Human platelets do not possess the FcεRI and FcεRII receptors for IgE

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Charles TACQUARD[1,2]*, Florian TUPIN[1]*, Stéphanie MAGNENAT[1], Nathalie BROUARD[1], Anita Eckly[1], Fabienne PROAMER[1], Carine METZ-FAVRE[3], Rodolphe STENGER[3], Anays PIOTIN[3], Frédéric DE BLAY[3], Didier G. EBO[4,5], Jessy ELST[4], Paul Michel MERTES[1,2], Béatrice HECHLER[1]

[1] Université de Strasbourg, INSERM, Établissement Français du Sang (EFS) Grand Est, BPPS UMR_S1255, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Strasbourg, France.

[2] Department of Anesthesia and Intensive Care, Strasbourg University Hospital, Strasbourg, France.

[3] Department of Pneumology and Allergology, Strasbourg University Hospital, Strasbourg, France.

[4] Faculty of Medicine and Health Sciences, Department of Immunology, Allergology, Rheumatology and the Infla-Med Centre of Excellence, University of Antwerp and Antwerp University Hospital, Antwerpen, Belgium.

[5] AZ Jan Palfijn Gent, Department of Immunology and Allergology, Ghent, Belgium. *First co-authorship.

Corresponding Author: Beatrice Hechler Ph.D., Université de Strasbourg, INSERM, Établissement Français du Sang (EFS) Grand Est, BPPS UMR_S1255, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Strasbourg, France. Phone: +033 3 88 21 25 25. Email: beatrice.hechler@efs.sante.fr.

To the Editor,

Anaphylaxis primarily involves antigen-specific immunoglobulins (IgE) and their high-affinity Fc receptor (FcεRI), while their low-affinity receptor FcεRII/CD23 might act as a regulator of IgE levels and immune responses. It has been suggested that blood platelets may contribute to IgE-mediated anaphylaxis, particularly the most severe reactions, but the mechanism of their activation is not established. In view of these uncertainties, we reassessed the effective presence of the FcεRI and FcεRII receptors on platelets from healthy donors as well as allergic patients (characteristics are detailed in **Supplementary Table 1**), given the potential stabilization of receptors on the cell surface in the presence of IgE[2].

Presence of FcεRI on platelets was evaluated using our well-established method to isolate washed platelets in their non-activated state[1], followed by flow cytometric analysis with a reliable method enabling a clear discrimination between specific and non-specific staining (**Fig. 1A**) and the latest analytical tools with validated monoclonal antibodies (mAbs) against FcεR1 and FcεRII, and a mouse mAb against platelet GPIX serving as positive control (**Fig. 1B**). FcεRI-α was undetectable on the surface of washed platelets from healthy donors and allergic patients using a mouse anti-human FcεRI alpha-chain (FcεRI-α) mAb (clone CRA-1) specifically recognizing an extracellular domain that does not overlap the region of the IgE binding site (**Fig. 1C**), whereas this antibody detected FcεRI-α on human basophils (**Fig. 1D**). Similar results were obtained using two other mouse anti-human FcεRI-α mAbs (clones 9E1 and CRA-2) reacting with an extracellular region and the IgE binding site on FcεR1-α, respectively (**Fig. 1C-D**). In addition, FcεRI-α was undetectable in platelet lysates from healthy donors and allergic patients by Western blot analyses using CRA-1 and 9E1, whereas both antibodies detected FcεRI-α (54 kDa) in human mast cell lysate (**Fig. 1E and S1**).

Since it cannot be excluded that FcεRI is present in very low quantities, below the detection threshold of Western blot or flow cytometry analyses, we next tested the functionality of the IgE-FcεRI pathway in washed platelets from healthy donors and allergic patients. Incubation of washed platelets with anti-TNP (trinitro-phenyl) IgE and challenge with TNP-BSA did not induce platelet aggregation (**Fig. 1Fa**). Similarly, stimulation of washed platelets from allergic patients with increasing concentrations of their specific allergen (protein or drugs), or with the anti-human IgE mAb G7-18, a specific FcεRI activator, did not induce platelet aggregation (**Fig. 1Fa-b**) or release of serotonin (**Fig. 1Fc**). In addition, exposure of whole blood from allergic patients to their specific allergen or to G7-18 did not induce platelet P-selectin exposure or integrin αIIbβ3 activation (**Fig. 1Ga-b**), whereas basophil activation was evidenced by CD63 exposure (**Fig. 1Gc**). Overall, neither platelets from healthy donors nor those from allergic patients responded directly to IgE stimulation.

Concerning FcεRII, its presence was also undetectable on platelets from healthy donors and allergic patients in flow cytometric analyses using a mouse anti-human CD23/FcεRII mAb (clone MAB123) directed against a large portion (aa 150-321) of the 274 aa extracellular region (**Fig. 2A**), with human B lymphocytes ($CD20^+$ cells) serving as a positive control (**Fig. 2B**). The full-length FcεRII receptor (~45 kDa) was also undetectable in platelet lysates from healthy donors and allergic patients by Western blot analysis, while three bands at ~25-30 kDa were detected (**Fig. 2C and S2**), potentially corresponding to cleaved fragments of FcεRII [3]. As it is unlikely that these fragments were generated during preparation of platelet lysates performed in the presence of a protease inhibitor cocktail, and soluble fragments would have been washed away, the possibility remains that FcεRII is normally present in a cleaved form on the surface of circulating platelets and that soluble FcεRII fragments have been internalized

into platelets. Indeed, confocal immunofluorescence microscopy (**Fig. 2D**) and immunogold electron microscopy (**Fig. 2E**) using MAB123 mAb on washed platelets indicated intracellular localization of FcεRII in their cytoplasm and on alpha-granule membranes but not on plasma membrane. Whether this constitutes a negative regulatory mechanism of the functions of soluble FcεRII in the regulation of IgE levels and immune responses is unknown.

