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IgG subclasses determine pathways of anaphylaxis in mice

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39

40 **ABSTRACT**

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

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

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

53 **Results:** Activating FcγRIII is the receptor primarily responsible for all three models of
54 anaphylaxis, and subsequent down regulation of this receptor was observed. These models
55 differentially relied on histamine release and on the contribution of mast cells, basophils,
56 macrophages and neutrophils. Strikingly, basophil contribution and histamine predominance in
57 IgG1- and IgG2b-mediated anaphylaxis correlated with the ability of inhibitory FcγRIIB to
58 negatively regulate these models of anaphylaxis.

59 **Conclusion:** We propose that the differential expression of inhibitory FcγRIIB on myeloid cells
60 and its differential binding of IgG subclasses controls the contributions of mast cells, basophils,
61 neutrophils and macrophages to IgG subclass-dependent anaphylaxis. Collectively, our results
62 unravel novel complexities in the involvement and regulation of cell populations in IgG-mediated
63 reactions *in vivo*.

64

CLINICAL IMPLICATIONS

65 Anaphylactic pathways induced by different IgG subclasses in mice vary in terms of
66 contributions by different cell types, mediators and antibody receptors. These results may help in
67 the design of efforts to understand and treat IgG-mediated anaphylaxis in humans, e.g., as seen
68 following intravenous IgG or administration of therapeutic IgG antibodies.

69

70

CAPSULE SUMMARY

71

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

75

76

77

KEY WORDS

78

79 Anaphylaxis; IgG; mouse model; basophil; neutrophil; monocyte; macrophage; Fc γ R; Platelet-
80 activating Factor; Histamine.

81

82 **ABBREVIATIONS USED**

83

84 Fc γ R: IgG Fc receptor

85 PAF: Platelet-activating factor

86 K_A: Affinity constant

87 WT: C57Bl/6 Wild-type

88 PSA: Passive systemic anaphylaxis

89 TNP: Trinitrophenyl

90 BSA: Bovine serum albumin

91 mAb: Monoclonal antibody

92 PBS: Phosphate Buffered Saline

93 Gfi1: Growth Factor Independence-1

94 GeoMean: Geometric Mean

95 SEM: Standard error of the mean

96

INTRODUCTION

97
98
99 Anaphylaxis is a hyperacute allergic reaction that occurs with increasing incidence in the
100 population and can be of fatal consequence. Symptoms include skin rashes, hypotension,
101 hypothermia, abdominal pain, bronchospasm and heart and lung failure that may lead to asphyxia
102 and sometimes death¹. The main treatment remains epinephrine (adrenaline) injection to restore
103 heart and lung function. Since anaphylaxis represents an emergency situation, few clinical studies
104 have been possible to address the mechanisms leading to anaphylaxis in patients. Experimental
105 models of anaphylaxis identified mechanisms involving allergen-specific antibodies that trigger
106 activating antibody receptors on myeloid cells, leading to the release of mediators. These
107 mediators can, by themselves, recapitulate the symptoms of anaphylaxis as observed in humans²
108 ³.

109 The “classical” mechanism of anaphylaxis states that allergen-specific IgE binds the
110 activating IgE receptor FcεRI on mast cells, which upon allergen encounter become activated and
111 release histamine, among other mediators. Notably, histamine injection suffices to induce the
112 signs of anaphylaxis in animal models⁴. In many cases, detectable allergen-specific IgE and
113 elevated histamine levels do not accompany anaphylaxis in humans (discussed in ⁵), leading to
114 the notion that “atypical” or “alternate” mechanisms of induction could explain these cases. One
115 of these atypical/alternate models proposes a similar cascade of events, but instead based on
116 allergen-specific IgG binding to allergen, forming IgG-allergen immune complexes that trigger
117 activating IgG receptors (FcγRs) expressed on myeloid cells (*i.e.* macrophages, basophils and/or
118 neutrophils), which in turn release Platelet-Activating Factor (PAF)^{2,3}. Importantly, PAF
119 injection suffices to induce the signs of anaphylaxis in animal models ⁶. IgG-induced anaphylaxis

120 can be elicited by intravenous injection of allergen-specific IgG followed by allergen
121 administration, and is termed IgG-induced passive systemic anaphylaxis (PSA).

122 IgG receptors in the mouse comprise four “classical“ IgG receptors termed FcγRs, but
123 also the neonatal IgG receptor (FcRn) and the intracellular FcR tripartite motif-containing protein
124 21 (TRIM21)^{7, 8}. Whereas FcRn and TRIM21 both participate in the intracellular routing of IgG,
125 and FcRn in protection from catabolism and distribution to tissues⁹, FcγRs control cell activation
126 in the presence of immune complexes. FcγRs in mice are subdivided into i) activating FcγRs, *i.e.*
127 FcγRI, FcγRIII and FcγRIV, that lead to cell activation upon immune complex binding, and ii) an
128 inhibitory FcγR, *i.e.* FcγRIIB, that inhibits cell activation when co-engaged by an immune
129 complex with an activating FcγR co-expressed on the same cell¹⁰. Inhibition of cell activation by
130 FcγRIIB thus requires that the immune complex contains IgG that are bound both by the
131 activating and by the inhibitory FcγR.

132 Four IgG subclasses exist in mice, IgG1, IgG2a, IgG2b and IgG3. Among those, only
133 IgG2a and IgG2b bind to all FcγRs, whereas IgG1 binds only to FcγRIIB and FcγRIII. It remains
134 under debate whether IgG3 binds to FcγRs, particularly FcγRI^{11, 12}. The affinities of these FcγRs
135 towards IgG subclasses are strikingly different (Table 1) leading to the notion of “high-affinity”
136 receptors that retain monomeric IgG and “low-affinity” receptors that do not⁸. The avidity of
137 IgG-immune complexes, however, enables both types of receptors to retain IgG-immune
138 complexes, leading to receptor clustering, intracellular signaling events and, eventually, to cell
139 activation. FcγRI is a high-affinity receptor for IgG2a¹³, and FcγRIV is a high-affinity receptor
140 for IgG2a and IgG2b¹⁴. All other FcγR-IgG interactions are of low affinity (reviewed in ⁷).

141 Three out of the four IgG subclasses in the mouse, *i.e.* IgG1, IgG2a and IgG2b, have been
142 reported to enable the induction of systemic anaphylaxis, inducing mild to severe hypothermia⁵,
143 ^{15, 16}. This is rather surprising for IgG1, considering that inhibitory FcγRIIB binds IgG1 with a

144 10-fold higher affinity ($K_A=3.3 \times 10^6 \text{ M}^{-1}$) than activating $\text{Fc}\gamma\text{RIII}$ ($K_A=3.1 \times 10^5 \text{ M}^{-1}$)¹⁷ (Table 1),
145 implying that inhibition should dominate over activation. WT mice, indeed, develop a very mild
146 anaphylactic reaction during IgG1-PSA compared to $\text{Fc}\gamma\text{RIIB}^{-/-}$ mice¹⁸, indicating that inhibition
147 by $\text{Fc}\gamma\text{RIIB}$ occurs in WT mice during IgG1-PSA, reducing, but not protecting from,
148 anaphylaxis. IgG1-PSA has been reported to rely on basophils¹⁹ that co-express $\text{Fc}\gamma\text{RIIB}$ and
149 $\text{Fc}\gamma\text{RIII}^{20}$. In this apparently simple situation, only one activating receptor and one inhibitory
150 receptor are engaged on a single cell type that, once activated, produces an anaphylactogenic
151 mediator, like PAF¹⁹.

152 IgG2a and IgG2b, however, bind three activating $\text{Fc}\gamma\text{Rs}$ and inhibitory $\text{Fc}\gamma\text{RIIB}$ with
153 different affinities ranging over 2 logs. In particular, the affinity of $\text{Fc}\gamma\text{RIIB}$ for IgG2a is
154 significantly lower than for IgG2b, whereas activating IgG receptors $\text{Fc}\gamma\text{RIII}$ and $\text{Fc}\gamma\text{RIV}$ bind
155 IgG2a and IgG2b with similar affinities, respectively (Table 1). Notably, $\text{Fc}\gamma\text{RIV}$ is not expressed
156 on basophils, but on monocytes/macrophages and neutrophils²¹ that have both been reported to
157 contribute to experimental anaphylaxis^{16, 22-24}. In addition, mice expressing only $\text{Fc}\gamma\text{RIV}$ can
158 develop IgG-PSA¹⁶. Together with expression and binding data, one would therefore hypothesize
159 that $\text{Fc}\gamma\text{RIV}$ contributes predominantly to IgG2a- and IgG2b-PSA. In this work, we present
160 evidence contrary to this hypothesis, and reveal which activating $\text{Fc}\gamma\text{R}$ on which cell type(s)
161 releasing which mediator(s) are responsible for IgG2a-PSA and IgG2b-PSA, and the differential
162 regulation of these models of anaphylaxis by $\text{Fc}\gamma\text{RIIB}$. Our results unravel a complex balance
163 determined by $\text{Fc}\gamma\text{R}$ expression patterns, inhibition potential by $\text{Fc}\gamma\text{RIIB}$ and respective affinities
164 of activating and inhibitory $\text{Fc}\gamma\text{Rs}$ for IgG subclasses that, altogether, regulate the contribution of
165 cells and anaphylactogenic mediators to a given model of IgG-induced anaphylaxis.

