

Allergen Induces the Migration of Platelets to Lung Tissue in Allergic Asthma

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Rationale: Platelets are essential for pulmonary leukocyte recruitment, airway hyperresponsiveness, and bronchial remodeling in animals with allergic inflammation and can be found in bronchoalveolar lavage of sensitized animals. No studies, however, have explored the direct migration of platelets to lungs.

Objectives: To assess whether platelets migrate into lung parenchyma in response to inhaled allergen in ovalbumin-sensitized mice; to assess the role of the FcεRI receptor in this phenomenon; and to evaluate whether platelets from patients with asthma, or from sensitized mice, undergo chemotaxis *in vitro* in response to relevant antigens.

Methods: Ovalbumin-sensitized wild-type (WT) mice, or FcRγ^{-/-} mice lacking the FcεRIγ, were challenged with aerosolized allergen and lungs analyzed by platelet-specific immunohistochemistry. In some experiments, mice were depleted of platelets and cross-transfused with either WT or FcRγ^{-/-} platelets to assess the role of platelet FcRγ^{-/-}. Chemotaxis of platelets from patients with asthma or from sensitized mice was studied *in vitro*.

Measurements and Main Results: Histology of lungs revealed isolated platelets, migrating out of vessels and localizing underneath the airways after allergen challenge in WT but not in FcRγ^{-/-} mice. Platelets from patients with asthma and from sensitized WT mice, but not from sensitized FcRγ^{-/-} mice, migrated *in vitro* toward the relevant allergen or an anti-IgE. Platelets from normal mice were found to express FcεRIγ and platelet-bound IgEs were increased in sensitized mice.

Conclusions: Platelets migrate extravascularly in response to a sensitizing allergen via a mechanism dependent on the interaction among allergen, allergen-specific IgE, and the FcεRI, and this may allow them to participate directly in allergic tissue inflammation.

Keywords: allergen; chemotaxis; FcεRI; IgE; inflammation; platelets

Extensive clinical data demonstrate that platelet activation accompanies allergen-induced bronchoconstriction in humans (1–4). Studies in animal models of allergic asthma have revealed

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Platelets are essential for pulmonary leukocyte recruitment, airway hyperresponsiveness, and airway wall remodeling in animal models of allergic inflammation. However, no studies have explored the direct migration of platelets in tissue.

What This Study Adds to the Field

We show that platelets are capable of directed migration both *in vitro* and in asthmatic lung.

the importance of platelets for acute bronchoconstriction, airway hyperresponsiveness, and bronchial wall remodeling (5–7).

Current research emphasizes a role of both acute (cellular infiltrate) and chronic (airway remodeling) inflammatory events in the pathophysiology of asthma (8).

We have previously reported that platelets influence leukocyte trafficking from blood vessels into lung tissue, because platelets are necessary for the pulmonary recruitment of eosinophils and lymphocytes in murine allergic inflammation (4, 9). Furthermore, platelet-leukocyte complexes are increased in blood from patients with asthma and allergen-sensitized mice, and leukocytes involved in these complexes migrate through endothelium into tissue with platelets adjacent to them (4, 9–11). We have also shown that airway wall remodeling fails to occur in allergen-sensitized mice depleted of platelets (12).

These observations suggest that platelets may act as inflammatory cells directly contributing to tissue damage and remodeling. However, to participate in such processes, cell migration through tissue is fundamental (13, 14). The ability of leukocytes to traffic into tissues in response to inflammatory stimuli is a central paradigm of inflammation and contributes to tissue remodeling (8, 13). We have previously detected platelets in the bronchoalveolar lavage fluid of allergen-challenged mice (12); these results strongly suggested that platelets were actively penetrating into lung tissue.

Indeed, platelets have been previously demonstrated to actively migrate through vascular endothelial cells in the skin of guinea pigs injected with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (15), and the presence of platelets in the extracellular compartment and on the surface of damaged respiratory epithelium has been reported in bronchial biopsies of allergic subjects with asthma (1).

Human platelets express the high-affinity receptor for IgE (FcεRI), and its stimulation induces the release of cytokines and serotonin (16–18) and the expression of antiparasite cytotoxic activity (19, 20), suggesting that several aspects of the partici-

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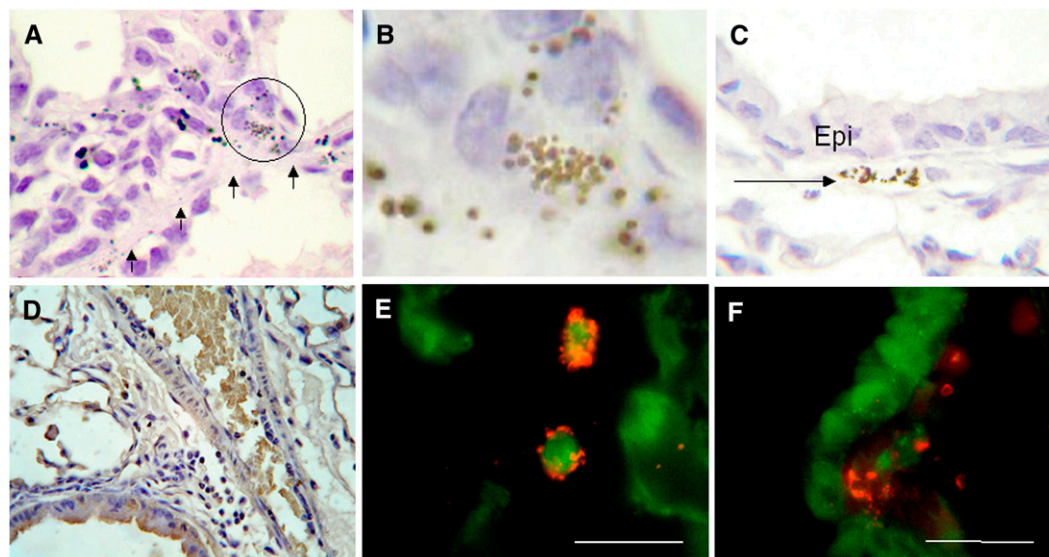


Figure 1. Histologic analysis of lungs from ovalbumin (OVA)-sensitized mice after allergen inhalation. Sections of lungs taken from OVA-immunized mice after allergen inhalation were stained for the platelet-specific antigen CD41 (integrin α IIb) using a specific goat anti-mouse polyclonal IgG antibody (sc-6604; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), via a horseradish peroxidase-streptavidin complex (sc-2053; Santa Cruz Biotechnology, Inc.); sections were background stained using Gill's hematoxylin (A–D), or (frozen sections) immunostained for CD41 (red) for platelet localization, and CD45 (green) for leukocyte localization within tissue (E, F).

