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IgG subclasses determine pathways of anaphylaxis in mice

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ABSTRACT

Background: Animal models have demonstrated that allergen-specific IgG confers sensitivity to systemic anaphylaxis that relies on IgG receptors (FcγRs). Mouse IgG2a and IgG2b bind activating FcγRI, FcγRIII and FcγRIV, and inhibitory FcγRIIB; mouse IgG1 binds only FcγRIII and FcγRIIB. Although these interactions are of strikingly different affinities, these three IgG subclasses have been shown to enable induction of systemic anaphylaxis.

Objective: Determine which pathways control the induction of IgG1-, IgG2a- and IgG2b-passive systemic anaphylaxis.

Methods: Mice were sensitized with IgG1, IgG2a or IgG2b anti-TNP mAbs and challenged with TNP-BSA intravenously to induce systemic anaphylaxis that was monitored using rectal temperature. Anaphylaxis was evaluated in mice deficient for FcγRs, injected with mediator antagonists or in which basophils, monocyte/macrophages or neutrophils had been depleted. The expression of FcγRs was evaluated on these cells before and after anaphylaxis.

Results: Activating FcγRIII is the receptor primarily responsible for all three models of anaphylaxis, and subsequent down regulation of this receptor was observed. These models differentially relied on histamine release and on the contribution of mast cells, basophils, macrophages and neutrophils. Strikingly, basophil contribution and histamine predominance in IgG1- and IgG2b-mediated 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-mediated reactions in vivo.
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 may help in the design of efforts to understand and treat IgG-mediated anaphylaxis in humans, e.g., as seen following intravenous IgG or administration of therapeutic IgG antibodies.

CAPSULE SUMMARY

Antibodies of the IgG class can contribute to anaphylaxis. This report reveals pathways induced by each IgG subclass in experimental anaphylaxis, demonstrating varying contributions of cells, mediators and antibody receptors.

KEY WORDS

Anaphylaxis; IgG; mouse model; basophil; neutrophil; monocyte; macrophage; FcγR; Platelet-activating Factor; Histamine.
ABBREVIATIONS USED

FcγR: IgG Fc receptor

PAF: Platelet-activating factor

Kₐ: Affinity constant

WT: C57Bl/6 Wild-type

PSA: Passive systemic anaphylaxis

TNP: Trinitrophenyl

BSA: Bovine serum albumin

mAb: Monoclonal antibody

PBS: Phosphate Buffered Saline

Gfi1: Growth Factor Independence-1

GeoMean: Geometric Mean

SEM: Standard error of the mean
INTRODUCTION

Anaphylaxis is a hyperacute allergic reaction that occurs with increasing incidence in the population and can be of fatal consequence. Symptoms include skin rashes, hypotension, hypothermia, abdominal pain, bronchospasm and heart and lung failure that may lead to asphyxia and sometimes death\(^1\). The main treatment remains epinephrine (adrenaline) injection to restore heart and lung function. Since 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 the release of mediators. These mediators can, by themselves, recapitulate the symptoms of anaphylaxis as observed in humans\(^2,3\).

The “classical” mechanism of anaphylaxis states that allergen-specific IgE binds the activating IgE receptor FceRI on mast cells, which upon allergen encounter become activated and release histamine, among other mediators. Notably, histamine injection suffices to induce the signs of anaphylaxis in animal models\(^4\). In many cases, detectable allergen-specific IgE and elevated histamine levels do not accompany anaphylaxis in humans (discussed in \(^5\)), 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 receptors (FcγRs) expressed on myeloid cells (\emph{i.e.} macrophages, basophils and/or neutrophils), which in turn release Platelet-Activating Factor (PAF)\(^2,3\). Importantly, PAF injection suffices to induce the 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 four “classical” IgG receptors termed FcγRs, but also the neonatal IgG receptor (FcRn) and the intracellular FcR tripartite motif-containing protein 21 (TRIM21)\(^7,\)\(^8\). Whereas 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 i) activating FcγRs, \(i.e.\) FcγRI, FcγRII and FcγRIIIB, that lead to cell activation upon immune complex binding, and ii) an inhibitory FcγR, \(i.e.\) FcγRIIB, that inhibits cell activation when co-engaged by an immune complex with an activating FcγR co-expressed on the same cell\(^10\). Inhibition of cell activation by FcγRIIB thus requires that the immune complex contains IgG that are bound both by the activating and by the inhibitory FcγR.

Four IgG subclasses exist in mice, IgG1, IgG2a, IgG2b and IgG3. Among those, only IgG2a and IgG2b bind to all FcγRs, whereas IgG1 binds only to FcγRIIB and FcγRIII. It remains under debate whether IgG3 binds to FcγRs, particularly FcγRI\(^11,\)\(^12\). The affinities of these FcγRs towards IgG subclasses are strikingly different (Table 1) leading to the notion of “high-affinity” receptors that retain monomeric IgG and “low-affinity” receptors that do not\(^8\). The avidity of IgG-immune complexes, however, enables both types of receptors to retain IgG-immune complexes, leading to receptor clustering, intracellular signaling events and, eventually, to cell activation. FcγRI is a high-affinity receptor for IgG2a\(^13\), and FcγRIV is a high-affinity receptor for IgG2a and IgG2b\(^14\). All other FcγR-IgG interactions are of low affinity (reviewed in \(^7\)).

Three out of the four IgG subclasses in the mouse, \(i.e.\) IgG1, IgG2a and IgG2b, have been reported to enable the induction of systemic anaphylaxis, inducing mild to severe hypothermia\(^5\), \(^15,\)\(^16\). This is rather surprising for IgG1, considering that inhibitory FcγRIIB binds IgG1 with a
10-fold higher affinity ($K_A=3.3\times10^6\text{ M}^{-1}$) than activating FcγRIII ($K_A=3.1\times10^5\text{ M}^{-1}$)\(^{17}\) (Table 1), implying that inhibition should dominate over activation. WT mice, indeed, develop a very mild anaphylactic reaction during IgG1-PSA compared to FcγRIIB\(^{-/-}\) mice\(^{18}\), indicating that inhibition by FcγRIIB occurs in WT mice during IgG1-PSA, reducing, but not protecting from, anaphylaxis. IgG1-PSA has been reported to rely on basophils\(^{19}\) that co-express FcγRIIB and FcγRIII\(^{20}\). In this apparently simple situation, only one activating receptor and one inhibitory receptor are engaged on a single cell type that, once activated, produces an anaphylactogenic mediator, like PAF\(^{19}\).

IgG2a and IgG2b, however, bind three activating FcγRs and inhibitory FcγRIIB with different affinities ranging over 2 logs. In particular, the affinity of FcγRIIB for IgG2a is significantly lower than for IgG2b, whereas activating IgG receptors FcγRIII and FcγRIV bind IgG2a and IgG2b with similar affinities, respectively (Table 1). Notably, FcγRIV is not expressed on basophils, but on monocytes/macrophages and neutrophils\(^{21}\) that have both been reported to contribute to experimental anaphylaxis\(^{16, 22-24}\). In addition, mice expressing only FcγRIV can develop IgG-PSA\(^{16}\). Together with expression and binding data, one would therefore hypothesize that FcγRIV contributes predominantly to IgG2a- and IgG2b-PSA. In this work, we present evidence contrary to this hypothesis, and reveal which activating FcγR on which cell type(s) releasing which mediator(s) are responsible for IgG2a-PSA and IgG2b-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, altogether, 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”) were purchased from Charles River, female Balb/cJRj mice from Janvier Labs, FcγRIIB−/− (MGI:1857166), FcγRIII−/− mice (MGI: 3620982) and Rosa26-YFP mice from Jackson Laboratories. FcγRI−/− mice (MGI: 3664782) were provided by J. Leusen (University Medical Center, Utrecht, The Netherlands), FcγRIII−/− mice (MGI: 5428684) by J.V. Ravetch (The Rockefeller University, New York, NY, USA), Gfi1−/− mice by T. Moroy (Montreal University, Montreal, QC, Canada) and MRP8-cre mice by Clifford Lowell (University of California at San Francisco, CA, USA). MRP8-cre and Rosa26-YFP mice were intercrossed to generate MRP8-cre; Rosa26-YFP mice. Cpa3-Cre; Mcl-1fl/fl mice (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 the Institutional Animal Care and Use Committee of Stanford University.