In contrast to previous studies reporting the presence of FcεRI and FcεRII on platelets [4-7], we used our rigorous, reliable and well-established preparation of washed platelets at 37°C (and not at 4°C, known to induce platelet activation), including addition of platelet activation inhibitors (PGI_2, heparin and apyrase), allowing isolation of platelets in their resting state, without formation of platelet/leukocyte aggregates[1]. Deviating from these strict conditions leads to pre-activation of platelets resulting in the formation of platelet-leukocyte aggregates likely responsible for false positive detection of FcεRI and FcεRII on the platelet surface by flow cytometry.

This study highlights that human platelets do not possess the FcεRI and full-length FcεRII receptors on their surface and are not able to directly respond to stimulation of the IgE pathway. However, indirect contribution of platelets, as secondary effector cells in IgE-mediated anaphylaxis cannot be excluded.

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Authorship Contributions

C.T. and B.H. designed the research. C.T., C.M.F., R.S., A.P. and F.D.B contributed to patients' selection and data collection. C.T., F.T., S.M., N.B., A.E., F.P., and J.E. performed experiments. C.T., F.T., S.M., N.B., A.E., D.G.E., P.M.M. and B.H. analyzed and interpreted the data; C.T. and B.H. draft the manuscript and B.H. supervised the study. All authors edited the manuscript and gave their final approval for submission.

Disclosure of Conflicts of Interest

The contributing authors have no conflicts of interest to disclose.

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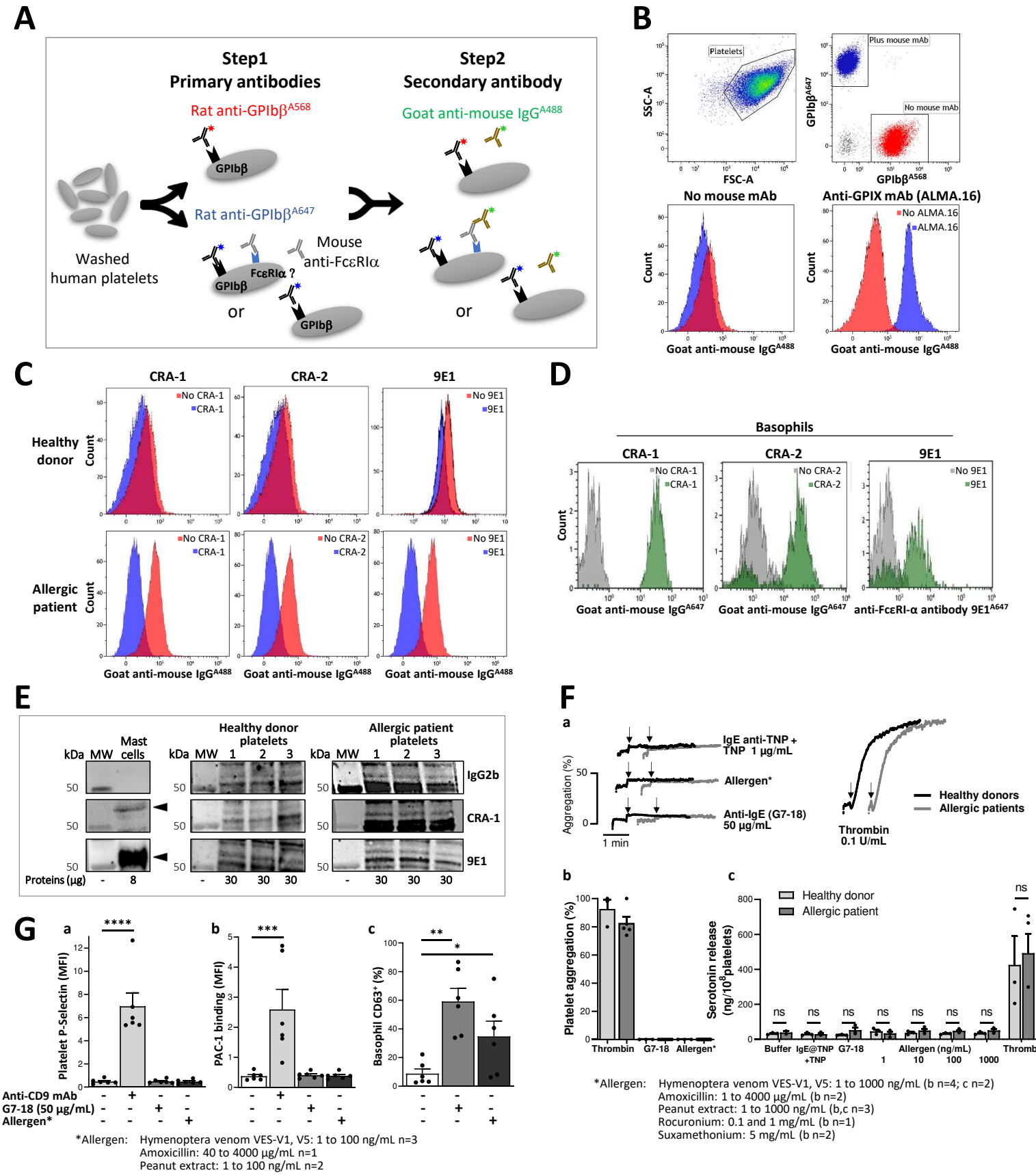

