ALLERGY

Platelets expressing IgG receptor FcγRIIA/CD32A determine the severity of experimental anaphylaxis

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Platelets are key regulators of vascular integrity; however, their role in anaphylaxis, a life-threatening systemic allergic reaction characterized by the loss of vascular integrity and vascular leakage, remains unknown. Anaphylaxis is a consequence of inappropriate cellular responses triggered by antibodies to generally harmless antigens, resulting in a massive mediator release and rapidly occurring organ dysfunction. Human platelets express receptors for immunoglobulin G (IgG) antibodies and can release potent mediators, yet their contribution to anaphylaxis has not been previously addressed in mouse models, probably because mice do not express IgG receptors on platelets. We investigated the contribution of platelets to IgG-dependent anaphylaxis in human IgG receptor–expressing mouse models and a cohort of patients suffering from drug-induced anaphylaxis. Platelet counts dropped immediately and markedly upon anaphylaxis induction only when they expressed the human IgG receptor FcγRIIA/CD32A. Platelet depletion attenuated anaphylaxis, whereas thrombocythemia substantially worsened its severity. FcγRIIA-expressing platelets were directly activated by IgG immune complexes in vivo and were sufficient to restore susceptibility to anaphylaxis in resistant mice. Serotonin released by activated platelets contributed to anaphylaxis severity. Data from a cohort of patients suffering from drug-induced anaphylaxis indicated that platelet activation was associated with anaphylaxis severity and was accompanied by a reduction in circulating platelet numbers. Our findings identify platelets as critical players in IgG-dependent anaphylaxis and provide a rationale for the design of platelet-targeting strategies to attenuate the severity of anaphylactic reactions.

INTRODUCTION

Anaphylaxis is an acute and life-threatening multisystem allergic reaction. It results from inappropriate cellular responses triggered by antibodies to generally inoffensive antigens and is characterized by massive mediator release (*1*) and by rapidly occurring vascular and organ dysfunction. Experimental models have elucidated two different antibody-dependent anaphylaxis pathways. The "classical" pathway relies on antibodies of the immunoglobulin E (IgE) class, which, when bound to the high-affinity FcεRI receptor on basophils and mast cells, trigger cell activation upon exposure to the causative allergen (*2*, *3*). The "alternative" anaphylaxis pathway is triggered by IgG antibodies, which, when aggregated by a specific antigen, activate myeloid cells via IgG receptors (FcγRs) (*4*–*6*). Although human anaphylaxis has been classically viewed as an IgE antibody–dependent reaction, accumulating evidence indicates that IgG antibody–dependent reactions may be responsible for human anaphylaxis cases, particularly in the context of drug-induced anaphylaxis (*7*).

Both clinical and experimental anaphylaxis are associated with immediate release of histamine and lipid mediators. The lipid mediator platelet-activating factor (PAF) has been proposed to contribute to the severity of anaphylaxis in humans (*8*) and in several experimental models (*9*–*11*). The cellular source and target(s) of PAF in such reactions remain unknown, but it is reasonably established that IgG-dependent anaphylaxis induction entails the FcγR-dependent activation of myeloid cells that can release PAF. In particular, both tissue-resident mast cells and macrophages and circulating neutrophils, monocytes, and basophils have been found to contribute to anaphylaxis but to largely varying degrees, depending on the animal model and the allergen used (*4*, *6*, *12*).

Among the five classical human FcγRs (FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA, and FcγRIIIB) (*13*), hFcγRIIA/CD32A is the most widely expressed FcγR in humans and is an activating IgG receptor present on all cells of myeloid origin, including platelets. When expressed in transgenic mice, hFcγRIIA can induce severe IgG-dependent anaphylaxis that relies on the contribution of neutrophils and monocytes/macrophages (*14*). Mice expressing four human FcγRs (FcγRIIA, FcγRIIB, FcγRIIIA, and FcγRIIIB) in place of endogenous mouse FcγRIIB, FcγRIII, and FcγRIV also demonstrate severe systemic IgG-induced anaphylaxis that can be abolished after hFcγRIIA blockade (*11*), suggesting a major role for hFcγRIIA in IgG-dependent anaphylaxis induction, even if other human FcγRs are expressed.

hFcγRIIA is the sole FcγR on human platelets (*15*). Wild-type (WT) mice do not express any IgG receptor on platelets, so we hypothesized that a putative platelet contribution to IgG-induced anaphylaxis might have been overlooked because of this inconsistency in classical mouse

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models. Platelets are equipped with many bioactive molecules that are released upon activation. Among them, serotonin is a prime candidate that may contribute to clinical signs of anaphylaxis because it can increase vascular permeability (*16*, *17*), trigger vasoconstriction or vasodilation (*18*), and induce bronchoconstriction in the context of allergic asthma (*19*).

To test whether hFcγRIIA-expressing platelets actively contribute to IgG-dependent anaphylaxis, we developed new mouse models either expressing only hFcγRIIA and no other FcγR or recapitulating the full complexity of human FcγRs in place of mouse FcγRs. We then corroborated our findings with patient samples from clinical cases of drug-induced anaphylaxis.

Our results demonstrate that platelets are critical players in hFcγRIIA-induced anaphylaxis. Human FcγRIIA-expressing platelets were directly activated by aggregated human IgG (hIgG) in vitro and in vivo and were sufficient to restore susceptibility to anaphylaxis in resistant mice. Activated platelets released serotonin, which contributed to the severity of anaphylaxis; accordingly, thrombocytemia aggravated IgG-dependent anaphylaxis, whereas thrombocytopenia was protective. Data from a clinical study on perioperative drug-induced anaphylaxis further support our findings that platelets contribute to human anaphylaxis.

RESULTS

Platelets are required for hFcγRIIA-mediated anaphylaxis

In mice expressing human FcγRs in place of endogenous mouse FcγRs, whether as transgenes or knock-in inserts, anaphylaxis can be induced by injection of heat-aggregated hIgG (HA-hIgG) as a surrogate for hIgG-immune complexes (*11*, *14*, *20*). As expected, hFcγRIIA^{tg} mice (on an FcγR^{null} background, thus expressing only hFcγRIIA and no other mouse or human FcγRs) developed rapid hypothermia upon HA-hIgG injection, a primary response associated with anaphylaxis due to changes in hemoconcentration and augmented vascular permeability (fig. S1, A and B). Unexpectedly, the reaction was accompanied by a profound thrombocytopenia (Fig. 1A), detectable as early as 5 min after anaphylaxis induction, and that persisted for 24 hours (Fig. 1B). As expected, FcγR^{null} mice (expressing no FcγRs) suffered no hypothermia, vascular leakage, or hemoconcentration and only a mild and transient decrease in platelet counts (Fig. 1, A and B, and fig. S1, A and B). To test whether thrombocytopenia was merely a marker of systemic cell activation or whether platelets were critical for anaphylaxis induction, we depleted platelets 72 hours before anaphylaxis induction using anti–glycoprotein Ibα (GPIbα) monoclonal antibodies (mAbs). Antibody-dependent platelet clearance was efficient even in FcγR^{null} mice (fig. S1C), demonstrating that this depletion method is FcγR-independent (*21*) and therefore should not interfere with FcγR-dependent cell activation thereafter. Platelet depletion abolished hypothermia in hFcγRIIA^{tg} mice (Fig. 1C and fig. S1, C and D), suggesting that platelets are required for IgG-dependent anaphylaxis. We then tested whether an increase in platelet numbers would lead to an escalation in anaphylaxis severity. hFcγRIIA^{tg} mice treated with a thrombopoietin receptor agonist, romiplostim, exhibited a threefold increase in circulating platelet numbers as compared with untreated mice (Fig. 1D) (*22*). This increase in platelet counts rendered hFcγRIIA^{tg} mice extremely sensitive to anaphylaxis induction, with a high accompanying mortality (Fig. 1E). Thrombocythemia alone was not sufficient to augment sensitivity to anaphylaxis, because thrombocythemic FcγR^{null} mice remained resistant (Fig. 1, D and E).

hFcγRIIA^{tg}-expressing platelets are sufficient to induce anaphylaxis in resistant mice

Platelet activation during anaphylaxis may result from direct engagement with circulating HA-hIgG and could therefore contribute to, or even be responsible for, anaphylaxis induction. Washed platelets from hFcγRIIA^{tg} mice aggregated in vitro upon incubation with HA-hIgG in a dose-dependent manner and to the extent reached by classical inducers of platelet aggregation, that is, thrombin or adenosine