166

167 **METHODS**

168 **Mice.** Female C57Bl/6J mice (herein referred to as “WT”) were purchased from Charles River,
169 female Balb/cJRj mice from Janvier Labs, FcγRIIB^{-/-} (MGI:1857166), FcγRIII^{-/-} mice (MGI:
170 3620982) and Rosa26-YFP mice from Jackson Laboratories. FcγRI^{-/-} mice (MGI: 3664782) were
171 provided by J. Leusen (University Medical Center, Utrecht, The Netherlands), FcγRIV^{-/-} mice
172 (MGI: 5428684) by J.V. Ravetch (The Rockefeller University, New York, NY, USA), Gfi1^{-/-}
173 mice by T. Moroy (Montreal University, Montreal, QC, Canada) and MRP8-cre mice by Clifford
174 Lowell (University of California at San Francisco, CA, USA). MRP8-cre and Rosa26-YFP mice
175 were intercrossed to generate MRP8-cre; Rosa26-YFP mice. Cpa3-Cre; Mcl-1^{fl/fl} mice²⁵
176 (backcrossed for at least 9 generations on a C57Bl/6J background) were kept in the Stanford
177 University animal facility. All mouse protocols were approved by the Animal Ethics committee
178 CETEA (Institut Pasteur, Paris, France) registered under #C2EA-89, and the Institutional Animal
179 Care and Use Committee of Stanford University.

180

181 **Antibodies and reagents.** PBS- and clodronate-liposomes were prepared as previously
182 described²⁶. TNP₍₂₁₋₃₁₎-BSA was obtained from Santa Cruz, ABT-491 from Sigma-Aldrich;
183 cetirizine DiHCl from Selleck Chemicals; anti-mouse FcγRIII (275003) from R&D Systems; rat
184 IgG2b isotype control (LTF-2) from Bio X Cell. Purified anti-CD200R3 (Ba103) was provided
185 by H. Karasuyama (Tokyo Medical and Dental University Graduate School, Tokyo, Japan). The
186 hybridoma producing mAbs anti-mouse FcγRIV (9E9) was provided by J.V. Ravetch
187 (Rockefeller University, New York, New York, USA), anti-Ly6G (NIMP-R14) by C. Leclerc
188 (Institut Pasteur, Paris, France), IgG1 anti-TNP (TIB-191) by D. Voehringer
189 (Universitätsklinikum, Erlangen, Germany), IgG2a anti-TNP (Hy1.2) by Shozo Izui (University
190 of Geneva, Geneva, Switzerland) and IgG2b anti-TNP (GORK) by B. Heyman (Uppsala

191 Universitet, Uppsala, Sweden): corresponding antibodies were purified as described¹⁶. Purified
192 mouse IgE anti-TNP was purchased from BD Pharmingen. MAb 9E9 was coupled to FITC using
193 the PierceTM FITC Antibody labeling kit (Life Technologies). The antibodies used for flow
194 cytometry staining of c-Kit (clone 2B8), CD49b (clone DX5), IgE (clone R35-72), CD11b (clone
195 M1/70), F4/80 (clone 6F12), CD115 (clone T38-320), Ly6G (clone 1A8) and Ly6C (clone AL-
196 21) were purchased from BD Pharmingen; CD45 (clone 30F11) and Gr1 (clone RB6-8C5) were
197 purchased from Miltenyi Biotec. FcγRIIB was detected using FITC-coupled mAb AT130-2
198 mIgG1 N297A²⁷.

199
200 ***Passive Systemic Anaphylaxis. IgG-induced PSA:*** IgG1, IgG2a or IgG2b anti-TNP antibodies
201 were administered intravenously at a dose of 500 µg, if not otherwise indicated, in 200 µL
202 physiological saline, followed by an intravenous challenge with 200 µg of the antigen (TNP-
203 BSA) in physiological saline 16 hours later. ***IgE-induced PSA:*** IgE anti-TNP antibodies were
204 administered intravenously at a dose of 50 µg in 200 µL physiological saline followed by an
205 intravenous challenge with 500 µg of TNP-BSA in physiological saline 24 hours later. The body
206 temperature of mice was monitored using a digital thermometer with rectal probe (YSI).

207
208 ***In vivo blocking and cellular depletion.*** 300 µg/mouse of PBS- or clodronate-liposomes, 300
209 µg/mouse of rat IgG2b isotype control or anti-Ly6G, and 30 µg/mouse of anti-CD200R3 mAbs
210 were injected i.v. 24 hours before challenge. Specificity of cell depletion was evaluated using
211 flow cytometry on blood, bone marrow, spleen and peritoneum taken from naïve WT mice 24
212 hours after injection of the depleting antibody or clodronate-liposomes (Examples are shown in
213 Supplemental Figures 1 & 2). 25 µg/mouse of ABT-491 or 300 µg/mouse of cetirizine were

214 injected intravenously 20 minutes or intraperitoneally 30 minutes before challenge, respectively.
215 200 µg/mouse of anti-FcγRIV mAb were injected intravenously 30 minutes before challenge.

216
217 **Flow cytometry analysis.** Freshly isolated cells were stained with indicated fluorescently labeled
218 mAbs for 30 minutes at 4°C. Cell populations were defined as follows: neutrophils
219 (CD45⁺/CD11b⁺/Ly6G^{hi}/Ly6C^{int}), monocytes (CD45⁺/CD11b⁺/Ly6G^{lo}/Ly6C^{lo} or ^{hi}), basophils
220 (CD45^{int}/DX5⁺/IgE⁺); spleen macrophages (CD45⁺/CD11b⁺/Gr-1^{lo}/CD115⁺/F4/80^{hi}); peritoneal
221 macrophages (CD45⁺/CD11b⁺/F4/80⁺); peritoneal mast cells (CD45⁺/c-Kit⁺/IgE⁺). Expression of
222 FcγR on indicated cell population is represented as Δ Geomean between specific and isotype
223 control staining. *NB:* In Figure 5: 1 or 0.5 mg IgG2b was injected to assess expression on
224 neutrophils/monocytes or basophils, respectively.

225
226 **Surface plasmon resonance analysis.** Experiments were performed at 25°C using a ProteOn
227 XPR36 real-time SPR biosensor (BioRad). Anti-TNP antibodies were immobilized covalently
228 through amine coupling on the surface of a GLC chip. TNP-BSA was then injected on the chip at
229 a flow rate of 25 µl.min⁻¹, with contact and dissociation time of 8 minutes each. Binding
230 responses were recorded in real time as resonance units (RU; 1 RU ≈ 1 pg/mm²). Background
231 signals were subtracted, and binding rates (k_{on} and k_{off}) and equilibrium constants (Kd) were
232 determined using the Biaevaluation software (GE Healthcare).

233
234 **ELISAs.** After the induction of IgG1-, IgG2a-, IgG2b- or IgE-induced PSA, plasma and serum
235 were collected at 5 minutes and 3 hours later to determine the histamine and mMCP-1 content,
236 respectively. Histamine and mMCP-1 concentration were determined using commercially
237 available ELISA kits (Beckman Coulter; eBioscience) following the manufacturer's instructions.

238 Relative binding affinity of IgG1, IgG2a and IgG2b anti-TNP antibodies to TNP-BSA was
239 determined by ELISA. Briefly, TNP-BSA-coated plates were incubated with dilutions of IgG1,
240 IgG2a or IgG2b anti-TNP antibodies. After washing, bound anti-TNP IgG were revealed using
241 the same HRP-coupled anti-mouse IgG and SIGMAFAST OPD solution.

242

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

248

249 ***Statistics.*** Data were analyzed using one-way or two-way ANOVA with Tukey's post-test. A *p*-
250 value less than .05 was considered significant: (**p* < .05; ***p* < .01; ****p* < .001; *****p* < .0001).

251 If not stated otherwise, data are represented as mean +/- SEM.

252

253

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^{3, 16, 19, 22, 28}, 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 $\approx 2^{\circ}\text{C}$, IgG2a-PSA of $\approx 4^{\circ}\text{C}$ and IgG2b-PSA of $\approx 3^{\circ}\text{C}$ 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

278 Fc γ RIII and Fc γ RIV); and a mild eosinophilia in Fc γ RIII^{-/-} mice, that also express significantly
279 more Fc γ RIIB on neutrophils and Ly6C^{hi} monocytes. Together, we think that these variations do
280 not explain the drastic phenotypes observed for PSA in Fc γ RIIB^{-/-} and Fc γ RIII^{-/-} mice compared
281 to WT mice. Thus, these data demonstrate that Fc γ RIII predominates in the induction of IgG1-,
282 IgG2a- and IgG2b-PSA, and that Fc γ RIIB specifically dampens anaphylaxis severity in IgG1-
283 and IgG2b-PSA.

284
285 **Basophils, mast cells, monocytes/macrophages and neutrophils contribute differentially to**
286 **IgG isotype-dependent anaphylaxis models**

287 Fc γ RIII is expressed by all myeloid cells^{7,20} and to a lesser extent by NK cells²⁹. One may
288 therefore anticipate that IgG immune complexes formed *in vivo* as a consequence of TNP-BSA
289 injection in anti-TNP sensitized mice would therefore engage Fc γ RIII on these cells, leading to
290 cell activation and possibly contributing to anaphylaxis. Basophils, mast cells, neutrophils and
291 monocyte/macrophages have indeed been reported to contribute to IgG-PSA^{16, 19, 22, 15}, however
292 the respective contribution of each of these different cell types remains debated^{2, 28}. To
293 investigate which cell types contribute to PSA induced by different IgG subclasses, we depleted
294 basophils (anti-CD200R3 mAb), monocytes/macrophages (clodronate-filled liposomes) or
295 neutrophils (anti-Ly6G) prior to anaphylaxis induction or evaluated anaphylaxis induction in
296 transgenic mice deficient in certain cell populations.