Figure 1: **Platelets from healthy donors or allergic patients do not possess the FcεRI receptor.** Analyses of platelets (**A-C,E-F, Ga-b**) and basophils (IgE$^+$ cells) (**D, Gc**) from healthy donors (**B-F**) or allergic patients (**C,E-G**). **A-C.** Flow cytometric protocol outline using a double-labeling method (with rat anti-mouse GPIbβ^{A568} and GPIbβ^{A647}) to distinguish specific from non-specific staining (**A**), gating of washed labeled platelets and staining with a mouse mAb against human GPIX (ALMA.16) (positive control) (**B**) or with mouse anti-human FcεRI-α mAbs CRA-1, CRA-2 and 9E1 (n=1 representative of n=5) (**C**). **D.** Flow cytometric detection of FcεRI-α on human basophils (IgE$^+$ cells) using CRA-1, CRA-2 or 9E1^{A647}. **E.** Reducing SDS-PAGE Western blot analyses of FcεR1-α (54 kDa) in platelet lysates (n=3 representative of n=5) and mast cells (positive control). Mouse IgG2b: non-immune control antibody for CRA-1 and 9E1. **F.** Aggregation profiles (**a**), maximal aggregations (mean ± s.e.m.) (**b**), platelet serotonin release (P>0.05, not significant ns) (**c**). **G.** Flow cytometric analyses of platelets (**a-b**) and basophils (IgE$^+$ cells) (**c**) in whole blood from allergic patients (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Expression of P-selectin (**a**) and the activated form of αIIbβ3 (PAC-1 binding) (**b**), and CD63 (**c**). Positive control: rat anti-human CD9 mAb (ALMA.1 10 µg/mL) activates platelets in a FcγRIIA-dependent manner. Data are representative of three independent experiments (B,D).