Antibodies and reagents. PBS- and clodronate-liposomes were prepared as previously described26. TNP21-31-BSA was obtained from Santa Cruz, ABT-491 from Sigma-Aldrich; cetirizine DiHCl from Selleck Chemicals; anti-mouse FcγRIII (275003) from R&D Systems; rat IgG2b isotype control (LTF-2) from Bio X Cell. Purified anti-CD200R3 (Ba103) was provided by H. Karasuyama (Tokyo Medical and Dental University Graduate School, Tokyo, Japan). The hybridoma producing mAbs anti-mouse FcγRIV (9E9) was provided by J.V. Ravetch (Rockefeller University, New York, New York, USA), anti-Ly6G (NIMP-R14) by C. Leclerc (Institut Pasteur, Paris, France), IgG1 anti-TNP (TIB-191) by D. Voehringer (Universitätsklinikum, Erlangen, Germany), IgG2a anti-TNP (Hy1.2) by Shozo Izui (University of Geneva, Geneva, Switzerland) and IgG2b anti-TNP (GORK) by B. Heyman (Uppsala...
Universitet, Uppsala, Sweden): corresponding antibodies were purified as described\textsuperscript{16}. Purified mouse IgE anti-TNP was purchased from BD Pharmingen. MAb 9E9 was coupled to FITC using the Pierce\textsuperscript{TM} FITC Antibody labeling kit (Life Technologies). The 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. Fc\textgamma RIIB was detected using FITC-coupled mAb AT130-2 mIgG1 N297A\textsuperscript{27}.

**Passive Systemic Anaphylaxis.** *IgG-inducted PSA:* IgG1, IgG2a or IgG2b anti-TNP antibodies were administered intravenously at a dose of 500 \( \mu \)g, if not otherwise indicated, in 200 \( \mu \)L physiological saline, followed by an intravenous challenge with 200 \( \mu \)g of the antigen (TNP-BSA) in physiological saline 16 hours later. *IgE-inducted PSA:* IgE anti-TNP antibodies were administered intravenously at a dose of 50 \( \mu \)g in 200 \( \mu \)L physiological saline followed by an intravenous challenge with 500 \( \mu \)g of TNP-BSA in physiological saline 24 hours later. The body temperature of mice was monitored using a digital thermometer with rectal probe (YSI).

**In vivo blocking and cellular depletion.** 300 \( \mu \)g/mouse of PBS- or clodronate-liposomes, 300 \( \mu \)g/mouse of rat IgG2b isotype control or anti-Ly6G, and 30 \( \mu \)g/mouse of anti-CD200R3 mAbs were injected i.v. 24 hours before challenge. Specificity of cell depletion was evaluated using flow cytometry on blood, bone marrow, spleen and peritoneum taken from naïve WT mice 24 hours after injection of the depleting antibody or clodronate-liposomes (Examples are shown in Supplemental Figures 1 & 2). 25 \( \mu \)g/mouse of ABT-491 or 300 \( \mu \)g/mouse of cetirizine were
injected intravenously 20 minutes or intraperitoneally 30 minutes before challenge, respectively. 200 µg/mouse of anti-FcγRIV mAb were injected intravenously 30 minutes before challenge.

**Flow cytometry 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^{hi}), monocytes (CD45+/CD11b+/Ly6G^{lo}/Ly6C^{lo} or ^{hi}), basophils (CD45^{int}/DX5+/IgE^{+}); spleen macrophages (CD45^{+}/CD11b^{+}/Gr-1^{lo}/CD115^{+}/F4/80^{hi}); peritoneal macrophages (CD45^{+}/CD11b^{+}/F4/80^{+}); peritoneal mast cells (CD45^{+}/c-Kit^{+}/IgE^{+}). Expression of FcγR on indicated cell population is represented as Δ Geomean between specific and isotype control staining. *NB:* In Figure 5: 1 or 0.5 mg IgG2b was injected to assess expression on neutrophils/monocytes or basophils, respectively.

**Surface plasmon resonance analysis.** Experiments were performed at 25°C using a ProteOn XPR36 real-time SPR biosensor (BioRad). 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 µl.min^{-1}, with contact and dissociation time of 8 minutes each. Binding responses were recorded in real time as resonance units (RU; 1 RU ≈ 1 pg/mm²). Background signals were subtracted, and binding rates (k_{on} and k_{off}) and equilibrium constants (K_{d}) were determined using the Biaevaluation software (GE Healthcare).

**ELISAs.** After the induction of IgG1-, IgG2a-, IgG2b- or IgE-induced PSA, plasma and serum were collected at 5 minutes and 3 hours later to determine the histamine and mMCP-1 content, respectively. Histamine and mMCP-1 concentration were determined using commercially available ELISA kits (Beckman Coulter; eBioscience) following the manufacturer’s instructions.
Relative binding affinity of IgG1, IgG2a and IgG2b anti-TNP antibodies to TNP-BSA was determined by ELISA. Briefly, TNP-BSA-coated plates were incubated with dilutions of IgG1, IgG2a or IgG2b anti-TNP antibodies. After washing, bound anti-TNP IgG were revealed using the same HRP-coupled anti-mouse IgG and SIGMAFAST OPD solution.

*Mast cell histology.* Mouse back skin biopsies were collected 24 hours after the induction of specific cell depletion and mouse ear skin biopsies were collected 30 minutes after IgE, IgG1, IgG2a or IgG2b-induced PSA, and embedded in paraffin prior to sectioning. Mast cells in toluidine blue-stained biopsies were counted visually in at least 15 FOV/mouse and > 6 mice per treatment (Supplemental Figure 1I).