297 Of note, the relatively mild temperature loss in IgG1-PSA in WT mice (Supplemental
298 Figure 4A), did not allow us to address reliably the contribution of either basophils or neutrophils
299 to this model of anaphylaxis. We therefore restricted our analysis of the contribution of myeloid
300 cell populations to IgG2a-PSA and IgG2b-PSA. Antibody-induced basophil depletion or
301 genetically-induced mast cell and basophil deficiency (Supplemental Figure 2H, Cpa3-Cre; Mcl-

302 $1^{fl/fl}$ mice²⁵), did not affect IgG2a-PSA (Figure 2A&B), but significantly inhibited IgG2b-PSA
303 (Figure 2F&G). Monocyte/macrophage depletion (Figure 2C&H) significantly inhibited both
304 IgG2a- and IgG2b-PSA. The absence of neutrophils, either following antibody-mediated
305 depletion (Figure 2D&I) or using neutropenic $Gfi1^{-/-}$ mice³⁰ (Figure 2E&J), significantly
306 inhibited both IgG2a- and IgG2b-PSA. Whereas monocytes/macrophages and neutrophils appear
307 to contribute to both models of anaphylaxis, basophils and possibly mast cells therefore
308 contribute specifically to IgG2b-PSA, but not to IgG2a-PSA.

309

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

311 Khodoun *et al* proposed to use the reduced expression level of FcγRIII on mouse
312 neutrophils as a marker to distinguish IgE- from IgG1-induced PSA, both of which required
313 priming with an antigen-specific IgG1 and challenge with that antigen³¹. We therefore wondered
314 if FcγRIII expression on neutrophils might also be a marker for IgG2a- and IgG2b-PSA. In
315 addition, reduced expression of FcγR(s) following IgG-PSA may document that a particular cell
316 population is activated following engagement of its FcγR(s) by IgG-immune complexes during
317 anaphylaxis. This parameter may thus be used to discriminate cell populations contributing to
318 anaphylaxis following direct activation by IgG-immune complexes from those contributing
319 following activation by mediators liberated by IgG-immune complex-activated cells (*e.g.*
320 histamine, PAF, leukotrienes and prostaglandins).

321 Among mouse IgG receptors, only FcγRIIB, FcγRIII and FcγRIV are significantly
322 expressed on circulating myeloid cells, but not FcγRI^{7, 32, 33}. Of circulating monocyte populations,
323 “classical” Ly6C^{hi} monocytes are FcγRIIB^{med}, FcγRIII^{med} FcγRIV⁻, whereas “non-classical”
324 Ly6C^{lo} monocytes are FcγRIIB^{lo}, FcγRIII^{lo} FcγRIV^{hi} ³⁴. We therefore determined the expression
325 of FcγRIIB, FcγRIII and FcγRIV before and after IgG2a-PSA induction on neutrophils and

326 monocyte subsets. The expression of Fc γ RIII was down regulated on neutrophils, but not on
327 Ly6C^{hi} monocytes, during IgG2a-PSA (Figure 3A&D). The expression of Fc γ RIV was also down
328 regulated on neutrophils, but not on Ly6C^{lo} monocytes, during IgG2a-PSA (Figure 3B&D). This
329 was unexpected considering that Fc γ RIV does not significantly contribute to this PSA model
330 (Figure 1B). The expression of Fc γ RIIB, however, remained unchanged on Ly6C^{hi} and Ly6C^{lo}
331 monocytes and neutrophils (Figure 3C&D), in agreement with the lack of contribution of this
332 receptor to IgG2a-PSA (Figure 1B). Together these data suggest that neutrophils may directly be
333 activated through Fc γ RIII by immune complexes formed during IgG2a-PSA. They also suggest
334 that neutrophils, but not Ly6C^{lo} monocytes, may be similarly activated through Fc γ RIV, even if
335 no contribution of this receptor was identified in this model using Fc γ RIV^{-/-} mice (Figure 1B).

336

337 **Elevated IgG2 antibody doses reveal Fc γ RIV contribution to IgG2a-PSA and IgG2b-PSA**

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

345 To explore this possibility, we primed Fc γ RIII^{-/-} mice with various doses of anti-TNP
346 IgG2a before challenge with TNP-BSA, in order to induce a range of *in vivo* concentrations of
347 immune complexes. As expected, the low doses did not trigger Fc γ RIII^{-/-} mice to develop
348 anaphylaxis after challenge. Elevated doses (1 or 2 mg), however, enabled significant
349 temperature drops in Fc γ RIII^{-/-} mice, comparable to those observed in WT mice primed with 500

350 μg IgG2, particularly at the highest dose of IgG2a (2 mg) (Figure 4A). Already at a dose of 1mg
351 of IgG2, Fc γ RIII^{-/-} mice developed mild hypothermia in IgG2a-PSA but not in IgG2b-PSA
352 (Figure 4B&C). Unexpectedly in the same conditions, Fc γ RIV contributed to IgG2b-PSA that
353 was not anymore dampened by inhibitory Fc γ RIIB (Figure 4C). At a dose of 2 mg of IgG,
354 Fc γ RIII^{-/-} mice developed hypothermia in both IgG2a-PSA and IgG2b-PSA that was abolished
355 when Fc γ RIII^{-/-} mice were pre-treated with a blocking antibody against Fc γ RIV (Figure 4D&E).
356 Fc γ RI did not contribute to either model of IgG2-PSA at an elevated dose (Figure 4B&C).
357 Furthermore, the expression of Fc γ RIII was down regulated on neutrophils and basophils, but not
358 on Ly6C^{hi} monocytes, following IgG2b-PSA (Figure 5A&D). The expression of Fc γ RIV was
359 also down regulated on neutrophils, but not on Ly6C^{lo} monocytes (Figure 5B&D). The
360 expression of Fc γ RIIB, however, did not change on either neutrophils or Ly6C^{hi} and Ly6C^{lo}
361 monocytes even though this inhibitory receptor regulates IgG2b-PSA (Figures 1C and 5C&D).
362 This observation is in agreement with the report by Khodoun et al, reporting that Fc γ RIIB
363 expression did not change on neutrophils following IgG1-PSA³¹. Altogether high doses of
364 antigen-specific IgG2 reveal the contribution of Fc γ RIV to IgG2a-PSA and to IgG2b-PSA, and
365 suggest the direct activation of neutrophils and basophils by IgG2b-immune complexes.

366

367 **IgG1 PSA in the absence of inhibitory Fc γ RIIB**

368 The unexpected differences observed between IgG2a- and IgG2b-PSA induction
369 pathways prompted us to find a mouse model more sensitive to IgG1-PSA than WT mice, to be
370 able to evaluate the contribution of cell types and mediators also in this model. Indeed, as
371 mentioned earlier, WT mice respond poorly to IgG1-PSA (Figure 1A; Supplemental Figure
372 4A)¹⁸. Fc γ RIIB^{-/-} mice, however, develop a temperature drop of $\approx 4^{\circ}\text{C}$ during IgG1-PSA,
373 comparable to temperature losses observed in WT mice during IgG2a- or IgG2b-PSA (Figure

374 1B&C). We therefore analyzed the contribution of cell types to IgG1-PSA in $Fc\gamma RIIB^{-/-}$ mice.
375 Basophil depletion mildly - but significantly - inhibited IgG1-PSA (Figure 6A), in agreement
376 with previous data¹⁹. The depletion of neutrophils had the same effect, although not consistently
377 as strongly as basophil depletion (Figure 6B and data not shown). Monocyte/macrophage
378 depletion had only a tendency to ameliorate anaphylaxis that was reproducible but not significant
379 (Figure 6C). These results suggest that IgG1-PSA relies on basophils and neutrophils, and
380 possibly also on monocytes.

381

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

383 Because cell types contribute differently to IgG2-PSA models (*i.e.* IgG2a-PSA,
384 neutrophils and monocytes; IgG2b-PSA, basophils, neutrophils and monocytes), one can expect
385 that the mediators responsible for clinical signs also may differ between them. Platelet activating
386 factor (PAF) has been shown to be responsible for anaphylactic reactions that required basophil¹⁹,
387 neutrophil^{16, 24} and/or monocyte/macrophage²² activation, whereas histamine has been shown to
388 be responsible for mast cell- and basophil-dependent anaphylaxis^{35, 36}. Neutrophils are the main
389 producers of PAF³⁷, whereas mast cells and basophils are the main producers of histamine^{38, 39}.
390 We therefore analyzed the relative contribution of these two mediators to the three models of
391 PSA using the histamine-receptor 1 antagonist cetirizine and the PAF-R antagonist ABT-491.
392 Surprisingly, histamine-receptor 1 antagonist cetirizine significantly inhibited IgG1-PSA whereas
393 PAF-R antagonist ABT-491 had no significant effect, in opposition with previous data¹⁹. The
394 combination of both antagonists had an additive effect, and almost abolished IgG1-PSA (Figure
395 7A). These results obtained in $Fc\gamma RIIB^{-/-}$ mice were confirmed in WT mice (Figure 7A). Whereas
396 cetirizine mildly reduced hypothermia in IgG2a-PSA, it significantly inhibited IgG2b-PSA. ABT-
397 491 mildly reduced hypothermia in IgG2a-PSA, but had no significant effect on IgG2b-PSA

398 (Figure 7B&C). The combination of cetirizine and ABT-491, however, almost abolished both
399 IgG2a- and IgG2b-PSA. Elevated plasma histamine levels were detected 5 minutes post
400 challenge in all three IgG-PSA models, and particularly high levels were observed in mice
401 undergoing IgE-PSA (as a positive control) or undergoing IgG2a-PSA (Figures 7D&E). This
402 latter finding is surprising as IgG2a-PSA is unaffected by the absence of both mast cells and
403 basophils that are considered major sources of histamine. Mast cell protease-1 (mMCP-1), which
404 is released upon activation of mucosal mast cells, could be detected in the serum of mice
405 undergoing IgE-PSA, but not in those undergoing any one of the three models of IgG-PSA, 3
406 hours post-PSA induction (Figure 7F). Collectively these results suggest that histamine
407 predominantly contributes to IgG1- and IgG2b-PSA, whereas histamine and PAF, together, are
408 necessary for IgG2a-PSA.