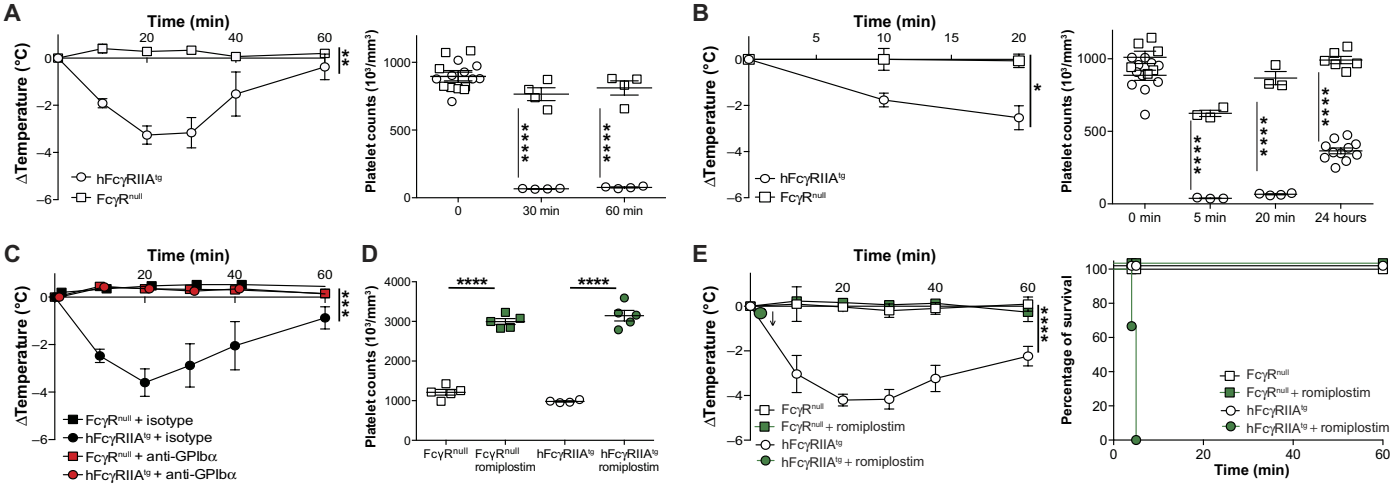


Fig. 1. Platelets are required for hFcγRIIA-induced anaphylaxis. (**A** and **B**) Change in body temperature (left) and platelet count (right) during HA-hIgG–dependent anaphylaxis in hFcγRIIA^{tg} (circles, $n = 4$) and FcγR^{null} (squares, $n = 4$) mice. (**C**) HA-hIgG–dependent anaphylaxis–induced changes in body temperature in hFcγRIIA^{tg} (circles) and FcγR^{null} (squares) mice ($n = 4$), pretreated with anti-GPIbα antibody (red symbols) or isotype control (black symbols, $n = 4$). (**D**) Platelet counts in hFcγRIIA^{tg} (circles) and FcγR^{null} (squares) mice, pretreated (green symbols, $n = 5$) or not (open symbols, $n = 4$ or 5) with romiplostim. (**E**) Change in body temperature (left) and survival (right) during HA-hIgG–dependent anaphylaxis in hFcγRIIA^{tg} (circles) and FcγR^{null} (squares) mice pretreated (green symbols) or not (open symbols) with romiplostim ($n = 3$). ↓: Death of all romiplostim-treated hFcγRIIA^{tg} mice before the first temperature readout. (A to E) Temperature data are means ± SEM, and platelet counts from individual mice are indicated together with the means ± SEM. Data in (A) to (E) are representative of at least two independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

5′-diphosphate (ADP) (Fig. 2A). Markers of platelet activation accompanied in vitro aggregation: Expression of both CD62P and activated αIIbβ3 integrin was significantly increased as compared to unstimulated controls on the platelet surface (Fig. 2B). Washed platelets from FcγR^{null} mice aggregated and exhibited markers of activation only upon stimulation with thrombin or ADP but not with HA-hIgG (fig. S2, A and B), indicating that the effect of the latter requires IgG receptor expression. Supporting this observation, blocking hFcγRIIA with an anti-hFcγRIIA Fab fragment prevented activation and aggregation of washed platelets from hFcγRIIA^{tg} mice by HA-hIgG (Fig. 2, A and B). These data demonstrate that IgG immune complexes can directly activate hFcγRIIA^{tg}-expressing mouse platelets in vitro and suggest that this may also occur in vivo.

Accordingly, 30 min after injection of HA-hIgG, circulating platelets in hFcγRIIA^{tg} mice, but not in FcγR^{null} mice, exhibited increased CD62P expression whether present as single platelets or as platelet-neutrophil aggregates (Fig. 2, C and D). About 80% of circulating neutrophils (Fig. 2E) and >90% of Ly6C^{hi} monocytes (fig. S2, C and E) in hFcγRIIA^{tg} but not FcγR^{null} mice were covered with platelets during anaphylaxis (t = 30 min) but not after the reaction (t = 5 hours). In addition, single neutrophils in circulation of hFcγRIIA^{tg} mice, but not of FcγR^{null} mice, displayed an activated phenotype as reflected by their reduced CD62L expression (Fig. 2F). This suggests that both hFcγRIIA^{tg}-expressing platelets and neutrophils become activated during HA-hIgG–dependent anaphylaxis, independent of their association.