(A) Platelets migrating into lung parenchyma from the vascular endothelium (arrows denote edge of endothelium). (B) An enlarged image of the area of tissue encompassed in the circle shown in (A) reveals nonaggregated platelets migrating not adjacent to leukocytes. (C) Platelets (arrow) localized underneath the airway epithelium (Epi) 30 hours after allergen exposure. (D) Staining for erythrocytes in inflamed lung tissue, using an anti-glycophorin A antibody: no erythrocytes can be seen outside blood vessels. (E) Platelets "orbiting" around leukocytes within the parenchyma. (F) Platelets within the proximity to airway walls. Horizontal bars represent 20 μ m. Original magnification, $\times 100$ objective, except for (D), which is $\times 40$.

pation of platelets in allergic inflammation are mediated by the activation of IgE receptors.

Other inflammatory cells, such as eosinophils, basophils and mast cells, were shown to migrate in response to allergen via an IgE-mediated mechanism involving Fc ϵ RI (21–23). We thus hypothesized that platelets could penetrate into lung tissue in response to allergen via an IgE-mediated mechanism.

Here we provide evidence for the first time that platelets migrate out of blood vessels into lung tissue *in vivo*, in allergen-sensitized mice, by a mechanism mediated by Fc ϵ RI γ , and that platelets from allergen-sensitized mice and from allergic patients with asthma undergo chemotaxis *in vitro* in response to the sensitizing allergen; we also show for the first time that the Fc ϵ RI γ is expressed on mouse platelets, that its surface expression is increased on platelets from allergen-sensitized mice, and that platelet-bound IgE is increased in allergen-sensitized mice. Our results show that platelets may make a previously unappreciated direct contribution to tissue inflammation in allergic conditions.

Some of the results of these studies have been previously reported in abstract form (24).

METHODS

Pulmonary Platelet Migration *In Vivo*: Studies in a Murine Model of Allergic Inflammation

Animals. Male C57BL/6 mice (20–25 g) were purchased from Charles River Italia (Como, Italy). For some selected experiments, mice deficient in the Fc receptor γ chains (FcR $\gamma^{-/-}$), thus lacking the Fc γ RI, Fc γ RIII, and Fc ϵ RI proteins (model 00583M, background C57BL/6) (25), were purchased from Taconic Europe A/S (Lille Skensved, Denmark) (*see details in the online supplement*).

Experimental procedure and tissue processing. Male C57BL/6 or FcR $\gamma^{-/-}$ mice were immunized to chicken egg albumin (ovalbumin [OVA]) as previously described (4). Fifteen days later, mice were exposed to aerosolized OVA (10 mg/ml) for three consecutive 15-minute periods separated from each other by 1 hour (model 104; FASET Aerosol Prisma, Milan, Italy) and lungs were then removed for histologic analysis at various time points afterward ($n = 8$ animals/group) using platelet antigen-specific antibodies (*see details in the*

online supplement). All studies were carried out under local ethical approval from the University of Perugia, Italy, and the Italian Ministry of Public Health (protocol no. 044/03) (*see details in the online supplement*). In some experiments, wild-type (WT) mice and FcR $\gamma^{-/-}$ mice were depleted of platelets, using a rabbit anti-mouse platelet serum (0.1 ml intramuscularly), and 24 hours later, cross-transfused with platelets from FcR $\gamma^{-/-}$ or WT mice, and vice versa, as previously described (4, 9). Briefly, platelet-rich plasma (PRP) from mice exposed to the above immunization protocol was injected intravenously into immunized and naive mice rendered thrombocytopenic, 1 hour before the start of allergen challenge. PRP was prepared from blood taken from anesthetized animals via cardiac puncture on acid citrate dextrose by centrifugation at 200 g for 10 minutes. The top layer was aspirated gently and added to 0.2 μ M prostaglandin I_2 (PGI $_2$). After 30 minutes' incubation at room temperature, to allow for the decay of PGI $_2$ activity, platelets were counted and 0.2 ml PRP was injected intravenously into thrombocytopenic animals.

Morphometry of airway tissue. Quantitative image analysis was carried out by counting the platelets present in lung parenchyma to a distance of 200 μ m outside blood vessels and to a depth of 50 μ m beneath the subepithelial basement membrane of distal airways. The area of parenchyma (μ m ^{2}) was measured using a $\times 100$ objective and an image analysis program (Scion Image; National Institutes of Health, Bethesda, MD) (*see details in the online supplement*) (9).

Analysis of Fc ϵ RI Expression in Mouse Platelets

Blood collected in sodium citrate 4% was centrifuged at 150 $\times g$ for 15 minutes and the supernatant PRP was centrifuged at 1,000 $\times g$ for 15 minutes to obtain platelet pellets. Fc ϵ RI γ chain mRNA expression was assessed by reverse transcriptase–polymerase chain reaction (RT-PCR); Fc ϵ RI γ -chain protein on the platelet surface was assessed by Western blotting or flow cytometry (*see details in the online supplement*).

Serum and Platelet-bound IgE

Non-anti-coagulated blood was collected from OVA-sensitized mice immediately after the last challenge on Day 15 and serum prepared and stored. Total serum IgE levels were then measured using an ELISA (Alpha Diagnostics, San Antonio, TX).