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411

412

DISCUSSION

413
414 Our work suggests that the activating IgG receptor FcγRIII predominantly contributes to
415 IgG-dependent passive systemic anaphylaxis, whether induced by IgG1, IgG2a or IgG2b
416 antibodies. A contribution of the activating IgG receptor FcγRIV was only identified when using
417 very high amounts of IgG2 antibodies, whereas the activating IgG receptor FcγRI played no
418 detectable role. Remarkably, the inhibitory IgG receptor FcγRIIB controlled the severity of IgG1-
419 and IgG2b-, but not IgG2a-induced anaphylaxis. The ability of FcγRIIB to inhibit a given model
420 of IgG-induced anaphylaxis correlated with the contribution of basophils and histamine to that
421 model. Indeed, basophils, and possibly mast cells, contributed with neutrophils to IgG1-PSA, and
422 with neutrophils and monocytes to IgG2b-PSA, but not to IgG2a-PSA that appeared to depend
423 entirely on neutrophils and monocytes/macrophages. Altogether our data propose that the three
424 IgG subclasses IgG1, IgG2a and IgG2b induce three qualitatively different pathways of
425 anaphylaxis that are nevertheless triggered primarily by a single IgG receptor, FcγRIII.

426
427 FcγRIII is a low-affinity receptor for IgG1, IgG2a and IgG2b, whereas FcγRI is a high-
428 affinity receptor for IgG2a, and FcγRIV is a high affinity receptor for IgG2a and IgG2b. One
429 would therefore assume that FcγRIII predominates in IgG1-PSA, FcγRI and FcγRIV in IgG2a-
430 PSA, and FcγRIV in IgG2b-PSA. However, our data from FcγRIII^{-/-} mice indicate that this
431 receptor predominates in all three models. Notably, we found an increased expression of FcγRIIB
432 on neutrophils and Ly6C^{hi} monocytes in FcγRIII^{-/-} mice, which could mask a potential
433 contribution of FcγRIV in these conditions. In support of the notion that FcγRIII predominates
434 IgG-PSA induction, an alternative model of PSA induced by sensitization and challenge with
435 goat antibodies was found to be driven by FcγRIII²² and blocking antibodies against FcγRIII
436 were protective in a model of PSA induced by IgG immune complexes¹⁶. In addition, IgG2a-PSA

437 in Fc γ RIIB^{-/-} mice was abolished following injection of anti-Fc γ RIIB/III blocking mAbs⁵.
438 Fc γ RIII is the only activating IgG receptor in the mouse that does not bind an IgG subclass with
439 high affinity, thus it remains unoccupied by monomeric IgG and accessible for binding of
440 immune complexes. This is theoretically not the case for Fc γ RI and Fc γ RIV, which at
441 physiological serum concentrations of IgG2a (\approx 2.5 mg/mL) and IgG2b (\approx 1.5 mg/mL), are likely
442 occupied *in vivo*, particularly on circulating cells. Of note, C57Bl/6 mice produce IgG2c, but not
443 IgG2a antibodies, whose amino acid sequence varies by about 15%. Experiments performed in
444 Balb/c mice that express endogenous IgG2a (but no IgG2c) gave similar results regarding the
445 contribution of basophils, neutrophils and monocytes to IgG2a (Supplemental Figure 4B),
446 indicating that IgG2a and IgG2c sequence variations probably do not affect the mechanisms of
447 anaphylaxis induction that we describe herein.

448 Adult female mice of 20 g, as used in this study, possess a circulating blood volume of
449 1.4-1.5 mL. Injection of 500 μ g antibody thus corresponds to \approx 330 μ g/mL of circulating
450 antibody, injection of 1 mg to \approx 660 μ g/mL, and injection of 2 mg to \approx 1,3 mg/mL. In cases of
451 anaphylaxis the circulating concentration of allergen-specific IgG has not been evaluated due to
452 lack of testing and appropriate controls (*i.e.* monoclonal anti-allergen antibodies); although we
453 have reported high circulating antigen-specific IgG levels in an autoimmune model of arthritis³³.
454 It seems rather unlikely that patients suffering from anaphylaxis possess such elevated circulating
455 levels of IgG anti-allergen as in the mice receiving the high doses we used in this study.
456 Nevertheless, our results in high-dose IgG2a- and IgG2b-PSA demonstrate that Fc γ RIV can by
457 itself (*i.e.* in the absence of Fc γ RIII) trigger anaphylaxis. Similar results have been obtained in
458 mice expressing only Fc γ RIV: “Fc γ RIV-only” mice developed IgG2b-PSA after injection of pre-
459 formed IgG2b immune complexes and also upon injection of polyclonal anti-sera followed by a
460 challenge with the antigen¹⁶. We reported previously that IgG2b-PSA triggered by the injection

461 of preformed IgG2b-immune complexes in WT mice was abolished following injection of anti-
462 Fc γ RIV blocking mAb 9E9. This contrasts with the findings of the current study, in which we
463 show that Fc γ RIII is the major activating receptor in all models of IgG-PSA, and Fc γ RIV
464 contributes only at high antibody concentrations. Two hypotheses may explain these discrepant
465 results: i) the injection of preformed IgG2b-immune complexes leads to an immediate circulating
466 bolus of immune complexes, which are similarly formed only after injection of high amounts of
467 IgG2b and antigen, thus triggering Fc γ RIV; 2) as recently reported⁴⁰ mAb 9E9 may not only
468 block Fc γ RIV through its Fab portions, but also Fc γ RIII via its Fc portion once 9E9 is bound to
469 Fc γ RIV. In our view, it is likely that a combination of these mechanisms reconcile our previous
470 and herein described results, and suggest that IgG2b-PSA induced following injection of
471 preformed IgG2b-immune complexes relies rather on both Fc γ RIII and Fc γ RIV than on Fc γ RIV
472 alone as we reported previously¹⁶. Together this body of evidence supports the notion that
473 Fc γ RIV is capable of triggering cell activation leading to anaphylaxis, yet in restricted conditions,
474 *i.e.* in the absence/blockade of Fc γ RIII or in presence of large amounts of IgG2a and/or IgG2b
475 antibodies.

476
477 The differential contribution of Fc γ Rs to IgG-PSA may rely on their respective expression
478 patterns on myeloid cells. Indeed, Fc γ RI is not^{32, 33} or only barely³⁴ expressed on circulating
479 monocytes, and its expression is largely restricted to tissue-resident macrophages. The level of its
480 expression on cells reported to contribute to anaphylaxis (*i.e.* monocytes in this case) may
481 therefore not suffice to induce their activation. This notion is supported by the absence of any
482 detectable effect of Fc γ RI deficiency in IgG2-PSA that we report in this study, even at high doses
483 of IgG2 antibodies. Fc γ RIII, however, is expressed on all myeloid cells⁷ and moreover at
484 comparably high levels on all those cell types that have been reported to contribute to

485 anaphylaxis; basophils, monocytes and neutrophils²⁰. This pattern of cellular expression may
486 explain its predominant contribution to all models of IgG-induced anaphylaxis. FcγRIV is
487 expressed on neutrophils and Ly6C^{lo} monocytes. It remains unclear, however, if Ly6C^{lo}, Ly6C^{hi}
488 or both monocyte subsets contribute to anaphylaxis. FcγRIV could contribute to PSA induction in
489 exceptional conditions (FcγRIII deficiency or high IgG2 antibody doses). The lack of FcγRIV
490 contribution in classical conditions of PSA may suggest that its expression level is not sufficient
491 in WT mice. Notably, it has been reported previously that particular FcγR deficiencies modify the
492 expression levels of other FcγRs. In particular FcγRIII^{-/-} mice, but not FcγRI^{-/-} mice, presented a
493 significant increase in FcγRIV expression levels on neutrophils^{16, 41, 42} and a tendency for
494 increased expression on Ly6C^{lo} monocytes (Supplemental Figure 5B). This could explain why
495 the contribution of FcγRIV to IgG2-PSA becomes apparent in FcγRIII^{-/-} mice. FcγRIV^{-/-} mice did
496 not, conversely, present alterations of FcγRIII expression on neutrophils or Ly6C^{hi} monocytes
497 compared to WT littermates (Supplemental Figure 5A). FcγRIIB^{-/-} mice expressed significantly
498 higher levels of FcγRIII and FcγRIV on neutrophils and increased FcγRIII on Ly6C^{hi} monocytes
499 that may, altogether, contribute to their higher susceptibility to anaphylaxis induction
500 (Supplemental Figure 5A&B).