*Statistics.* Data were analyzed using one-way or two-way ANOVA with Tukey’s post-test. A p-value less than .05 was considered significant: (*p < .05; **p < .01; ***p < .001; ****p < .0001). If not stated otherwise, data are represented as mean +/- SEM.
RESULTS

FcγRIII dominates anaphylaxis induced by IgG subclasses

Passive systemic anaphylaxis was induced by an intravenous injection of one of the different anti-TNP IgG isotypes (IgG1, IgG2a, IgG2b) followed by an intravenous challenge with TNP-BSA 16 h later. This protocol induces a transient decrease in body temperature that is most pronounced between 30 and 40 minutes. As reported previously, all three IgG isotypes were capable of inducing anaphylaxis in WT mice (Figure 1A-C). In these experimental conditions IgG1-PSA triggered a maximum temperature loss of ≈2°C, IgG2a-PSA of ≈4°C and IgG2b-PSA of ≈3°C in WT mice. Using single FcγR-knockout mice we evaluated the contribution of each of the four mouse FcγRs to these anaphylaxis models. The absence of either FcγRIV (with the exception of a single time point in IgG2b-PSA) or FcγRI had no significant impact on IgG-PSA-induced hypothermia, regardless of the subclass of IgG antibodies used to induce anaphylaxis (Figure 1A-C). The lack of FcγRIII, however, protected mice from anaphylaxis in all models. Mice lacking the inhibitory receptor FcγRIIB had a significantly more severe temperature drop than WT mice in both IgG1- and IgG2b-PSA, but showed no significant difference in the severity of IgG2a-PSA (Figure 1A-C). Even though the three anti-TNP IgG mAbs used are not switch variants of a unique anti-TNP antibody, they show comparable binding to TNP-BSA by ELISA, similar affinity (nanomolar range) and dissociation rates (k_{off}) by surface plasmon resonance analysis, particularly the IgG2a and IgG2b anti-TNP antibodies (Supplemental Figures 3A, B & C). Of note, untreated FcγR-deficient mice presented modest variations in FcγR expression levels (Supplemental Figure 5) and leukocyte representation among blood cells compared to WT mice (Supplemental Figure 6). In particular, a mild lymphopenia in FcγRIV^{−/−} mice and in FcγRIIB^{−/−} mice (the latter also have a tendency to express higher levels of
FcγRIII and FcγRIV); and a mild eosinophilia in FcγRIII−/− mice, that also express significantly more FcγRIIB on neutrophils and Ly6C hi monocytes. Together, we think that these variations do not explain the drastic phenotypes observed for PSA in FcγRIIB−/− and FcγRIII−/− mice compared to WT mice. Thus, these data demonstrate that FcγRIII predominates in the induction of IgG1-, IgG2a- and IgG2b-PSA, and that FcγRIIB specifically dampens anaphylaxis severity in IgG1- and IgG2b-PSA.

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

FcγRIII is expressed by all myeloid cells7, 20 and to a lesser extent by NK cells29. One may therefore anticipate that IgG immune complexes formed in vivo as a consequence of TNP-BSA injection in anti-TNP sensitized mice would therefore 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-PSA16, 19, 22, 15, however the respective contribution of each of these different cell types remains debated2–28. 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) prior to anaphylaxis induction or evaluated anaphylaxis induction in transgenic mice deficient in certain cell populations.

Of note, the relatively mild temperature loss in IgG1-PSA in WT mice (Supplemental Figure 4A), did not allow us to address reliably the contribution of either basophils or neutrophils to this model of anaphylaxis. We therefore restricted our analysis of the contribution of myeloid cell populations to IgG2a-PSA and IgG2b-PSA. Antibody-induced basophil depletion or genetically-induced mast cell and basophil deficiency (Supplemental Figure 2H, Cpa3-Cre; Mcl-
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15/16 mice did not affect IgG2a–PSA (Figure 2A&B), but significantly inhibited IgG2b-PSA (Figure 2F&G). Monocyte/macrophage depletion (Figure 2C&H) significantly inhibited both IgG2a- and IgG2b-PSA. The absence of neutrophils, either following antibody-mediated depletion (Figure 2D&I) or using neutropenic Gfi1⁻/⁻ mice (Figure 2E&J), significantly inhibited both IgG2a- and IgG2b-PSA. Whereas monocytes/macrophages and neutrophils appear to contribute to both models of anaphylaxis, basophils and possibly mast cells therefore contribute specifically to IgG2b-PSA, but not to IgG2a-PSA.

309 **FcγRIII is down-regulated specifically on neutrophils following IgG2a PSA**

Khodoun *et al* proposed to use the reduced expression level of FcγRIII on mouse neutrophils as a marker to distinguish IgE- from IgG1-induced PSA, both of which required priming with an antigen-specific IgG1 and challenge with that antigen. We therefore wondered if FcγRIII expression on neutrophils might also be a marker for IgG2a- and IgG2b-PSA. In addition, reduced expression of FcγR(s) following IgG-PSA may document that a particular cell population is activated following engagement of its FcγR(s) by IgG-immune complexes during anaphylaxis. This parameter may thus be used to discriminate cell populations contributing to anaphylaxis following direct activation by IgG-immune complexes from those contributing following activation by mediators liberated by IgG-immune complex-activated cells (e.g. histamine, PAF, leukotrienes and prostaglandins).

Among mouse IgG receptors, only FcγRIIB, FcγRIII and FcγRIV are significantly expressed on circulating myeloid cells, but not FcγRI. Of circulating monocyte populations, “classical” Ly6C⁺ monocytes are FcγRIIB⁺, FcγRIII⁺ FcγRIV⁻, whereas “non-classical” Ly6C⁻ monocytes are FcγRIIB⁺, FcγRIII⁻ FcγRIV⁺. We therefore determined the expression of FcγRIIB, FcγRIII and FcγRIV before and after IgG2a-PSA induction on neutrophils and
monocyte subsets. The expression of FcγRIII was down regulated on neutrophils, but not on Ly6C^{hi} monocytes, during IgG2a-PSA (Figure 3A&D). The expression of FcγRIV was also down regulated on neutrophils, but not on Ly6C^{lo} monocytes, during IgG2a-PSA (Figure 3B&D). This was unexpected considering that FcγRIV does not significantly contribute to this PSA model (Figure 1B). The expression of FcγRIIB, however, remained unchanged on Ly6C^{hi} and Ly6C^{lo} monocytes and neutrophils (Figure 3C&D), in agreement with the lack of contribution of this receptor to IgG2a-PSA (Figure 1B). Together these data suggest that neutrophils may directly be activated through FcγRIII by immune complexes formed during IgG2a-PSA. They also suggest that neutrophils, but not Ly6C^{lo} monocytes, may be similarly activated through FcγRIV, even if no contribution of this receptor was identified in this model using FcγRIV^{-/-} mice (Figure 1B).

**Elevated IgG2 antibody doses reveal FcγRIV contribution to IgG2a-PSA and IgG2b-PSA**

In mice, FcγRIV binds monomeric IgG2a and IgG2b. At physiological concentrations of IgG2a (≈ 2.5 mg/mL) and IgG2b (≈ 1.5 mg/mL) in the serum, FcγRIV may therefore be occupied *in vivo*, particularly on circulating neutrophils and monocytes. Nevertheless, the short binding half-lives of monomeric IgG2a (t_{1/2} ≈ 3 min) and monomeric IgG2b (t_{1/2} ≈ 10 min) by FcγRIV, and their ability to be displaced from this receptor by immune complexes,^14 may enable IgG2-immune complexes to interact with FcγRIV during anaphylaxis and therefore contribute to its induction and/or severity.

To explore this possibility, we primed FcγRIII^{-/-} mice with various doses of anti-TNP IgG2a before challenge with TNP-BSA, in order to induce a range of *in vivo* concentrations of immune complexes. As expected, the low doses did not trigger FcγRIII^{-/-} mice to develop anaphylaxis after challenge. Elevated doses (1 or 2 mg), however, enabled significant temperature drops in FcγRIII^{-/-} mice, comparable to those observed in WT mice primed with 500
µg IgG2, particularly at the highest dose of IgG2a (2 mg) (Figure 4A). Already at a dose of 1mg of IgG2, FcγRIII\(^{−/−}\) mice developed mild hypothermia in IgG2a-PSA but not in IgG2b-PSA (Figure 4B&C). Unexpectedly in the same conditions, FcγRIV contributed to IgG2b-PSA that was not anymore dampened by inhibitory FcγRIIB (Figure 4C). At a dose of 2 mg of IgG, FcγRIII\(^{−/−}\) mice developed hypothermia in both IgG2a-PSA and IgG2b-PSA that was abolished when FcγRII\(^{−/−}\) mice were pre-treated with a blocking antibody against FcγRIV (Figure 4D&E).