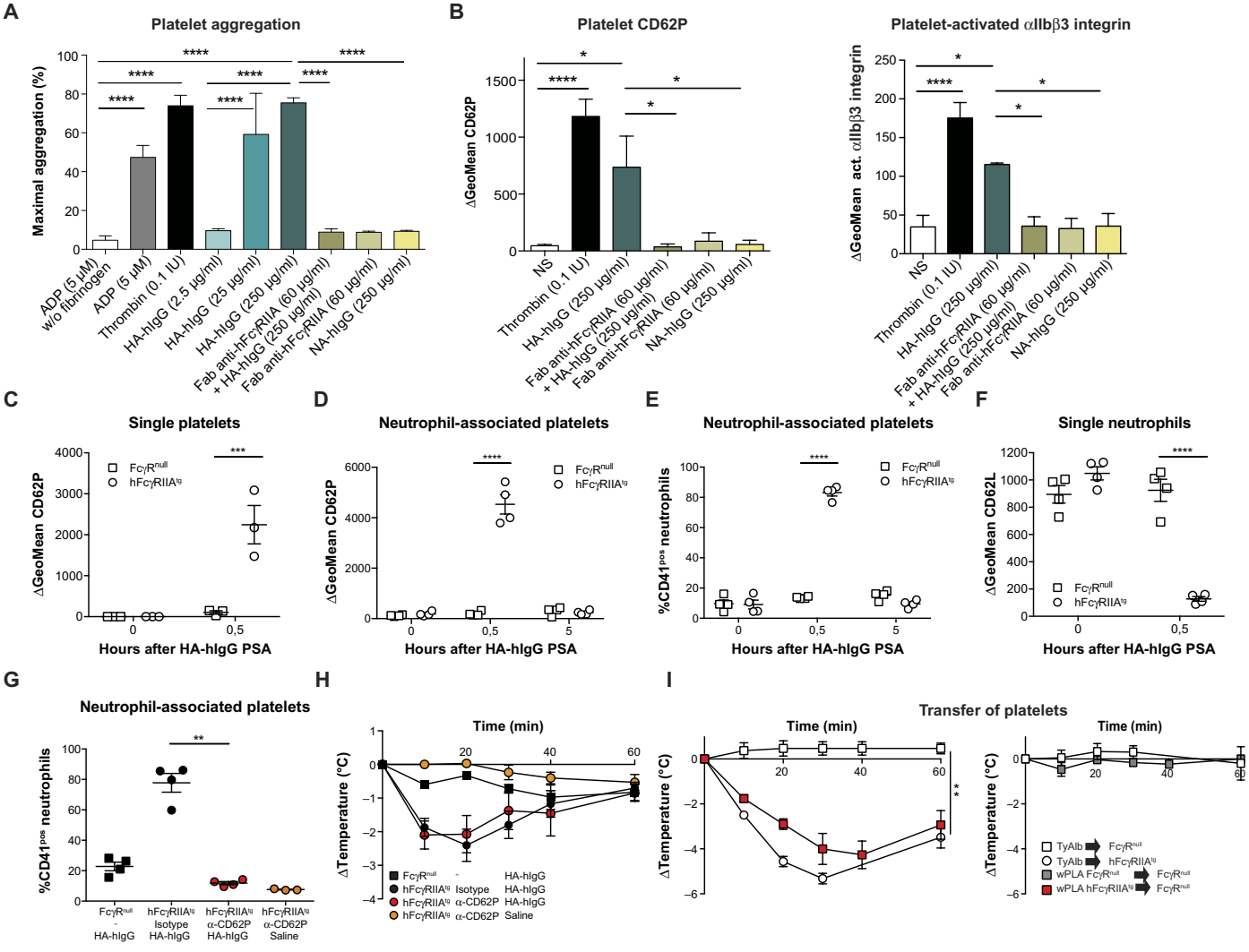


Fig. 2. hFcγRIIA-expressing platelets are sufficient to induce anaphylaxis in resistant mice. (**A**) Maximal aggregation (%) of washed platelets from hFcγRIIA^{tg} mice compared with baseline and (**B**) expression of platelet activation markers after incubation with indicated agonist. Data are pooled from three independent experiments. NS, not stimulated. (**C** to **F**) hFcγRIIA^{tg} (circles) and FcγR^{null} (squares) mice were challenged or not with HA-hIgG (n = 4). (C and D) ΔGeoMean CD62P expression (C) on single platelets and (D) within platelet-neutrophil aggregates, (E) percentage of neutrophils associated to CD41^{+} platelets, and (F) ΔGeoMean CD62L expression on single neutrophils. PSA, passive systemic anaphylaxis. (**G** and **H**) Indicated groups of mice were pretreated with anti-CD62P mAb or isotype control (black circles, n = 4) and challenged or not with HA-hIgG (all groups, n = 4). (G) Percentage of neutrophils associated to CD41^{+} platelets 30 min after challenge and (H) changes in body temperature over time. (**I**) Washed platelets from hFcγRIIA^{tg} mice (red squares, left) or from FcγR^{null} mice (gray squares, right), or Tyrode's albumin buffer (TyAlb) alone (open squares), were transferred intravenously into FcγR^{null} mice before challenge with HA-hIgG (all groups, n = 3). hFcγRIIA^{tg} mice receiving intravenous injection of TyAlb, followed by challenge with HA-hIgG, served as a positive control (open circles, n = 5). wPLA, washed platelets. Changes in body temperature were recorded. (C to I) Data are representative of at least two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Injection of an anti-CD62P antibody abolished the formation of platelet-myeloid cell aggregates after injection of HA-hIgG (Fig. 2G and fig. S2E), without affecting anaphylaxis severity (Fig. 2H), or neutrophil (fig. S2F) and platelet activation (fig. S2, G and H).

We evaluated whether expression of hFcγRIIA exclusively on platelets may be sufficient to induce anaphylaxis without the requirement of other FcγR-mediated myeloid cell activation and therefore transferred washed platelets from hFcγRIIA^{tg} mice, or from FcγR^{null} mice as a negative control, into FcγR^{null} mice before injection with HA-hIgG. Platelets from hFcγRIIA^{tg} mice, but not from FcγR^{null} mice, conferred FcγR^{null} mice with a susceptibility to hypothermia in response to HA-hIgG injection (Fig. 2I). Therefore, hFcγRIIA-mediated activation of platelets is sufficient for anaphylaxis induction in resistant mice, in the absence of other FcγR-mediated myeloid cell activation.

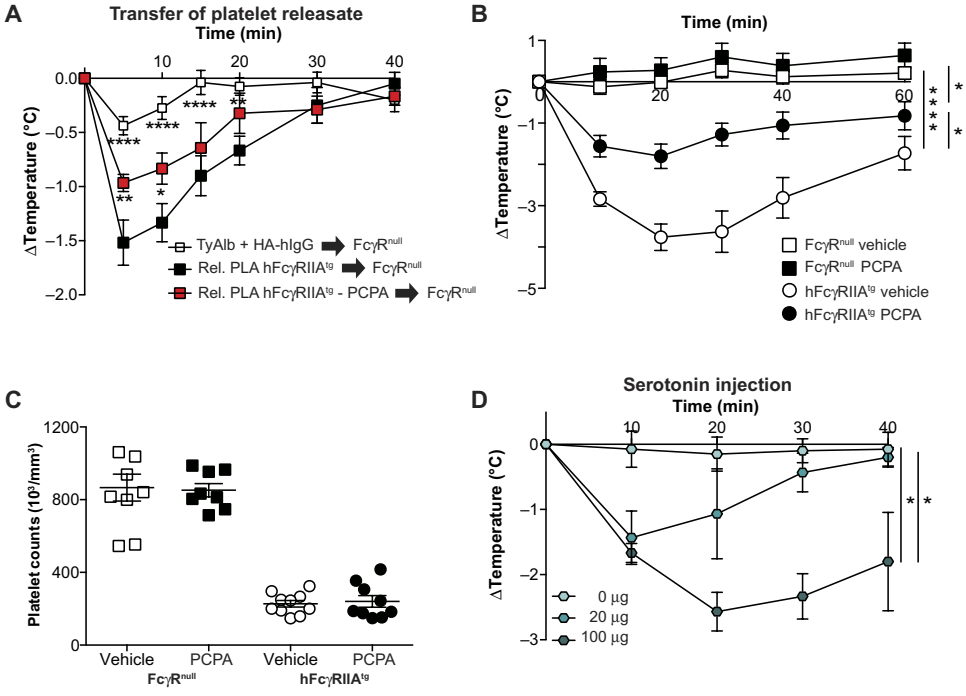


Fig. 3. Platelet-released serotonin contributes to hypothermia in mice undergoing HA-hIgG–dependent anaphylaxis. (**A**) Releasates (Rel.) from HA-hIgG–activated washed platelets from hFcγRIIA^{tg} mice left untreated (black squares, $n = 6$) or treated with PCPA (red squares, $n = 8$) or HA-hIgG–containing TyAlb alone (open squares, $n = 9$) were transferred intravenously into FcγR^{null} mice, and changes in body temperature were recorded. (**B**) Changes in body temperature during HA-hIgG–dependent anaphylaxis in hFcγRIIA^{tg} (circles) and FcγR^{null} (squares) mice, pretreated with PCPA (black symbols, $n \geq 8$) or vehicle control (open symbols, $n \geq 8$). (**C**) Platelet counts from the experiment represented in (B) 70 min after anaphylaxis induction. (**D**) Changes in body temperature in WT mice injected intravenously with 0 ($n = 4$), 20 ($n = 3$), or 100 µg ($n = 3$) of serotonin. Data are representative of three independent experiments. (A to C) Data are pooled from three (A) or two (B and C) independent experiments. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$.

Platelet-released serotonin contributes to hypothermia in mice undergoing HA-hIgG–dependent anaphylaxis

We next investigated whether platelets activated via hFcγRIIA release mediators that are able to directly induce a hypothermic response. Isolated platelets from hFcγRIIA^{tg} mice were stimulated in vitro with HA-hIgG to induce platelet aggregation and secretion, and the supernatant containing the platelet releasate was recovered by centrifugation. The releasate from HA-hIgG–activated platelets, but not the buffer alone, induced significant hypothermia when injected intravenously into recipient FcγR^{null} mice (Fig. 3A). This releasate contained substantial amounts of serotonin but only minute quantities of histamine (337 ± 52 ng of serotonin versus 1.7 ± 0.2 ng of histamine per 10^9 platelets) (fig. S3A). Furthermore, a 15-fold increase in serotonin concentration was observed in the circulation of hFcγRIIA^{tg} mice 5 min after anaphylaxis onset, whereas histamine levels increased very little (fig. S3B). To test whether serotonin contributes to HA-hIgG–induced anaphylaxis, we inhibited serotonin biosynthesis in hFcγRIIA^{tg} mice before anaphylaxis induction using 4-chloro-phenylalanine (PCPA), an inhibitor of the essential enzyme tryptophan hydroxylase (*23*). Pretreatment with PCPA reduced anaphylactic hypothermia in hFcγRIIA^{tg} mice more than twofold (Fig. 3B) without altering platelet consumption (Fig. 3C), indicating that serotonin contributes to HA-hIgG–dependent anaphylaxis. Accordingly, intravenous injection of serotonin led to a dose-dependent hypothermia in WT mice (Fig. 3D). The transfer of the releasate from serotonin-deprived HA-hIgG–activated washed platelets (fig. S3C) induced a milder temperature drop in recipient FcγR^{null} mice than that from serotonin-sufficient platelets (Fig. 3A). Together, these results indicate that hFcγRIIA-expressing platelets release serotonin upon activation with HA-hIgG, which contributes to hypothermic symptoms of HA-hIgG–dependent anaphylaxis.

