IgG subclasses determine pathways of anaphylaxis in mice

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Background: Animal models have demonstrated that allergen-specific IgG confers sensitivity to systemic anaphylaxis that relies on IgG Fc receptors (FcγRs). Mouse IgG_{2a} and IgG_{2b} bind activating FcγRI, FcγRIII, and FcγRIV and inhibitory FcγRIIB; mouse IgG_1 binds only FcγRIII and FcγRIIB. Although these interactions are of strikingly different affinities, these 3 IgG subclasses have been shown to enable induction of systemic anaphylaxis.

Objective: We sought to determine which pathways control the induction of IgG_1-, IgG_{2a}-, and IgG_{2b}-dependent passive systemic anaphylaxis.

Methods: Mice were sensitized with IgG_1, IgG_{2a}, or IgG_{2b} anti-trinitrophenyl mAbs and challenged with trinitrophenyl-BSA intravenously to induce systemic anaphylaxis that was monitored by using rectal temperature. Anaphylaxis was evaluated in mice deficient for FcγRs injected with mediator antagonists or in which basophils, monocytes/macrophages, or neutrophils had been depleted. FcγR expression was evaluated on these cells before and after anaphylaxis.

Results: Activating FcγRIII is the receptor primarily responsible for all 3 models of anaphylaxis, and subsequent downregulation of this receptor was observed. These models differentially relied on histamine release and the contribution of mast cells, basophils, macrophages, and neutrophils. Strikingly, basophil contribution and histamine predominance in mice with IgG_1- and IgG_{2b}-induced anaphylaxis correlated with the ability of inhibitory FcγRIIB to negatively regulate these models of anaphylaxis.

Conclusion: We propose that the differential expression of inhibitory FcγRIIB on myeloid cells and its differential binding of IgG subclasses controls the contributions of mast cells, basophils, neutrophils, and macrophages to IgG subclass–dependent anaphylaxis. Collectively, our results unravel novel complexities in the involvement and regulation of cell populations in IgG-dependent reactions *in vivo*. (J Allergy Clin Immunol 2017;139:269-80.)

Key words: *Anaphylaxis, IgG, mouse model, basophil, neutrophil, monocyte, macrophage, IgG Fc receptor, platelet-activating factor, histamine*

Anaphylaxis is a hyperacute allergic reaction that occurs with increasing incidence in the population and can be of fatal consequence. Symptoms include skin rash, hypotension, hypothermia, abdominal pain, bronchospasm, and heart and lung failure, which can lead to asphyxia and sometimes death.[1] The main treatment remains epinephrine (adrenaline) injection to restore heart and lung function. Because anaphylaxis represents an emergency situation, few clinical studies have been possible to address the mechanisms leading to anaphylaxis in patients. Experimental models of anaphylaxis identified mechanisms involving allergen-specific antibodies that trigger activating antibody receptors on myeloid cells, leading to mediator release. These mediators can, by themselves, recapitulate the symptoms of anaphylaxis observed in human subjects.[2,3]

The "classical" mechanism of anaphylaxis states that allergen-specific IgE binds the activating IgE receptor FcεRI on mast cells, which, on allergen encounter, become activated and release histamine, among other mediators. Notably, histamine injection suffices to induce signs of anaphylaxis in animal models.[4] In many cases detectable allergen-specific IgE and increased histamine levels do not accompany anaphylaxis in human subjects (discussed in Khodoun et al[5]), leading to the notion that "atypical" or "alternate" mechanisms of induction could explain these cases. One of these atypical/alternate models proposes a similar cascade of events but instead based on allergen-specific IgG binding to allergen, forming IgG-allergen immune complexes that trigger activating IgG Fc receptors (FcγRs) expressed on myeloid cells (ie, macrophages,

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Abbreviations used
FcγR: IgG Fc receptor
FcRn: Neonatal IgG receptor
FITC: Fluorescein isothiocyanate
Gfi1: Growth factor independence 1
K_A: Affinity constant
K_D: Dissociation equilibrium constant
K_{off}: Dissociation rate
K_{on}: Association rate
mMCP-1: Mast cell protease 1
PAF: Platelet-activating factor
PSA: Passive systemic anaphylaxis
RU: Resonance units
TNP: Trinitrophenyl
TRIM21: Tripartite motif-containing protein 21
WT: C57Bl/6 wild-type

basophils, and/or neutrophils), which in turn release platelet-activating factor (PAF).[2,3] Importantly, PAF injection suffices to induce signs of anaphylaxis in animal models.[6] IgG-induced anaphylaxis can be elicited by intravenous injection of allergen-specific IgG followed by allergen administration and is termed IgG-induced passive systemic anaphylaxis (PSA).

IgG receptors in the mouse comprise 4 "classical" IgG receptors termed FcγRs but also the neonatal IgG receptor (FcRn) and the intracellular FcR tripartite motif-containing protein 21 (TRIM21).[7,8] Although FcRn and TRIM21 both participate in the intracellular routing of IgG and FcRn in protection from catabolism and distribution to tissues,[9] FcγRs control cell activation in the presence of immune complexes. FcγRs in mice are subdivided into (1) activating FcγRs (ie, FcγRI, FcγRIII, and FcγRIV), which lead to cell activation on immune complex binding, and (2) an inhibitory FcγR (ie, FcγRIIB), which inhibits cell activation when coengaged by an immune complex with an activating FcγR coexpressed on the same cell.[10] Thus inhibition of cell activation by FcγRIIB requires that the immune complex contains IgG bound by both the activating and inhibitory FcγR.

Four IgG subclasses exist in mice: IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3. Among those, only IgG_{2a} and IgG_{2b} bind to all FcγRs, whereas IgG_1 binds only to FcγRIIB and FcγRIII. It remains under debate whether IgG_3 binds to FcγRs, particularly FcγRI.[11,12] The affinities of these FcγRs toward IgG subclasses are strikingly different (Table I),[11-14] leading to the notion of high-affinity receptors that retain monomeric IgG and low-affinity receptors that do not.[8] However, the avidity of IgG-immune complexes enables both types of receptors to retain IgG-immune complexes, leading to receptor clustering, intracellular signaling events, and, eventually, cell activation. FcγRI is a high-affinity receptor for IgG_{2a},[15] and FcγRIV is a high-affinity receptor for IgG_{2a} and IgG_{2b}.[16] All other FcγR-IgG interactions are of low affinity (reviewed in Bruhns[7]).

Three of the 4 IgG subclasses in the mouse, IgG_1, IgG_{2a}, and IgG_{2b}, have been reported to enable the induction of systemic anaphylaxis, inducing mild-to-severe hypothermia.[5,17,18] This is rather surprising for IgG_1, considering that inhibitory FcγRIIB binds IgG1 with a 10-fold higher affinity (affinity constant [K_A], 3.3×10^6 M^{-1}) than activating FcγRIII (K_A, 3.1×10^5 M^{-1}; Table I),[13] implying that inhibition should dominate over activation. C57Bl/6 wild-type (WT) mice experience a very mild anaphylactic reaction during IgG_1-induced PSA compared to FcγRIIB$^{-/-}$ mice,[19] indicating that inhibition

TABLE I. Affinities of mouse FcγR-IgG subclass interactions (K_A values in M^{-1})

	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3
FcγRI	−	1×10^8	1×10^5	(+)
FcγRIIB	3.3×10^6	4.2×10^5	2.2×10^6	−
FcγRIII	3.1×10^5	6.8×10^5	6.4×10^5	−
FcγRIV	−	2.9×10^7	1.7×10^7	−

Data were compiled from Nimmerjahn and Ravetch[13] and Nimmerjahn et al.[14]
−, No detectable affinity; *(+)*, under debate.[11,12]

by FcγRIIB occurs in WT mice during IgG_1-induced PSA, reducing but not protecting against anaphylaxis. IgG_1-dependent PSA has been reported to rely on basophils[20] that coexpress FcγRIIB and FcγRIII.[21] In this apparently simple situation, only 1 activating receptor and 1 inhibitory receptor are engaged on a single cell type that, once activated, produces an anaphylactogenic mediator, such as PAF.[20]

However, IgG_{2a} and IgG_{2b} bind 3 activating FcγRs and inhibitory FcγRIIB with different affinities, ranging over 2 logs. In particular, the affinity of FcγRIIB for IgG_{2a} is significantly lower than that for IgG_{2b}, whereas the activating IgG receptors FcγRIII and FcγRIV bind IgG_{2a} and IgG_{2b} with similar affinities, respectively (Table I). Notably, FcγRIV is not expressed on basophils but on monocytes/macrophages and neutrophils,[14] which have both been reported to contribute to experimental anaphylaxis.[18,22-24] In addition, mice expressing only FcγRIV can develop IgG-dependent PSA.[16] Therefore, together with expression and binding data, one would hypothesize that FcγRIV contributes predominantly to IgG_{2a}- and IgG_{2b}-induced PSA.

In this work we present evidence contrary to this hypothesis and reveal which activating FcγR on which cell types releasing which mediators are responsible for IgG_{2a}-dependent PSA and IgG_{2b}-dependent PSA and the differential regulation of these models of anaphylaxis by FcγRIIB. Our results unravel a complex balance determined by FcγR expression patterns, inhibition potential by FcγRIIB, and respective affinities of activating and inhibitory FcγRs for IgG subclasses that, together, regulate the contribution of cells and anaphylactogenic mediators to a given model of IgG-induced anaphylaxis.

METHODS

Mice

Female C57Bl/6J mice (herein referred to as WT mice) were purchased from Charles River (Wilmington, Mass), female BALB/cJRj mice were from Janvier Labs (Le Genest-Saint-Isle, France), and FcγRIIB$^{-/-}$ (MGI:1857166), FcγRIII$^{-/-}$ (MGI: 3620982) and Rosa26-YFP mice were from Jackson Laboratories (Bar Harbor, Me). FcγRI$^{-/-}$ mice (MGI: 3664782) were provided by J. Leusen (University Medical Center, Utrecht, The Netherlands), FcγRIV$^{-/-}$ mice (MGI: 5428684) were provided by J. V. Ravetch (Rockefeller University, New York, NY), growth factor independence 1 (Gfi1)$^{-/-}$ mice were provided by T. Moroy (Montreal University, Montreal, Quebec, Canada), and MRP8-cre mice were provided by Clifford Lowell (University of California at San Francisco, San Francisco, Calif). MRP8-cre and Rosa26-YFP mice were intercrossed to generate MRP8-cre; Rosa26-YFP mice. Cpa3-Cre; Mcl-1$^{fl/fl}$ mice[25] (backcrossed for at least 9 generations on a C57Bl/6J background) were kept in the Stanford University animal facility. All mouse protocols were approved by the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #C2EA-89 and/or the Institutional Animal Care and Use Committee of Stanford University.

Antibodies and reagents

PBS and clodronate liposomes were prepared as previously described.[26] Trinitrophenyl ($TNP_{[21-31]}$)–BSA was obtained from Santa Cruz Biotechnology (Dallas, Tex), ABT-491 was obtained from Sigma-Aldrich (St Louis, Mo), cetirizine DiHCl was obtained from Selleck Chemicals (Houston, Tex), anti-mouse FcγRIII (275003) was obtained from R&D Systems (Minneapolis, Minn), and rat IgG_{2b} isotype control (LTF-2) was obtained from Bio X Cell (West Lebanon, NH). Purified anti-CD200R3 (Ba103) was provided by H. Karasuyama (Tokyo Medical and Dental University Graduate School, Tokyo, Japan). The hybridoma producing mAb anti-mouse FcγRIV (9E9) was provided by J. V. Ravetch (Rockefeller University), anti-Ly6G (NIMP-R14) was provided by C. Leclerc (Institut Pasteur), IgG_1 anti-TNP (TIB-191) was provided by D. Voehringer (Universitätsklinikum, Erlangen, Germany), IgG_{2a} anti-TNP (Hy1.2) was provided by Shozo Izui (University of Geneva, Geneva, Switzerland), and IgG_{2b} anti-TNP (GORK) was provided by B. Heyman (Uppsala Universitet, Uppsala, Sweden); corresponding antibodies were purified, as previously described.[18] Purified mouse IgE anti-TNP was purchased from BD PharMingen (San Jose, Calif). The mAb 9E9 was coupled to fluorescein isothiocyanate (FITC) by using the Pierce FITC Antibody labeling kit (Life Technologies, Grand Island, NY). Antibodies used for flow cytometry staining of c-Kit (clone 2B8), CD49b (clone DX5), IgE (clone R35-72), CD11b (clone M1/70), F4/80 (clone 6F12), CD115 (clone T38-320), Ly6G (clone 1A8), and Ly6C (clone AL-21) were purchased from BD PharMingen; CD45 (clone 30F11) and Gr1 (clone RB6-8C5) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). FcγRIIB was detected by using the FITC-coupled mAb AT130-2 mIgG1 N297A.[27]

PSA

IgG-induced PSA. IgG_1, IgG_{2a}, or IgG_{2b} anti-TNP antibodies were administered intravenously at a dose of 500 μg, if not otherwise indicated, in 200 μL of physiologic saline, followed by an intravenous challenge with 200 μg of the antigen (TNP-BSA) in physiologic saline 16 hours later.