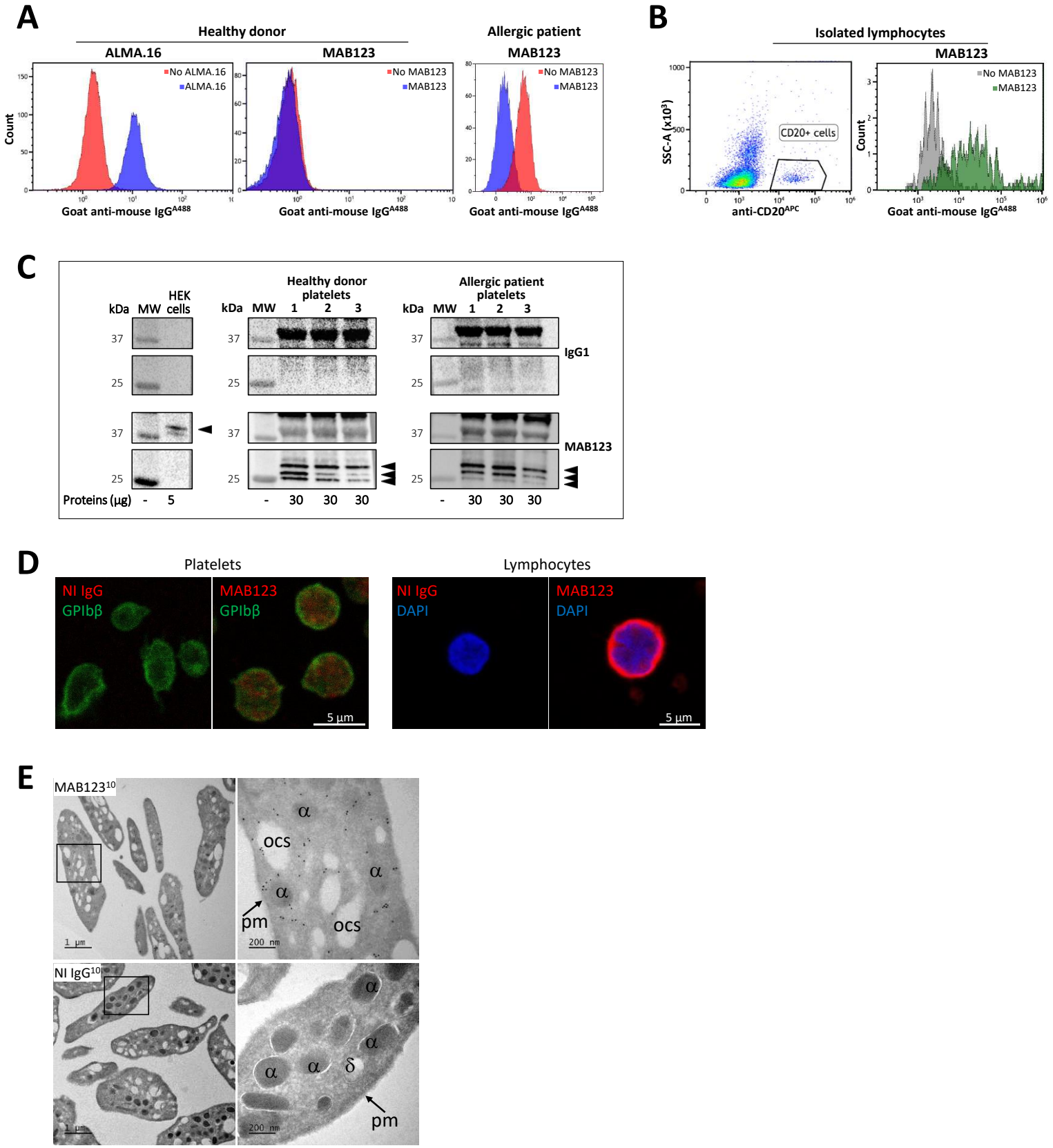

Figure 2: The full-length FcεRII receptor is undetectable on platelets from healthy donors or allergic patients. A-B. Flow cytometric analyses of washed platelets (**A**) or human B lymphocytes (CD20⁺ cells) (**B**). **A.** Double-labeling method described in Fig.1 to distinguish specific from non-specific staining, followed by staining with mouse anti-human GPIX mAb ALMA.16 (positive control) or with the mouse anti-human CD23/FcεRII mAb (MAB123) (n=1 representative of n=5). **B.** Detection of CD23/FcεRII using MAB123 followed by a goat anti-mouse IgG^{A488}. **C.** Reducing SDS-PAGE Western blot analyses of FcεRII in platelet lysates (n=3 representative of n=5) and HEK cells overexpressing human FcεRII (positive control). Mouse IgG1: non-immune control antibody for MAB123. **D**. Confocal observation of washed platelets immunolabeled with the anti-FcεRII mAb (MAB123) showing intracellular localization of FcεRII (red) as compared to GPIbβ-positive plasma membranes (green). Human lymphocytes were also immunolabeled and imaged as a positive control and showed plasma membrane labeling of FcεRII (red) and nucleus (DAPI, blue). **E.** Transmission electron microscopy of washed platelets and immuno-gold labeling of FcεRII with MAB123 mAb (10 nm Protein A gold particles PAG). Platelets are shown to label positive for FcεRII in their cytoplasm and on alpha-granule membranes but not on plasma membrane. Data are representative of three independent experiments (B,D,E).

Supplementary Table 1: Characteristics of allergic patients. CT: challenge test; F: female; H: concordant clinical history; M: male; sIgE: serum allergen-specific IgE; ST: skin test; yo: years-old.

Sex	Age (yo)	Specific Allergen	Clinical presentation	Allergy workup	sIgE	sIgE level (kUI/L)	Baseline serum tryptase (µg/L)	FcERI/FcERII labelling	Platelet/basophil activation test	Platelet aggregometry
M	30	Wasp venom	Anaphylaxis	H+ST+sIgE	Ves V1	1.13	5.2		✓	✓
M	61	Wasp venom	Anaphylaxis	H+ST+sIgE	Ves V5	0.53	2.86		✓	✓
F	73	Wasp venom	Anaphylaxis	H+ST+sIgE	Ves V1	1.90	2.93		✓	
F	21	Wasp venom	Anaphylaxis	H+ST+sIgE	Ves V5	1.26	2.05	✓		✓
M	26	Wasp venom	Anaphylaxis	H+ST+sIgE	Ves V1	6.33	4.87	✓		✓
F	19	Peanut	Anaphylaxis	H+ST+CT+sIgE	Ara h2	80	N/A		✓	
F	21	Peanut	Anaphylaxis	H+ST+CT+sIgE	Ara h2	89	6.86		✓	
F	17	Peanut	Anaphylaxis	H+ST+sIgE+CT	Ara h2	15.5	N/A	✓		✓
F	38	Peanut	Anaphylaxis	H+ST+sIgE	Ara h2	7.65	N/A	✓		✓
F	45	Peanut	Anaphylaxis	H+ST	N/A	N/A	N/A	✓		✓
F	63	Amoxicillin	Anaphylaxis	H+ST+sIgE	Amoxicillin	4.57	5.18		✓	
M	38	Amoxicillin	Anaphylaxis	H+ST+CT	Amoxicillin	<0.1	N/A			✓
M	65	Amoxicillin	Anaphylaxis	H+ST	N/A	N/A	N/A			✓
F	52	Suxamethonium	Anaphylaxis	H+ST+sIgE	C202	0.37	5.84			✓
M	71	Suxamethonium	Anaphylaxis	H+sIgE	C260	4.12	5			✓
F	58	Rocuronium	Anaphylaxis	H+sIgE	C260	22.4	1			✓