501
502 The contribution of a rather restricted subset of myeloid cells to these (and other) models
503 of anaphylaxis^{2, 3} appears to be determined by at least two factors: their capacity to release
504 anaphylactogenic mediators (*e.g.* histamine or PAF) and their expression of sufficient levels of
505 activating IgG receptors. Mast cells and basophils release histamine, and neutrophils, monocytes/
506 macrophages and basophils release PAF, upon FcγR-triggering. Other mediators may induce
507 anaphylaxis or contribute to its severity, among them lipid mediators like prostaglandins,
508 thromboxanes and leukotrienes. Some of these have indeed been reported to trigger

509 bronchoconstriction and an increase in vascular permeability⁴³. The release of such mediators is
510 sufficiently rapid to coincide with the celerity of hypothermia, which is detectable within minutes
511 after allergen challenge. It is therefore surprising that eosinophils do not contribute to IgG-PSA,
512 as they express high levels of activating FcγRIII and FcγRIIB²⁰ (but no FcγRI or FcγRIV), and
513 are capable of releasing Leukotriene C4, Prostaglandin E2, thromboxane and PAF upon
514 activation⁴³. Though eosinophils represent relatively low numbers among blood cells
515 ($\approx 2 \times 10^5$ /mL), this is an unlikely explanation because basophils are significantly less numerous
516 ($\approx 5 \times 10^4$ /mL) but do contribute to anaphylaxis models. Most revealingly, it has been reported that
517 eosinophils do not release PAF following IgG-dependent activation⁴⁴. Whether eosinophils
518 produce other potentially anaphylactogenic mediators following IgG-immune complex activation
519 has not been investigated, but the lack of such an effect appears the most reasonable hypothesis
520 to explain why eosinophils have not been found to contribute to IgG-induced anaphylaxis.

521 We investigated the contribution of neutrophils and monocytes to IgG-PSA models using
522 depletion approaches. Ly6G⁺ cell depletion using NIMP-R14 resulted in an efficient depletion of
523 neutrophils in the blood and the spleen (Supplemental Figures 1B&2B). The same treatment
524 resulted only in a partial depletion in the bone marrow, in which a proportion of Ly6G⁺ cells are
525 masked from fluorescent anti-Ly6G staining, but not depleted by NIMP-R14 treatment (refer to
526 bone marrow panels in Supplemental Figures 1C,D & 2C,D,I). Importantly, we found that NIMP-
527 R14 depletion has a significant impact on monocyte populations in the blood and to some extent
528 in the spleen. This should be taken into consideration when interpreting the results of NIMP-R14
529 depletion experiments. All IgG-PSA models were ameliorated following NIMP-R14 depletion,
530 but also when monocytes/macrophages were targeted using clodronate liposomes. Intravenous
531 injection of clodronate liposomes resulted in a significant depletion of monocytes from the blood
532 and monocytes/macrophages from the spleen and BM, but not from the skin (data not shown) and

533 peritoneum (Supplemental Figures 1&2, as reported²⁶), and to a significant increase in blood
534 leukocyte counts and particularly of neutrophils (Supplemental Figures 1&2). Thus the anti-
535 Ly6G and the clodronate liposome treatments alter also the monocytes and neutrophil
536 compartment, respectively, but reduced hypothermia in the three models of IgG-PSA studied.
537 Constitutive deficiency in neutrophils, studied using *Gfi1*^{-/-} mice, confirmed the role of
538 neutrophils in IgG2a- and IgG2b-PSA models. Both neutrophils and monocytes can therefore be
539 considered to contribute to IgG-induced anaphylaxis in mice, whether dependent on IgG1, IgG2a
540 or IgG2b. The role of macrophages in the different IgG-PSA models remains to be investigated
541 more deeply, as clodronate liposomes injected intravenously efficiently targeted macrophages in
542 the spleen, but not in other tissues like peritoneum or skin, and thus do not allow conclusions on
543 their contribution.

544
545 The contribution of basophils to models of anaphylaxis has been a recent matter of
546 debate. Tsujimura *et al* reported that depletion of basophils using anti-CD200R3 (clone Ba103)
547 monoclonal antibodies strongly inhibited IgG1-PSA and rescued mast cell-deficient mice from
548 active anaphylaxis¹⁹. Ohnmacht *et al*, however, found that basophil-deficient *Mcpt8*^{cre} mice
549 demonstrated slightly decreased but significant hypothermia in response to IgG1-PSA (induced
550 with the same antibody clone) when compared to WT mice⁴⁵. More recently, Reber *et al*.
551 reported that peanut-induced anaphylaxis was reduced following Diphtheria toxin injection in
552 *Mcpt8*^{DTR} mice that selectively depletes basophils, and confirmed that basophil depletion using
553 anti-CD200R3 mAbs inhibited anaphylaxis³⁶. Moreover, Khodoun *et al* found a contribution of
554 basophils to anaphylaxis mortality, but not to hypothermia, in a model of IgG2a-PSA following
555 anti-CD200R3 mAb injection⁵. It therefore appears that differences between inducible basophil
556 depletion using specific antibodies or toxin administration and a constitutive lack of basophils,

557 possibly leading to compensatory mechanisms during development of these mice, may account
558 for the divergent results observed. Intriguingly however, basophils have been reported to be
559 resistant to IgG-immune complex triggering *ex vivo* due to dominant inhibition by FcγRIIB over
560 activation by FcγRIII²⁰. In this study, we report that both basophil depletion following anti-
561 CD200R3 mAb (Ba103) injection or constitutive deficiency of basophils and mast cells in Cpa3-
562 Cre; Mcl-1^{fl/fl} mice inhibits IgG2b-PSA but not IgG2a-PSA, confirming a role for basophils (and
563 potentially mast cells) to specific IgG-PSA models. Of note, Ba103 efficiently depleted basophils
564 from the blood and partially from the spleen and the bone marrow, but had no significant effect
565 on mast cells in the peritoneum or skin (Supplemental Figures 1A&1E and 2A&2E). The
566 difference in the ability of basophils to respond to IgG-immune complex triggering *in vitro* and
567 the various *in vivo models* may be explained by functional alterations during basophil purification
568 or a requirement for co-stimulation by other cells or their products that are present *in vivo*, but not
569 *ex vivo*, for basophils to respond to IgG-immune complexes. Our results using Cpa3-Cre; Mcl-
570 1^{fl/fl} mice indicate that mast cells were not necessary for IgG2a-PSA. We could not formally
571 define their role in IgG2b-PSA as basophil depletion and deficiency in basophils and mast cells
572 lead to similar reduction in IgG2b-PSA. Notably, increased plasma histamine levels, but no
573 increase in mMCP-1 levels could be detected, suggesting that mucosal mast cells were not
574 activated during IgG-PSA. Intriguingly, however, dermal mast cells displayed a degranulated
575 morphology 30 minutes after challenge in all IgG PSA models tested (Supplemental Figure 7).
576 Whether their degranulation is a cause or a consequence of anaphylaxis remains however elusive.

577
578 The ability of cells expressing activating FcγRs to respond to IgG-immune complexes has
579 been proposed to be regulated by co-expression of FcγRIIB⁴⁶. FcγRIIB^{-/-} mice develop increased
580 hypersensitivity and anaphylactic reactions to IgG1-PSA (this report and^{16, 18}). Our results further

581 demonstrate that FcγRIIB inhibits IgG2b-, but not IgG2a-PSA. This latter finding is supported by
582 results from Khodoun *et al*⁵: these authors proposed that the lack of this inhibitory receptor may
583 lead to increased spontaneous formation of immune complexes in FcγRIIB^{-/-} mice, that could
584 compete with IgG2a-immune complexes. In light of our results comparing IgG1-, IgG2a- and
585 IgG2b-PSA, we rather propose that the significantly lower affinity of inhibitory FcγRIIB for
586 IgG2a ($K_A = 4.2 \cdot 10^5 \text{ M}^{-1}$) than for IgG1 ($K_A = 3.3 \cdot 10^6 \text{ M}^{-1}$) and IgG2b ($K_A = 2.2 \cdot 10^6 \text{ M}^{-1}$) is the
587 determining factor (Table 1). Another factor may be the variance in expression of FcγRIIB on
588 circulating myeloid cells: basophils > monocytes > eosinophils >> neutrophils²⁰. Whereas the
589 exact numbers of expressed activating FcγRIII and inhibitory FcγRIIB per cell remain unknown,
590 flow cytometric analysis allowed the estimation of their relative expression: indeed, the ratio
591 FcγRIII/FcγRIIB is higher on neutrophils than on monocytes and basophils. These differential
592 expression levels may thus explain why neutrophils contribute to anaphylaxis, as the receptor
593 balance is in favor of the activating receptor. Strikingly, FcγRIIB is co-expressed only with
594 FcγRIII on basophils and Ly6C^{hi} monocytes, whereas it is co-expressed with FcγRIII and FcγRIV
595 on neutrophils and Ly6C^{lo} monocytes³⁴. Contribution of a given cell type to anaphylaxis may
596 therefore be favored when inhibitory FcγRIIB is required to dampen the stimulatory potential of
597 two activating IgG receptors instead of one. This concept extends to IgG1-immune complexes
598 that only engage one activating receptor, FcγRIII.