Platelets contribute to hIgG-dependent anaphylaxis in a mouse model reproducing human FcγR complexity

hFcγRIIA is one of four human activating IgG receptors, along with hFcγRI, hFcγRIIIA, and hFcγRIIIB, in addition to the inhibitory IgG receptor hFcγRIIB. We therefore investigated whether platelet activation contributes also to anaphylaxis induction in the context of full human FcγR complexity by using a novel mouse model knocked-in for all human FcγRs (hFcγR^{KI}), which recapitulates hFcγR expression (figs. S4 and S5). These mice are susceptible to hIgG-dependent anaphylaxis, demonstrating rapid hypothermia after HA-hIgG injection, which was likewise accompanied by a profound thrombocytopenia (Fig. 4A). Blocking hFcγRIIA in vivo was sufficient to abolish hypothermia and thrombocytopenia in HA-hIgG–injected hFcγR^{KI} mice (Fig. 4B), indicating that hFcγRIIA is the dominant FcγR contributing to anaphylaxis induction, as supported by our previous studies using another mouse model (*11*). Platelet depletion in hFcγR^{KI} mice reduced the nadir of hypothermia twofold (Fig. 4C and fig. S1C), confirming the contribution of platelets to anaphylaxis. This effect was less pronounced than in hFcγRIIA^{tg} mice, in which platelet depletion abolished hypothermia (Fig. 1C). As observed in hFcγRIIA^{tg} mice (Fig. 2), platelets rapidly associated with neutrophils during HA-hIgG–dependent anaphylaxis in hFcγR^{KI} mice (fig. S6A), and pretreatment of hFcγR^{KI} mice with an anti-CD62P antibody abolished the formation of these aggregates (fig. S6A) without affecting the

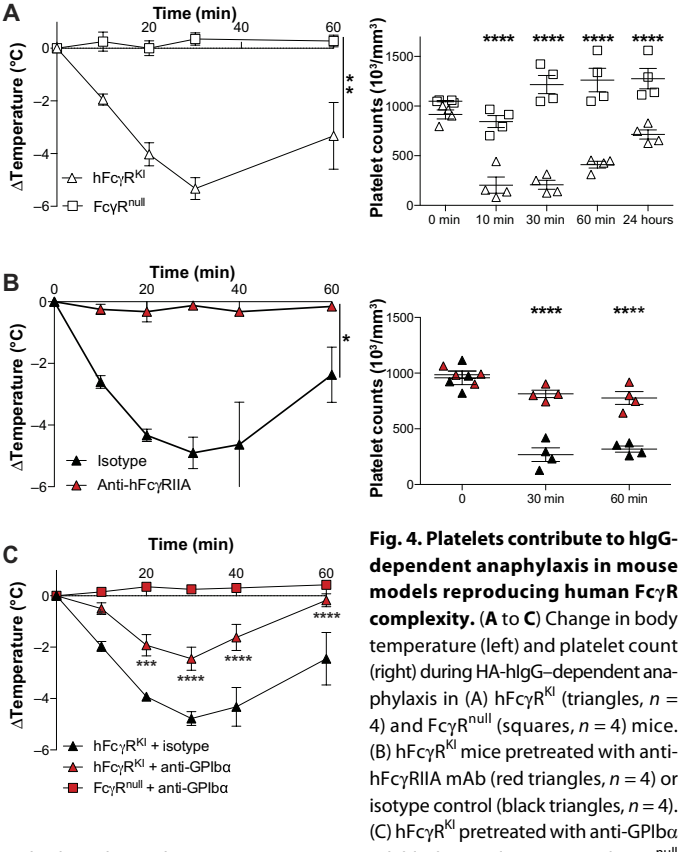


Fig. 4. Platelets contribute to hIgG-dependent anaphylaxis in mouse models reproducing human FcγR complexity. (**A** to **C**) Change in body temperature (left) and platelet count (right) during HA-hIgG–dependent anaphylaxis in (A) hFcγR^{KI} (triangles, n = 4) and FcγR^{null} (squares, n = 4) mice. (B) hFcγR^{KI} mice pretreated with anti-hFcγRIIA mAb (red triangles, n = 4) or isotype control (black triangles, n = 4). (C) hFcγR^{KI} pretreated with anti-GPIbα antibody (red triangles, n = 4) or isotype control (black triangles, n = 4) and FcγR^{null} mice pretreated with anti-GPIbα antibody (red squares, n = 4). (A to C) Temperature data are means ± SEM, and platelet counts from individual mice are indicated together with the means ± SEM. Data are representative of at least two independent experiments. *P < 0.05, ***P < 0.001, and ****P < 0.0001.

anaphylaxis outcome (fig. S6B) or platelet consumption (fig. S6C). These data confirm the contribution of platelets to anaphylaxis pathophysiology, in a more complex model of cognate hFcγR expression, while simultaneously arguing for the additional activation of other cellular pathways (*9*, *11*, *14*).

Releasate from HA-hIgG–activated human platelets induces hypothermia in mice

Having demonstrated the crucial role of hFcγRIIA-expressing mouse platelets in HA-hIgG–dependent anaphylaxis (Fig. 1), we investigated whether human platelets could get activated and thus contribute to anaphylaxis in a similar fashion. Washed human platelets aggregated in vitro upon incubation with HA-hIgG in a dose-dependent manner and to (a similar) extent as reached by classical inducers of platelet aggregation, that is, thrombin or ADP (Fig. 5A). In vitro aggregation was accompanied by up-regulation of platelet activation markers: expression of P-selectin (Fig. 5B) and of activated αIIbβ3 integrin as revealed by PAC-1 antibody binding (Fig. 5C). hFcγRIIA was required for the activation of human platelets by HA-hIgG because blocking hFcγRIIA prevented aggregation and expression of P-selectin and of activated αIIbβ3 integrin on the platelet surface (Fig. 5, A to C). These data demonstrate that IgG immune complexes can directly activate human platelets via hFcγRIIA in vitro and suggest that this may also occur during anaphylactic reactions in humans without the requirement of accessory cells.

We next investigated whether human platelets activated with HA-hIgG release mediators that can induce a temperature drop in vivo. Transfer of the releasate from washed human platelets activated with HA-hIgG induced significant hypothermia in recipient mice, which was not observed upon injection of HA-hIgG–containing buffer alone (Fig. 5D). As observed in the releasate of hFcγRIIA-expressing mouse platelets (Fig. 3), human platelet releasate contained significant serotonin concentrations (177 ± 45 ng per 10^9 platelets) but only a minute amount of histamine (7 ± 1 ng per 10^9 platelets) (Fig. 5E). Thus, serotonin released from HA-hIgG–activated human platelets may contribute to hypothermia in recipient mice.