To detect IgE bound to platelets, diluted whole blood (1:10 in phosphate-buffered saline), from sham- and OVA-immunized mice, was incubated with a phycoerythrin-labeled anti-mouse integrin CD41

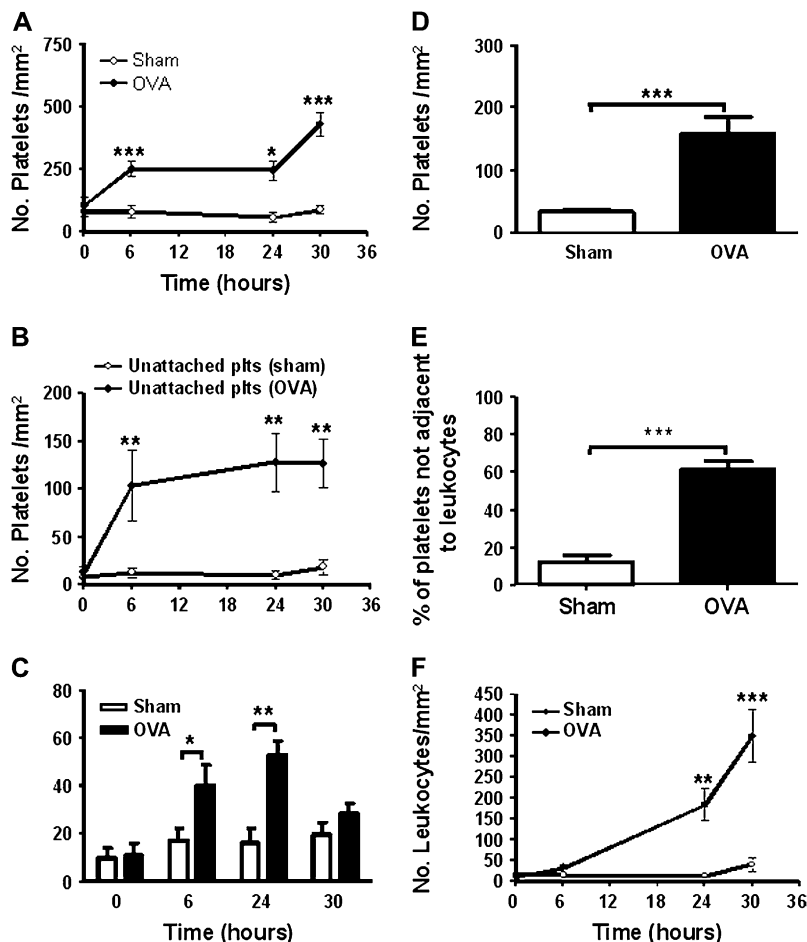


Figure 2. Morphometric analysis of platelet and leukocyte migration into lung tissue of ovalbumin (OVA)-immunized mice after allergen inhalation. Number of platelets and leukocytes in the lung parenchyma, to a distance of 200 μ m from blood vessel endothelium 0–36 hours post-allergen exposure, comparing sham- and OVA-immunized mice. (A) Total number of platelets. (B) Platelets not adjacent to leukocytes. (C) Percentage of platelets not adjacent to leukocytes compared with total. (D) Number of platelets/mm² within a distance of 50 μ m beneath the epithelial basement membrane of airways, 30 hours post-allergen exposure, comparing sham- and OVA-immunized mice. (E) Percentage of platelets not adjacent to leukocytes compared with total, localized beneath the airway wall (≤ 50 μ m). (F) Total number of leukocytes. $n = 16$ to 24 observations from lungs taken from eight mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; A, B, and F versus time 0; C, D, and F versus sham-immunized mice. plts = platelets.

monoclonal antibody (catalog no. M020-2; Emfret Analytics, Wuerzburg, Germany) and a fluorescein isothiocyanate-labeled rat anti-mouse IgE antibody (catalog no. 553415; BD Pharmingen, San Jose, CA). Samples were then analyzed by flow cytometry.

In Vitro Chemotaxis

The migration of murine and human platelets was studied *in vitro* using a modified Boyden chamber (26–28), as described in detail in the online supplement. Migration was expressed as “chemotactic index” defined as the average number of platelets that migrated in response to a chemotactic stimulus divided by the average number of platelets that migrated in response to vehicle (28).

Statistical Analysis

Data are expressed as arithmetic means $\pm$ SEM, and were analyzed with one-way analysis of variance, followed by the Bonferroni’s multiple comparison tests between all groups, using the GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). A P value of less than 0.05 was considered statistically significant. For the morphometry data, three slides for each mouse lung ($n = 8$ /group) were assessed and the mean value obtained was used for statistical analysis purposes.

RESULTS

Platelets Penetrate into Lung Tissue in Sensitized Mice after Allergen Inhalation

Platelets were detected in lung tissue of OVA-immunized mice 30 hours after allergen challenge; platelets were typically at the front of cellular migration away from the blood vessel (Figure 1A), mostly not bound to any other cells and not aggregated

(Figure 1B). Furthermore, platelets were visualized underneath the subepithelial basement membrane of airway walls (Figure 1C), suggesting that they were migrating in the direction of the focus of the inflammatory stimulus. Platelets were not observed in noninflamed tissues surrounding lungs, such as adipose tissue, lymph nodes, or cardiac tissue. In lungs of sham-immunized mice, platelets were virtually absent or present in low number in association with mononuclear cells and in close proximity (<10 μ m) of blood vessels. Platelets were not detected in lung sections taken from OVA-immunized mice previously depleted of platelets. Red blood cells (glycophorin A) were observed only inside blood vessels, and never in tissue, excluding the possibility that the platelets observed in tissues were brought there passively by unwanted hemorrhage (Figure 1D).

Immunofluorescent staining confirmed the presence in lung tissue both of isolated platelets, in proximity to the airway walls (Figure 1E), and of platelets adjacent to leukocytes. Indeed, when platelets were associated with leukocytes, this interaction appeared as a tightly bound attachment, with platelets spread on the leukocyte surface (Figure 1F).