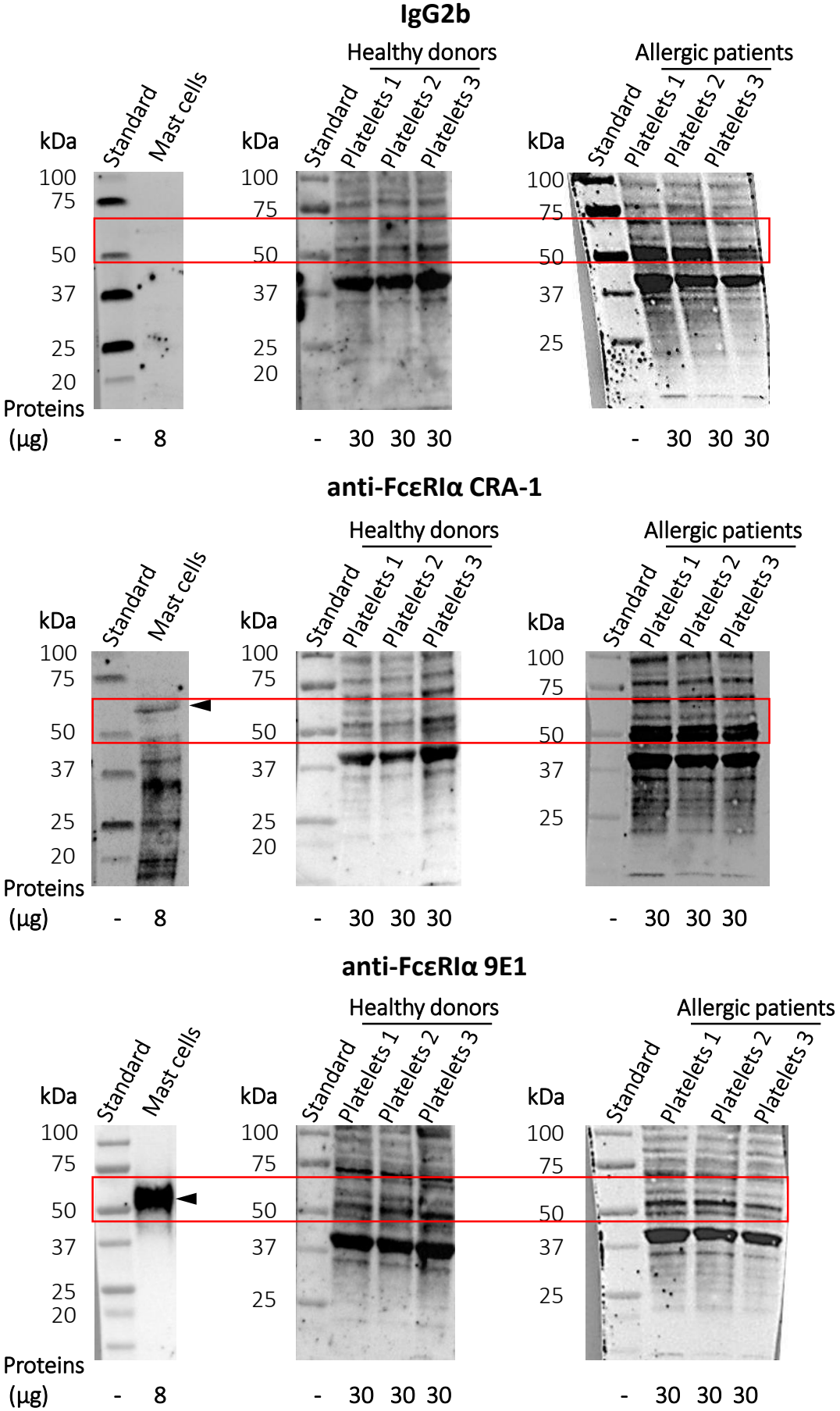

Figure S1: Full pictures of the reducing SDS-PAGE Western blot analyses shown in Figure 1 looking for the presence of FcεRI-α protein in lysates of human platelets and mast cells.