Evidence for platelet activation during human drug-induced anaphylaxis

Having demonstrated that hFcγRIIA-expressing platelets contribute to IgG-dependent anaphylaxis severity in mouse models, we sought to evaluate the translation of these findings to the clinic. To this end, we investigated circulating platelet numbers and activation status in blood samples from 67 patients suffering from mild or severe anaphylaxis to perioperative neuromuscular blocking agent (NMBA) administration and their matched controls in the case-control "NASA" (Neutrophil Activation in Systemic Anaphylaxis) study (https://clinicaltrials.gov/ct2/show/NCT01637220). Significantly fewer single platelets, identified as CD61$^+$/FSC^{lo} cells by flow cytometry (fig. S7), were detected 30 min after anaphylaxis induction (i.e., NMBA injection) in the blood of patients suffering from severe versus mild anaphylaxis (1.30 ± 0.14 × 10^4/μl versus 1.76 ± 0.18 × 10^4/μl) or compared with matched control patients undergoing anesthesia without anaphylaxis (1.80 ± 0.09 × 10^4/μl). Six to 8 weeks after anaphylaxis, platelet counts from patients who had suffered a severe anaphylactic reaction were comparable (2.06 ± 0.15 × 10^4/μl) to those of controls (Fig. 6A), indicating that this phenotype was not inherent to this patient group. The platelet counts represented herein are lower than expected because only single CD61$^+$/FSC^{lo} cells were considered (thus excluding platelets in aggregates) and because blood was drawn on heparin in this clinical protocol, which is known to affect platelet responsiveness (*24*). However, the sampling method should not be responsible for the variations that we observe between patient groups because all blood was sampled identically. Nonetheless, to complement this analysis, we took advantage of four case-control pairs among all patients in this study who underwent cardiac surgery and therefore assessment of their differential blood counts before, during, and after surgery. In this patient subgroup, we could confirm thrombocytopenia in anaphylactic patients compared with controls, particularly in the 3- to 4-hour interval after anesthesia (Fig. 6B). This effect could not be accounted for merely by hemodilution because simultaneously monitored leukocyte counts did not change significantly (Fig. 6C). Last, and similar to results from our preclinical models, platelet CD62P expression and the percentage of CD62P-positive platelets were significantly higher in patients suffering from severe versus mild NMBA-induced anaphylaxis, or versus control groups (Fig. 6D), indicating that human platelets are activated during anaphylaxis.

DISCUSSION

This work identifies platelets as critical effectors of IgG-dependent anaphylaxis using two different preclinical mouse models in which activating hIgG receptor hFcγRIIA (CD32A) is expressed on platelets. Severe thrombocytopenia and platelet activation required hFcγRIIA expression on platelets, whereas anaphylaxis severity increased with augmented

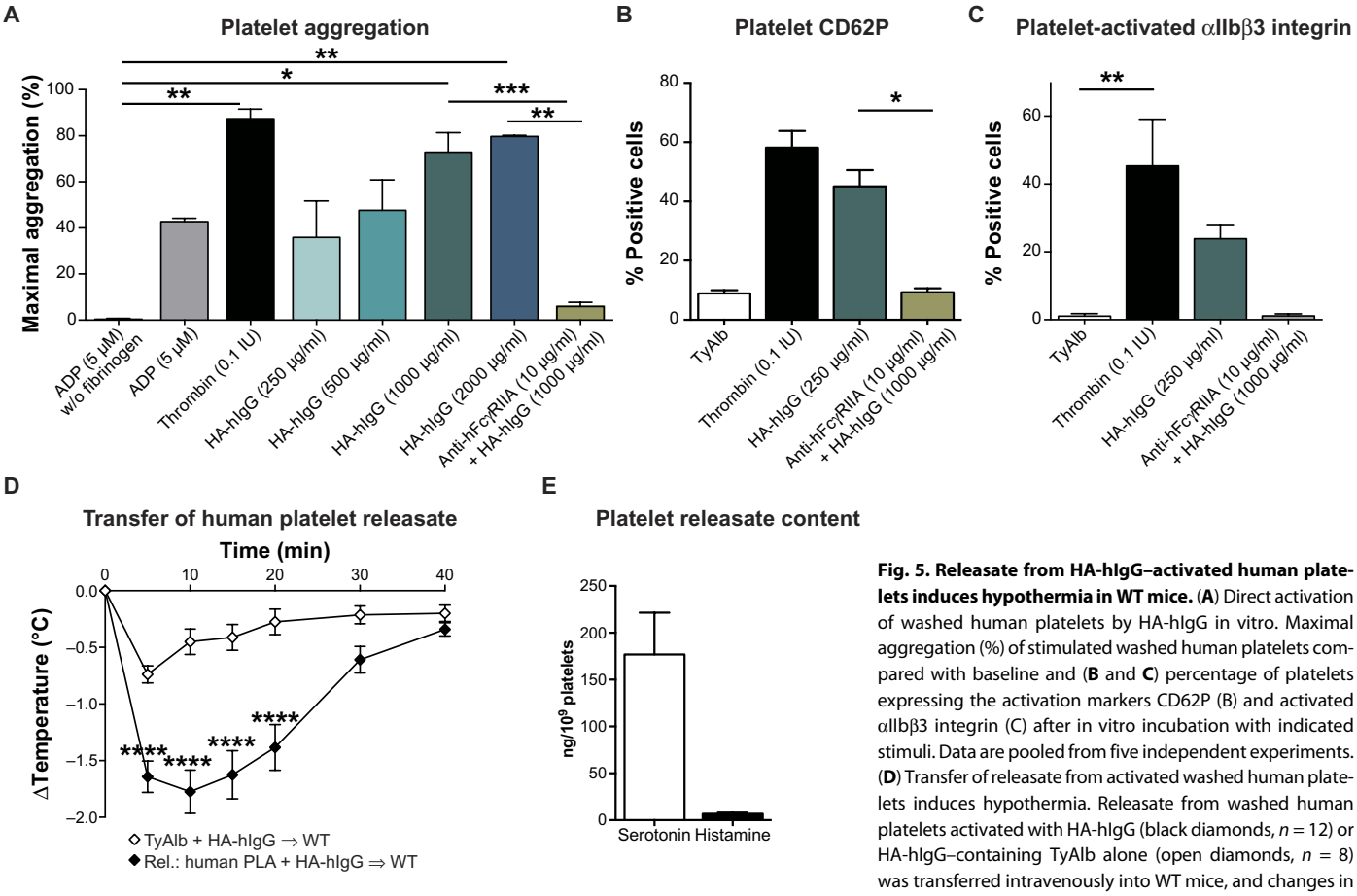


Fig. 5. Releasate from HA-hIgG–activated human platelets induces hypothermia in WT mice. (**A**) Direct activation of washed human platelets by HA-hIgG in vitro. Maximal aggregation (%) of stimulated washed human platelets compared with baseline and (**B** and **C**) percentage of platelets expressing the activation markers CD62P (B) and activated αIIbβ3 integrin (C) after in vitro incubation with indicated stimuli. Data are pooled from five independent experiments. (**D**) Transfer of releasate from activated washed human platelets induces hypothermia. Releasate from washed human platelets activated with HA-hIgG (black diamonds, $n = 12$) or HA-hIgG–containing TyAlb alone (open diamonds, $n = 8$) was transferred intravenously into WT mice, and changes in body temperature were immediately recorded. Data are pooled from three independent experiments. (**E**) Serotonin ($n = 4$) and histamine ($n = 5$) content in the releasate of HA-hIgG–stimulated washed human platelets. (A to E) Data are means ± SEM and are representative of at least three independent experiments. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

platelet numbers and was reduced in their absence. hFcγRIIA-expressing platelets were directly activated by aggregated hIgG in vitro and in vivo and were sufficient to restore susceptibility to anaphylaxis in resistant mice. Activated platelets released serotonin, which contributed to anaphylaxis severity. These observations were corroborated in part by clinical data from a cohort of patients suffering from drug-induced anaphylaxis, showing that platelet activation was associated with anaphylaxis severity and that anaphylaxis occurrence was accompanied by a reduction in circulating platelet numbers.

Our analysis identifies platelet-derived serotonin as a mediator in hFcγRIIA-induced anaphylaxis. Serotonin is released by both human and mouse platelets after HA-hIgG activation: The releasate from HA-hIgG–activated human or mouse platelets contained high concentrations of serotonin and was able to induce significant hypothermia when transferred into recipient mice, whereas the releasate from serotonin-depleted platelets induced only a mild hypothermia. Mice exhibited elevated circulating serotonin concentrations rapidly after anaphylaxis induction and developed only a mild hypothermia when serotonin biosynthesis was inhibited in vivo before anaphylaxis induction. Furthermore, injection of serotonin alone into mice induced a hypothermic response. Supporting our findings, release of platelet serotonin was observed after incubation of neuromuscular blockers with blood samples of patients who had previously had an anaphylactic reaction to a neuromuscular blocker (*25*). Serotonin has also been described to trigger bronchoconstriction when released in the lungs of asthmatic patients (*19*), to induce either vasoconstriction or vasodilation depending on the type of serotonin receptor and vascular bed (*18*), and to increase vascular permeability through the formation of gaps in the endothelium (*16*, *17*), suggestive of pleiotropic effects when released during anaphylaxis. Depletion of platelet serotonin stores by inhibition of serotonin biosynthesis attenuated but did not completely protect mice from anaphylaxis, indicating that serotonin is not the sole mediator of anaphylaxis in the preclinical models tested here. Circulating histamine concentrations did not change significantly during anaphylaxis in our models, and antihistamine pretreatment had no effect on anaphylaxis severity (Fig. S6D). However, PAF receptor antagonists attenuated HA-hIgG–induced anaphylaxis in $hFc\gamma R^{KI}$ mice, as reported for other models of IgG-dependent anaphylaxis (*9*–*11*), suggesting that PAF, but not histamine, may contribute along with serotonin to IgG-dependent anaphylaxis. Whether PAF originates from platelets and/or from independently activated myeloid cells remains an open question.