FcγRI did not contribute to either model of IgG2-PSA at an elevated dose (Figure 4B&C). Furthermore, the expression of FcγRIII was down regulated on neutrophils and basophils, but not on Ly6C\(^{hi}\) monocytes, following IgG2b-PSA (Figure 5A&D). The expression of FcγRIV was also down regulated on neutrophils, but not on Ly6C\(^{lo}\) monocytes (Figure 5B&D). The expression of FcγRIIB, however, did not change on either neutrophils or Ly6C\(^{hi}\) and Ly6C\(^{lo}\) monocytes even though this inhibitory receptor regulates IgG2b-PSA (Figures 1C and 5C&D).

This observation is in agreement with the report by Khodoun et al, reporting that FcγRIIB expression did not change on neutrophils following IgG1-PSA. Altogether high doses of antigen-specific IgG2 reveal the contribution of FcγRIV to IgG2a-PSA and to IgG2b-PSA, and suggest the direct activation of neutrophils and basophils by IgG2b-immune complexes.

### IgG1 PSA in the absence of inhibitory FcγRIIB

The unexpected differences observed between IgG2a- and IgG2b-PSA induction pathways prompted us to find a mouse model more sensitive to IgG1-PSA than WT mice, to be able to evaluate the contribution of cell types and mediators also in this model. Indeed, as mentioned earlier, WT mice respond poorly to IgG1-PSA (Figure 1A; Supplemental Figure 4A). FcγRIIB\(^{−/−}\) mice, however, develop a temperature drop of ≈4°C during IgG1-PSA, comparable to temperature losses observed in WT mice during IgG2a- or IgG2b-PSA (Figure
We therefore analyzed the contribution of cell types to IgG1-PSA in FcγRIIB-/- mice. Basophil depletion mildly but significantly inhibited IgG1-PSA (Figure 6A), in agreement with previous data. The depletion of neutrophils had the same effect, although not consistently as strongly as basophil depletion (Figure 6B and data not shown). Monocyte/macrophage depletion had only a tendency to ameliorate anaphylaxis that was reproducible but not significant (Figure 6C). These results suggest that IgG1-PSA relies on basophils and neutrophils, and possibly also on monocytes.

**PAF and histamine contribute differentially to IgG2a- and IgG2b-PSA**

Because cell types contribute differently to IgG2-PSA models (i.e. IgG2a-PSA, neutrophils and monocytes; IgG2b-PSA, basophils, neutrophils and monocytes), one can expect that the mediators responsible for clinical signs also may differ between them. Platelet activating factor (PAF) has been shown to be responsible for anaphylactic reactions that required basophil, neutrophil and/or monocyte/macrophage activation, whereas histamine has been shown to be responsible for mast cell- and basophil-dependent anaphylaxis. Neutrophils are the main producers of PAF, whereas mast cells and basophils are the main producers of histamine. We therefore analyzed the relative contribution of these two mediators to the three models of PSA using the histamine-receptor 1 antagonist cetirizine and the PAF-R antagonist ABT-491. Surprisingly, histamine-receptor 1 antagonist cetirizine significantly inhibited IgG1-PSA whereas PAF-R antagonist ABT-491 had no significant effect, in opposition with previous data. The combination of both antagonists had an additive effect, and almost abolished IgG1-PSA (Figure 7A). These results obtained in FcγRIIB-/- mice were confirmed in WT mice (Figure 7A). Whereas cetirizine mildly reduced hypothermia in IgG2a-PSA, it significantly inhibited IgG2b-PSA. ABT-491 mildly reduced hypothermia in IgG2a-PSA, but had no significant effect on IgG2b-PSA.
The combination of cetirizine and ABT-491, however, almost abolished both IgG2a- and IgG2b-PSA. Elevated plasma histamine levels were detected 5 minutes post challenge in all three IgG-PSA models, and particularly high levels were observed in mice undergoing IgE-PSA (as a positive control) or undergoing IgG2a-PSA (Figures 7D&E). This latter finding is surprising as IgG2a-PSA is unaffected by the absence of both mast cells and basophils that are considered major sources of histamine. Mast cell protease-1 (mMCP-1), which is released upon activation of mucosal mast cells, could be detected in the serum of mice undergoing IgE-PSA, but not in those undergoing any one of the three models of IgG-PSA, 3 hours post-PSA induction (Figure 7F). Collectively these results suggest that histamine predominantly contributes to IgG1- and IgG2b-PSA, whereas histamine and PAF, together, are necessary for IgG2a-PSA.
DISCUSSION

Our work suggests that the activating IgG receptor FcγRIII predominantly contributes to IgG-dependent passive systemic anaphylaxis, whether induced by IgG1, IgG2a or IgG2b antibodies. A contribution of the activating IgG receptor FcγRIV was only identified when using very high amounts of IgG2 antibodies, whereas the activating IgG receptor FcγRI played no detectable role. Remarkably, the inhibitory IgG receptor FcγRIIB controlled the severity of IgG1- and IgG2b-, but not IgG2a-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 IgG1-PSA, and with neutrophils and monocytes to IgG2b-PSA, but not to IgG2a-PSA that appeared to depend entirely on neutrophils and monocytes/macrophages. Altogether our data propose that the three IgG subclasses IgG1, IgG2a and IgG2b induce three qualitatively different pathways of anaphylaxis that are nevertheless triggered primarily by a single IgG receptor, FcγRIII.