Quantitative morphometric analysis from lungs of allergen-immunized mice undergoing allergen challenge revealed the penetration of a significantly higher number of platelets from blood vessels to a distance of 200 μ m into lung parenchyma, as compared with sham-immunized mice (Figure 2A). A significant number of the platelets detected in lung tissue appeared to be isolated, not bound to leukocytes (Figure 2C), although the fraction of platelets not adjacent to leukocytes at 30 hours post-challenge decreased, as compared with 6 and 24 hours post-challenge (Figure 2C), concomitantly to the increase of the

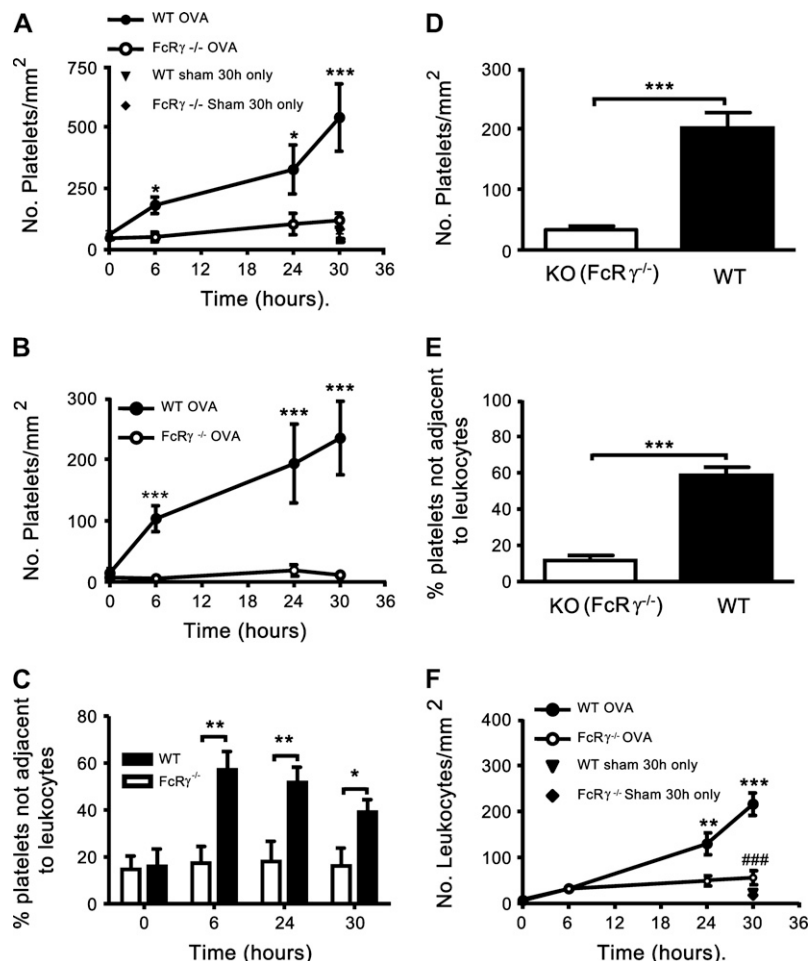


Figure 3. Morphometric analysis of platelet and leukocyte migration into lung tissue of ovalbumin (OVA)-immunized wild-type (WT) and FcRγ^{-/-} mice after allergen inhalation. Number of platelets and leukocytes in the lung parenchyma, to a distance of 200 μm from blood vessel endothelium, 0–36 hours post-allergen exposure, comparing OVA-immunized WT and FcRγ^{-/-} mice. (A) Total number of platelets. (B) Number of platelets not adjacent to leukocytes. (C) Percentage of platelets not adjacent to leukocytes compared with total. (D) Number of platelets/mm² within a distance of 50 μm beneath the epithelial basement membrane of airways 30 hours post-allergen exposure, comparing sham- and OVA-immunized mice. (E) Percentage of platelets not adjacent to leukocytes compared with total, localized beneath the airway wall (≤50 μm). (F) Total number of leukocytes. *n* = 8 to 12 observations from lungs taken from four mice. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; A, B, and F versus time 0; C, D, and E versus FcRγ^{-/-}.

number of leukocytes migrated into tissue at this time point (Figure 2F). The number of platelets in tissue within a distance of 50 μm from the surface of the subepithelial basement membrane at 30 hours post-allergen challenge was also significantly higher in OVA-immunized mice compared with sham-immunized mice (*n* = 8, *P* < 0.001), suggesting directed migration toward the airways (Figure 2D). A significant fraction of these platelets, too, were not adjacent to leukocytes (Figure 2E).

Leukocyte migration into tissue in OVA-immunized mice was evident at 24 and 30 hours after allergen challenge, and not at 6 hours (Figure 2F), suggesting that platelet migration preceded leukocyte trafficking into lung tissue. Interestingly, in mice made thrombocytopenic by an antiplatelet antiserum, the number of leukocytes in tissue 24 hours after allergen challenge was significantly reduced compared with normal mice (17.5 ± 6.2 vs. 156.2 ± 35.0 cells/mm²; *n* = 8, *P* < 0.001).

Platelet FcεRIγ Is Required for Platelet Penetration in Lung Tissue

The early localization of platelets in airway tissue after allergen challenge suggested a possible direct activation by allergen, thus via an IgE-dependent mechanism, similarly to what has been reported for mast cells (21). Therefore, we examined lung tissue from OVA-immunized mice deficient in the FcRγ chain, thus lacking FcγRI, FcγRIII, and FcεRI, undergoing allergen challenge. Platelet migration into lungs of OVA-immunized FcRγ^{-/-} mice was significantly lower compared with WT mice (Figure 3A). Here, too, a consistent fraction of platelets detected in the lung parenchyma of WT mice were not adjacent to leukocytes

(Figure 3C). The number of platelets detected immediately beneath the airways was also significantly reduced in FcRγ^{-/-} mice, both when evaluated as total number of platelets (Figure 3D) and as number of platelets not adjacent to leukocytes (Figure 3E).

Leukocyte migration into lung tissue was significantly lower in FcRγ^{-/-} mice as compared with WT mice at 24 and 30 hours after allergen challenge (Figure 3F), although a significant increase in the number of leukocytes in lung tissue was still observed 30 hours after allergen as compared with baseline (Figure 3F).

To further elucidate whether the Fc receptors of platelets, and not of other types of cells, are necessary for tissue penetration in response to allergen exposure, WT mice were selectively depleted of platelets and cross-transfused with platelets from FcRγ^{-/-} mice, and vice versa; a control group in which WT mice were cross-transfused with WT platelets was also studied. The number of circulating platelets and leukocytes before and after the cross-transfusion are shown in Table 1.

