunderson KA, Busse WW, Jarjour NN, Mosher DF. Platelet activation, P-selectin, and eosinophil β_1 -integrin activation in asthma. *Am J Respir Crit Care Med* 2012;185:498-507.
- 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.
- Knauer KA, Lichtenstein LM, Adkinson NF Jr, Fish JE. Platelet activation during antigen-induced airway reactions in asthmatic subjects. *N Engl J Med* 1981;304:1404-1407.
- 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;36:426-432.
- 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;145:571-576.
- Sullivan PJ, Jafar ZH, Harbinson PL, Restrict LJ, Costello JF, Page CP. Platelet dynamics following allergen challenge in allergic asthmatics. *Respiration* 2000;67:514-517.
- Pan D, Amison RT, Rizzo-Vasquez Y, Spina D, Cleary SJ, Wakelam MJ, et al. P-Rex and Vav Rac-GEFs in platelets control leukocyte recruitment to sites of inflammation. *Blood* 2015;125:1146-1158.

11. 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: 587–593.
12. 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: 948–954.
13. Pitchford SC, Riffo-Vasquez Y, Sousa A, Momi S, Gresele P, Spina D, et al. Platelets are necessary for airway wall remodeling in a murine model of chronic allergic inflammation. *Blood* 2004;103: 639–647.
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;21:151–159.
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: 1745–1753.
16. 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;177:604–612.
17. 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: 146–152.
18. 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 synthetic peptides derived from the allergen Der p 1. *Int Arch Allergy Immunol* 1992;98:127–134.
19. 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;187:476–485.
20. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015;16:45–56.
21. van Rijt LS, Vos N, Willart M, Kleinjan A, Coyle AJ, Hoogsteden HC, et al. Essential role of dendritic cell CD80/CD86 costimulation in the induction, but not reactivation, of T_H2 effector responses in a mouse model of asthma. *J Allergy Clin Immunol* 2004;114:166–173.
22. Lambrecht BN, Hammad H. Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. *Annu Rev Immunol* 2012;30:243–270.
23. Lambrecht BN, Hammad H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. *Lancet* 2010; 376:835–843.
24. Tian J, Zhu T, Liu J, Guo Z, Cao X. Platelets promote allergic asthma through the expression of CD154. *Cell Mol Immunol* 2015;12: 700–707.
25. 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;105:2074–2081.
26. Platzer B, Stout M, Fiebiger E. Functions of dendritic-cell-bound IgE in allergy. *Mol Immunol* 2015;68:116–119.
27. Amison RT, Momi S, Morris A, Manni G, Keir S, Gresele P, et al. RhoA signaling through platelet P2Y₁ receptor controls leukocyte recruitment in allergic mice. *J Allergy Clin Immunol* 2015;135: 528–538.
28. Baravalle G, Greer AM, LaFlam TN, Shin JS. Antigen-conjugated human IgE induces antigen-specific T cell tolerance in a humanized mouse model. *J Immunol* 2014;192:3280–3288.
29. Maurer D, Fiebiger S, Ebner C, Reininger B, Fischer GF, Wichlas S, et al. Peripheral blood dendritic cells express FcεRI as a complex composed of FcεRI α- and FcεRI γ-chains and can use this receptor for IgE-mediated allergen presentation. *J Immunol* 1996;157: 607–616.
30. Platzer B, Baker K, Vera MP, Singer K, Panduro M, Lexmond WS, et al. Dendritic cell-bound IgE functions to restrain allergic inflammation at mucosal sites. *Mucosal Immunol* 2015;8:516–532.
31. Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, et al. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential Toll-like receptor ligation. *J Exp Med* 2003;197:101–109.
32. Czapiga M, Kirk AD, Lektstrom-Himes J. Platelets deliver costimulatory signals to antigen-presenting cells: a potential bridge between injury and immune activation. *Exp Hematol* 2004;32:135–139.
33. Takeda T, Unno H, Morita H, Futamura K, Emi-Sugie M, Arae K, et al. Platelets constitutively express IL-33 protein and modulate eosinophilic airway inflammation. *J Allergy Clin Immunol* 2016;138: 1395–1403.e6.
34. Chae WJ, Ehrlich AK, Chan PY, Teixeira AM, Henegariu O, Hao L, et al. The Wnt antagonist Dickkopf-1 promotes pathological type 2 cell-mediated inflammation. *Immunity* 2016;44:246–258.
35. Zufferey A, Speck ER, Guo L, Aslam R, Kapur R, Semple JW. Murine bone marrow-derived megakaryocytes are capable of antigen cross-presentation on major histocompatibility class (MHC) I molecules. *Blood* 2015;126:3465.