FcγRIII is a low-affinity receptor for IgG1, IgG2a and IgG2b, whereas FcγRI is a high-affinity receptor for IgG2a, and FcγRIV is a high affinity receptor for IgG2a and IgG2b. One would therefore assume that FcγRIII predominates in IgG1-PSA, FcγRI and FcγRIV in IgG2a-PSA, and FcγRIV in IgG2b-PSA. However, our data from FcγRIII−/− mice indicate that this receptor predominates in all three models. Notably, we found an 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 IgG-PSA induction, an alternative model of PSA induced by sensitization and challenge with goat antibodies was found to be driven by FcγRIII22 and blocking antibodies against FcγRIII were protective in a model of PSA induced by IgG immune complexes16. In addition, IgG2a-PSA
in FcγRIIB−/− mice was abolished following injection of anti-FcγRIIB/III blocking mAbs. FcγRIII is the only activating IgG receptor in the mouse that does not bind an IgG subclass with high affinity, 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 physiological serum concentrations of IgG2a (≈ 2.5 mg/mL) and IgG2b (≈ 1.5 mg/mL), are likely occupied in vivo, particularly on circulating cells. Of note, C57Bl/6 mice produce IgG2c, but not IgG2a antibodies, whose amino acid sequence varies by about 15%. Experiments performed in Balb/c mice that express endogenous IgG2a (but no IgG2c) gave similar results regarding the contribution of basophils, neutrophils and monocytes to IgG2a (Supplemental Figure 4B), indicating that IgG2a and IgG2c 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 1.4-1.5 mL. Injection of 500 µg antibody thus corresponds to ≈330 µg/mL of circulating antibody, injection of 1 mg to ≈660 µg/mL, and injection of 2 mg to ≈13 mg/mL. In cases of anaphylaxis the circulating concentration of allergen-specific IgG has not been evaluated due to lack of testing and appropriate controls (i.e. monoclonal anti-allergen antibodies); although we have reported high circulating antigen-specific IgG levels in an autoimmune model of arthritis. It seems rather unlikely that patients suffering from anaphylaxis possess such elevated circulating levels of IgG anti-allergen as in the mice receiving the high doses we used in this study. Nevertheless, our results in high-dose IgG2a- and IgG2b-PSA demonstrate that FcγRIV can by itself (i.e. in the absence of FcγRIII) trigger anaphylaxis. Similar results have been obtained in mice expressing only FcγRIV: “FcγRIV-only” mice developed IgG2b-PSA after injection of pre-formed IgG2b immune complexes and also upon injection of polyclonal anti-sera followed by a challenge with the antigen. We reported previously that IgG2b-PSA triggered by the injection
of preformed IgG2b-immune complexes in WT mice was abolished following injection of anti-FcγRIV blocking mAb 9E9. 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-PSA, and FcγRIV contributes only at high antibody concentrations. Two hypotheses may explain these discrepant results: i) the injection of preformed IgG2b-immune complexes leads to an immediate circulating bolus of immune complexes, which are similarly formed only after injection of high amounts of IgG2b and antigen, thus triggering FcγRIV; 2) as recently reported mAb 9E9 may not only block FcγRIV through its Fab portions, but also FcγRIII via its Fc portion once 9E9 is bound to FcγRIV. In our view, it is likely that a combination of these mechanisms reconcile our previous and herein described results, and suggest that IgG2b-PSA induced following injection of preformed IgG2b-immune complexes relies rather on both FcγRIII and FcγRIV than on FcγRIV alone as we reported previously. Together this body of evidence supports the notion that FcγRIV is capable of triggering cell activation leading to anaphylaxis, yet in restricted conditions, i.e. in the absence/blockade of FcγRIII or in presence of large amounts of IgG2a and/or IgG2b antibodies.

The differential contribution of FcγRs to IgG-PSA may rely on their respective expression patterns on myeloid cells. Indeed, FcγRI is not or only barely expressed on circulating monocytes, and its expression is largely restricted to tissue-resident macrophages. The level of its expression on cells reported to contribute to anaphylaxis (i.e. monocytes in this case) may therefore not suffice to induce their activation. This notion is supported by the absence of any detectable effect of FcγRI deficiency in IgG2-PSA that we report in this study, even at high doses of IgG2 antibodies. FcγRIII, however, is expressed on all myeloid cells and moreover at comparably high levels on all those cell types that have been reported to contribute to
anaphylaxis; basophils, monocytes and neutrophils\textsuperscript{20}. This pattern of cellular expression may explain its predominant contribution to all models of IgG-induced anaphylaxis. FcγRIIV is expressed on neutrophils and Ly6C\textsuperscript{lo} monocytes. It remains unclear, however, if Ly6C\textsuperscript{lo}, Ly6C\textsuperscript{hi} or both monocyte subsets contribute to anaphylaxis. FcγRIIV could contribute to PSA induction in exceptional conditions (FcγRIII deficiency or high IgG2 antibody doses). The lack of FcγRIIV contribution in classical conditions of PSA may 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 FcγRIII\textsuperscript{−/−} mice, but not FcγRI\textsuperscript{−/−} mice, presented a significant increase in FcγRIIV expression levels on neutrophils\textsuperscript{16, 41, 42} and a tendency for increased expression on Ly6C\textsuperscript{lo} monocytes (Supplemental Figure 5B). This could explain why the contribution of FcγRIIV to IgG2-PSA becomes apparent in FcγRIII\textsuperscript{−/−} mice. FcγRIIV\textsuperscript{−/−} mice did not, conversely, present alterations of FcγRIII expression on neutrophils or Ly6C\textsuperscript{hi} monocytes compared to WT littermates (Supplemental Figure 5A). FcγRIIIB\textsuperscript{−/−} mice expressed significantly higher levels of FcγRIII and FcγRIIV on neutrophils and increased FcγRIII on Ly6C\textsuperscript{hi} monocytes that may, altogether, contribute to their higher susceptibility to anaphylaxis induction (Supplemental Figure 5A&B).

The contribution of a rather restricted subset of myeloid cells to these (and other) models of anaphylaxis\textsuperscript{2, 3} appears to be determined by at least two factors: their capacity to release anaphylactogenic mediators (e.g. 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, upon FcγR-triggering. Other mediators may induce anaphylaxis or contribute to its severity, among them lipid mediators like prostaglandins, thromboxanes and leukotrienes. Some of these have indeed been reported to trigger
bronchoconstriction and an increase in vascular permeability. The release of such mediators is sufficiently rapid to coincide with the celerity of hypothermia, which is detectable within minutes after allergen challenge. It is therefore surprising that eosinophils do not contribute to IgG-PSA, as they express high levels of activating FcγRIII and FcγRIIB (but no FcγRI or FcγRIV), and are capable of releasing Leukotriene C4, Prostaglandin E2, thromboxane and PAF upon activation. Though eosinophils represent relatively low numbers among blood cells (∼2x10⁵/mL), this is an unlikely explanation because basophils are significantly less numerous (∼5x10⁴/mL) but do contribute to anaphylaxis models. Most revealingly, it has been reported that eosinophils do not release PAF following IgG-dependent activation. Whether eosinophils produce other potentially anaphylactogenic mediators following 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-PSA models using depletion approaches. Ly6G⁺ cell depletion using NIMP-R14 resulted in an efficient depletion of neutrophils in the blood and the spleen (Supplemental Figures 1B&2B). The same treatment resulted only in a 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 Supplemental Figures 1C,D & 2C,D,I). Importantly, we found that NIMP-R14 depletion has a significant impact 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-PSA models were ameliorated following NIMP-R14 depletion, but also when monocytes/macrophages were targeted 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 BM, but not from the skin (data not shown) and
peritoneum (Supplemental Figures 1&2, as reported\textsuperscript{26}), and to a significant increase in blood
leukocyte counts and particularly of neutrophils (Supplemental Figures 1&2). Thus the anti-
Ly6G and the clodronate liposome treatments alter also the monocytes and neutrophil
compartment, respectively, but reduced hypothermia in the three models of IgG-PSA studied.
Constitutive deficiency in neutrophils, studied using Gfi1\textsuperscript{−/−} mice, confirmed the role of
neutrophils in IgG2a- and IgG2b-PSA models. Both neutrophils and monocytes can therefore be
considered to contribute to IgG-induced anaphylaxis in mice, whether dependent on IgG1, IgG2a
or IgG2b. The role of macrophages in the different IgG-PSA models remains to be investigated
more deeply, as clodronate liposomes injected intravenously efficiently targeted macrophages in
the spleen, but not in other tissues like peritoneum or skin, and thus do not allow conclusions on
their contribution.