IgE-induced PSA. IgE anti-TNP antibodies were administered intravenously at a dose of 50 μg in 200 μL of physiologic saline, followed by an intravenous challenge with 500 μg of TNP-BSA in physiologic saline 24 hours later. The body temperature of mice was monitored with a digital thermometer with a rectal probe (YSI, Yellow Springs, Ohio).

In vivo blocking and cellular depletion

Three hundred microlitres per mouse of PBS or clodronate liposomes, 300 μg/mouse of rat IgG_{2b} isotype control or anti-Ly6G, and 30 μg/mouse of anti-CD200R3 mAbs were injected intravenously 24 hours before challenge. The specificity of cell depletion was evaluated by using flow cytometry on blood, bone marrow, and splenic and peritoneal cells taken from naive WT mice 24 hours after injection of the depleting antibody or clodronate-liposomes (examples are shown in Figs E1 and E2 in this article's Online Repository at www.jacionline.org). Twenty-five micrograms per mouse of ABT-491 or 300 μg/mouse of cetirizine was injected intravenously 20 minutes or intraperitoneally 30 minutes before challenge, respectively. Two hundred micrograms per mouse of anti-FcγRIV mAb was injected intravenously 30 minutes before challenge.

Flow cytometric analysis

Freshly isolated cells were stained with indicated fluorescently labeled mAbs for 30 minutes at 4°C. Cell populations were defined as follows: neutrophils ($CD45^+/CD11b^+/Ly6G^{hi}/Ly6C^{int}$), monocytes ($CD45^+/CD11b^+/Ly6G^{lo}/Ly6C^{lo/hi}$), basophils ($CD45^{int}/CD49b^+/IgE^+$), spleen macrophages ($CD45^+/CD11b^+/Gr\text{-}1^{lo}/CD115^+/F4/80^{hi}$), peritoneal macrophages ($CD45^+/CD11b^+/F4/80^+$), and peritoneal mast cells ($CD45^+/c\text{-}Kit^+/IgE^+$). FcγR expression on the indicated cell population is represented as Δ geometric mean between specific and isotype control staining.

Surface plasmon resonance analysis

Experiments were performed at 25°C with a ProteOn XPR36 real-time SPR biosensor (Bio-Rad Laboratories, Hercules, Calif). Anti-TNP antibodies were immobilized covalently through amine coupling on the surface of a GLC chip. TNP-BSA was then injected on the chip at a flow rate of 25 $\mu L \cdot min^{-1}$, with contact and dissociation times of 8 minutes each. Binding responses were recorded in real time as resonance units (RU; 1 RU ≈ 1 pg/mm^2). Background signals were subtracted, and binding rates (k_{on} [association rate] and k_{off} [dissociation rate]) and equilibrium constants (Kd [dissociation equilibrium constant]) were determined with BIAevaluation software (GE Healthcare, Fairfield, Conn).

ELISAs

After induction of IgG_1-, IgG_{2a}-, IgG_{2b}-, or IgE-induced PSA, plasma and sera were collected at 5 minutes and 3 hours later to determine the histamine and mast cell protease 1 (mMCP-1) content, respectively. Histamine and mMCP-1 concentrations were determined with commercially available ELISA kits (Beckman Coulter, Fullerton, Calif, and eBioscience, San Diego, Calif), according to the manufacturer's instructions. The relative binding affinity of IgG_1, IgG_{2a}, and IgG_{2b} anti-TNP antibodies to TNP-BSA was determined by using ELISA. Briefly, TNP-BSA–coated plates were incubated with dilutions of IgG_1, IgG_{2a}, or IgG_{2b} anti-TNP antibodies. After washing, bound anti-TNP IgG was revealed by using the same horseradish peroxidase–coupled anti-mouse IgG and SIGMAFAST OPD Sigma-Aldrich (St Louis, Mo) solution.

Mast cell histology

Mouse back skin biopsy specimens were collected 24 hours after induction of specific cell depletion, and mouse ear skin biopsy specimens were collected 30 minutes after IgE-, IgG_1-, IgG_{2a}-, or IgG_{2b}-induced PSA and embedded in paraffin before sectioning. Mast cells in toluidine blue–stained biopsy specimens were counted visually in at least 15 fields of view per mouse and more than 6 mice per treatment (see Fig E1, *I*).

Statistics

Data were analyzed by using 1-way or 2-way ANOVA with the Tukey posttest. A P value of less than .05 was considered significant. If not stated otherwise, data are represented as means ± SEMs.

RESULTS

FcγRIII dominates anaphylaxis induced by IgG subclasses

PSA was induced by means of an intravenous injection of one of the different anti-TNP IgG isotypes (IgG_1, IgG_{2a}, and IgG_{2b}), followed by an intravenous challenge with TNP-BSA 16 hours later. This protocol induces a transient decrease in body temperature that is most pronounced between 30 and 40 minutes. As reported previously,[3,18,20,22,28] all 3 IgG isotypes were capable of inducing anaphylaxis in WT mice (Fig 1). In these experimental conditions IgG_1-induced PSA triggered a maximum temperature loss of approximately 2°C, IgG_{2a}-induced PSA triggered a maximum temperature loss of approximately 4°C, and IgG_{2b}-induced PSA triggered a maximum temperature loss of approximately 3°C in WT mice. Using single FcγR knockout mice we evaluated the contribution of each of the 4 mouse FcγRs to these anaphylaxis models. The absence of either FcγRIV (with the exception of a single time point in IgG_{2b}-induced PSA) or FcγRI had no significant effect on IgG- PSA–induced hypothermia, regardless of the subclass of IgG antibodies used to induce anaphylaxis (Fig 1). However, the lack of FcγRIII protected mice from anaphylaxis in all models.

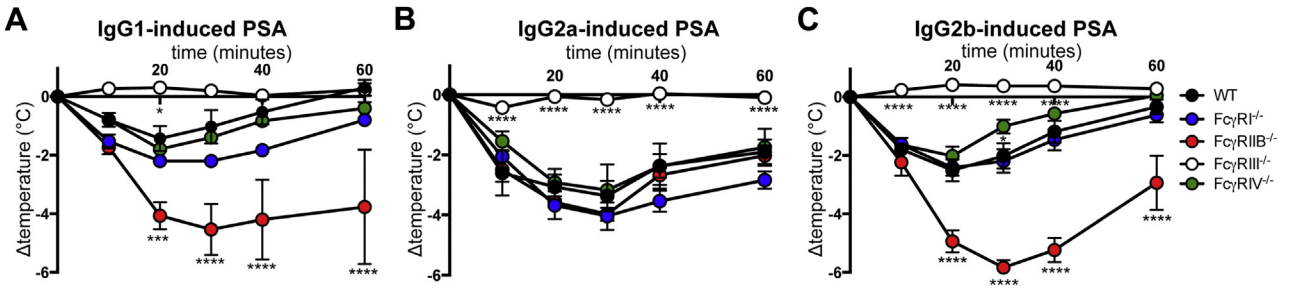


FIG 1. FcγRIII dominates in IgG-induced PSA models. Mice injected with anti-TNP mAbs were challenged with TNP-BSA, and body temperatures were monitored: IgG_1-induced PSA **(A)**, IgG_{2a}-induced PSA **(B)**, or IgG_{2b}-induced PSA **(C)** in indicated mice (n ≥ 3 per group). Data are representative of at least 2 independent experiments (Fig 1, *A*: n = 2; Fig 1, *B*: n = 3; Fig 1, *C*: n = 2). Significant differences compared with the WT group are indicated. **P* < .05, ****P* < .001, and *****P* < .0001.

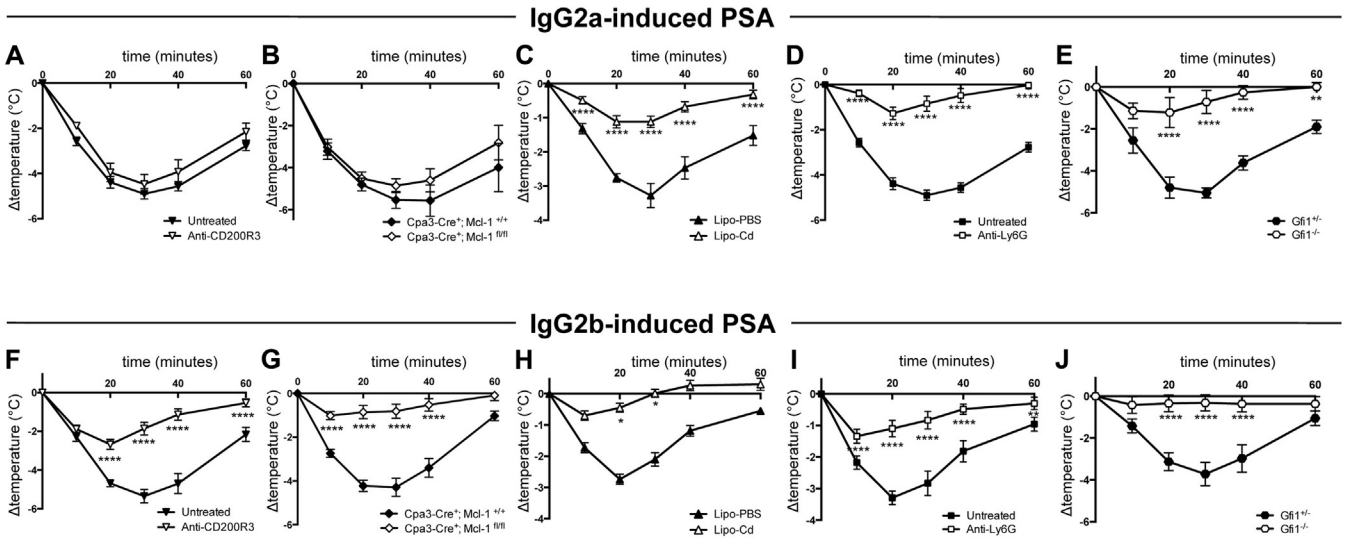


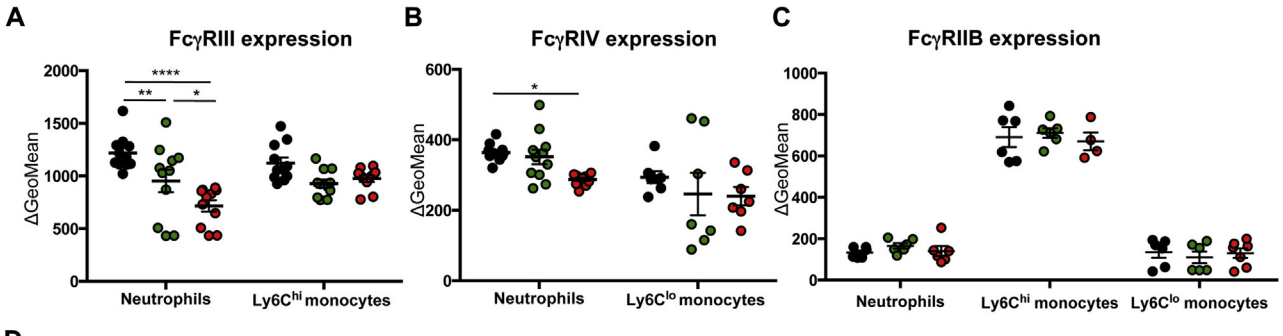
FIG 2. Basophils, mast cells, monocytes/macrophages, and neutrophils contribute differentially to IgG-induced PSA models. Indicated mice (n ≥ 8 per group) were injected with IgG_{2a} **(A-E)** or IgG_{2b} **(F-J)** anti-TNP mAbs and challenged with TNP-BSA, and body temperatures were monitored. WT mice (n = 8 per group) were pretreated, as indicated (Fig 2, *A*, *C*, *D*, *F*, *H*, and *I*). *Lipo-PBS*, PBS liposomes; *Lipo-Cd*, clodronate liposomes. Data are pooled from at least 2 independent experiments. **P* <.05, ***P* <.01, and *****P* < .0001.