Experimental anaphylaxis in hFcγRIIA-expressing mice was associated with transient hypothermia, augmented vascular permeability, and hemoconcentration and was accompanied by a rapid and severe thrombocytopenia. Anaphylaxis-induced thrombocytopenia

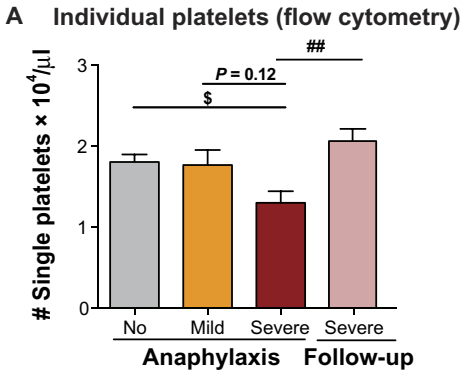
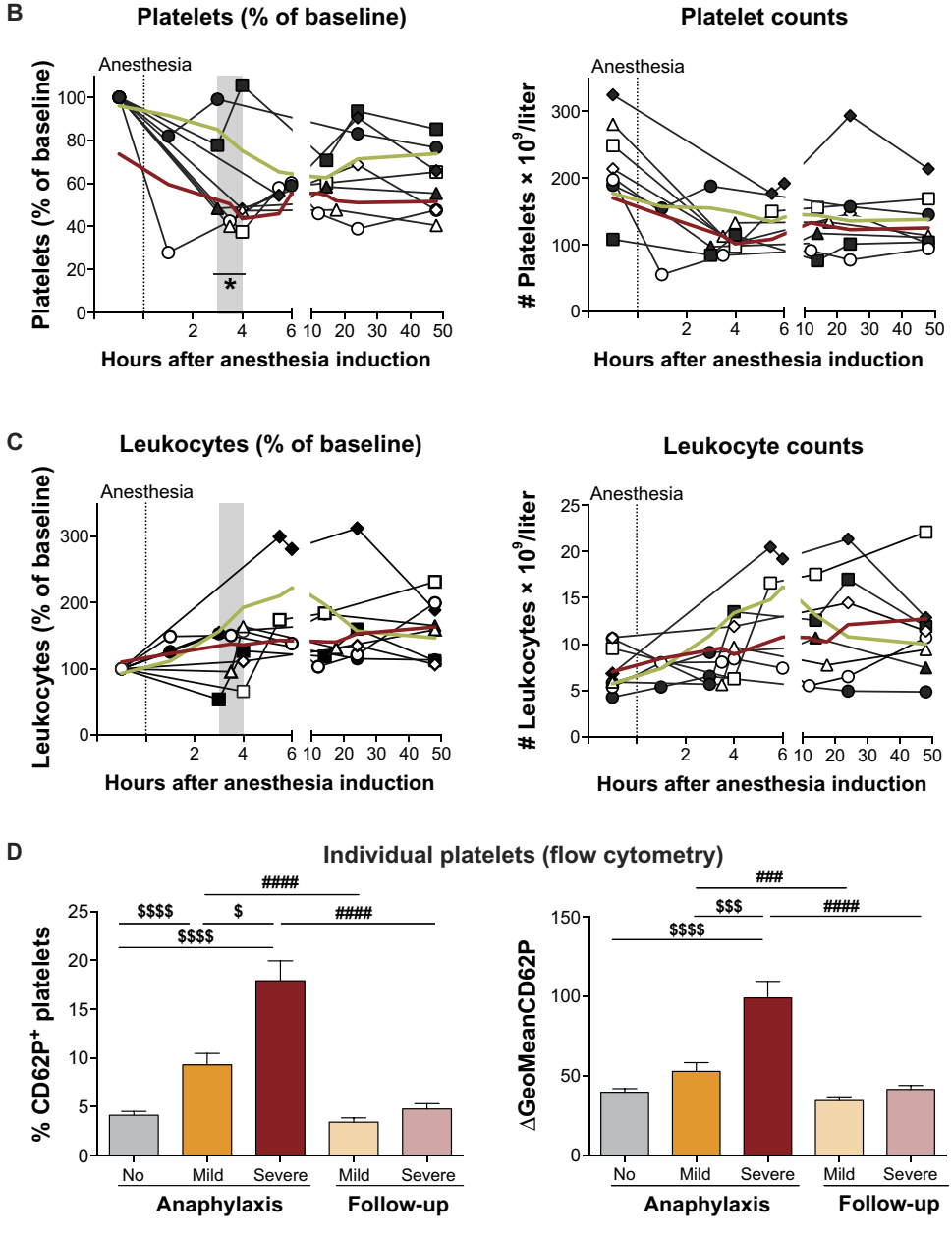


Fig. 6. Evidence for platelet activation during human drug-induced anaphylaxis. (**A**) Platelet counts in blood of individuals from the NASA study presenting no ($n = 67$), mild ($n = 37$), or severe ($n = 30$) anesthesia-induced anaphylaxis and the same severe patients during follow-up visit ($n = 30$). (**B**) Platelet and (**C**) leukocyte counts of four cardiac surgery cases (black symbols) and their matched control (open symbols) pairs (identical symbols within a pair) from the NASA cohort indicated as percentage of baseline (left) and absolute counts (right). A smoothed trend line has been added to indicate average behavior of each group (second-order polynomic, three neighbors: red, patients; green, controls). *$P < 0.05$, unpaired t test of cases versus controls, considering all values in the 3- to 4-hour interval (gray area). (**D**) CD62P-expressing platelets (left, percentage; right, geometric mean CD62P minus isotype staining) from the NASA study patients presenting no ($n = 73$), mild ($n = 35$), or severe ($n = 29$) anesthesia-induced anaphylaxis or the same mild ($n = 35$) or severe ($n = 29$) patients during follow-up visit. (A and D) Statistically significant differences between groups of anesthetized subjects ($\$P < 0.05$, $\$\$\$P < 0.001$, and $\$\$\$\$P < 0.0001$) and within a group of subjects comparing two different time points ($\#\#P < 0.01$, $\#\#\#P < 0.001$, and $\#\#\#\#P < 0.0001$.

occurred within minutes and preceded significant hypothermia in our models, suggesting that thrombocytopenia is an early sign of anaphylaxis triggered by direct platelet activation. In support of the notion that platelets may trigger anaphylaxis induction, IgG receptor expression exclusively on platelets was sufficient to induce hypothermia. Furthermore, these results may explain the dominance of hFcγRIIA over other human FcγRs in anaphylaxis induction in hFcγR^{KI} mice (expressing hFcγRI, hFcγRIIA, hFcγRIIB, hFcγRIIIA, and hFcγRIIIB) or in mice expressing a more restricted set of hFcγRs, as reported previously (*11*), because hFcγRIIA is the only activating IgG receptor expressed by platelets (*15*). The role of hFcγRIIA-induced platelet activation is best established in the pathophysiology of immune-mediated thrombocytopenia and thrombosis, a group of pathologies that encompass thrombosis triggered by certain therapeutic mAbs and sepsis-associated or heparin-induced thrombocytopenia (*26*–*29*). In most of these conditions, disease-causing IgG is binding via its antigen recognition site to the surface of platelets, thereby facilitating interactions of its Fc portion with platelet hFcγRIIA. Whether this is a general requirement for hFcγRIIA activation is not known. However, it may imply that, in the context of IgG-dependent anaphylaxis, IgG complexes must first be retained by one hFcγRIIA molecule on the platelet surface to then be able to trigger platelet activation via another hFcγRIIA molecule on the same or adjacent platelet (i.e., in cis or trans). hIgG readily bound to the platelet surface in hFcγRIIA^{tg} mice injected with HA-hIgG, in a dose-dependent manner, which led to a corresponding



reduction in detectable hFcγRIIA expression on platelets (fig. S8). Other studies investigating IgG-induced inflammatory reactions reported that platelets might rather play a regulatory role, preventing exaggerated neutrophil-mediated inflammation or inducing anti-inflammatory cytokine (e.g., interleukin-10) production by monocytes (*30*, *31*). However, these studies have been performed in mice expressing mouse FcγRs and therefore in the absence of the sole activating IgG receptor on platelets, hFcγRIIA. In light of the data presented herein and previous reports on hFcγRIIA function in vivo, it may be pertinent to reevaluate the role of platelets during inflammation using hFcγRIIA-expressing mice to ascertain whether protective or regulatory functions are preserved when platelets express hFcγRIIA.