Remarkably, at 30 hours post-allergen challenge, platelets from FcRγ^{-/-} mice transfused into WT mice were unable to penetrate into the lung parenchyma, whereas platelets from WT mice transfused into FcRγ^{-/-} mice migrated in lungs in response to allergen to levels comparable to those observed in WT mice transfused with WT platelets (Figure 4A); a significant number of platelets detected in lungs of FcRγ^{-/-} mice transfused with WT platelets were not adjacent to leukocytes (Figure 4B) and were localized beneath the airway wall (Figures 4C and 4D). Interestingly, the transfusion of WT platelets into FcRγ^{-/-} mice restored leukocyte migration into lung parenchyma to levels comparable to those observed in WT mice transfused

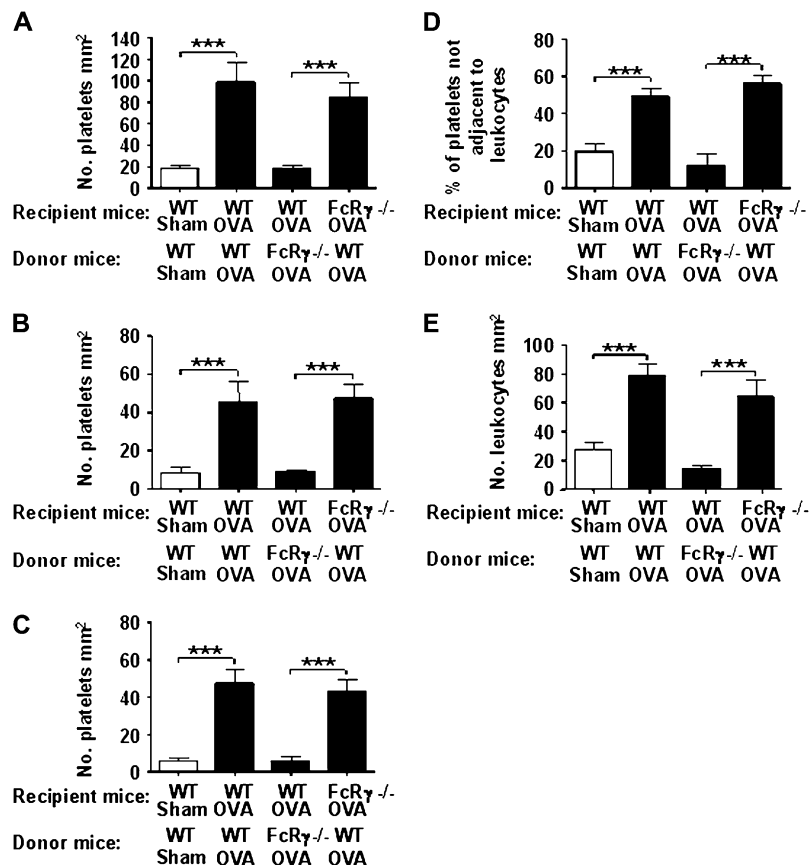


Figure 4. Morphometric analysis of platelet and leukocyte migration into lung tissue of ovalbumin (OVA)-immunized wild-type (WT) and FcγR^{-/-} mice depleted of platelets and subsequently cross-transfused with platelets from alternate WT or FcγR^{-/-} mice after allergen inhalation. Number of platelets and leukocytes in the lung parenchyma, to a distance of 200 μm from blood vessel endothelium, 30 hours post-allergen exposure, comparing OVA-immunized WT and FcγR^{-/-} mice. (A) Total number of platelets. (B) Number of platelets not adjacent to leukocytes. (C) Number of platelets/mm² within a distance of 50 μm beneath the epithelial basement membrane of airways, 30 hours post-allergen exposure, comparing sham- and OVA-immunized mice. (D) Percentage of platelets not adjacent to leukocytes compared with total, localized beneath the airway wall (≤50 μm). (E) Total number of leukocytes. n = 8 to 12 observations from lungs taken from eight mice. ***P < 0.001.

with WT platelets (Figure 4E). Thus, at early time points after allergen challenge, the presence of FcγR^{-/-} on platelets is an essential requirement for the recruitment in lungs of both leukocytes and platelets.

FcεRI and FcεRII (CD23) Expression in Mouse Platelets

We therefore assessed whether murine platelets express the high-affinity receptor for IgE (FcεRI), because this has pre-

viously been reported only for human platelets (16, 29), and whether this expression changes in sensitized mice, similarly to what has been observed in platelets of patients with allergic asthma (29).

The presence of FcεRIγ on mouse platelets was confirmed by several methods. RT-PCR showed the presence of mRNA for FcεRIγ chain (118 bp); the degree of expression was not significantly different in sham- and OVA-immunized mice (n = 3)

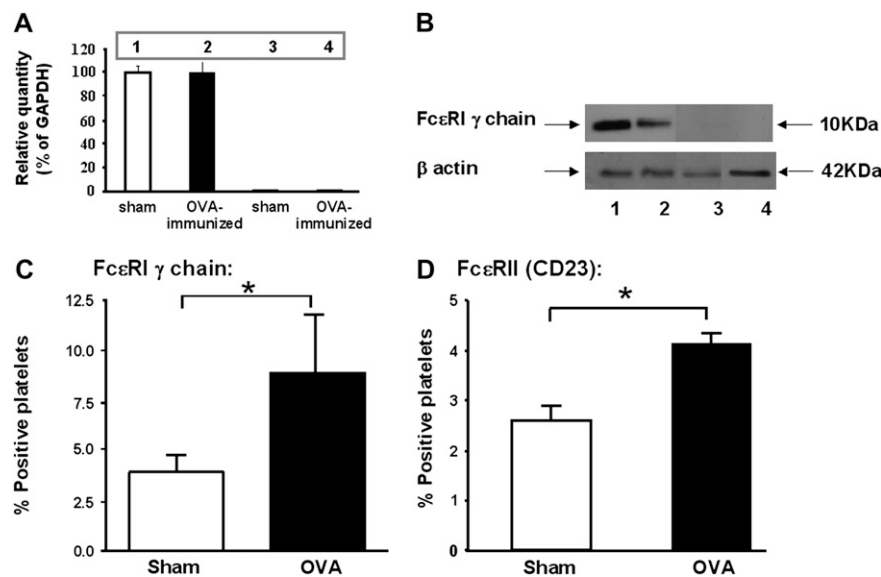


Figure 5. Demonstration of the FcεRIγ chain in mouse platelets. (A) mRNA FcεRIγ expression in platelets of sham- and ovalbumin (OVA)-immunized wild-type (WT) mice (n = 7) assessed by reverse transcriptase-polymerase chain reaction. Column 1 and 2: FcεRIγ chain gene expression in mRNA extracted from platelets from sham- and OVA-immunized WT mice; column 3 and 4: CD45 gene expression in mRNA from platelets of sham- and OVA-immunized mice. Each sample was tested in triplicate and the expression of the gene was then calculated by the ΔΔC_T method; data are given as relative quantity in percentage of GAPDH expression. (B) Western blotting identification of FcεRIγ chain in murine platelets. Platelet lysates from (1) sham- and (2) OVA-immunized WT mice and platelet lysates from (3) sham- and (4) OVA-immunized FcγR^{-/-} mice. Mouse anti-β-actin was used as an internal control. (C) FcεRIγ and (D) FcεRII (CD23) expression on the platelet surface of sham- and OVA-immunized WT mice assessed by flow cytometry. Data are expressed as percentage of positive cells. *P < 0.05 OVA- versus sham-immunized mice.