Mice lacking the inhibitory receptor FcγRIIB had a significantly more severe temperature decrease than seen in WT mice with IgG_1- or IgG_{2b}-induced PSA but showed no significant difference in the severity of IgG_{2a}-induced PSA (Fig 1). Even though the 3 anti-TNP IgG mAbs used are not switch variants of a unique anti-TNP antibody, they show comparable binding to TNP-BSA, as determined by using ELISA, and similar affinity (nanomolar range) and dissociation rates (k_{off}), as determined by using surface plasmon resonance analysis, particularly the IgG_{2a} and IgG_{2b} anti-TNP antibodies (see Fig E3, *A-C*, in this article's Online Repository at www.jacionline.org).

Of note, untreated FcγR-deficient mice presented modest variations in FcγR expression levels (see Fig E4 in this article's Online Repository at www.jacionline.org) and leukocyte representation among blood cells compared with WT mice (see Fig E5 in this article's Online Repository at www.jacionline.org). In particular, a mild lymphopenia in FcγRIV$^{-/-}$ and FcγRIIB$^{-/-}$ mice (the latter also have a tendency to express higher levels of FcγRIII and FcγRIV) and a mild eosinophilia in FcγRIII$^{-/-}$ mice, which also express significantly more FcγRIIB on neutrophils and Ly6C^{hi} monocytes, were seen. Together, we think that these variations do not explain the drastic phenotypes observed for PSA in FcγRIIB$^{-/-}$ and FcγRIII$^{-/-}$ mice compared with WT mice. Thus these data indicate that FcγRIII predominates in the induction of IgG_1-, IgG_{2a}-, and IgG_{2b}-induced PSA and that FcγRIIB specifically dampens anaphylaxis severity in mice with IgG_1- and IgG_{2b}-induced PSA.

Basophils, mast cells, monocytes/macrophages and neutrophils contribute differentially to IgG isotype-dependent anaphylaxis models

FcγRIII is expressed by all myeloid cells[7,21] and, to a lesser extent, by natural killer (NK) cells.[29] Therefore one might anticipate that IgG immune complexes formed *in vivo* as a consequence of TNP-BSA injection in anti-TNP–sensitized mice would engage FcγRIII on these cells, leading to cell activation and possibly contributing to anaphylaxis. Basophils, mast cells, neutrophils, and monocyte/macrophages have indeed been reported to contribute to IgG-induced PSA,[17,18,20,22]; however, the



D

FcγRIII expression	Untreated	19h post-IgG2a	19h post-IgG2a, 3h post-Ag
Neutrophils	1217 ± 49	952 ± 106	715 ± 54
Ly6C^{hi} monocytes	1123 ± 53	927 ± 39	975 ± 31

FcγRIV expression	Untreated	19h post-IgG2a	19h post-IgG2a, 3h post-Ag
Neutrophils	363 ± 7.3	352 ± 21	287 ± 5
Ly6C^{lo} monocytes	294 ± 17	246 ± 60	240 ± 26

FcγRIIB expression	Untreated	19h post-IgG2a	19h post-IgG2a, 3h post Ag
Neutrophils	133 ± 10	165 ± 13	140 ± 24
Ly6C^{hi} monocytes	691 ± 48	711 ± 23	670 ± 43
Ly6C^{lo} monocytes	134 ± 27	110 ± 28	130 ± 23

FIG 3. Reduced expression of FcγRIII and FcγRIV, but not FcγRIIB, on neutrophils after IgG_{2a}-induced PSA. **A-C,** FcγRIII (Fig 3, *A*), FcγRIV (Fig 3, *B*), and FcγRIIB (Fig 3, *C*) expression on blood cells from WT mice (Fig 3, *A* and *B*: n = 11 per group; Fig 3, *C*: n ≥ 6 per group) left untreated, injected with IgG_{2a} anti-TNP mAbs, or injected with IgG_{2a} anti-TNP mAbs and challenged with TNP-BSA is shown. **D,** Compilation of Δ geometric mean *(GeoMean)* ± SEM data from Fig 3, *A-C*. *Ag*, Antigen. *$P < .05$, **$P < .01$, and ****$P < .0001$.

respective contribution of each of these different cell types remains debated.[2,28] To investigate which cell types contribute to PSA induced by different IgG subclasses, we depleted basophils (anti-CD200R3 mAb), monocytes/macrophages (clodronate-filled liposomes), or neutrophils (anti-Ly6G) before anaphylaxis induction or evaluated anaphylaxis induction in transgenic mice deficient in certain cell populations.

Of note, the relatively mild temperature loss in WT mice with IgG_1-induced PSA (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org) did not allow us to address reliably the contribution of either basophils or neutrophils to this model of anaphylaxis. Therefore we restricted our analysis of the contribution of myeloid cell populations to IgG_{2a}- and IgG_{2b}-induced PSA. Antibody-induced basophil depletion or genetically induced mast cell and basophil deficiency (see Fig E2, *H*: Cpa3-Cre; Mcl-$1^{fl/fl}$ mice[25]) did not affect IgG_{2a}-induced PSA (Fig 2, *A* and *B*) but significantly inhibited IgG_{2b}-induced PSA (Fig 2, *F* and *G*). Monocyte/macrophage depletion (Fig 2, *C* and *H*) significantly inhibited both IgG_{2a}- and IgG_{2b}-induced PSA. The absence of neutrophils, either after antibody-mediated depletion (Fig 2, *D* and *I*) or with neutropenic Gfi$1^{-/-}$ mice (Fig 2, *E* and *J*),[30] significantly inhibited both IgG_{2a}- and IgG_{2b}-induced PSA. Although monocytes/macrophages and neutrophils appear to contribute to both models of anaphylaxis, basophils and possibly mast cells contribute specifically to IgG_{2b}- but not IgG_{2a}-induced PSA.

FcγRIII is downregulated specifically on neutrophils after IgG_{2a}-induced PSA

Khodoun et al[31] proposed to use the reduced expression of FcγRIII on mouse neutrophils as a marker to distinguish IgE- from IgG_1-induced PSA, both of which required priming with an antigen-specific antibody and challenge with the recognized antigen. Therefore we wondered whether FcγRIII expression on neutrophils might also be a marker for IgG_{2a}- and IgG_{2b}-induced PSA. In addition, reduced expression of FcγRs after IgG-induced PSA might document that a particular cell population is activated after engagement of its FcγRs by IgG-immune complexes during anaphylaxis. Thus this parameter can be used to discriminate cell populations contributing to anaphylaxis after direct activation by IgG-immune complexes from those contributing after activation by mediators liberated by IgG-immune complex–activated cells (eg, histamine, PAF, leukotrienes, and prostaglandins).

Among mouse IgG receptors, only FcγRIIB, FcγRIII, and FcγRIV are significantly expressed on circulating myeloid cells but not FcγRI.[7,32,33] Of circulating monocyte populations, "classical" Ly6C^{hi} monocytes are FcγRIIB^{med}FcγRIII^{med}FcγRIV^{-}, whereas "nonclassical" Ly6C^{lo} monocytes are FcγRIIB^{lo}FcγRIII^{lo}FcγRIV^{hi}.[34] Therefore we determined the expression of FcγRIIB, FcγRIII, and FcγRIV before and after IgG_{2a}-induced PSA induction on neutrophils and monocyte subsets. Expression of FcγRIII was downregulated on neutrophils, but not on Ly6C^{hi} monocytes, during IgG_{2a}-induced PSA (Fig 3, *A* and *D*). Expression of FcγRIV was also downregulated on neutrophils, but not on Ly6C^{lo} monocytes, during IgG_{2a}-induced PSA (Fig 3, *B* and *D*). This was unexpected considering that FcγRIV does not significantly contribute to this PSA model (Fig 1, *B*). However, FcγRIIB expression remained unchanged on Ly6C^{hi} and Ly6C^{lo} monocytes and neutrophils (Fig 3, *C* and *D*), which is in agreement with the lack of contribution of this receptor to IgG_{2a}-induced PSA (Fig 1, *B*).

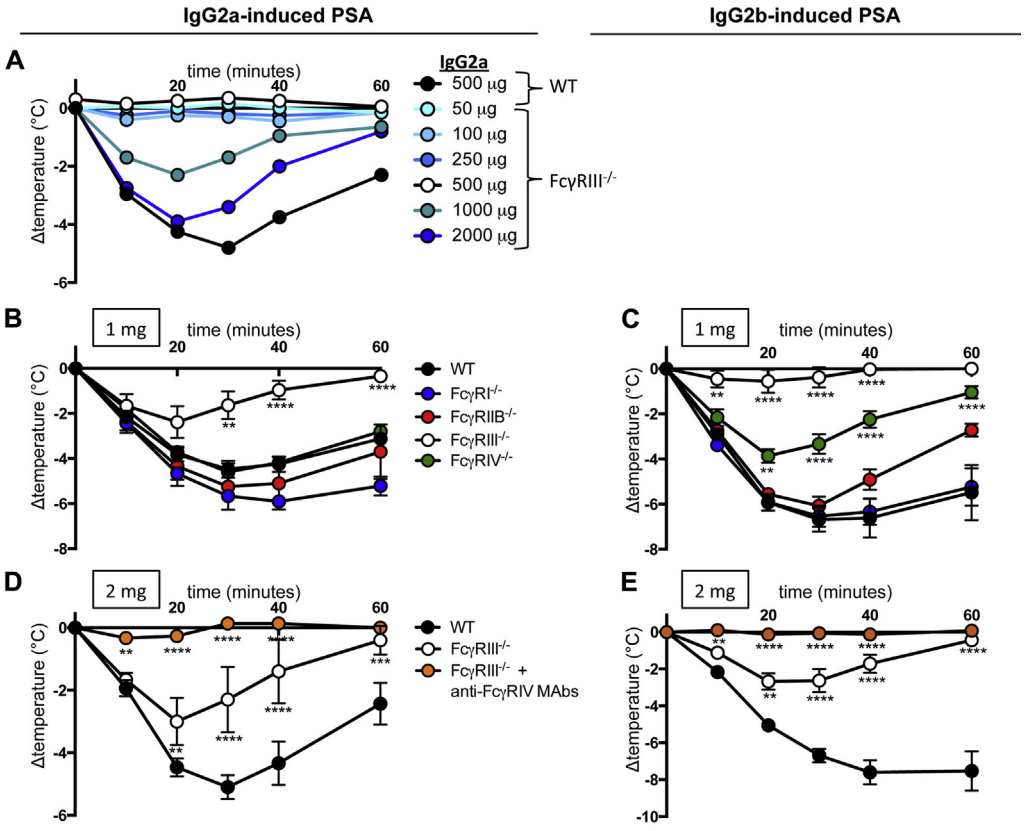


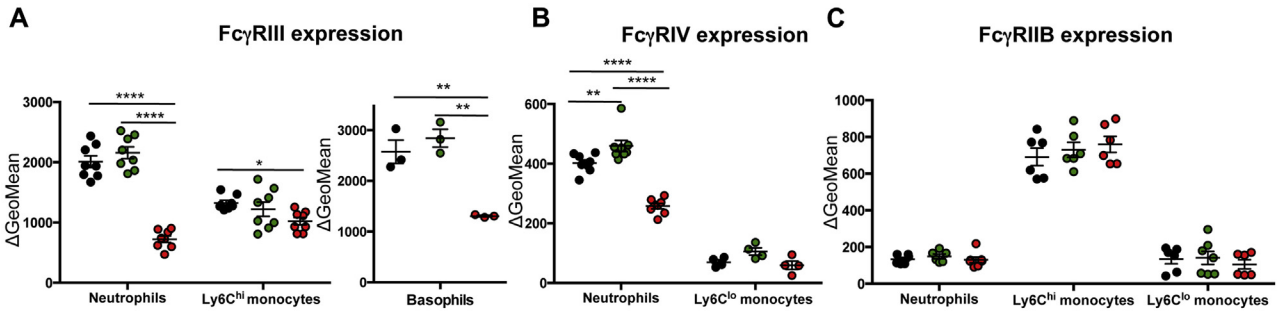
FIG 4. High doses of IgG_2 antibodies reveal FcγRIV contribution to IgG_2-induced PSA. **A,** PSA in indicated mice injected with various doses of IgG_{2a} anti-TNP mAbs (n = 2 per group). **B-E,** PSA in indicated mice (Fig 4, *B* and *C*: n = 8 per group; Fig 4, *D* and *E*: n ≥ 3 per group) injected with indicated doses of anti-TNP mAbs. Data are pooled from 2 independent experiments. Significant differences compared with the untreated WT group are indicated. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

Together, these data suggest that neutrophils might be activated directly through FcγRIII by immune complexes formed during IgG_{2a}-induced PSA. They also suggest that neutrophils, but not $Ly6C^{lo}$ monocytes, can be similarly activated through FcγRIV, even if no contribution of this receptor was identified in this model using FcγRIV$^{-/-}$ mice (Fig 1, *B*).