We found that the large majority of circulating neutrophils and Ly6C^{hi} monocytes were covered with activated platelets, which, however, accounted neither for the reduction in circulating platelet numbers nor for the activation of either platelets or myeloid cells, because anti-CD62P antibodies blocked these interactions yet had no significant effect on anaphylaxis severity or thrombocytopenia. Other phenomena in vivo such as platelet-platelet aggregation, phagocytosis, or adhesion to the vascular endothelium (*32*) may contribute to the thrombocytopenia observed during anaphylaxis. In addition, our data imply that activation of platelets (increased CD62P expression) and neutrophils (CD62L shedding) occurs independently of their association. Previous studies on platelet-neutrophil interactions have suggested that platelet binding to neutrophils may facilitate neutrophil effector functions, such as extravasation into inflamed tissues and the formation of neutrophil extracellular traps (*33*–*35*). Our data indicate that these physical interactions are not required for anaphylaxis induction but rather that platelets and myeloid cells are each activated through direct FcγR engagement. We have shown previously that antibody-mediated neutrophil depletion had a beneficial effect on IgG-dependent anaphylaxis in hFcγRIIA^{tg} mice (*14*) and in mice expressing a more restricted set of human FcγRs (*11*) than that of hFcγR^{KI} mice.

This study presents several limitations: Inherently, data obtained using engineered mouse models may over- or underestimate pathways leading to disease. Platelet contribution to anaphylaxis was observed to be greater in the hFcγRIIA^{tg} mice than in the hFcγR^{KI} mice. This may be attributed to differences in hFcγRIIA expression levels on platelets, which is greater in hFcγRIIA^{tg} mice than in hFcγR^{KI} mice. Another possibility is that expression of multiple hIgG receptors compared with expression of a single IgG receptor (hFcγRIIA) might lead to the concurrent activation of additional cellular pathways in hFcγR^{KI} mice compared with hFcγRIIA^{tg} mice. Our data indicate that, regardless of whether high or moderate, hFcγRIIA expression on platelets promotes platelet contribution to anaphylaxis severity. Our in vivo data were supported by our in vitro studies on human washed platelets, which, like their mouse counterparts, aggregated and expressed activation markers in response to hFcγRIIA engagement by HA-hIgG. We also observed reduced numbers of circulating platelets in severe anaphylactic cases, along with an activated platelet phenotype. However, the setup for this study was not optimized for the analysis of platelets and therefore limited to the analysis of single platelets. Further clinical studies will be necessary to confirm these observations and evaluate whether platelets or platelet-derived mediators such as serotonin may represent previously unknown targets to intervene and ameliorate anaphylactic reactions in patients. Our observations are consistent with the sole other report in the literature that studied platelet numbers during human anaphylaxis, in which two anaphylactic cases were accompanied by >60% reduction in circulating platelet counts (*36*). Together, clinical data are in line with our data obtained in preclinical mouse models and substantiate the premise that platelets contribute to human anaphylaxis.

This report offers previously unknown insights into the mechanisms underlying anaphylaxis as it may occur in humans and identifies platelets and their serotonin release as critical players. Our work provides a rationale for future studies to assess the role of platelets in more complex clinical and preclinical settings, in which both IgE and IgG anti-allergen antibodies may contribute to anaphylaxis.

MATERIALS AND METHODS

Clinical study design

Detailed description of the NASA clinical study design and cohort can be found in (*37*). In brief, the multicenter NASA study involved 11 hospital anesthesia departments in the Ile-de-France region in France (https://clinicaltrials.gov/ct2/show/NCT01637220). The purpose of the NASA study was to evaluate the role of neutrophils during anaphylactic reactions to NMBA in humans through a case-control study design, with a 6 to 8 weeks' follow-up visit for cases, with clinical signs consistent with intra-anesthetic anaphylaxis to NMBA whatever the grade of the reaction. Patients were retrospectively classified in severity grade according to the Ring and Messmer (*38*) classification by two independent evaluations of clinician experts. A severe acute hypersensitivity reaction (AHR) was defined as a grade 3 or 4 (i.e., a severe organ failure or a cardiac/respiratory arrest). Because case patients were not in a medical and psychological state to provide study consent during the AHR, their consent was obtained as soon as they were judged able. If the patients did not recover the day after the AHR, then consent was obtained from a next of kin. Noninclusion criteria were the absence of consent. The study protocol was approved by a local ethics committee [committee for the protection of individuals ("Ile-de-France X")] and prospectively registered at ClinicalTrials.gov (identifier: NCT0163722). The biological collection was approved and labeled by the National Health Authority and registered within the French Ministry of Research and Agences Régionales de l'Hospitalisation. As recommended by international/national guidelines (*39*–*41*) on management of perioperative AHR, a venous blood sample was collected 30 min after the onset of the AHR when the patient's condition had been stabilized. Platelet enumerations were performed by flow cytometry on diluted total human blood using anti-CD61 and anti-CD62P antibodies. All patients who suffered an AHR were treated following the French and international practice guidelines (*39*, *40*, *42*, *43*).

Mice

hFcγR^{KI} mice were generated by intercrossing of VG1543 mice (*11*) with VG6074 mice (fig. S4A) designed and generated by Regeneron Pharmaceuticals Inc. on a mixed 62.5% C57BL/6N and 37.5% 129S6/SvEv genetic background and backcrossed one generation to C57BL/6N. FcγR^{null} mice were described previously (*44*). Mice expressing the *fcgr2a* transgene (*45*) were bred to FcγR^{null} mice to generate FcγRIIA^{tg} FcγR^{null} mice (referred to as hFcγRIIA^{tg} herein). All mice were bred at Institut Pasteur and used for experiments at 7 to 11 weeks of age. hFcγR^{KI}, FcγR^{null}, or hFcγRIIA^{tg} mice demonstrated normal development and breeding patterns. Experiments using mice were validated by the comité d'éthique en expérimentation animale (CETEA) #89 (Institut Pasteur, Paris, France) under #2013-0103 and by the French Ministry of Research under agreement #00513.02.

Antibodies and reagents

ADP, prostacyclin, apyrase, PAF, acid-citrate-dextrose anticoagulant, and PCPA were obtained from Sigma-Aldrich, trinitrophenol$_{(21–31)}$–bovine serum albumin from Santa Cruz Biotechnology, thrombin from Roche, anti-GPIbα (polyclonal rat IgG) and isotype control from emfret ANALYTICS, and heparin from Tocris. Human albumin and fibrinogen were provided by LFB Biomédicaments (2014-01029). Anti-FcγRIIA (mouse IgG2b, clone IV.3) and isotype control mIgG2b [clone Gork; hybridoma from B. Heyman (Uppsala Universitet, Uppsala, Sweden)] antibodies were purified as described (*12*). The antibodies used for flow cytometry staining of human CD61 (clone VI-PL2), CD62P (clone AK-4 or CLBThromb/6), CD64 (clone 10.1), and CD16 (clone 3G8) and of activated human αIIbβ3 integrin (clone PAC-1), mouse CD62P (clone KO2.3), CD41 (clone MWReg30), Ly6G (clone 1A8), Ly6C (clone AL-21), CD11b (clone M1/70), and CD45 (clone 30-F11) were purchased from BD Pharmingen. CD62L (clone MEL14-H2.100) was purchased from Miltenyi Biotec, αIIbβ3 (clone JON/A) antibody from emfret ANALYTICS, anti-human CD32A (clone IV.3) from STEMCELL Technologies, and isotype controls mIgG1 (clone P3.6.2.8.1) from eBioscience and mIgG2b (clone MPC-11) from BioLegend. Serotonin Fast Track and histamine enzyme-linked immunosorbent assay kits were obtained from Labor Diagnostika Nord and Abcam, respectively.