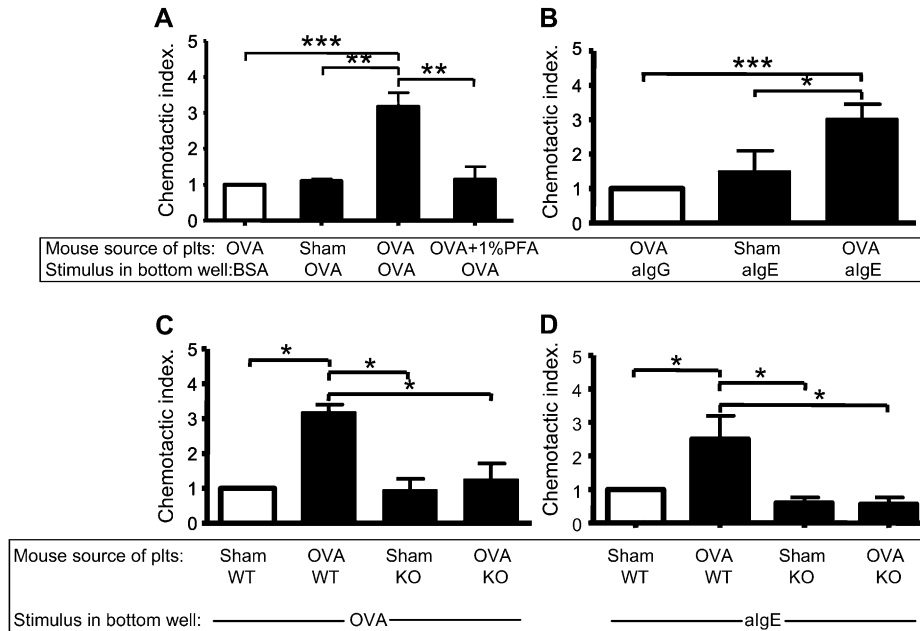


Figure 6. *In vitro* chemotaxis of murine platelets in response to allergen and anti-IgE and the role of FcεRI. (A) Chemotaxis in response to ovalbumin (OVA) or bovine serum albumin (BSA) of platelets from sham- or OVA-sensitized mice, or of platelets from OVA-sensitized mice fixed with paraformaldehyde (PFA). (B) Chemotaxis of platelets from sham- or OVA-sensitized mice in response to rat anti-mouse antibody anti-IgE or anti-IgG. (C) Chemotaxis in response to OVA (1 μg/ml) of platelets from OVA-immunized (OVA) or sham-immunized (Sham) animals, either of the wild-type (WT) or the FcRγ^{-/-} (model 00583M; Taconic Europe) genetic strain. (D) Chemotaxis in response to rat anti-mouse monoclonal anti-IgE antibody (10 μg/ml) of platelets from sham- or OVA-immunized animals, either of the WT or the FcRγ^{-/-} genetic strain. n = 12 field views of platelets taken from the blood of six mice. ***p < 0.001, **p < 0.01, *p < 0.05.

(Figure 5A). The absence of contamination of the platelet preparation by leukocytes was confirmed using primers for a leukocyte antigen (CD45) (Figure 5A) (*see also* Figure E1 in the online supplement).

Western blotting showed the presence of FcεRIγ in platelets from normal WT mice, without any significant difference between sham- and OVA-immunized mice, but not in platelets from FcRγ^{-/-} mice (29), whereas β-actin expression, evaluated as an internal control, was comparable in WT and FcRγ^{-/-} mice (Figure 5B).

Flow cytometry clearly showed the presence of a signal for FcεRIγ chain on the surface of mouse platelets; the receptor was significantly more expressed on platelets from OVA-immunized mice as compared with platelets of sham-immunized mice (9.8 ± 2.9 vs. 4 ± 0.9% of positive cells, *P* < 0.05) (Figure 5C).

Confirming previous data with rats (30), the low-affinity IgE receptor (FcεRII or CD23) was expressed on platelets from WT normal mice, and its expression was increased in OVA-immunized animals (Figure 5D).

Total Serum IgE and Platelet-bound IgE

Total serum IgE were significantly higher in OVA-sensitized as compared with sham-sensitized mice (1,121.5 ± 3.4 vs. 236.7 ± 3.7 ng/ml; n = 12, *P* < 0.001). Moreover, 50% of platelets of OVA-immunized mice and only 11% of platelets of sham-immunized mice (n = 6, *P* < 0.001) were bound to IgE when assessed by flow cytometry.

TABLE 1. CIRCULATING PLATELETS AND LEUKOCYTES MEASURED BEFORE AND AFTER CROSS-TRANSFUSION OF PLATELETS

Experimental Groups	Circulating Blood Cells in the Different Experimental Groups	
	Platelets (No./μl)	WBC (No./μl)
WT, untransfused	992,000 ± 65,961	3,762 ± 402
FcRγ ^{-/-} , untransfused	923,000 ± 30,769	4,500 ± 265
Donor WT, recipient FcRγ ^{-/-}	742,000 ± 20,099	3,650 ± 206
Donor FcRγ ^{-/-} , recipient WT	802,000 ± 33,041	3,560 ± 116
Donor WT, recipient WT	719,166 ± 33,041	3,150 ± 222

Definition of abbreviations: WBC = white blood cells; WT = wild-type.

Recipient mice were made thrombocytopenic by the injection of an anti-mouse antiplatelet serum and then cross-transfused with platelets taken from the donor group.