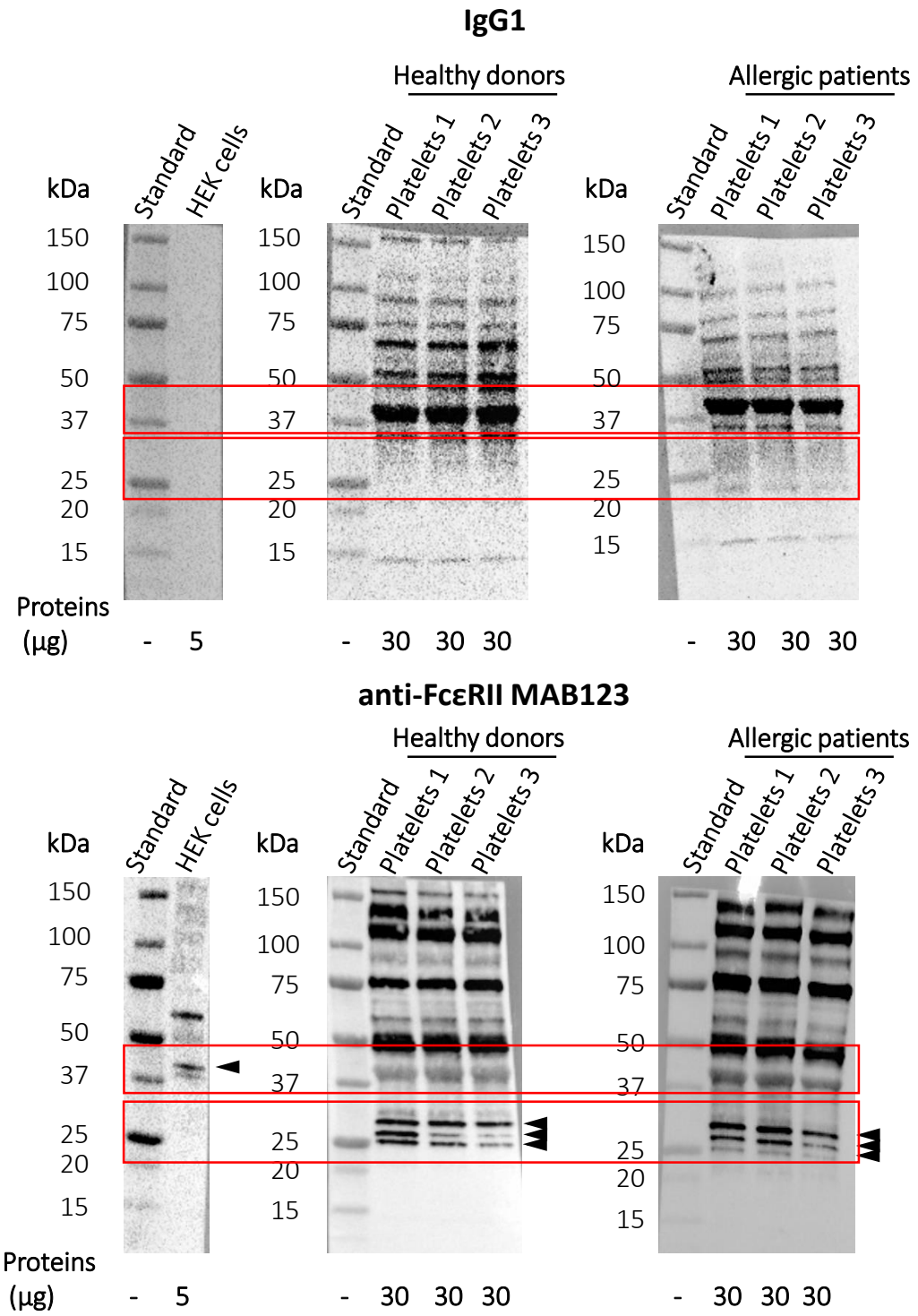

Figure S2: Full pictures of the reducing SDS-PAGE Western blot analyses shown in Figure 2 looking for the presence of FcεRII protein in lysates of human platelets and HEK cells overexpressing the human FcεRII receptor.

Supplementary methods

Antibodies and reagents. Mouse anti-human FcεRI alpha-chain (FcεRI-α) monoclonal antibodies (mAbs): clone CRA-1 (IgG2b) was purchased from Biolegend, clone 9E1 (IgG2b) from Aviva Systems Biology and clone CRA-2 (IgG1) from AbNOVA. A mouse anti-human CD23/FcεRII mAb (IgG1, clone MAB123) was from BioTechne. TNP (trinitro-phenyl)-BSA (LSL-LG-1117) was purchased from Cosmo Bio Ltd., while mouse anti-TNP IgE (C48-2, 557080) and a mouse anti-human IgE mAb (G7-18, 555894) were purchased from BD Pharmingen™. A FITC-conjugated mouse anti-human IgE antibody (SAB4700354) was from Sigma-Aldrich. APC-conjugated mouse anti-human CD203c (324610) and PE-conjugated mouse anti-human CD63 (353004) mAbs, and FcR blocking reagent (Trustain) were purchased from Biolegend. A FITC-conjugated PAC-1 mAb and a FITC-conjugated anti-P-selectin mAb (CLB-Thromb/6) were from Beckman Coulter, and an APC-conjugated mouse anti-human CD20 antibody (2H7) and an A488 or A647-conjugated goat anti-mouse antibodies were from Invitrogen. IL-3 (200-03) was purchased from PeproTech. A568- or A647-conjugated rat anti-mouse GPIbβ (clone RAM.1), rat anti-human GPIX (clone ALMA.16) and rat anti-human CD9 (clone ALMA.1) mAbs were produced in our laboratory. VES-V1 came from INDOOR Technology, VES-V5 from Biomay AG, amoxicillin from Panpharma, rocuronium from B. Braun and suxamethonium from Biocodex. Lysate from HEK293T cells transiently overexpressing human FcεRII/CD23 was obtained from Novus Biologicals. Serotonin Fast Track ELISA kit was obtained from ImmuSmol.

Blood sampling. The protocol was approved by the ethical committee from the Strasbourg medical university (CE-2023-33). Blood samples from healthy donors were obtained from the French National Blood Donor Service (EFS Grand Est) with the informed consent of the donors. Blood samples from allergic patients were obtained in collaboration with the Allergology Service of the University Hospital of Strasbourg with the informed consent of the patients. Blood was drawn into 3.8% (v/v) sodium citrate (1:9) anticoagulant or acid-citrate-dextrose (ACD) (1:6) anticoagulant to prepare washed platelets.