Passive systemic anaphylaxis

Human intravenous IgGs (Gamunex, Grifols) were heat-aggregated (HA-hIgG) by incubation at 20 or 25 mg/ml in borate-buffered saline [0.17 M H_3BO_3 and 0.12 M NaCl (pH 8)] for 1 hour at 63°C and then diluted to 10 mg/ml in 0.9% NaCl for intravenous injection at 100 μL per mouse, resulting in a dose of 0.05 mg/g of body weight. Central temperature was monitored using a digital thermometer (YSI) with a rectal probe. Control nonaggregated hIgG (NA-hIgG) was similarly diluted without heating. For the illustration of vascular leakage, 100 μL of 0.5% Evans Blue was injected intravenously 5 min before anaphylaxis induction. Fifteen minutes later, mice were sacrificed, and front paws were prepared for extraction in formamide [48 hours at room temperature (RT)]. Optical density of extracted dye was measured at 620 nm for quantification.

Platelet enumerations and hematocrit determination

In selected patients (four cardiac surgery anaphylactic cases and four paired controls), platelets from EDTA-treated whole blood were enumerated using the Sysmex TS 500 Hemocytometer. For analysis of mouse blood, 10 μL of blood was collected at the tail vein directly in EDTA (20 mM final), and platelet numbers and hematocrit were assessed using a hematometer (scil Vet abc Plus, Horiba Medical).

In vivo blocking, depletion, and romiplostim treatment

For platelet depletion, mice were injected intravenously with 50 μg per mouse of anti-GPIbα (anti-CD42b) or isotype control (rat IgG) 72 hours before challenge. Blocking of FcγRIIA was achieved by intravenous injection of 40 μg per mouse of blocking anti-FcγRIIA mAb (clone IV.3) or isotype control (mIgG2b) once at 24 and once at 12 hours before challenge. For CD62P blocking experiments, 50 μg per mouse of anti-CD62P REAffinity (REA) antibody (clone REA344, Miltenyi) or an isotype control was injected 30 min before challenge. To induce neoproduction of platelets, mice received subcutaneous injections of romiplostim (50 μg/kg of body weight; Nplate, Amgen) on days 0 and 3 and were used for experiments on day 6, adapted from (*22*). For in vivo serotonin depletion, mice received intraperitoneal injections of PCPA (300 mg/kg) or vehicle [phosphate-buffered saline (PBS)–Tween 20, 5% (v/v)] for 7 consecutive days. On the third day of treatment, a mild thrombocytopenia was induced with 5 μg per mouse of anti-GPIbα mAb to remove serotonin-loaded platelets from the circulation. Mice were used for experiments on day 7 when platelet counts had recovered.

Preparation of washed platelet suspensions, in vitro stimulation, and transfer

Preparation of washed human or mouse platelets

Washed platelets from mouse blood were prepared as previously described (*46*). Platelets were suspended 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, and 5 mM Hepes (pH 7.3)] containing 0.35% human serum albumin and apyrase (0.02 U/ml). Suspension of washed platelets was adjusted at the indicated concentration and maintained at 37°C until use. For generation of serotonin-depleted washed platelets, mice received intraperitoneal injections of PCPA (300 mg/kg) or vehicle [PBS-Tween 20, 5% (v/v)] for 5 consecutive days and were used for experiments on day 6.

In vitro stimulation

Platelet aggregation was measured at 37°C in an aggregometer APACT 4004 (ELITechGroup). A 270-μl aliquot of washed human (2×10^6 platelets/μl) or mouse (2×10^5 platelets/μl) platelets was stirred at 1100 revolutions per minute and activated by addition of the appropriate agonist and human fibrinogen (0.8 mg/ml) in a final volume of 300 μl. The extent of aggregation was quantified by measuring the maximum curve height above baseline. For flow cytometric analysis, washed human or mouse platelets were stimulated for 10 min at 37°C.

Transfer of washed mouse platelets

Washed mouse platelets (1.7×10^9) in 300 μL Tyrode's albumin buffer (or buffer alone) were injected intravenously into recipient mice 20 min before anaphylaxis induction.

Transfer of platelet releasates

Washed human or mouse platelets (6×10^6 platelets/μl) were activated by HA-hIgG (1 mg/ml) for 30 min, followed by centrifugation at 15,000*g* for 10 min, and releasates were collected for intravenous injection into recipient mice (200 μl per mouse). Tyrode's albumin buffer incubated with HA-hIgG (1 mg/ml) served as negative control.

Flow cytometric analysis of platelets, leukocytes, and their aggregates

For the flow cytometric analysis of NASA study patients, as recommended by International/National Guidelines (*39–41*) on management of perioperative AHR, a venous blood sample was collected 30 min after the onset of the AHR. Platelet analysis was not an original objective of the NASA study and that blood samples were taken using heparin as anticoagulant. Platelet enumerations and assessment of CD62P expression were performed by flow cytometry. Diluted total human blood was incubated for 15 min at RT in the dark in the presence of anti-CD61 antibodies, together with anti-CD62P antibodies or its isotype control (mIgG1). Mouse blood cells or washed human or mouse platelets were stained with indicated fluorescently labeled mAbs for 15 min at RT. In mouse whole blood, single platelets were defined as $FSC^{lo}/CD41^+$ cells, neutrophils as $CD45^+/CD11b^+/Ly6G^{hi}$ cells, and $Ly6C^{hi}$ monocytes as $CD45^+/CD11b^+/Ly6G^{lo}/Ly6C^{hi}$ cells. Within these populations, aggregates were identified using $CD41^+$ as a marker. Platelet activation of single platelets and

on aggregates was assessed by CD62P expression and neutrophil activation by CD62L shedding.

Statistical analysis

Mouse data and experiments with human washed platelets were analyzed using one-way or two-way analysis of variance (ANOVA) with Tukey's post hoc test or unpaired *t* test for comparison of two groups of measurements. A *P* value less than 0.05 was considered significant: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. If not stated otherwise, then data are means ± SEM. Statistical differences between different patient groups were assessed using a Kruskal-Wallis test with Dunn's correction for multiple testing ($), whereas differences between measures acquired at two different time points in the same patients were assessed using a Wilcoxon test (#). If not stated otherwise, then data are means ± SEM.

SUPPLEMENTARY MATERIALS

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NASA study group

Fig. S1. Clinical signs of HA-hIgG–dependent anaphylaxis and platelet depletion using anti-GPIbα antibodies.

Fig. S2. Platelets associate with Ly6C^{hi} monocytes during HA-hIgG–dependent anaphylaxis and require FcγRIIA expression for activation.

Fig. S3. Serotonin but not histamine concentration is augmented during HA-hIgG–dependent anaphylaxis.

Fig. S4. Generation of hFcγR^{KI} mice.

Fig. S5. Expression of hFcγRs in blood of humans or transgenic mice.

Fig. S6. Formation of platelet/neutrophil aggregates is dispensable for HA-hIgG–dependent anaphylaxis in hFcγR^{KI} mice.

Fig. S7. Platelet gating in NASA study.

Fig. S8. Platelet-bound hIgG and hFcγRIIA expression on platelets ex vivo after HA-hIgG injection into hFcγRIIA^{tg} mice.

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Platelets expressing IgG receptor FcγRIIA/CD32A determine the severity of experimental anaphylaxis

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Potent platelets

Anaphylaxis results from inappropriate immune responses to allergens. Human platelets express the IgG receptor Fc γRIIA/CD32A and release inflammatory mediators in response to their engagement, but their contribution to anaphylaxis is not well understood. Beutier *et al*. developed mouse models that express either human FcγRIIA/CD32A alone or the full human IgG receptor complexity to understand the role of platelets in anaphylaxis. Anaphylaxis induced a marked decrease in platelet levels, but preventive platelet depletion reduced anaphylaxis severity. A clinical study of patients with drug-induced anaphylaxis revealed that a severe reaction was likewise associated with fewer circulating platelets. Activated platelets released serotonin, which contributed to anaphylaxis severity. These results reveal a critical role for platelets in IgG-mediated anaphylaxis.