Increased IgG_2 antibody doses reveal the contribution of FcγRIV to IgG_{2a} and IgG_{2b}-induced PSA

In mice FcγRIV binds monomeric IgG_{2a} and IgG_{2b}. Therefore at physiologic concentrations of IgG_{2a} (approximately 2.5 mg/mL) and IgG_{2b} (approximately 1.5 mg/mL) in serum, FcγRIV might be occupied *in vivo*, particularly on circulating neutrophils and monocytes. Nevertheless, the short binding half-lives of monomeric IgG_{2a} (half-life, approximately 3 minutes) and monomeric IgG_{2b} (half-life, approximately 10 minutes) by FcγRIV and their ability to be displaced from this receptor by immune complexes[16] might enable IgG_2-immune complexes to interact with FcγRIV during anaphylaxis and therefore contribute to its induction, severity, or both.

To explore this possibility, we primed FcγRIII$^{-/-}$ mice with various doses of anti-TNP IgG_{2a} before challenge with TNP-BSA to induce a range of *in vivo* concentrations of immune complexes. As expected, the low doses did not trigger FcγRIII$^{-/-}$ mice to have anaphylaxis after challenge. However, increased doses (1 or 2 mg) enabled significant temperature decreases in FcγRIII$^{-/-}$ mice comparable with those observed in WT mice primed with 500 µg of IgG_2, particularly at the highest dose of IgG_{2a} (2 mg; Fig 4, *A*). Already at a dose of 1 mg of IgG_2, FcγRIII$^{-/-}$ mice had mild hypothermia with IgG_{2a}- but not IgG_{2b}-induced PSA (Fig 4, *B* and *C*). Unexpectedly, in the same conditions FcγRIV contributed to IgG_{2b}-induced PSA, which was no longer dampened by inhibitory FcγRIIB (Fig 4, *C*). At a dose of 2 mg of IgG, FcγRIII$^{-/-}$ mice had hypothermia with both IgG_{2a}- and IgG_{2b}-induced PSA, which was abolished when FcγRIII$^{-/-}$ mice were pretreated with a blocking antibody against FcγRIV (Fig 4, *D* and *E*). FcγRI did not contribute to either model of IgG_2-induced PSA at an increased dose (Fig 4, *B* and *C*). Furthermore, FcγRIII expression was downregulated on neutrophils and basophils, but not on $Ly6C^{hi}$ monocytes, after IgG_{2b}-induced PSA (Fig 5, *A* and *D*). FcγRIV expression was also downregulated on neutrophils, but not on $Ly6C^{lo}$ monocytes (Fig 5, *B* and *D*). However, FcγRIIB expression did not change on either neutrophils or $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes, even though this inhibitory receptor regulates IgG_{2b}-induced PSA (Figs 1, *C*, and 5, *C* and *D*). This observation is in agreement with the study by Khodoun et al,[31] which reported that FcγRIIB expression did not change on neutrophils after IgG_1-induced PSA. Altogether, high doses of antigen-specific IgG_2 reveal the contribution of FcγRIV to IgG_{2a}-induced PSA and IgG_{2b}-induced PSA and



D

FcγRIII expression	Untreated	19h post-IgG2b	19h post-IgG2b, 3h post Ag
Neutrophils	2008 ± 97	2158 ± 98	724 ± 54
Ly6C^{hi} monocytes	1326 ± 42	1222 ± 117	1021 ± 60
BM basophils	2574 ± 231	2842 ± 176	1307 ± 15
FcγRIV expression	**Untreated**	**19h post-IgG2b**	**19h post-IgG2b, 3h post Ag**
Neutrophils	402 ± 11	459 ± 19	258 ± 9
Ly6C^{lo} monocytes	70 ± 8	106 ± 12	59 ± 14
FcγRIIB expression	**Untreated**	**19h post-IgG2b**	**19h post-IgG2b, 3h post Ag**
Neutrophils	133 ± 10	149 ± 12	130 ±16
Ly6C^{hi} monocytes	691 ± 48	730 ± 41	759 ± 44
Ly6C^{lo} monocytes	135 ± 27	141 ± 35	105 ± 25

FIG 5. Expression of FcγRs on myeloid cells after IgG_{2b}-induced PSA. **A-C,** FcγRIII (*left*: n = 8 per group, *right*: n = 3 per group; Fig 5, *A*), FcγRIV (n = 8 per group; Fig 5, *B*), and FcγRIIB expression (n ≥ 6 per group; Fig 5, *C*) on cells from WT mice (n = 8 per group) left untreated, injected with IgG_{2b} anti-TNP mAbs, or injected with IgG_{2b} anti-TNP mAbs and challenged with TNP-BSA. **D,** Compilation of Δ geometric mean *(GeoMean)* ± SEM data from Fig 5, *A-C. Ag*, Antigen. In this figure 1 or 0.5 mg of IgG_{2b} was injected to assess expression on neutrophils/monocytes or basophils, respectively. **P* <.05, ***P* <.01, and *****P* <.0001.

suggest the direct activation of neutrophils and basophils by IgG_{2b}-immune complexes.

IgG_1-induced PSA in the absence of inhibitory FcγRIIB

The unexpected differences observed between IgG_{2a}- and IgG_{2b}-induced PSA induction pathways prompted us to find a mouse model more sensitive to IgG_1-induced PSA than WT mice to be able to evaluate the contribution of cell types and mediators in this model. Indeed, as mentioned earlier, WT mice respond poorly in a model of IgG_1-induced PSA (Fig 1, *A*, and see Fig E6, *A*).[19] However, FcγRIIB$^{-/-}$ mice experience a temperature decrease of approximately 4°C during IgG_1-induced PSA, which is comparable with temperature losses observed in WT mice during IgG_{2a}- or IgG_{2b}-induced PSA (Fig 1, *B* and *C*). Therefore we analyzed the contribution of cell types to IgG_1-induced PSA in FcγRIIB$^{-/-}$ mice. Basophil depletion mildly but significantly inhibited IgG_1-induced PSA (Fig 6, *A*), which is in agreement with previous data.[20] The depletion of neutrophils had the same effect, although not consistently as strongly as basophil depletion (Fig 6, *B*, and data not shown). Monocyte/macrophage depletion had a tendency to ameliorate anaphylaxis that was reproducible but not significant (Fig 6, *C*). These results suggest that IgG_1-induced PSA relies on basophils and neutrophils and possibly also monocytes.

PAF and histamine contribute differentially to IgG_{2a}- and IgG_{2b}-induced PSA

Because cell types contribute differently to IgG_2-induced PSA models (ie, IgG_{2a}-induced PSA for neutrophils and monocytes and IgG_{2b}-induced PSA for basophils, neutrophils, and monocytes), one can expect that the mediators responsible for clinical signs also might differ between them. PAF has been shown to be responsible for anaphylactic reactions that required basophil,[20] neutrophil,[18,24] and/or monocyte/macrophage[22] activation, whereas histamine has been shown to be responsible for mast cell– and basophil-dependent anaphylaxis.[35,36] Neutrophils are the main producers of PAF,[37] whereas mast cells and basophils are the main producers of histamine.[38,39] Therefore we analyzed the relative contribution of these 2 mediators to the 3 models of PSA by using the histamine receptor 1 antagonist cetirizine and the PAF receptor antagonist ABT-491. Surprisingly, the histamine receptor 1 antagonist cetirizine significantly inhibited IgG_1-induced PSA, whereas the PAF receptor antagonist ABT-491 had no significant effect, which is in opposition to previous data.[20] The combination of both antagonists had an additive effect and almost abolished IgG_1-induced PSA (Fig 7, *A*). The results obtained in FcγRIIB$^{-/-}$ mice were confirmed in WT mice (Fig 7, *A*). Whereas cetirizine mildly reduced hypothermia in IgG_{2a}-induced PSA, it significantly inhibited IgG_{2b}-induced PSA. ABT-491 mildly reduced hypothermia in mice with IgG_{2a}-induced PSA but had no significant effect on mice with IgG_{2b}-induced PSA (Fig 7, *B* and *C*). However, the combination

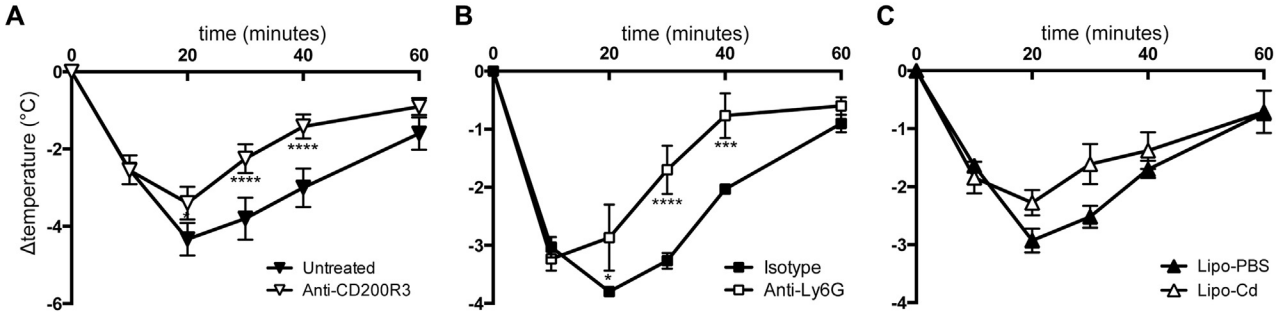


FIG 6. Cell contributions to IgG_1-induced PSA in the absence of inhibitory FcγRIIB. FcγRIIB$^{-/-}$ mice were pretreated as indicated, injected with IgG_1 anti-TNP mAbs, and challenged with TNP-BSA, and central temperatures were monitored (**A**: n = 8 per group; **B**: n = 7 per group; **C**: n = 10 per group). Data are represented as means ± SEMs. Data are pooled from 2 independent experiments. *P < .05, ***P < .001, and ****P < .0001.

of cetirizine and ABT-491 almost abolished both IgG_{2a}- and IgG_{2b}-induced PSA. Increased plasma histamine levels were detected 5 minutes after challenge in all 3 IgG-induced PSA models, and particularly high levels were observed in mice undergoing IgE-induced PSA (as a positive control) or IgG_{2a}-induced PSA (Fig 7, *D* and *E*). This latter finding is surprising because IgG_{2a}-induced PSA is unaffected by the absence of both mast cells and basophils, which are considered major sources of histamine. mMCP-1, which is released on activation of mucosal mast cells, could be detected in the sera of mice undergoing IgE-induced PSA but not in those undergoing any one of the 3 models of IgG-induced PSA 3 hours after PSA induction (Fig 7, *F*). Collectively, these results suggest that histamine predominantly contributes to IgG_1- and IgG_{2b}-induced PSA, whereas histamine and PAF together are necessary for IgG_{2a}-induced PSA.