Preparation of washed platelets. Washed platelets were prepared as previously described [1]. Briefly, platelets were isolated by differential centrifugation and washed twice at 37°C in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, 5 mM HEPES, pH 7.3, 295 mOsm/L) containing 0.35% purified human serum albumin (HSA), 10 U/mL heparin and 0.5 µM prostaglandin I2 (PGI_2). The platelets were finally suspended in Tyrode's buffer containing 0.35% HSA and 0.02 U/mL of the adenine nucleotide scavenger apyrase, a concentration sufficient to prevent the desensitization of platelet responses to ADP [2]. The final suspension contained no PGI_2 and was adjusted to 3.10^5 platelets/µL. Washed platelets were kept at 37°C throughout all experiments.

Aggregation assays and measurement of secretion. Aggregation was measured at 37°C by a turbidimetric method in an APACT 4004 aggregometer (ELITechGroup). A 450 µL aliquot of washed platelet suspension was stirred at 1100 rpm and activated by the addition of 50 µL of the appropriate agonist: thrombin (0.1 U/mL), a specific FcεRI activating anti-human IgE mAb (G7-18, 50 µg/mL), or various concentrations of specific allergens (hymenoptera venom VES-V1 or Ves-V5 1, 10, 100 and 1000 ng/mL; amoxicillin 1, 10, 100 µg/mL and 4 mg/mL, peanut extract 1 to 1000 ng/mL, rocuronium 0.1 and 1 mg/mL, suxamethonium 5 mg/mL). In the

passive sensitization assay, washed platelets were incubated with anti-TNP (trinitro-phenyl) IgE (50 µg/mL) for 1 hour during the second washing step, washed in Tyrode's buffer containing 0.35% HSA and then exposed to TNP (1 µg/mL). The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline. For measurement of secreted serotonin, platelets were stimulated with the indicated agonist and centrifuged after 3 minutes. Serotonin in the supernatant was measured by ELISA.

Western blot analyses. Human mast cells were derived from $CD34^+$ progenitor cells isolated from peripheral blood as previously described [3]. To prepare mast cell lysates, mast cells were pelleted, washed in PBS, resuspended in lysis buffer (10 mM Tris HCl, 150 mM NaCl, 5 mM Na_3VO_4, 1 mM EGTA, 1 mM EDTA, 100 mM NaF, pH 7.5) containing 1% SDS and a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Boehringer-Mannheim) and incubated at 95°C for 5 min. After centrifugation at 16,000 g for 45 min at 4°C, the supernatants were collected, aliquoted and stored at -80°C until use. To prepare platelet lysates, platelets in Tyrode's buffer containing HEPES were washed twice in PBS, resuspended in lysis buffer containing 1% SDS and a protease inhibitor cocktail, and incubated at 95°C for 5 min. After centrifugation at 16,000 g for 45 min at 4°C, the supernatants were collected and stored at -80°C until use. Protein samples were mixed with SDS sample Laemmli buffer and boiled for 3 minutes at 96.5°C under reducing (10 mM DTT) conditions, separated on 4-15% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were incubated with the indicated antibodies overnight at 4°C and immunoreactive bands were revealed with an enhanced chemiluminescence (ECL) detection kit (Amersham).

Flow cytometric analyses. *Detection of FcεRI and FcεRII on the surface of human platelets*. A double-labeling strategy was used to discriminate between specific and non-specific staining. The protocol is described in **Fig. 1A**. Briefly, washed platelets (10 µL, 3.10^5 platelets/µL) from the same donor were incubated with either 1 µg/mL Alexa 568-conjugated rat anti-mouse GPIbβ (RAM.1) alone, or with 1 µg/mL Alexa 647-conjugated RAM.1 together with each of the three mouse anti-human FcεRI-α mAbs CRA-1, CRA-2 and 9E1 or the mouse anti-human FcεRII mAb MAB123 at increasing concentrations (0, 1, 2 and 5 µg/mL). A mouse mAb against the extracellular domain of human GPIX (clone ALMA.16 1, 2 and 5 µg/mL) was used as a positive control. After incubation for 20 min at RT and washing, 20 µL aliquots of the platelet suspensions were mixed and incubated for 20 min at RT with an Alexa 488-conjugated goat anti-mouse antibody (5 µg/mL). The samples were finally diluted in 300 µL of Tyrode's buffer and analyzed using a Fortessa X-20 flow cytometer and Diva software (BD Biosciences). The light scattering and fluorescence intensity of 10,000 platelets were collected with a logarithmic gain.

Detection of FcεRI on human basophils: citrated (3.8%) whole blood was incubated for 10 min at 4°C with FcR blocking reagent, followed by incubation for 20 min at 4°C with Alexa 647-conjugated mouse anti-FcεRI-α antibody (9E1) (0, 1, 2 and 5 µg/mL), or with mouse anti-FcεRI-α mAbs CRA-1 or CRA-2 (0, 1, 2, and 5 µg/mL), followed by washing and incubation for 20 min at 4°C with an Alexa 647-conjugated goat anti-mouse antibody (1 µg/mL). After washing, the three samples were incubated for 20 min at RT with a FITC-conjugated mouse anti-human IgE (1.25 µg/mL) and then for 10 min at 4°C with a red blood cell lysis solution (BD FACS™ Lysing Solution). Phosphate buffered saline (PBS, 1000 µL) was added to the samples, which were centrifuged for 5 min at 300 g and resuspended in 300 µL of PBS for flow cytometric analysis.