Allergen or an Anti-IgE Antibody Induces Migration of Murine Platelets *In Vitro*

Platelets of OVA-immunized mice migrated through micropore filters in response to OVA in a concentration-dependent manner (chemotactic index: OVA, 0 ng/ml: 1.0 ± 0.0; 10 ng/ml: 1.42 ± 0.11; 100 ng/ml: 2.12 ± 0.15, *P* < 0.01; 1,000 ng/ml: 3.17 ± 0.39, *P* < 0.01), differently from platelets of sham-immunized mice (1.1 ± 0.06, *P* < 0.01) (Figure 6A). This phenomenon was specific because platelets taken from OVA-immunized animals did not migrate in response to a nonsensitizing protein (bovine serum albumin, 1 μg/ml) (chemotactic index: 1.0 ± 0.0, *P* < 0.001, compared with OVA-challenged platelets) (Figure 6A). No migration occurred when platelets were fixed with 1% paraformaldehyde (chemotactic index: 1.15 ± 0.35) (Figure 6A), confirming that filter penetration is an active phenomenon requiring viable, activated cells. When allergen was incubated with platelets in the top well, migration was no longer evident, indicating the lack of induction of chemokinesis (data not shown).

Platelets from sensitized mice migrated through the filter in response to an anti-IgE antibody, significantly more than platelets from sham-sensitized control animals (Figure 6B). This phenomenon was specific for anti-IgE, because no migration was seen in response to an anti-IgG (30.0 μg/ml) (Figure 6B). No chemokinesis was observed when the anti-IgE was added to platelets in the top well (data not shown).

Platelets from OVA-immunized FcRγ^{-/-} mice did not migrate through filters in response to OVA or to an anti-IgE (Figures 6C and 6D).

Allergen or an Anti-IgE Antibody Induces Migration of Platelets from Patients with Asthma *In Vitro*

Platelets taken from subjects with allergic asthma migrated through micropore filters in response to the specific allergen to which the patient was sensitized, in a concentration-dependent manner and significantly more than platelets isolated from control subjects (Figure 7A). On the contrary, platelets from subjects with asthma did not undergo chemotaxis in response to a nonsensitizing allergen chosen among those giving a negative response to prick-puncture skin testing (data not shown).

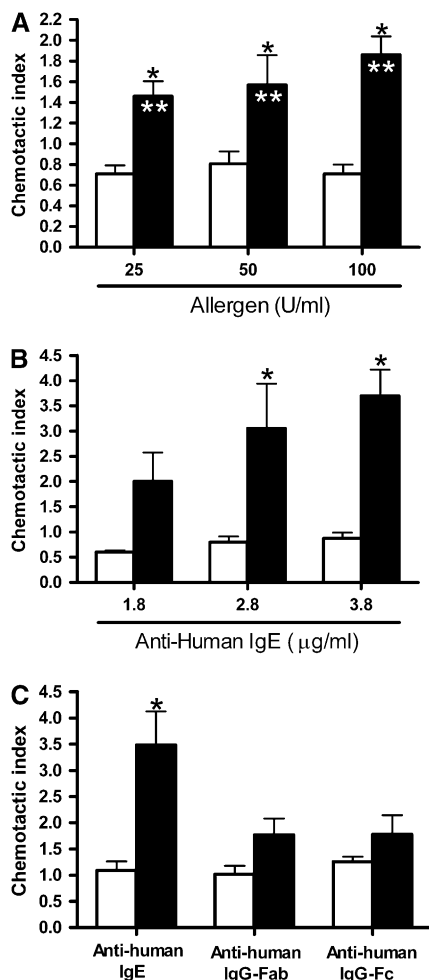


Figure 7. *In vitro* chemotaxis of platelets from allergic patients with asthma in response to sensitizing allergen or to monoclonal antibody anti IgE. (A) Dose-dependent platelet migration in response to sensitizing allergen of platelets from patients with asthma (solid bars) or from healthy control subjects (open bars). Data are expressed as chemotaxis index. (B) Platelet migration against different doses of monoclonal antibody (mAb) anti-human IgE. (C) Platelet migration against mAb anti-human IgE, anti-human IgG-Fc, and anti-IgG-Fab (3.8 µg/ml each). $n = 28$ field views of platelets. *Outside bars: $P < 0.01$ between platelet migration against specific allergen and medium, or between platelet migration against mAb anti-IgE and the relative control; **inside bars: $P < 0.01$ between subjects with asthma and healthy control subjects.

Platelets of patients with allergic asthma migrated in response to an anti-human IgE, in a concentration-dependent manner (Figure 7B). In contrast, incubation with anti-human IgG (either Fab or Fc) did not induce migration (Figure 7C). When the anti-IgE antibody was incubated with platelets in the upper chamber, migration was not significantly different from medium alone, suggesting lack of induction of chemokinesis (data not shown).

DISCUSSION

Our results provide for the first time evidence that platelets of allergen-sensitized mice undergo chemotaxis in response to the sensitizing allergen *in vivo* and *in vitro*.

In vivo, platelets of OVA-immunized mice were observed to migrate out of blood vessels and to localize in lung tissue directly

underneath the airways after the exposure to allergen. The penetration of platelets into lungs was not a consequence of hemorrhage, because no erythrocyte antigens (glycophorin A) were detectable extravascularly in lung tissue. Platelet influx preceded leukocyte influx into inflamed tissue, and a high proportion of platelets did not seem to be adjacent to leukocytes. However, some role for leukocytes in platelet migration into lungs cannot be definitely excluded from this histologic analysis.

Allergen-sensitized mice deficient in the Fc γ RI chain, and thus lacking the Fc γ RI, Fc γ RIII, and Fc ϵ RI cell surface receptors (25), did not show platelet migration in lung tissue upon allergen inhalation. Furthermore, platelets deficient in the Fc γ RI chain transfused into WT mice were unable to penetrate into lung parenchyma, demonstrating that direct activation of platelets via IgE receptors is required for allergen-triggered platelet migration *in vivo*.

Human platelets express both the high- and low-affinity receptors for IgE (Fc ϵ RI and Fc ϵ RII/CD23, respectively) (16, 29, 30), and a larger proportion of platelets from allergic subjects with asthma, as compared with healthy control subjects, express IgE binding sites on their surface (29). We reveal here for the first time that murine platelets also possess the high-affinity receptor for IgE (Fc ϵ RI) and that Fc ϵ RI is up-regulated in allergen-sensitized mice. Enhanced surface expression in sensitized animals, despite unchanged mRNA, may be the consequence of receptor stabilization by IgEs, which prevent receptor internalization and degradation, as already reported for monocytes, fibroblasts (31), or immature dendritic cells (32).