DISCUSSION

Our work suggests that the activating IgG receptor FcγRIII predominantly contributes to IgG-dependent PSA, irrespective of whether induced by IgG_1, IgG_{2a}, or IgG_{2b} antibodies. A contribution of the activating IgG receptor FcγRIV was only identified when using very high amounts of IgG_2 antibodies, whereas the activating IgG receptor FcγRI played no detectable role. Remarkably, the inhibitory IgG receptor FcγRIIB controlled the severity of IgG_1- and IgG_{2b}- but not IgG_{2a}-induced anaphylaxis. The ability of FcγRIIB to inhibit a given model of IgG-induced anaphylaxis correlated with the contribution of basophils and histamine to that model. Indeed, basophils, and possibly mast cells, contributed with neutrophils to IgG_1-induced PSA and with neutrophils and monocytes to IgG_{2b}-induced PSA but not to IgG_{2a}-induced PSA, which appeared to depend entirely on neutrophils and monocytes/macrophages. Altogether, our data propose that the 3 IgG subclasses, IgG_1, IgG_{2a}, and IgG_{2b}, induce 3 qualitatively different pathways of anaphylaxis that are nevertheless triggered primarily by a single IgG receptor, FcγRIII.

FcγRIII is a low-affinity receptor for IgG_1, IgG_{2a}, and IgG_{2b}, whereas FcγRI is a high-affinity receptor for IgG_{2a}, and FcγRIV is a high-affinity receptor for IgG_{2a} and IgG_{2b}. Therefore one would assume that FcγRIII predominates in IgG_1-induced PSA, FcγRI and FcγRIV predominate in IgG_{2a}-induced PSA, and FcγRIV predominates in IgG_{2b}-induced PSA. However, our data from FcγRIII$^{-/-}$ mice indicate that this receptor predominates in all 3 models. Notably, we found increased expression of FcγRIIB on neutrophils and Ly6C^{hi} monocytes in FcγRIII$^{-/-}$ mice, which could mask a potential contribution of FcγRIV in these conditions.

In support of the notion that FcγRIII predominates in IgG-induced PSA induction, an alternative model of PSA induced by sensitization and challenge with goat antibodies was found to be driven by FcγRIII,[22] and blocking antibodies against FcγRIII were protective in a model of PSA induced by IgG immune complexes.[18] In addition, IgG_{2a}-induced PSA in FcγRIIB$^{-/-}$ mice was abolished after injection of anti-FcγRIIB/III blocking mAbs.[5] FcγRIII is the only activating IgG receptor in the mouse that does not bind an IgG subclass with high affinity, and thus it remains unoccupied by monomeric IgG and accessible for binding of immune complexes. This is theoretically not the case for FcγRI and FcγRIV, which at physiologic serum concentrations of IgG_{2a} (approximately 2.5 mg/mL) and IgG_{2b} (approximately 1.5 mg/mL), are likely occupied *in vivo*, particularly on circulating cells. Of note, C57Bl/6 mice produce IgG_{2c} but not IgG_{2a} antibodies, the amino acid sequence of which varies by about 15%. Experiments performed in BALB/c mice that express endogenous IgG_{2a} (but no IgG_{2c}) produced similar results regarding the contribution of basophils, neutrophils, and monocytes to IgG_{2a} (see Fig E6, *B*), indicating that IgG_{2a} and IgG_{2c} sequence variations probably do not affect the mechanisms of anaphylaxis induction that we describe herein.

Adult female mice of 20 g, as used in this study, possess a circulating blood volume of 1.4 to 1.5 mL. Injection of 500 μg of antibody thus corresponds to approximately 330 μg/mL of circulating antibody, injection of 1 mg corresponds to approximately 660 μg/mL, and injection of 2 mg corresponds to approximately 1.3 mg/mL. In cases of anaphylaxis, the circulating concentration of allergen-specific IgG has not been evaluated because of a lack of testing and appropriate controls (ie, anti-allergen mAbs), although we have reported high circulating antigen-specific IgG levels in an autoimmune model of arthritis.[33] It seems rather unlikely that patients with anaphylaxis possess such increased circulating levels of IgG anti-allergen as mice receiving the high doses we used in this study. Nevertheless, our results in high-dose IgG_{2a}- and IgG_{2b}-induced PSA demonstrate that FcγRIV can trigger anaphylaxis by itself (ie, in the absence of FcγRIII). Similar results have been obtained in mice expressing only FcγRIV: "FcγRIV-only" mice had IgG_{2b}-induced PSA after injection of preformed IgG_{2b} immune complexes and also on

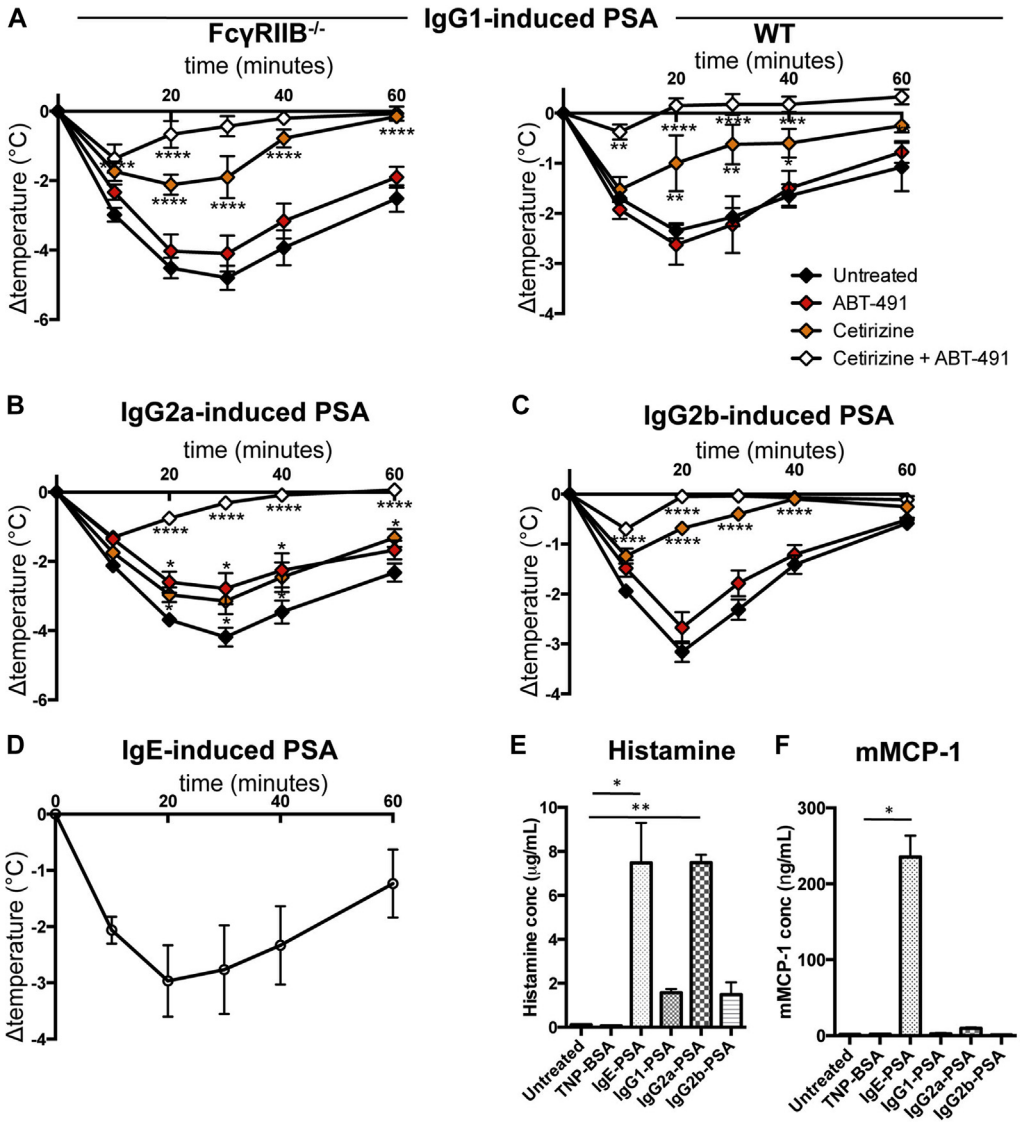


FIG 7. Contributions of histamine and PAF to IgG-induced PSA. **A-D,** Body temperatures of pretreated mice during IgG_1-induced PSA in FcγRIIB$^{-/-}$ (n = 6 per group) or WT (n = 4 per group) mice (Fig 7, *A*), IgG_{2a}-induced PSA (Fig 7, *B*), IgG_{2b}-induced PSA (Fig 7, *C*) or IgE-induced PSA (Fig 7, *D*) in WT mice (n ≥ 7 per group). **E** and **F,** Histamine (Fig 7, *E*) and mMCP-1 (Fig 7, *F*) concentrations after PSA (n = 3 per group). Data are representative of at least 2 independent experiments, except for Fig 7, *A* and *C* (pooled from 2 independent experiments). **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

injection of polyclonal anti-sera, followed by antigen challenge.[18] We reported previously that IgG_{2b}-induced PSA triggered by injection of preformed IgG_{2b}-immune complexes in WT mice was abolished after injection of anti-FcγRIV blocking mAb 9E9.[18]

This contrasts with the findings of the current study, in which we show that FcγRIII is the major activating receptor in all models of IgG-induced PSA and FcγRIV contributes only at high antibody concentrations. Two hypotheses might explain these discrepant results: (1) the injection of preformed IgG_{2b}-immune complexes leads to an immediate circulating bolus of immune complexes, which are similarly formed only after injection of high amounts of IgG_{2b} and antigen, thus triggering FcγRIV, and (2) as recently reported,[40] mAb 9E9 might not only block FcγRIV through its Fab portions but also FcγRIII through its Fc portion once 9E9 is bound to FcγRIV. In our view it is likely that a combination of these mechanisms reconciles our previous and herein described results and suggest that IgG_{2b} PSA induced after injection of preformed IgG_{2b}-immune complexes relies on both FcγRIII and FcγRIV rather than on FcγRIV alone, as we reported previously.[18] Together, this body of evidence supports the notion that FcγRIV is capable of triggering cell activation leading to anaphylaxis, although in restricted conditions (ie, in the absence/blockade of FcγRIII or presence of large amounts of IgG_{2a} and/or IgG_{2b} antibodies).

The differential contribution of FcγRs to IgG-induced PSA might rely on their respective expression patterns on myeloid cells. Indeed, FcγRI is not[32,33] or is only barely[34] expressed on circulating monocytes, and its expression is largely restricted to tissue-resident macrophages. Therefore the level of its expression on cells reported to contribute to anaphylaxis (ie, monocytes in

this case) might not suffice to induce their activation. This notion is supported by the absence of any detectable effect of FcγRI deficiency in the mice with IgG_2-induced PSA on which we report in this study, even at high doses of IgG_2 antibodies. However, FcγRIII is expressed on all myeloid cells[7] and, moreover, at comparably high levels on all those cell types that have been reported to contribute to anaphylaxis: basophils, monocytes, and neutrophils.[21] This pattern of cellular expression might explain its predominant contribution to all models of IgG-induced anaphylaxis. FcγRIV is expressed on neutrophils and $Ly6C^{lo}$ monocytes. However, it remains unclear whether $Ly6C^{lo}$, $Ly6C^{hi}$, or both monocyte subsets contribute to anaphylaxis. FcγRIV could contribute to PSA induction in exceptional conditions (FcγRIII deficiency or high IgG_2 antibody doses). The lack of FcγRIV contribution in classical conditions of PSA might suggest that its expression level is not sufficient in WT mice. Notably, it has been reported previously that particular FcγR deficiencies modify the expression levels of other FcγRs. In particular, $Fc\gamma RIII^{-/-}$ mice, but not $Fc\gamma RI^{-/-}$ mice, presented a significant increase in FcγRIV expression levels on neutrophils[18,41,42] and a tendency for increased expression on $Ly6C^{lo}$ monocytes (see Fig E4, *B*). This could explain why the contribution of FcγRIV to IgG_2-induced PSA becomes apparent in $Fc\gamma RIII^{-/-}$ mice. Conversely, $Fc\gamma RIV^{-/-}$ mice did not present alterations of FcγRIII expression on neutrophils or $Ly6C^{hi}$ monocytes compared with WT littermates (see Fig E4, *A*). $Fc\gamma RIIB^{-/-}$ mice expressed significantly higher levels of FcγRIII and FcγRIV on neutrophils and increased FcγRIII levels on $Ly6C^{hi}$ monocytes that might, altogether, contribute to their higher susceptibility to anaphylaxis induction (see Fig E4, *A* and *B*).