The light scattering and fluorescence intensity of 500 basophils (IgE^+) were collected with a logarithmic gain.

Detection of FcεRII on human B lymphocytes: Citrated (3.8%) whole blood was carefully layered onto the upper Histopaque-1077 medium of a double gradient formed by layering an equal volume of Histopaque-1077 over Histopaque-1119 (Sigma). Following centrifugation at 1100 g for 30 min at RT, lymphocytes and other mononuclear cells were retrieved at the upper plasma/Histopaque-1077 interface. After washing in PBS, the cell suspension was incubated for 10 min at 4°C with FcR blocking reagent, followed by incubation for 20 min at 4°C with the mouse anti-human FcεRII mAb MAB123 at increasing concentrations (0, 1, 2 and 5 µg/mL), followed by washing and incubation for 20 min at 4°C with an Alexa 488-conjugated goat anti-mouse antibody (1 µg/mL). After washing, the samples were incubated for 20 min at 4°C with an APC-conjugated mouse anti-human CD20 antibody (10 µg/mL). The samples were finally diluted in 300 µL of PBS for flow cytometric analysis using a Gallios flow cytometer and Kalusa software (Beckmann Coulter, Villepinte, France). The light scattering and fluorescence intensity of 500 B lymphocytes ($CD20^+$) were collected with a logarithmic gain.

Basophil and platelet activation tests. Citrated whole blood from allergic patients was incubated for 10 min at 37°C with IL-3 (10 ng/mL) and then for 30 min at 37°C with either buffer alone, a mouse anti-human IgE mAb (G7-18, 50 µg/mL), a rat anti-human CD9 mAb (clone ALMA.1, 10 µg/mL) or three different concentrations of their specific allergen (hymenoptera venom VES-V1 or VES-V5 1, 10 and 100 ng/mL, peanut extract 1, 10 and 100 ng/mL, amoxicillin 0.04, 0.4 and 4 mg/mL). To analyze basophils, citrated whole blood was incubated for 20 min at 4°C with 1.25 µg/mL FITC-conjugated mouse anti-human IgE, APC-conjugated anti-CD203c and PE-conjugated mouse anti-human CD63, and then for 10 min at 4°C with a red blood cell lysis solution. PBS (1 mL) was added to the samples, which were centrifuged for 5 min at 300 g and resuspended in 300 µL of PBS for flow cytometric analysis. To analyze platelets, citrated whole blood was incubated for 20 min at RT with 1 µg/mL Alexa 647-conjugated rat anti-mouse GPIbβ (RAM.1) and 5 µg/mL FITC-conjugated mouse anti-activated human GPIIbIIIa (PAC-1) or FITC-conjugated mouse anti-human CD62P (P-selectin) or their respective non-immune controls. The samples were then diluted in 300 µL of Tyrode's buffer for flow cytometric analysis using a Gallios flow cytometer and Kalusa software (Beckmann Coulter, Villepinte, France). The light scattering and fluorescence intensity of 500 basophils or 5,000 platelets were collected with a logarithmic gain.

Immunofluorescence and confocal microscopy imaging. Paraformaldehyde (4%) fixed platelets were deposited on polylysine-coated glass slides, permeabilized with 0.1% triton, incubated with the anti-FcεRII mAb MAB123 (5 µg/mL) or the corresponding non-immune control IgG, followed by a secondary antibody (goat anti mouse-A647), and an Alexa488-conjugated anti-GPIbβ antibody (1 µg/mL). Isolated human lymphocytes were used as a positive control to demonstrate the specificity of the anti-FcεRII mAb (MAB123). Platelets or lymphocytes were observed under a confocal microscope (Leica TCS SP8, Leica Microsystems) with oil objective (HC PL APO CS2 63x/1,40 OIL).

Immunoelectron microscopy. Washed platelets were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde, infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen. Ultrathin cryosections of 100 nm were obtained at -110°C with a LEICA Ultracut UCT cryo-ultramicrotome (Leica Microsystems). The thin sections were rinsed for 15 min in PBS containing 150 mM glycine, in PBS again and for 15 min in PBS containing 1% BSA, before

incubation for 30 min with the anti-FcεRI-α mAb CRA-1 (5 µg/mL in PBS/1% BSA) or the corresponding non-immune control. After incubation for 15 min with protein A coupled to 10 nm colloidal gold particles (PAG, Cell Microscopy Center, University Medical Center Utrecht, The Netherlands), the sections were postfixed in 1% glutaraldehyde, embedded in 1.8% uranyl acetate/0.2% methylcellulose and examined under a MET at 120 kV (Jeol 2100-Plus).

Statistical analyses. Statistical analyses were performed with GraphPad software (Prism 9.2.0). Data were reported as the mean ± SEM and were analyzed by one-way analysis of variance. A P value of <0.05 was considered to be statistically significant.

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