The contribution of basophils to models of anaphylaxis has been a recent matter of
debate. Tsujimura \textit{et al} reported that depletion of basophils using anti-CD200R3 (clone Ba103)
monoclonal antibodies strongly inhibited IgG1-PSA and rescued mast cell-deficient mice from
active anaphylaxis\textsuperscript{19}. Ohnmacht \textit{et al}, however, found that basophil-deficient Mcpt8\textsuperscript{cre} mice
demonstrated slightly decreased but significant hypothermia in response to IgG1-PSA (induced
with the same antibody clone) when compared to WT mice\textsuperscript{45}. More recently, Reber \textit{et al.}
reported that peanut-induced anaphylaxis was reduced following Diphtheria toxin injection in
Mcpt8\textsuperscript{DTR} mice that selectively depletes basophils, and confirmed that basophil depletion using
anti-CD200R3 mAbs inhibited anaphylaxis\textsuperscript{36}. Moreover, Khodoun \textit{et al} found a contribution of
basophils to anaphylaxis mortality, but not to hypothermia, in a model of IgG2a-PSA following
anti-CD200R3 mAb injection\textsuperscript{5}. It therefore appears that differences between inducible basophil
depletion using specific antibodies or toxin administration and a constitutive lack of basophils,
possibly leading to compensatory mechanisms during development of these mice, may account for the divergent results observed. Intriguingly however, basophils have been reported to be resistant to IgG-immune complex triggering \textit{ex vivo} due to dominant inhibition by Fc\(\gamma\)RIIB over activation by Fc\(\gamma\)RIII\(^{20}\). In this study, we report that both basophil depletion following anti-CD200R3 mAb (Ba103) injection or constitutive deficiency of basophils and mast cells in Cpa3-Cre; Mcl-1\(^{fl/fl}\) mice inhibits IgG2b-PSA but not IgG2a-PSA, confirming a role for basophils (and potentially mast cells) to specific IgG-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 (Supplemental Figures 1A&1E and 2A&2E). The difference in the ability of basophils to respond to IgG-immune complex triggering \textit{in vitro} and the various \textit{in vivo} models may be explained by functional alterations during basophil purification or a requirement for co-stimulation by other cells or their products that are present \textit{in vivo}, but not \textit{ex vivo}, for basophils to respond to IgG-immune complexes. Our results using Cpa3-Cre; Mcl-1\(^{fl/fl}\) mice indicate that mast cells were not necessary for IgG2a-PSA. We could not formally define their role in IgG2b-PSA as basophil depletion and deficiency in basophils and mast cells lead to similar reduction in IgG2b-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-PSA. Intriguingly, however, dermal mast cells displayed a degranulated morphology 30 minutes after challenge in all IgG PSA models tested (Supplemental Figure 7). Whether their degranulation is a cause or a consequence of anaphylaxis remains however elusive.

The ability of cells expressing activating Fc\(\gamma\)Rs to respond to IgG-immune complexes has been proposed to be regulated by co-expression of Fc\(\gamma\)RIIB\(^{46}\). Fc\(\gamma\)RIIB\(^{-/-}\) mice develop increased hypersensitivity and anaphylactic reactions to IgG1-PSA (this report and\(^{16,18}\)). Our results further
demonstrate that FcγRIIB inhibits IgG2b-, but not IgG2a-PSA. This latter finding is supported by results from Khodoun et al\(^5\): these authors proposed that the lack of this inhibitory receptor may lead to increased spontaneous formation of immune complexes in FcγRIIB\(^{−\circ}\) mice, that could compete with IgG2a-immune complexes. In light of our results comparing IgG1-, IgG2a- and IgG2b-PSA, we rather propose that the significantly lower affinity of inhibitory FcγRIIB for IgG2a \((K_{A} = 4.2 \times 10^{5} \text{ M}^{-1})\) than for IgG1 \((K_{A} = 3.3 \times 10^{6} \text{ M}^{-1})\) and IgG2b \((K_{A} = 2.2 \times 10^{6} \text{ M}^{-1})\) is the determining factor (Table 1). Another factor may be the variance in expression of FcγRIIB on circulating myeloid cells: basophils > monocytes > eosinophils >> neutrophils\(^20\). Whereas 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 ratio FcγRIII/FcγRIIB is higher on neutrophils than on monocytes and basophils. These differential expression levels may thus explain why neutrophils contribute to anaphylaxis, as the receptor balance is in favor of the activating receptor. Strikingly, FcγRIIB is co-expressed only with FcγRIII on basophils and Ly6C\(^{hi}\) monocytes, whereas it is co-expressed with FcγRIII and FcγRIV on neutrophils and Ly6C\(^{lo}\) monocytes\(^34\). Contribution of a given cell type to anaphylaxis may therefore be favored when inhibitory FcγRIIB is required to dampen the stimulatory potential of two activating IgG receptors instead of one. This concept extends to IgG1-immune complexes that only engage one activating receptor, FcγRII.

Our results on the contribution of mouse IgG receptors, cells and mediators in IgG-induced anaphylaxis can potentially be translated to human IgG-mediated anaphylaxis, e.g. following intravenous IgG or therapeutic IgG antibody administration. Indeed, even though IgG receptors are different in the two species, we have already reported that human FcγRI (hFcγRI) and human FcγRIIA (hFcγRIIA) can induce anaphylaxis when expressed under the control of
their own promoter in transgenic mice\textsuperscript{23, 24}. hFcγRI (CD64) is the equivalent of mouse FcγRI whereas hFcγRIIA (CD32A) can be regarded as the equivalent of mouse FcγRIII, and hFcγRIIIA (CD16A) the equivalent of mouse FcγRIV\textsuperscript{7}. hFcγRIIA, like mouse FcγRIII, is expressed on all myeloid cells and could therefore act as the principal IgG receptor responsible for anaphylaxis in humans. hFcγRIIB, the equivalent of mouse FcγRIIB, is scarcely expressed on most circulating myeloid cells\textsuperscript{47} except for its high expression on basophils\textsuperscript{20}, suggesting that among myeloid cells only human basophils are highly sensitive to hFcγRIIB-mediated inhibition. In contrast to mouse FcγRI, hFcγRI is constitutively expressed on circulating monocytes and inducibly on neutrophils, allowing this receptor to induce anaphylaxis\textsuperscript{24}. The binding of human IgG subclasses to hFcγRs differs strikingly from the binding of mouse IgG subclasses to mouse FcγRs. Noticeably, the affinity of hFcγRIIB for any human IgG subclass is the lowest among human IgG-hFcγR interactions. For example, human IgG1, the equivalent of mouse IgG2a, is bound by all activating hFcγRs (K\textsubscript{A} > 10\textsuperscript{6} M\textsuperscript{-1}) with at least a ten-fold higher affinity than by inhibitory hFcγRIIB (K\textsubscript{A} \approx 10\textsuperscript{5} M\textsuperscript{-1})\textsuperscript{48}. If we consider the translation of our results obtained in the mouse to human IgG-induced anaphylaxis, one could anticipate that hFcγRIIB-mediated inhibition of IgG-induced anaphylaxis is inefficient in human neutrophils and monocytes, and efficient only in human basophils for which the elevated hFcγRIIB expression may compensate for the low-affinity of this receptor for human IgG subclasses. Certainly, FcγR-engagement by IgG immune complexes on human basophils could not trigger any detectable basophil activation \textit{in vitro}\textsuperscript{20}, similar to the results we reported for mouse basophil activation. Our data altogether 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-mediated reactions \textit{in vivo}. 
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AUTHORSHIP AND CONFLICT OF INTEREST STATEMENTS

H.B. performed all experiments at the Institut Pasteur with contributions from P.E, C.M.G, F.J.; R.S. and L.L.R contributed experiments using Cpa3-Cre; Mcl-10/fl mice; B.I. and
O.G. genotyped mice and produced reagents; M.S.C., S.J.G. and N.v.R. provided reagents; H.B., P.B., P.E., C.M.G., S.J.G, F.J., D.A.M., L.L.R. and R.S. analyzed and discussed results; F.J., P.B. and D.A.M. supervised and designed the research; P.B. and F.J. wrote the manuscript. All authors read and had an opportunity to contribute to the editing of the manuscript, and declare no competing financial interests.