The contribution of a rather restricted subset of myeloid cells to these (and other) models of anaphylaxis[2,3] appears to be determined by at least 2 factors: their capacity to release anaphylactogenic mediators (eg, histamine or PAF) and their expression of sufficient levels of activating IgG receptors. Mast cells and basophils release histamine, and neutrophils monocytes/macrophages, and basophils release PAF on FcγR triggering. Other mediators might induce anaphylaxis or contribute to its severity, among them lipid mediators, such as prostaglandins, thromboxanes, and leukotrienes. Indeed, some of these have been reported to trigger bronchoconstriction and an increase in vascular permeability.[43] The release of such mediators is sufficiently rapid to coincide with the celerity of hypothermia, which is detectable within minutes after allergen challenge. Therefore it is surprising that eosinophils do not contribute to IgG-induced PSA because they express high levels of activating FcγRIII and FcγRIIB[21] (but no FcγRI or FcγRIV) and are capable of releasing leukotriene C_4, prostaglandin E_2, thromboxane, and PAF on activation.[43] Although eosinophils appear in relatively low numbers among blood cells (approximately 2×10^5/mL), this is an unlikely explanation because basophils are significantly less numerous (approximately 5×10^4/mL) but do contribute to anaphylaxis models. Most revealingly, it has been reported that eosinophils do not release PAF after IgG-dependent activation.[44] Whether eosinophils produce other potentially anaphylactogenic mediators after IgG-immune complex activation has not been investigated, but the lack of such an effect appears the most reasonable hypothesis to explain why eosinophils have not been found to contribute to IgG-induced anaphylaxis.

We investigated the contribution of neutrophils and monocytes to IgG-induced PSA models by using depletion approaches. $Ly6G^+$ cell depletion with NIMP-R14 resulted in an efficient depletion of neutrophils in the blood and spleen (see Figs E1, *B*, and E2, *B*). The same treatment resulted only in partial depletion in the bone marrow, in which a proportion of $Ly6G^+$ cells are masked from fluorescent anti-Ly6G staining but not depleted by NIMP-R14 treatment (refer to bone marrow panels in Figs E1, *C* and *D*, and E2, *C*, *D*, and *I*). Importantly, we found that NIMP-R14 depletion has a significant effect on monocyte populations in the blood and, to some extent, in the spleen. This should be taken into consideration when interpreting the results of NIMP-R14 depletion experiments. All IgG-induced PSA models were ameliorated after NIMP-R14 depletion but also when monocytes/macrophages were targeted by using clodronate liposomes. Intravenous injection of clodronate liposomes resulted in a significant depletion of monocytes from the blood and monocytes/macrophages from the spleen and bone marrow but not from the skin (data not shown) and peritoneum (see Figs E1 and E2, as previously reported[26]) and to a significant increase in blood leukocyte counts, particularly neutrophils (see Figs E1 and E2). Thus the anti-Ly6G and clodronate liposome treatments alter also monocytes and the neutrophil compartment, respectively, but reduce hypothermia in the 3 models of IgG-induced PSA studied. Constitutive deficiency in neutrophils, as studied with $Gfi1^{-/-}$ mice, confirmed the role of neutrophils in IgG_{2a}- and IgG_{2b}-induced PSA models. Therefore both neutrophils and monocytes can be considered to contribute to IgG-induced anaphylaxis in mice, whether dependent on IgG_1, IgG_{2a}, or IgG_{2b}. The role of macrophages in the different IgG-induced PSA models remains to be investigated more deeply because clodronate liposomes injected intravenously efficiently targeted macrophages in the spleen but not in other tissues, such as the peritoneum or skin, and this does not allow conclusions on their contribution.

The contribution of basophils to models of anaphylaxis has been a recent matter of debate. Tsujimura et al[20] reported that depletion of basophils with anti-CD200R3 (clone Ba103) mAbs strongly inhibited IgG_1-induced PSA and rescued mast cell–deficient mice from active anaphylaxis. However, Ohnmacht et al[45] found that basophil-deficient $Mcpt8^{cre}$ mice demonstrated slightly decreased but significant hypothermia in response to IgG_1-induced PSA (induced with the same antibody clone) when compared with WT mice. More recently, Reber et al[36] reported that peanut-induced anaphylaxis was reduced after diphtheria toxin injection in $Mcpt8^{DTR}$ mice, which selectively depletes basophils, and confirmed that basophil depletion with anti-CD200R3 mAbs inhibited anaphylaxis. Moreover, Khodoun et al[5] found a contribution of basophils to anaphylaxis-related mortality but not to hypothermia in a model of IgG_{2a}-induced PSA after anti-CD200R3 mAb injection. Therefore it appears that differences between inducible basophil depletion with specific antibodies or toxin administration and a constitutive lack of basophils, possibly leading to compensatory mechanisms during development of these mice, might account for the divergent results observed. However, intriguingly, basophils have been reported to be resistant to IgG-immune complex triggering *ex vivo* because of dominant inhibition by FcγRIIB over activation by FcγRIII.[21]

In this study we report that both basophil depletion after anti-CD200R3 mAb (Ba103) injection or constitutive deficiency of basophils and mast cells in Cpa3-Cre; $Mcl\text{-}1^{fl/fl}$ mice inhibits IgG_{2b}-induced PSA but not IgG_{2a}-induced PSA, confirming a role for basophils (and potentially mast cells) to specific IgG-induced PSA models. Of note, Ba103 efficiently depleted

basophils from the blood and partially from the spleen and the bone marrow but had no significant effect on mast cells in the peritoneum or skin (see Figs E1, *A* and *E*, and E2, *A* and *E*). The difference in the ability of basophils to respond to IgG-immune complex triggering *in vitro* and the various *in vivo* models might be explained by functional alterations during basophil purification or a requirement for costimulation by other cells or their products that are present *in vivo*, but not *ex vivo*, for basophils to respond to IgG-immune complexes.

Our results with Cpa3-Cre; Mcl-$1^{fl/fl}$ mice indicate that mast cells were not necessary for IgG_{2a}-induced PSA. We could not formally define their role in IgG_{2b}-induced PSA because basophil depletion and deficiency in basophils and mast cells lead to similar reduction in IgG_{2b}-induced PSA. Notably, increased plasma histamine levels, but no increase in mMCP-1 levels, could be detected, suggesting that mucosal mast cells were not activated during IgG-induced PSA. Intriguingly, however, some dermal mast cells displayed a degranulated morphology 30 minutes after challenge in all IgG-induced PSA models tested (for examples see Fig E7 in this article's Online Repository at www.jacionline.org). However, whether their degranulation is a cause or a consequence of anaphylaxis remains elusive.[17]

The ability of cells expressing activating FcγRs to respond to IgG-immune complexes has been proposed to be regulated by coexpression of FcγRIIB.[46] FcγRIIB$^{-/-}$ mice experience increased hypersensitivity and anaphylactic reactions to IgG_1-induced PSA (as seen in this report).[18,19] Our results further demonstrate that FcγRIIB inhibits IgG_{2b}-induced PSA but not IgG_{2a}-induced PSA. This latter finding is supported by results from Khodoun et al,[5] who proposed that the lack of this inhibitory receptor can lead to increased spontaneous formation of immune complexes in FcγRIIB$^{-/-}$ mice, which could compete with IgG_{2a}-immune complexes. In light of our results comparing IgG_1-, IgG_{2a}-, and IgG_{2b}-induced PSA, we propose that the significantly lower affinity of inhibitory FcγRIIB for IgG_{2a} (K_A, 4.2×10^5 M^{-1}) than for IgG_1 (K_A, 3.3×10^6 M^{-1}) and IgG_{2b} (K_A, 2.2×10^6 M^{-1}) is the determining factor (Table I). Another factor might be the variance in expression of FcγRIIB on circulating myeloid cells as follows: basophils > monocytes > eosinophils >> neutrophils.[21] Although the exact numbers of expressed activating FcγRIII and inhibitory FcγRIIB per cell remain unknown, flow cytometric analysis allowed the estimation of their relative expression: indeed, the FcγRIII/FcγRIIB ratio is higher on neutrophils than on monocytes and basophils. Thus these differential expression levels might explain why neutrophils contribute to anaphylaxis because the receptor balance is in favor of the activating receptor. Strikingly, FcγRIIB is coexpressed only with FcγRIII on basophils and $Ly6C^{hi}$ monocytes, whereas it is coexpressed with FcγRIII and FcγRIV on neutrophils and $Ly6C^{lo}$ monocytes.[34] Therefore contribution of a given cell type to anaphylaxis might be favored when inhibitory FcγRIIB is required to dampen the stimulatory potential of 2 activating IgG receptors instead of 1. This concept extends to IgG_1-immune complexes that only engage one activating receptor, FcγRIII.

Our results on the contribution of mouse IgG receptors, cells, and mediators in the setting of IgG-induced anaphylaxis can potentially be translated to human IgG-dependent anaphylaxis (eg, after intravenous IgG or therapeutic IgG antibody administration). Indeed, even though IgG receptors are different in the 2 species, we have already reported that human FcγRI and human FcγRIIA can induce anaphylaxis when expressed under the control of their own promoter in transgenic mice.[23,24] Human FcγRI (CD64) is the equivalent of mouse FcγRI, whereas human FcγRIIA (CD32A) can be regarded as the equivalent of mouse FcγRIII, and human FcγRIIIA (CD16A) is the equivalent of mouse FcγRIV.[7] Human FcγRIIA, like mouse FcγRIII, is expressed on all myeloid cells and could therefore act as the principal IgG receptor responsible for anaphylaxis in human subjects. Human FcγRIIB, the equivalent of mouse FcγRIIB, is scarcely expressed on most circulating myeloid cells,[47] except for its high expression on basophils,[21] suggesting that among myeloid cells, only human basophils are highly sensitive to human FcγRIIB-mediated inhibition. In contrast to mouse FcγRI, human FcγRI is constitutively expressed on circulating monocytes and inducibly on neutrophils, allowing this receptor to induce anaphylaxis.[24] The binding of human IgG subclasses to human FcγRs differs strikingly from the binding of mouse IgG subclasses to mouse FcγRs. Noticeably, the affinity of human FcγRIIB for any human IgG subclass is the lowest among human IgG–human FcγR interactions. For example, human IgG_1, the equivalent of mouse IgG_{2a}, is bound by all activating human FcγRs (K_A, $>10^6$ M^{-1}) with at least a 10-fold higher affinity than by inhibitory human FcγRIIB (K_A, 10^5 M^{-1}).[48] If we consider the translation of our results obtained in the mouse to human IgG-induced anaphylaxis, one could anticipate that human FcγRIIB-mediated inhibition of IgG-induced anaphylaxis is inefficient in human neutrophils and monocytes and efficient only in human basophils for which increased human FcγRIIB expression might compensate for the low-affinity version of this receptor for human IgG subclasses. Certainly, FcγR engagement by IgG immune complexes on human basophils could not trigger any detectable basophil activation *in vitro*,[21] which is similar to the results we reported for mouse basophil activation. Altogether, our data propose that the differential expression of inhibitory FcγRIIB on myeloid cells and its differential binding of IgG subclasses control the contribution of basophils, neutrophils, and monocytes to IgG-dependent anaphylaxis, thus revealing novel complexities in the mechanism of regulation of cell populations and therefore their contribution to IgG-induced reactions *in vivo*.

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Clinical implications: Anaphylactic pathways induced by different IgG subclasses in mice vary in terms of contributions by different cell types, mediators, and antibody receptors. These results might help in the design of efforts to understand and treat IgG-induced anaphylaxis in human subjects, such as those seen after intravenous IgG or administration of therapeutic IgG antibodies.