599
600 Our results on the contribution of mouse IgG receptors, cells and mediators in IgG-
601 induced anaphylaxis can potentially be translated to human IgG-mediated anaphylaxis, *e.g.*
602 following intravenous IgG or therapeutic IgG antibody administration. Indeed, even though IgG
603 receptors are different in the two species, we have already reported that human FcγRI (hFcγRI)
604 and human FcγRIIA (hFcγRIIA) can induce anaphylaxis when expressed under the control of

605 their own promoter in transgenic mice^{23, 24}. hFcγRI (CD64) is the equivalent of mouse FcγRI
606 whereas hFcγRIIA (CD32A) can be regarded as the equivalent of mouse FcγRIII, and hFcγRIIA
607 (CD16A) the equivalent of mouse FcγRIV⁷. hFcγRIIA, like mouse FcγRIII, is expressed on all
608 myeloid cells and could therefore act as the principal IgG receptor responsible for anaphylaxis in
609 humans. hFcγRIIB, the equivalent of mouse FcγRIIB, is scarcely expressed on most circulating
610 myeloid cells⁴⁷ except for its high expression on basophils²⁰, suggesting that among myeloid cells
611 only human basophils are highly sensitive to hFcγRIIB-mediated inhibition. In contrast to mouse
612 FcγRI, hFcγRI is constitutively expressed on circulating monocytes and inducibly on neutrophils,
613 allowing this receptor to induce anaphylaxis²⁴. The binding of human IgG subclasses to hFcγRs
614 differs strikingly from the binding of mouse IgG subclasses to mouse FcγRs. Noticeably, the
615 affinity of hFcγRIIB for any human IgG subclass is the lowest among human IgG-hFcγR
616 interactions. For example, human IgG1, the equivalent of mouse IgG2a, is bound by all activating
617 hFcγRs ($K_A > 10^6 \text{ M}^{-1}$) with at least a ten-fold higher affinity than by inhibitory hFcγRIIB ($K_A \approx$
618 10^5 M^{-1})⁴⁸. If we consider the translation of our results obtained in the mouse to human IgG-
619 induced anaphylaxis, one could anticipate that hFcγRIIB-mediated inhibition of IgG-induced
620 anaphylaxis is inefficient in human neutrophils and monocytes, and efficient only in human
621 basophils for which the elevated hFcγRIIB expression may compensate for the low-affinity of
622 this receptor for human IgG subclasses. Certainly, FcγR-engagement by IgG immune complexes
623 on human basophils could not trigger any detectable basophil activation *in vitro*²⁰, similar to the
624 results we reported for mouse basophil activation. Our data altogether propose that the
625 differential expression of inhibitory FcγRIIB on myeloid cells and its differential binding of IgG
626 subclasses control the contribution of basophils, neutrophils and monocytes to IgG-dependent
627 anaphylaxis, thus revealing novel complexities in the mechanism of regulation of cell
628 populations, and therefore their contribution to IgG-mediated reactions *in vivo*.

629

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781

AUTHORSHIP AND CONFLICT OF INTEREST STATEMENTS

782
783 H.B. performed all experiments at the Institut Pasteur with contributions from P.E,
784 C.M.G, F.J.; R.S. and L.L.R contributed experiments using Cpa3-Cre; Mcl-1^{fl/fl} mice; B.I. and

785 O.G. genotyped mice and produced reagents; M.S.C., S.J.G. and N.v.R. provided reagents; H.B.,
 786 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.
 787 and D.A.M. supervised and designed the research; P.B. and F.J. wrote the manuscript. All authors
 788 read and had an opportunity to contribute to the editing of the manuscript, and declare no
 789 competing financial interests.

790 TABLES

791

792 **Table 1:** Affinities of mouse FcγR-IgG subclass interactions (K_A values in M^{-1})

793

	IgG1	IgG2a	IgG2b	IgG3
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	-

794 “-”, no detectable affinity.

795 “(+)”, under debate^{11, 12}.

796 *Data compiled from* ^{17, 21}

797

798

799 **FIGURE LEGENDS**

800

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

806

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

813

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

819

820 **Figure 4. High doses of IgG2 antibodies reveal FcγRIV contribution to IgG2-PSA.** **(A)** PSA
821 in indicated mice injected with various doses of IgG2a anti-TNP mAbs ($n=2$ /group). **(B-E)** PSA

822 in indicated mice (B&C: n=8/group; D&E: n \geq 3/group) injected with indicated doses of anti-TNP
823 mAbs. Data are pooled from two independent experiments. Significant differences compared to
824 the untreated WT group are indicated.

825
826 **Figure 5. Expression of Fc γ Rs on myeloid cells following IgG2b-PSA.** (A) Fc γ RIII (left:
827 n=8/group, right: n=3/group), (B) Fc γ RIV (n=8/group) and (C) Fc γ RIIB expression (n \geq 6/group)
828 on cells from WT mice (n=8/group) left untreated, injected with IgG2b anti-TNP mAbs, or
829 injected with IgG2b anti-TNP mAbs and challenged with TNP-BSA. (D) Compilation of Δ
830 Geomean \pm SEM data from A-C.

831
832 **Figure 6. Cell contributions to IgG1-PSA in the absence of inhibitory Fc γ RIIB.** Fc γ RIIB^{-/-}
833 mice were pretreated as indicated, then injected with IgG1 anti-TNP mAbs, challenged with
834 TNP-BSA and central temperatures were monitored (A: n=8/group; B: n=7/group; C:
835 n=10/group). Data are represented as mean \pm SEM. Data are pooled from two independent
836 experiments.

837
838 **Figure 7. Contributions of histamine and PAF to IgG-PSA.** Body temperatures of pretreated
839 mice during (A) IgG1-PSA in Fc γ RIIB^{-/-} (n=6/group) or WT mice (n=4/group), (B) IgG2a-PSA,
840 (C) IgG2b-PSA or (D) IgE-PSA in WT mice (n \geq 7/group). (E) Histamine and (F) mMCP-1
841 concentrations post-PSA (n=3/group). Data are representative of at least two independent
842 experiments, except for A&C (pooled from two independent experiments).

843

844

Figure 1

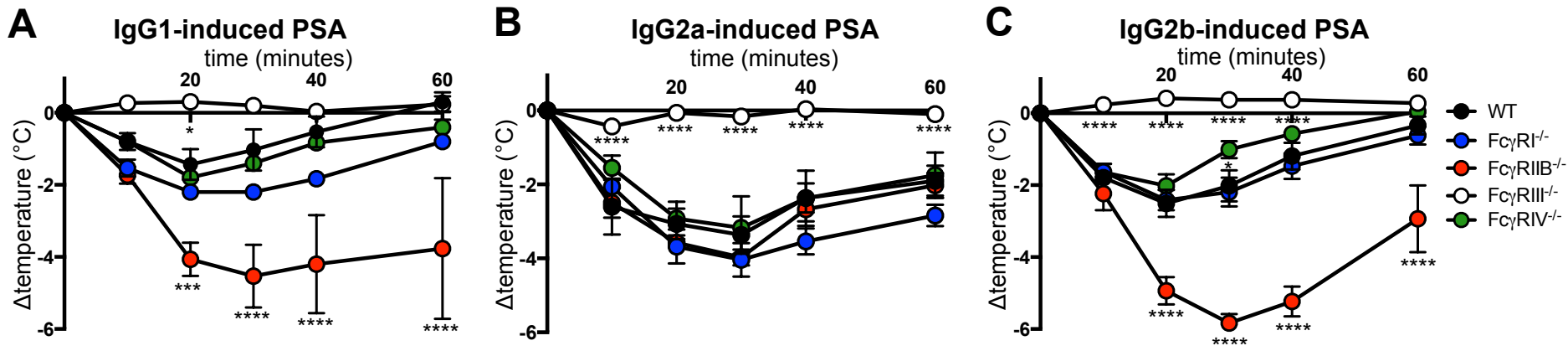
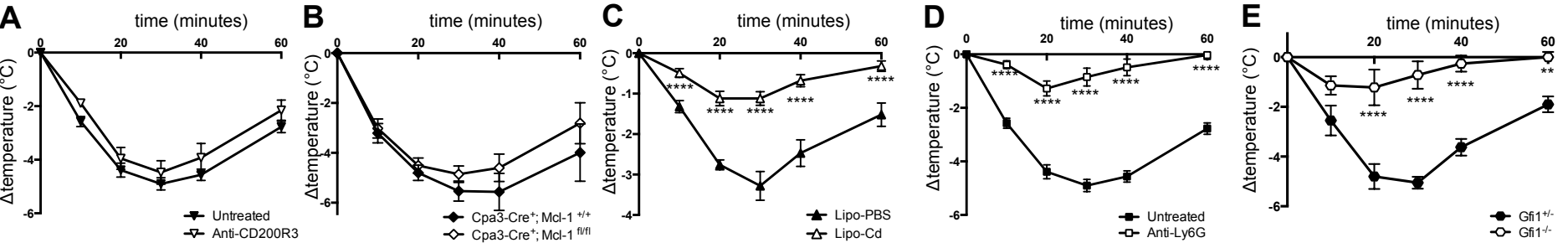


Figure 2

IgG2a-induced PSA



IgG2b-induced PSA

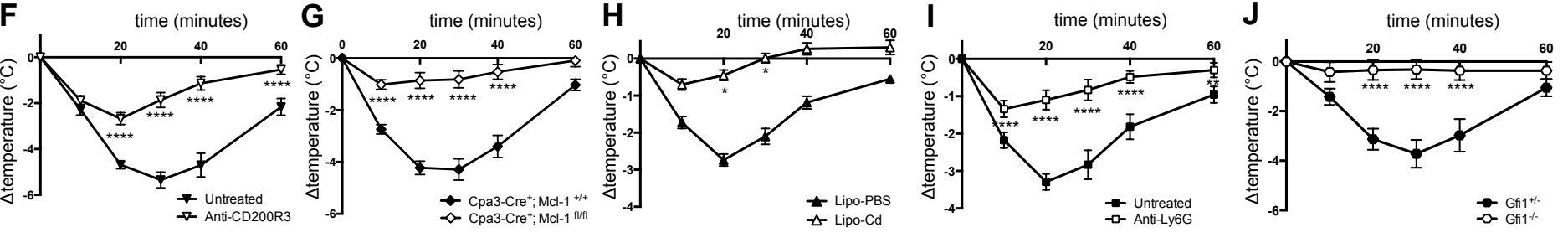
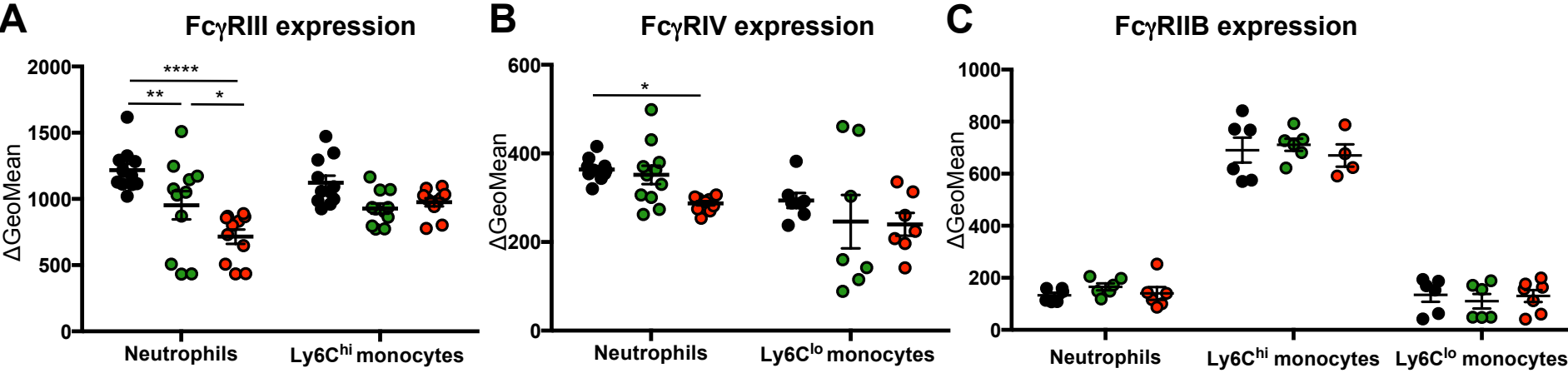