Mice lacking the Fc γ RI chain had a reduction of leukocyte migration into lungs early after allergen challenge compared with WT mice, in accordance with previous observations (33). This finding apparently contradicts previous evidence suggesting that eosinophil recruitment, and airway hyperresponsiveness, develop independently of IgE in murine models of allergic asthma (34, 35). However, others have shown a suppression of early leukocyte recruitment to the lungs after allergen exposure in mast cell-deficient mice (36), and antibodies against IgE have been reported to inhibit early antigen-induced eosinophilia (37). These contradicting results suggest that both IgE-dependent and IgE-independent (Th2-dependent) mechanisms can induce the recruitment of eosinophils to the airways, with IgE-dependent pathways accounting for early eosinophil recruitment and CD4 T-cell-dependent pathways accounting for late inflammatory responses (38). Indeed, we observed that, although reduced, leukocyte migration in lungs 30 hours after allergen challenge was significant in mice lacking the Fc γ RI chain. It is therefore likely that leukocyte infiltration in lungs develops later in time in mice deficient in the Fc γ RI chain, and/or upon repeated allergen exposure.

In vitro, platelets isolated from allergen-sensitized mice migrated toward the sensitizing antigen, as well as toward an anti-mouse IgE antibody, whereas platelets from Fc γ RI $^{-/-}$ animals did not undergo chemotaxis upon stimulation with either antigen or anti-IgE antibody, revealing a specific requirement for Fc ϵ RI in this phenomenon. Similarly, platelets of patients with allergic asthma migrated *in vitro* toward the specific sensitizing allergen and toward an anti-IgE antibody, whereas platelets from nonallergic donors did not.

The activation of platelets of patients with asthma via IgE was previously shown to result in the release of inflammatory mediators, such as RANTES (regulated upon activation, normal T-cell expressed and secreted), serotonin, thromboxane A₂, platelet-derived growth factor, or platelet factor (PF) 4 (16, 19, 29). Moreover, platelets from patients allergic to *Dermatophagoides pteronyssinus* were shown to be activated by synthetic peptides derived from the allergen through a process mediated by IgE (20).

Indeed, the cross-linking of receptor-bound IgE on platelets has been shown to trigger a number of platelet reactions, such as cytotoxicity against parasites (39), oxygen radical formation, and release of chemokines (16, 40).

The cross-linking of allergen-specific IgE bound to the FcεRI of mast cells or basophils was shown to result not only in the release of mediators but also in cell migration toward the specific allergen (21, 23).

It is thus conceivable that, in sensitized mice and in patients with asthma, allergen-specific IgEs produced after previous contact with the sensitizing antigen are bound to platelet IgE receptors and that, upon inhalation exposure, their binding to the allergen induces the cross-linking of contiguous receptors and the consequent triggering of cell migration (16–19, 21, 23). Indeed, we showed strikingly increased levels of total serum IgEs and a significant higher percentage of platelets with IgEs bound to their surface in allergen-sensitized animals. An anti-IgE antibody may mimic this phenomenon by binding to contiguous Fc portions of IgE receptor-bound IgEs, thus inducing cross-linking of the receptors. In accordance with this hypothesis, platelets of sensitized mice lacking the γ-chain subunit of the FcεRI receptors, which is required for their surface expression (41), did not undergo chemotaxis in response to either OVA or an anti-IgE.

Platelets have been previously reported to undergo chemotaxis *in vitro*, in response to prostaglandins (26), autoantibodies (42), the chemotactic peptide fMLP, or necrotic cells (43). Platelets have also been reported to penetrate *in vivo* in the skin of guinea pigs injected subcutaneously with fMLP (15) or of sensitized rabbits injected with anti-IgE antibodies (44), but ours is the first demonstration of a concentration-dependent (*in vitro*) and time-dependent (*in vivo*) platelet migration in response to an allergic stimulus.

The ability to extend pseudopod-like processes and the presence of an actin cytoskeleton allow platelets to undergo chemotaxis. Moreover, platelets contain releasable enzymes that may contribute to movement through the endothelium and connective tissue, such as glycohydrolases (cathepsin, heparinase, β-hexosaminidase), capable of degrading glycoproteins, glycolipids, and glycosaminoglycans (45), or matrix metalloproteinases (MMP2 and MMP9), capable of degrading collagen and fibronectin in the extracellular matrix (46, 47).

Our data suggest that platelets may actively penetrate in lungs in response to specific stimuli and this may allow them to participate in the pathogenesis of allergic asthma, although some role for leukocytes in platelet transmigration cannot be excluded. One consequence of persistent, chronic inflammation of the airways in asthma is alterations in anatomic structure, referred to as airway remodeling. Our previous studies have demonstrated an absolute requirement of platelets not only for the recruitment of inflammatory cells (9), confirmed by the present results, but also for the changes of airway architecture in murine asthma (12), suggesting a similarity to the contribution that platelets can make to the remodeling of blood vessels in atherosclerosis (48). In particular, platelet P-selectin appears to play a crucial role because P-selectin blocking antibodies, or the use of platelets from P-selectin knockout mice, were able to blunt inflammation in different models of lung injury (9, 49). However, platelet depletion did not alter the development of airway hyperresponsiveness (12). This may depend on a species-specific effect—in fact, platelets have been shown to be necessary for the development of airway hyperresponsiveness in guinea pigs and rabbits (5, 50)—and on the peculiar mechanisms regulating airway hyperresponsiveness in this allergic asthma model in the mouse in which alterations in lung tissue resistance and in airway nerves, rather than airway inflammation and remodeling, are involved (51, 52).

In conclusion, platelets behave as inflammatory cells *per se* in allergic asthma and not only as passive bystanders. Although the molecular events that follow to the interaction between allergen and FcεRI-bound IgEs in platelets remain to be elucidated, the unraveling of a direct role of platelets in tissue inflammation may represent the first step in the development of new therapeutic strategies aimed at preventing this initial triggering event in tissue inflammation.

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