ONLINE SUPPLEMENTARY MATERIAL

Platelets play a central role in sensitization to allergen.

Richard T Amison, PhD ¹; Simon Cleary, BSc ¹; Yanira Riffo-Vasquez, PhD ¹; Maida Bajwa, MSc ¹; Clive P Page, PhD ¹; Simon C Pitchford, PhD ¹

¹Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, London. SE1 9NH

A. Blood microsampling

For blood microsampling to enumerate circulating platelet and leukocyte numbers, mice were placed in a heated chamber (37 °C) for 10 minutes before a 1-2 mm cut was made with a scalpel blade across the lateral tail vein. Using a micropipette, 2 µl of blood was diluted 1:100 into stromatol solution for platelet counts, a further 2 µl diluted 1:50 in Turk's solution for leukocyte counts (both using a Neubauer improved haemocytometer). Manual counts were made using a Zeiss Axiovert microscope.

B. Immunohistochemistry of lungs.

At 0h, 24h, and 48h, post one day of allergen challenge on day 15, lungs were recovered from terminally anaesthetized mice. Lungs were inflated with a tracheal cannula with 4% PFA and then immersed to fix in 4% PFA for 1 hour. The lungs were then incubated in 10% sucrose solution (in PBS) overnight at 4°C. The sucrose solution was then replaced with 25% OCT (in PBS) for 1 hour, and then with 100% OCT for 1 hour. Lungs were then stored at -80°C, after which they were sectioned (6µm) with a Cryostat and placed on poly-L-lysine coated microscope slides. Tissue sections were stained with primary antibodies to detect CD11c⁺ cells (hamster anti-mouse CD11c Integrin α x chain, HL3, BD Biosciences) and

platelets (Goat polyclonal IgG Integrin α Ib. k-18, Santa Cruz Biotech), for 2 hours. Slides containing lung sections were then washed and incubated with secondary fluorescent antibodies: 1. Donkey anti-goat IgG-Cy3 for 1 hour, before PBS wash followed by 2. Goat-anti Armenian hamster IgG-FITC for 1 hour. Finally, slides were cover slipped using a photo bleaching protecting mountant and stored in the dark at 4°C until required for microscopic analysis.

C. Microscopic analysis and Lung morphometry

Lung section slides were analysed under a fluorescent microscope (Zeiss Axioskop) using a x100 objective, where CD11c+ cell localisation was measured around airway walls to a depth of 50 μ m beneath the sub-epithelial basement membrane, in a five field area. CD11c+ cells were investigated for platelet co-localisation (CD41+ events) and the extent of those with positive results quantified per airway using an image analysis programme (Scion Image; National Institutes of Health, Bethesda, MD).

D. Flow cytometric analysis of CD11c+ cells and platelets

All experiments were analysed on a flow cytometer (FC500 series Beckman Coulter) as previously described.^{10,16} Single cell suspensions isolated from lung digestion at 0 and 24 hours after allergen challenge on day 15 were incubated with the DC marker, anti-CD11c-fluorescein mAb, in a saturating concentration for 30 minutes at room temperature away from direct light. For quantification of CD11c+ cells, samples were also treated with Beckman coulter Flow CountTM fluorosphere beads (Beckman Coulter). Specific gating parameters were set up to calibrate for the assayed concentration of the fluorospheres to

allow absolute quantification of anti-CD11c-fluorescein positive events. For analysis of Platelet FcεRI expression, citrated blood was obtained via cardiac puncture on other groups of mice on day 15 in the absence allergen challenge (0 hours). Blood was incubated with platelet specific anti-CD41-phycoerythrin mAb and anti-FcεRI-fluorescein mAb in saturating concentrations for 30 minutes at room temperature and platelets were gated on size and FcεRI positive events were then measured on CD41 positive cells.

E. Quantification of IgE, IL-4, IL-5, and IFN-γ

Bronchoalveolar lavage samples taken at day 18 were centrifuged at 300 G for 5 minutes and supernatants harvested for quantification of *IL-4*, *IL-5*, and *IFN-γ* using specific ELISAs as per the manufacturer's instructions (Duoset, R&D systems). Total IgE was measured in plasma samples prepared from citrated blood harvested via cardiac puncture at day 18. A murine specific IgE ELISA was used as per the manufacturer's instructions (BD Pharmingen. Primary capture antibody: rat anti-mouse IgE clone R35-72).