Figure 3

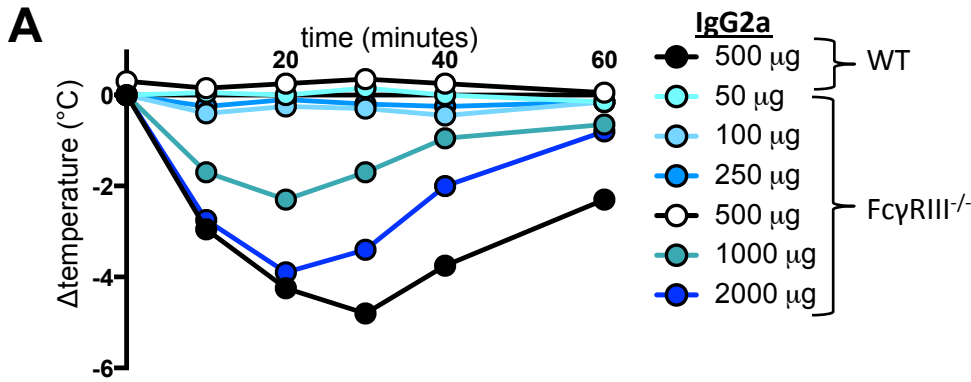


D	FcγRIII expression	Untreated	19h post-IgG2a	19h post-IgG2a, 3h post-Ag
	Neutrophils	1217 ± 49	952 ± 106	715 ± 54
	Ly6C^{hi} monocytes	1123 ± 53	927 ± 39	975 ± 31
	FcγRIV expression	Untreated	19h post-IgG2a	19h post-IgG2a, 3h post-Ag
	Neutrophils	363 ± 7.3	352 ± 21	287 ± 5
	Ly6C^{lo} monocytes	294 ± 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

- Untreated
- 19h post-IgG2a
- 19h post-IgG2a, 3h post-Ag

Figure 4

IgG2a-induced PSA



IgG2b-induced PSA

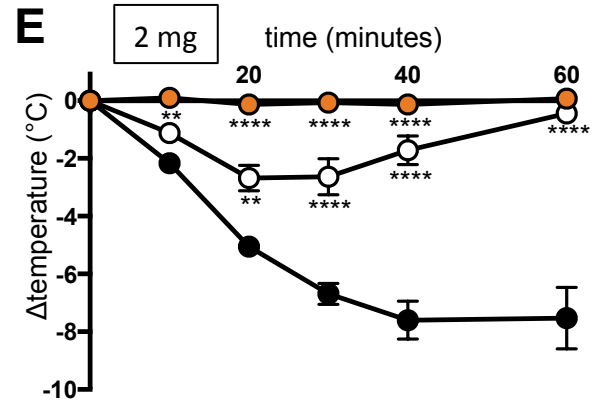
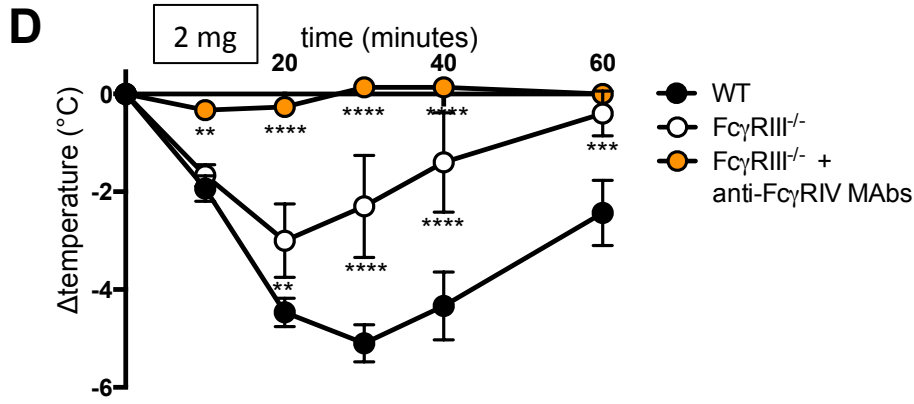
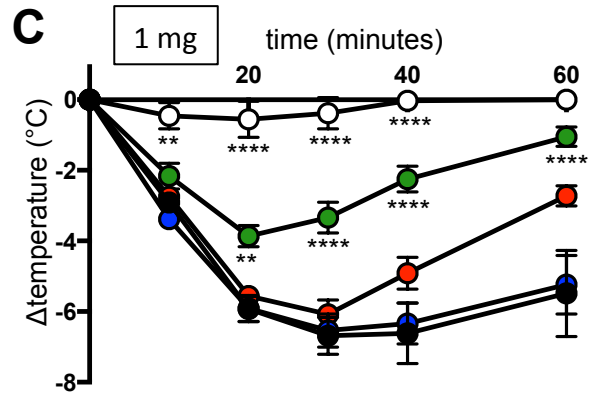
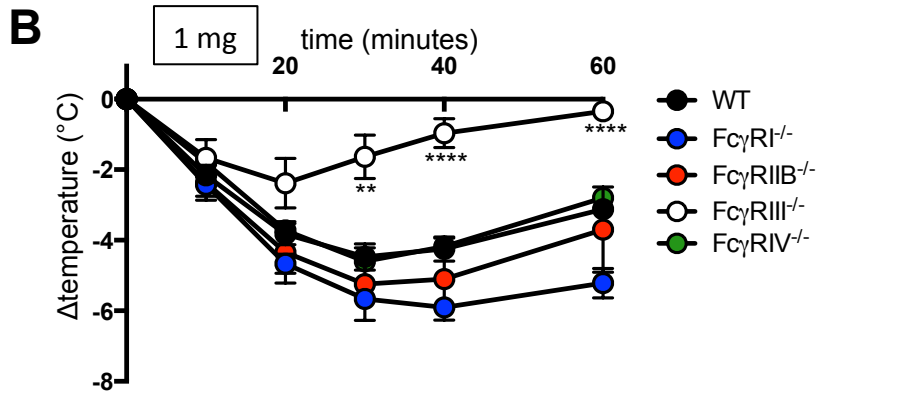
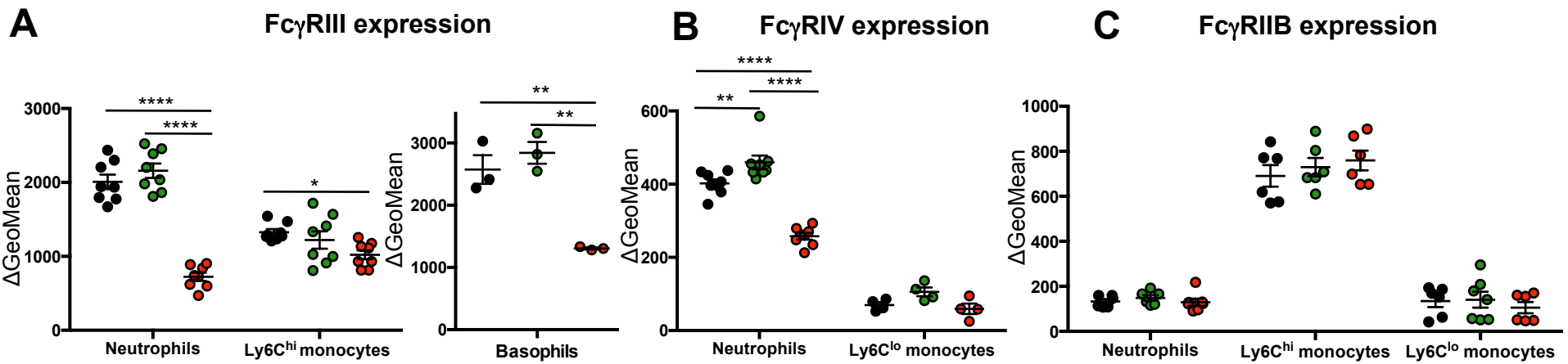