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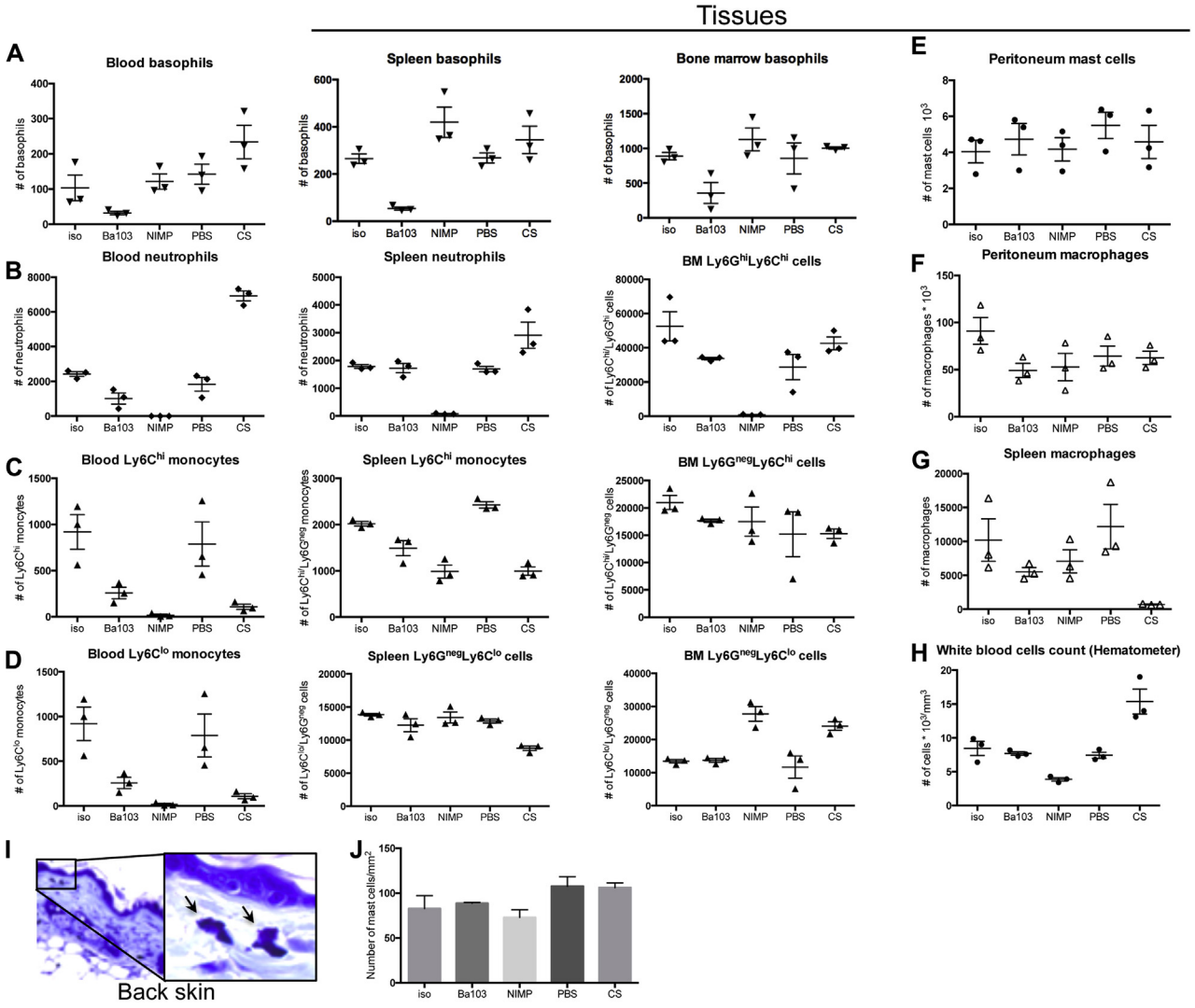


FIG E1. Effects of depletion strategies on myeloid cell populations: cell counts. WT mice were treated with indicated reagents. Twenty-four hours after injection, counts of specific cell populations were determined by means of flow cytometry **(A-G)** or histology (**I** and **J**), and leukocyte counts in total blood were measured with an automatic blood analyzer **(H)**: counts of basophils (Fig E1, *A*), neutrophils (Fig E1, *B*), Ly6C^{hi} monocytes (Fig E1, *C*), and Ly6C^{lo} monocytes (Fig E1, *D*) in blood, spleen, and bone marrow; peritoneal mast cells (Fig E1, *E*); peritoneal macrophages (Fig E1, *F*); and splenic macrophages (Fig E1, *G*). **I,** Representation of a toluidine blue–stained back skin section with 2 mast cells *(arrows)*. **J,** Counts of mast cells per square millimeter in the dermis of WT mice. Fig E1, *A-H*, show 1 of 3 independent experiments. Individual measurements and means ± SEMs are represented. *Ba103*, Anti-CD200R3 mAb; *CS*, clodronate liposomes; *Iso*, isotype rat IgG$_{2b}$; *NIMP*, anti-Ly6G mAb; *PBS*, PBS liposomes.

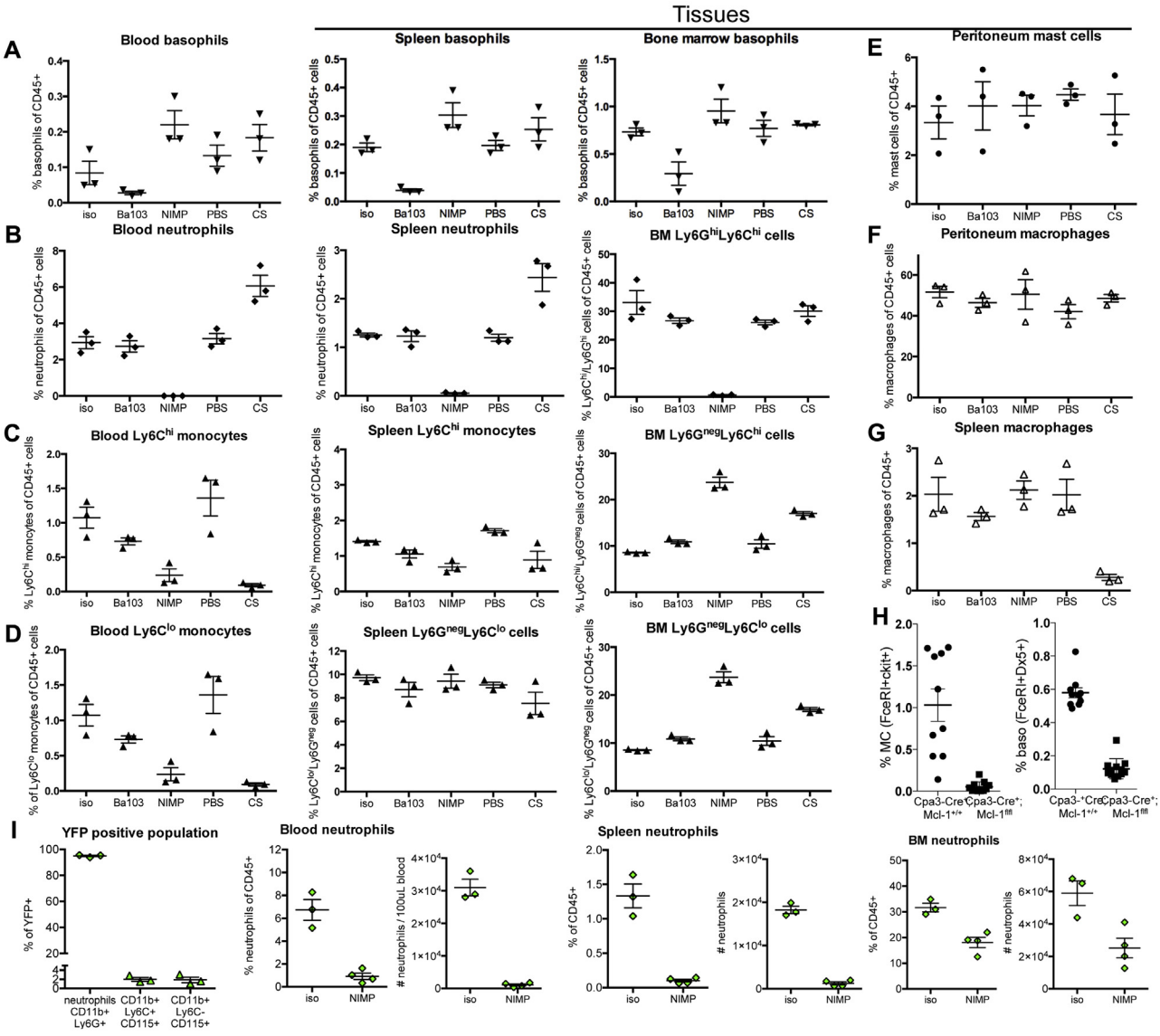
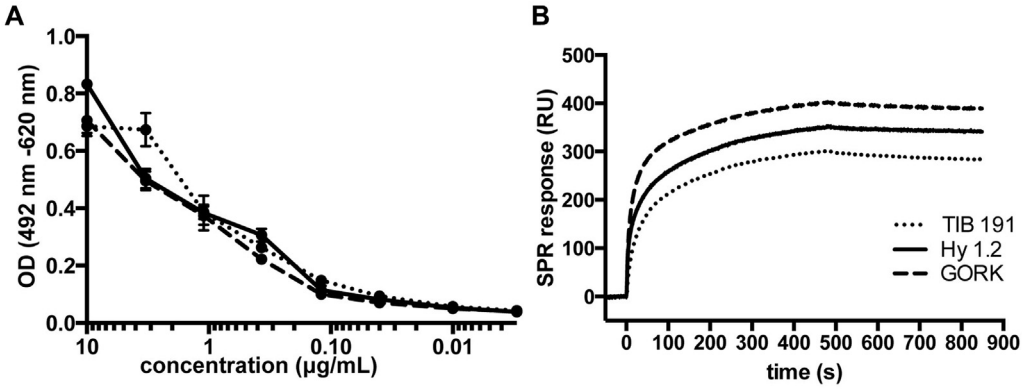


FIG E2. Effects of depletion strategies on myeloid cell populations: frequencies. WT mice were treated with indicated reagents. **A-G,** Twenty-four hours after injection, percentages of specific cell populations among $CD45^+$ cells were determined by means of flow cytometry: basophils (Fig E2, *A*), neutrophils (Fig E2, *B*), $Ly6C^{hi}$ monocytes (Fig E2, *C*), and $Ly6C^{lo}$ monocytes (Fig E2, *D*) in blood, spleen, and bone marrow; peritoneal mast cells (Fig E2, *E*); peritoneal macrophages (Fig E2, *F*); and splenic macrophages (Fig E2, *G*). Fig E2, *H*, Percentages of peritoneal mast cells (pMC $Fc\varepsilon RI^+$/c-Kit^+) and blood basophils ($Fc\varepsilon RI^+$/$CD49b^+$) in Cpa3-Cre; Mcl-$1^{fl/fl}$ and Cpa3-Cre; Mcl-$1^{+/+}$ mice. **I,** *Left*, Percentages of YFP-positive cells in MRP8-Cre; Rosa26-YFP mice. *Right*, Effect of NIMP-R14 injection on neutrophils (percentages and counts of $CD45^+$/YFP^+/$Ly6C^{neg}$/$CD115^{neg}$ cells) in blood, spleen, and bone marrow of MRP8-Cre; Rosa26-YFP mice. Fig E2, *A-H*, show corresponding percentages to cell counts shown in Fig E1 and display values for individually measured mice with means and SEMs. *Ba103*, Anti-CD200R3 mAb; *CS*, Clodronate liposomes; *Iso*, isotype rat IgG_{2b}, *NIMP*, anti-Ly6G mAb; *PBS*, PBS liposomes.



C

	k_{on} ($10^5 M^{-1}s^{-1}$)	k_{off} (10^{-4} s^{-1})	K_D (nM)
TIB 191 (IgG1)	0.97 (± 0.29)	2.27 (± 0.32)	2.34 (± 0.33)
Hy1.2 (IgG2a)	1.43 (± 0.43)	1.08 (± 0.30)	0.76 (± 0.31)
GORK (IgG2b)	2.15 (± 0.65)	1.19 (± 0.24)	0.55 (± 0.20)

FIG E3. Relative affinity of IgG_1 (TIB191), IgG_{2a} (Hy1.2), and IgG_{2b} (GORK) anti-TNP to TNP-BSA. **A,** ELISA anti-TNP. Comparison of binding capacity of TIB191, Hy1.2, or GORK to immobilized TNP-BSA. Data are presented as means ± SEMs and representative of results from 5 independent experiments. **B,** Surface plasmon resonance analysis. Comparison of binding affinity TNP-BSA to immobilized TIB191, Hy1.2 or GORK clones. **C,** The table shows the k_{on}, k_{off}, and K_D values for each condition.