**TABLES**

**Table 1:** Affinities of mouse FcγR-IgG subclass interactions (Kₐ values in M⁻¹)

<table>
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<tr>
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<td>FcγRIV</td>
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“-”, no detectable affinity.

“(+)”, under debate¹¹,¹².

Data compiled from¹⁷,²¹
**FIGURE LEGENDS**

**Figure 1.** FcγRIII dominates in IgG-PSA models. Mice injected with anti-TNP mAbs were challenged with TNP-BSA and body temperatures monitored. (A) IgG1-, (B) IgG2a- or (C) IgG2b-induced PSA in indicated mice (n≥3/group). Data are representative of at least two independent experiments (A: n=2; B: n=3; C: n=2). Significant differences compared to the WT group are indicated.

**Figure 2.** Basophils, mast cells, monocytes/macrophages and neutrophils contribute differentially to IgG-PSA models. Indicated mice (n≥8/group) were injected with IgG2a (A-E) or IgG2b (F-J) anti-TNP mAbs, challenged with TNP-BSA and body temperatures were monitored. WT mice (n=8/group) were pretreated as indicated (A, C-D, F, H-I). Lipo-PBS: PBS liposomes; Lipo-Cd: clodronate liposomes. Data are pooled from at least two independent experiments.

**Figure 3.** Reduced expression of FcγRIII and FcγRIV, but not FcγRIIB, on neutrophils following IgG2a-PSA. (A) FcγRIII, (B) FcγRIV and (C) FcγRIIB expression on blood cells from WT mice (A&B: n=11/group; C: n≥6/group) left untreated, injected with IgG2a anti-TNP mAbs, or injected with IgG2a anti-TNP mAbs and challenged with TNP-BSA. (D) Compilation of Δ Geomean +/- SEM data from A-C.

**Figure 4.** High doses of IgG2 antibodies reveal FcγRIV contribution to IgG2-PSA. (A) PSA in indicated mice injected with various doses of IgG2a anti-TNP mAbs (n=2/group). (B-E) PSA
in indicated mice (B&C: n=8/group; D&E: n≥3/group) injected with indicated doses of anti-TNP mAbs. Data are pooled from two independent experiments. Significant differences compared to the untreated WT group are indicated.

Figure 5. Expression of FcγRs on myeloid cells following IgG2b-PSA. (A) FcγRIII (left: n=8/group, right: n=3/group), (B) FcγRIV (n=8/group) and (C) FcγRIIB expression (n≥6/group) on cells from WT mice (n=8/group) left untreated, injected with IgG2b anti-TNP mAbs, or injected with IgG2b anti-TNP mAbs and challenged with TNP-BSA. (D) Compilation of Δ Geomean +/- SEM data from A-C.

Figure 6. Cell contributions to IgG1-PSA in the absence of inhibitory FcγRIIB. FcγRIIB−/− mice were pretreated as indicated, then injected with IgG1 anti-TNP mAbs, challenged with TNP-BSA and central temperatures were monitored (A: n=8/group; B: n=7/group; C: n=10/group). Data are represented as mean +/- SEM. Data are pooled from two independent experiments.

Figure 7. Contributions of histamine and PAF to IgG-PSA. Body temperatures of pretreated mice during (A) IgG1-PSA in FcγRIIB−/− (n=6/group) or WT mice (n=4/group), (B) IgG2a-PSA, (C) IgG2b-PSA or (D) IgE-PSA in WT mice (n≥7/group). (E) Histamine and (F) mMCP-1 concentrations post-PSA (n=3/group). Data are representative of at least two independent experiments, except for A&C (pooled from two independent experiments).
Figure 1

A) IgG1-induced PSA

B) IgG2a-induced PSA

C) IgG2b-induced PSA

Legend:
- WT
- $\text{Fc}^\gamma\text{R}^{Il-}$
- $\text{Fc}^\gamma\text{R}^{IIb-}$
- $\text{Fc}^\gamma\text{R}^{III-}$
- $\text{Fc}^\gamma\text{R}^{IV-}$
Figure 3

A. FcγRIII expression

B. FcγRIV expression

C. FcγRIIB expression

D. Summary of FcγR expression

<table>
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<td>FcγRIV expression</td>
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<td>246 ± 60</td>
<td>240 ± 26</td>
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<td>FcγRIIB expression</td>
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<tr>
<td>Neutrophils</td>
<td>133 ± 10</td>
<td>165 ± 13</td>
<td>140 ± 24</td>
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<tr>
<td>Ly6C&lt;sup&gt;hi&lt;/sup&gt; monocytes</td>
<td>691 ± 48</td>
<td>711 ± 23</td>
<td>670 ± 43</td>
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<tr>
<td>Ly6C&lt;sup&gt;lo&lt;/sup&gt; monocytes</td>
<td>134 ± 27</td>
<td>110 ± 28</td>
<td>130 ± 23</td>
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</tbody>
</table>
Figure 4

**IgG2a-induced PSA**

- A: 
  - Graph showing temperature changes over time (minutes) for different doses of IgG2a.
  - Legend shows WT and FcγRIII−/−.
  - Data points indicate different dose levels (50 µg, 100 µg, 250 µg, 500 µg, 1000 µg, 2000 µg).

- B: 
  - Graph showing temperature changes over time (minutes) for 1 mg IgG2a.
  - Legend shows different genotypes: WT, FcγRI−/−, FcγRIIB−/−, FcγRIII−/−, FcγRIV−/−.

- C: 
  - Graph showing temperature changes over time (minutes) for 1 mg IgG2b.

**IgG2b-induced PSA**

- D: 
  - Graph showing temperature changes over time (minutes) for 2 mg IgG2b.
  - Legend shows WT and FcγRIII−/−.
  - Additional data points for FcγRIII−/− + anti-FcγRIV MAbs.

- E: 
  - Graph showing temperature changes over time (minutes) for 2 mg IgG2b.

Legend:
- **:** p < 0.05
- **:** p < 0.01
- ****: p < 0.0001
Figure 5

A. FcγRIII expression

B. FcγRIV expression

C. FcγRIIB expression

D. Summary of Fcγ receptor expression

<table>
<thead>
<tr>
<th>FcγR expression</th>
<th>Untreated</th>
<th>19h post-IgG2b</th>
<th>19h post-IgG2b, 3h post Ag</th>
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<tr>
<td>Neutrophils</td>
<td>2008 ± 97</td>
<td>2158 ± 98</td>
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<td>Ly6C^hi monocytes</td>
<td>1326 ± 42</td>
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<td>BM basophils</td>
<td>2574 ± 231</td>
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<table>
<thead>
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<th>FcγR expression</th>
<th>Untreated</th>
<th>19h post-IgG2b</th>
<th>19h post-IgG2b, 3h post Ag</th>
</tr>
</thead>
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<td>Neutrophils</td>
<td>402 ± 11</td>
<td>459 ± 19</td>
<td>258 ± 9</td>
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<tr>
<td>Ly6C^lo monocytes</td>
<td>70 ± 8</td>
<td>106 ± 12</td>
<td>59 ± 14</td>
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<table>
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<tr>
<th>FcγR expression</th>
<th>Untreated</th>
<th>19h post-IgG2b</th>
<th>19h post-IgG2b, 3h post Ag</th>
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<tr>
<td>Neutrophils</td>
<td>133 ± 10</td>
<td>149 ± 12</td>
<td>130 ± 16</td>
</tr>
<tr>
<td>Ly6C^hi monocytes</td>
<td>691 ± 48</td>
<td>730 ± 41</td>
<td>759 ± 44</td>
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<tr>
<td>Ly6C^lo monocytes</td>
<td>135 ± 27</td>
<td>141 ± 35</td>
<td>105 ± 25</td>
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Figure 7