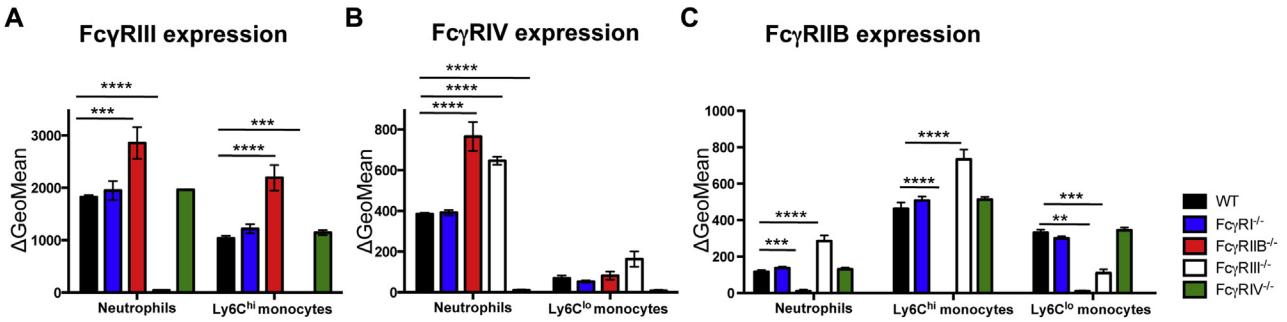


FIG E4. FcγR expression in FcγR-deficient mice. Expression of FcγRIII **(A)**, FcγRIV **(B)**, and FcγRIIB **(C)** is represented as the Δ geometric mean *(GeoMean)* of FcγR-specific staining compared with isotype control staining from blood leukocytes collected from untreated WT, FcγRI$^{-/-}$, FcγRIIB$^{-/-}$, FcγRIII$^{-/-}$, and FcγRIV$^{-/-}$ mice (n = 4 per group). Data are represented as means ± SEMs. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

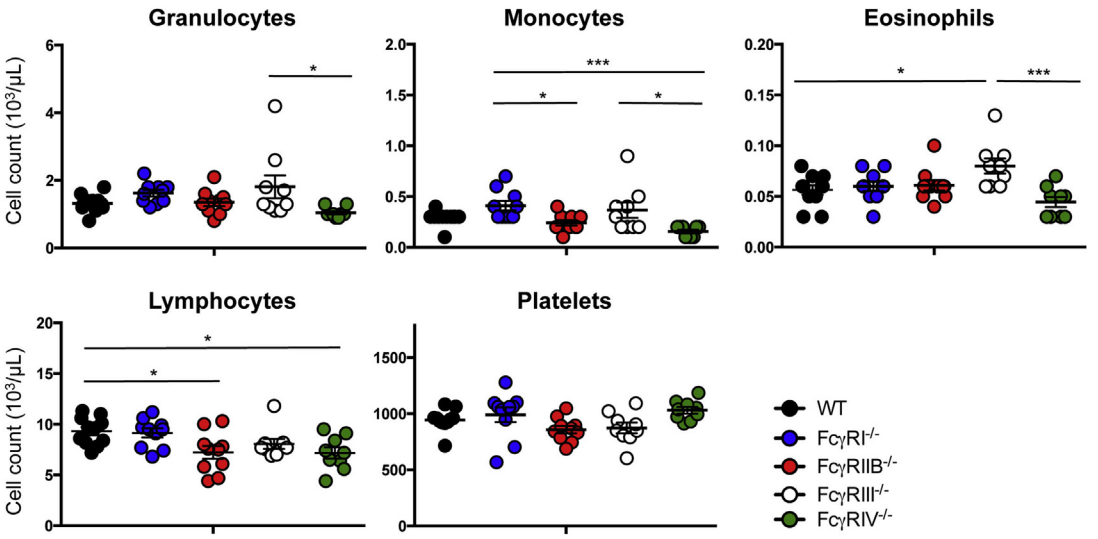


FIG E5. Blood leukocyte numbers in FcγR-deficient mice. Leukocyte populations were assessed by using an ABC Vet automatic blood analyzer (Horiba ABX, Irvine, Calif) from blood collected from untreated WT, FcγRI$^{-/-}$, FcγRIIB$^{-/-}$, FcγRIII$^{-/-}$, and FcγRIV$^{-/-}$ mice (n = 4 per group). Granulocytes represent mainly neutrophils (as judged by their size and granularity). Data are represented as means ± SEMs, and each point represents 1 mouse. *GeoMean*, Geometric mean. **P* < .05 and ****P* < .001.

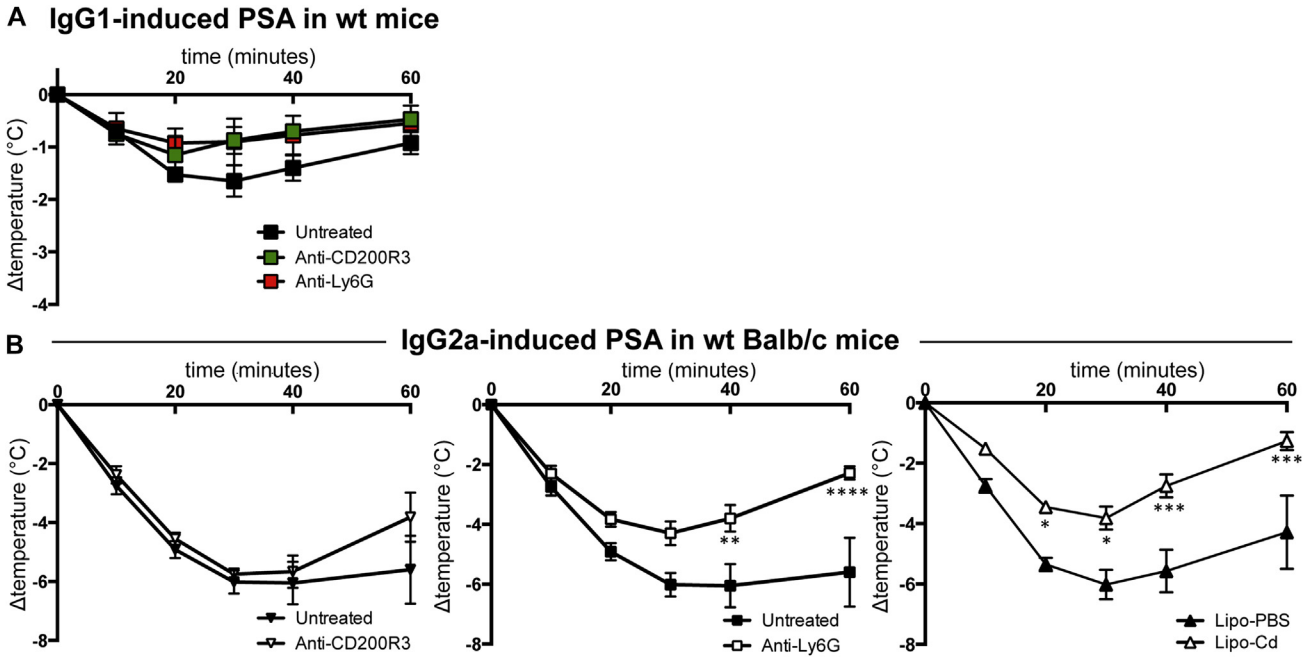


FIG E6. IgG_1-induced PSA induces mild hypothermia in WT mice, and monocytes/macrophages and neutrophils contribute to IgG_{2a}-induced PSA in BALB/c mice. **A,** WT mice were injected with IgG_1 anti-TNP mAbs and challenged with TNP-BSA, and body temperatures were monitored. PSA in mice left untreated or injected with anti-Ly6G or anti-CD200R3 (n = 4 per group) is shown. **B,** BALB/c mice were left untreated or injected with anti-Ly6G, anti-CD200R3 (n = 6 per group), lipo-PBS (n = 6 per group), or lipo-Cd (n = 6 per group) before IgG_{2a}-induced PSA induction. Body temperatures were monitored. Data are represented as means ± SEMs. Data are pooled from 2 independent experiments. Significant differences compared with the untreated group are indicated. **P* <.05, ***P* <.01,****P* <.001, and *****P* <.0001.

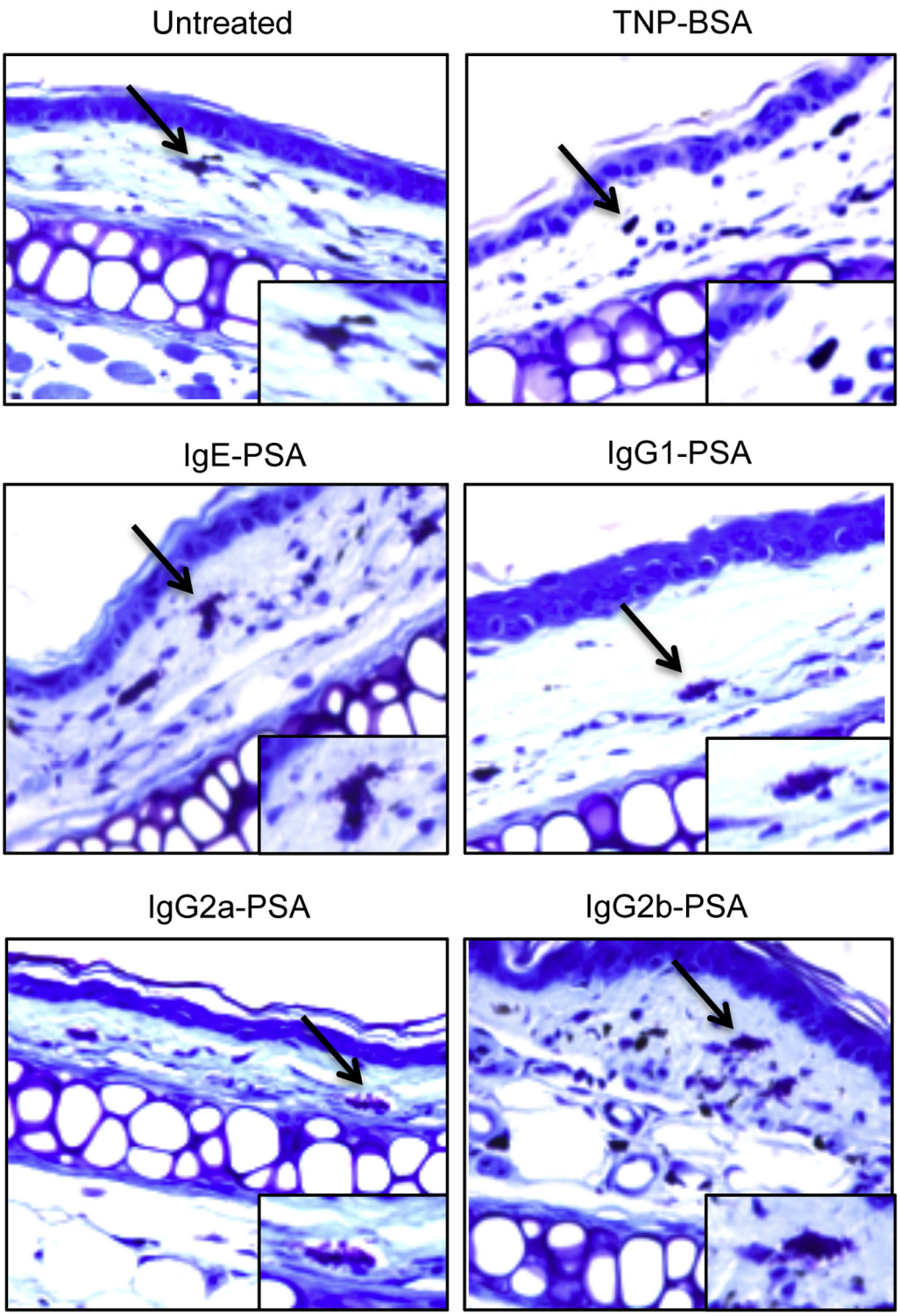


FIG E7. Examples of mast cell degranulation after IgG_1-, IgG_{2a}-, and IgG_{2b}-induced PSA. WT mice were injected with IgE, IgG_1, IgG_{2a}, and IgG_{2b} anti-TNP mAbs or left untreated (n = 3 for all groups) and challenged with TNP-BSA. Mouse ear skin biopsy specimens were collected 30 minutes after TNP-BSA injection. A representation of a toluidine blue–stained ear skin section with 1 mast cell (indicated by an *arrow*) for 1 mouse of each group of mice is shown.