A. FcγRIIB−/- IgG1-induced PSA

B. IgG2a-induced PSA

C. IgG2b-induced PSA

D. IgE-induced PSA

E. Histamine

F. mMCP-1
Supplemental Figure 1. Effects of depletion strategies on myeloid cell populations – Cell counts. WT mice were treated with indicated reagents. 24 hours after injection, counts of specific cell populations were determined by flow cytometry (A-G) or histology (I&J); leukocyte counts in total blood were measured with an automatic blood analyzer (H). Counts of (A) basophils, (B) neutrophils, (C) Ly6C<sup>hi</sup> monocytes and (D) Ly6C<sup>lo</sup> monocytes in blood, spleen and bone marrow, (E) peritoneal mast cells (F) peritoneal macrophages and (G) splenic macrophages. (I) Representation of a toluidine blue-stained back skin section with two mast cells (arrows). (J) Counts of mast cells/mm<sup>2</sup> in the dermis of WT mice. (A-H) Figures show one of three independent experiments. Individual measurements and mean +/- SEM are represented. Iso = isotype rat IgG2b, Ba103 = anti-CD200R3 mAb, NIMP = anti-Ly6G mAb, PBS = PBS liposomes, CS= clodronate liposomes.

Supplemental Figure 2. Effects of depletion strategies on myeloid cell populations – Frequencies. WT mice were treated with indicated reagents. 24 hours after injection, percentages of specific cell populations among CD45<sup>+</sup> cells were determined by flow cytometry (A-H): (A) basophils, (B) neutrophils, (C) Ly6C<sup>hi</sup> monocytes and (D) Ly6C<sup>lo</sup> monocytes in blood, spleen and bone marrow, (E) peritoneal mast cells (F) peritoneal macrophages and (G) splenic macrophages. (H) Percentages of peritoneal mast cells (pMC FcεRI<sup>+</sup>/cKit<sup>+</sup>) and blood basophils (FcεRI<sup>+</sup>/DX5<sup>+</sup>) in Cpa3-Cre; Mcl<sup>-1<sup>fl/fl</sup></sup> mice and in Cpa3-Cre; Mcl<sup>-1<sup>+/+</sup></sup> 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 CD45<sup>+</sup>/YFP<sup>+</sup>/Ly6C<sup>neg</sup>/CD115<sup>neg</sup> cells) in blood, spleen and bone marrow of MRP8-Cre; Rosa26-YFP mice. (A-H) Figures show corresponding percentages to cell counts shown in Supplemental Figure 1 and display values for individually measured mice.
Supplemental Figure 3. Relative affinity of IgG1 (TIB191), IgG2a (Hy1.2) and IgG2b (GORK) anti-TNP to TNP-BSA. (A) ELISA anti-TNP. Comparison of binding capacity of TIB 191, Hy1.2 or GORK to immobilized TNP-BSA. Data are represented as mean +/- SEM and representative of results from five independent experiments. (B) Surface plasmon resonance analysis. Comparison of binding affinity TNP-BSA to immobilized TIB 191, Hy1.2 or GORK clones. (C) The table recapitulates the $k_{on}$, $k_{off}$ and Kd for each condition.

Supplemental Figure 4. IgG1-PSA induces mild hypothermia in WT mice and monocytes/macrophages and neutrophils contribute to IgG2a-PSA in Balb/c mice. (A) WT mice were injected with IgG1 anti-TNP mAbs, challenged with TNP-BSA and body temperatures were monitored. PSA in mice left untreated, injected with anti-Ly6G or anti-CD200R3 (n=4/group). (B) Balb/c mice were left untreated, injected with anti-Ly6G, anti-CD200R3 (n=6/group), lipo-PBS (n=6/group) or lipo-Cd (n=6/group) prior to IgG2a-PSA induction. Body temperatures were monitored. Data are represented as mean +/- SEM. Data are pooled from two independent experiments. Significant differences compared to the untreated group are indicated.

Supplemental Figure 5. FcγR expression in FcγR-deficient mice. Expression of (A) FcγRIII, (B) FcγRIV and (C) FcγRIIB is represented as the ΔGeomean of FcγR-specific staining compared to isotype control staining from blood leukocytes collected from untreated WT, FcγRI−/−, FcγRIIB−/−, FcγRIII−/− and FcγRIV−/− mice (n=4/group). Data are represented as mean +/- SEM.
Supplemental Figure 6. Blood leukocyte numbers in FcγR-deficient mice. Leukocyte populations were assessed using an ABC Vet automatic blood analyzer (Horiba ABX) from blood collected from untreated WT, FcγRI\(^{+/−}\), FcγRIIB\(^{+/−}\), FcγRIII\(^{+/−}\) and FcγRIV\(^{+/−}\) mice (n=4/group). “Granulocytes” represent mainly neutrophils (as judged by their size and granularity). Data are represented as mean +/- SEM; each point represents one mouse.

Supplemental Figure 7. Mast cell degranulation after IgG1, IgG2a and IgG2b-induced PSA. WT mice were injected with IgE, IgG1, IgG2a, IgG2b anti-TNP mAbs or left untreated (n=3 for all groups) and challenged with TNP-BSA. Mouse ear skin biopsies were collected 30 minutes after TNP-BSA injection. Representation of a toluidine blue-stained ear skin sections with one mast cell (indicated by an arrow) for one mouse of each group of mice.
Supplemental Figure 3

A

OD (492 nm - 620 nm)

B

SPR response (RU)

C

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<tr>
<th>mAb</th>
<th>$k_{on} \times 10^5$ M$^{-1}$s$^{-1}$</th>
<th>$k_{off}$ s$^{-1}$</th>
<th>Kd (nM)</th>
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<tr>
<td>TIB 191 (IgG1)</td>
<td>0.97 (± 0.29)</td>
<td>2.27 (± 0.32)</td>
<td>2.34 (± 0.33)</td>
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<tr>
<td>Hy1.2 (IgG2a)</td>
<td>1.43 (± 0.43)</td>
<td>1.08 (± 0.30)</td>
<td>0.76 (± 0.31)</td>
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<td>GORK (IgG2b)</td>
<td>2.15 (± 0.65)</td>
<td>1.19 (± 0.24)</td>
<td>0.55 (± 0.20)</td>
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Supplemental Figure 6

A  IgG1-induced PSA in wt mice

B  IgG2a-induced PSA in wt Balb/c mice
Supplemental Figure 5

A  FcγRIII expression

B  FcγRIV expression

C  FcγRIIB expression

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<th>FcγRI tumor</th>
<th>FcγRIIB tumor</th>
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<tr>
<td>Neutrophils</td>
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<tr>
<td>Ly6C&lt;sup&gt;hi&lt;/sup&gt; monocytes</td>
<td>3000 ± 100</td>
<td>2000 ± 100</td>
<td>1000 ± 50</td>
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<td>800 ± 20</td>
<td>400 ± 10</td>
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*** P < 0.001, **** P < 0.0001
Supplemental Figure 7

Untreated

TNP-BSA

IgE-PSA

IgG1-PSA

IgG2a-PSA

IgG2b-PSA