Figure 5



D

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

● Untreated
 ● 19h post-IgG2b
 ● 19h post-IgG2b, 3h post-Ag

Figure 6

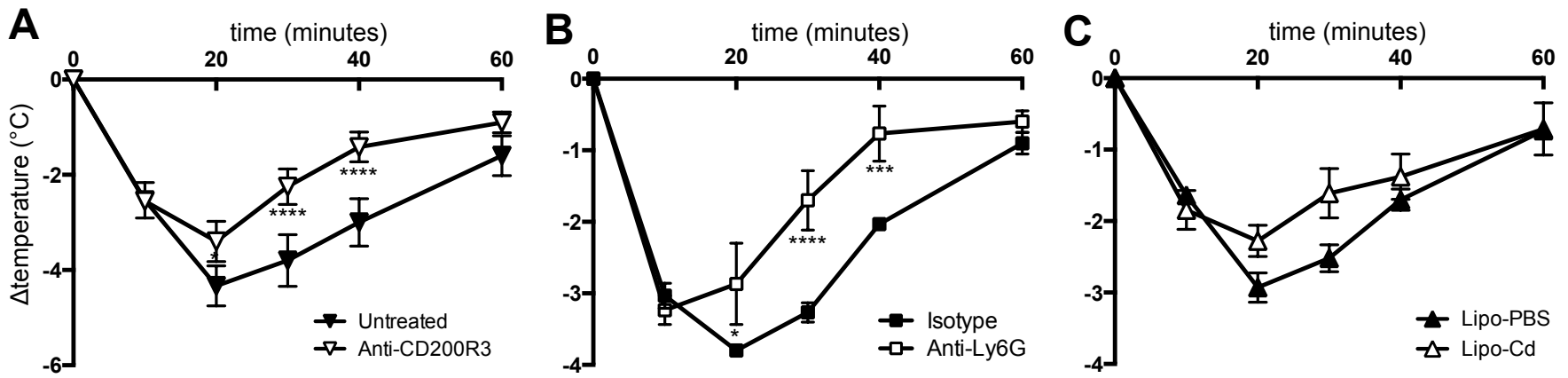
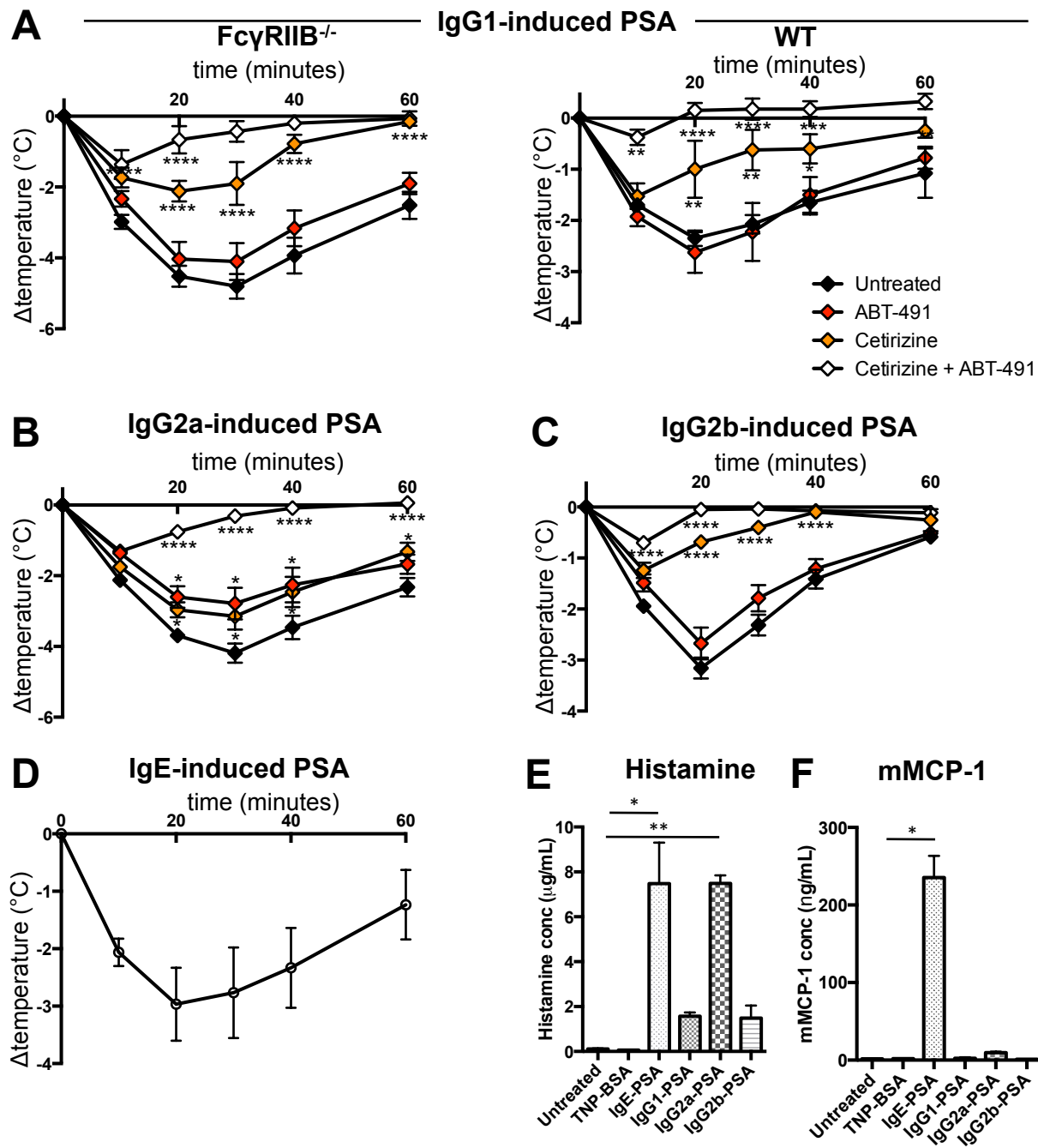


Figure 7

1 **Supplemental Figure 1. Effects of depletion strategies on myeloid cell populations – Cell**
2 **counts.** WT mice were treated with indicated reagents. 24 hours after injection, counts of specific
3 cell populations were determined by flow cytometry (A-G) or histology (I&J); leukocyte counts
4 in total blood were measured with an automatic blood analyzer (H). Counts of (A) basophils, (B)
5 neutrophils, (C) Ly6C^{hi} monocytes and (D) Ly6C^{lo} monocytes in blood, spleen and bone marrow,
6 (E) peritoneal mast cells (F) peritoneal macrophages and (G) splenic macrophages. (I)
7 Representation of a toluidine blue-stained back skin section with two mast cells (arrows). (J)
8 Counts of mast cells/mm² in the dermis of WT mice. (A-H) Figures show one of three
9 independent experiments. Individual measurements and mean +/- SEM are represented. Iso =
10 isotype rat IgG2b, Ba103 = anti-CD200R3 mAb, NIMP = anti-Ly6G mAb, PBS = PBS
11 liposomes, CS= clodronate liposomes.

12
13 **Supplemental Figure 2. Effects of depletion strategies on myeloid cell populations –**
14 **Frequencies.** WT mice were treated with indicated reagents. 24 hours after injection, percentages
15 of specific cell populations among CD45⁺ cells were determined by flow cytometry (A-H): (A)
16 basophils, (B) neutrophils, (C) Ly6C^{hi} monocytes and (D) Ly6C^{lo} monocytes in blood, spleen and
17 bone marrow, (E) peritoneal mast cells (F) peritoneal macrophages and (G) splenic macrophages.
18 (H) Percentages of peritoneal mast cells (pMC FcεRI⁺/cKit⁺) and blood basophils (FcεRI⁺/DX5⁺)
19 in Cpa3-Cre; Mcl-1^{fl/fl} mice and in Cpa3-Cre; Mcl-1^{+/+} mice. (I) Left: Percentages of YFP-
20 positive cells in MRP8-Cre; Rosa26-YFP mice. Right: Effect of NIMP-R14 injection on
21 neutrophils (percentages and counts CD45⁺/YFP⁺/Ly6C^{neg}/CD115^{neg} cells) in blood, spleen and
22 bone marrow of MRP8-Cre; Rosa26-YFP mice. (A-H) Figures show corresponding percentages
23 to cell counts shown in Supplemental Figure 1 and display values for individually measured mice

24 and the mean and SEM. Iso = isotype rat IgG2b, PBS = PBS liposomes, CS= clodronate
25 liposomes.

26
27 **Supplemental Figure 3. Relative affinity of IgG1 (TIB191), IgG2a (Hy1.2) and IgG2b**
28 **(GORK) anti-TNP to TNP-BSA. (A) ELISA anti-TNP.** Comparison of binding capacity of
29 TIB 191, Hy1.2 or GORK to immobilized TNP-BSA. Data are represented as mean +/- SEM and
30 representative of results from five independent experiments. **(B) Surface plasmon resonance**
31 **analysis.** Comparison of binding affinity TNP-BSA to immobilized TIB 191, Hy1.2 or GORK
32 clones. **(C)** The table recapitulates the k_{on} , k_{off} and K_d for each condition.

33
34 **Supplemental Figure 4. IgG1-PSA induces mild hypothermia in WT mice and**
35 **monocytes/macrophages and neutrophils contribute to IgG2a-PSA in Balb/c mice. (A) WT**
36 mice were injected with IgG1 anti-TNP mAbs, challenged with TNP-BSA and body temperatures
37 were monitored. PSA in mice left untreated, injected with anti-Ly6G or anti-CD200R3
38 (n=4/group). **(B)** Balb/c mice were left untreated, injected with anti-Ly6G, anti-CD200R3
39 (n=6/group), lipo-PBS (n=6/group) or lipo-Cd (n=6/group) prior to IgG2a-PSA induction. Body
40 temperatures were monitored. Data are represented as mean +/- SEM. Data are pooled from two
41 independent experiments. Significant differences compared to the untreated group are indicated.

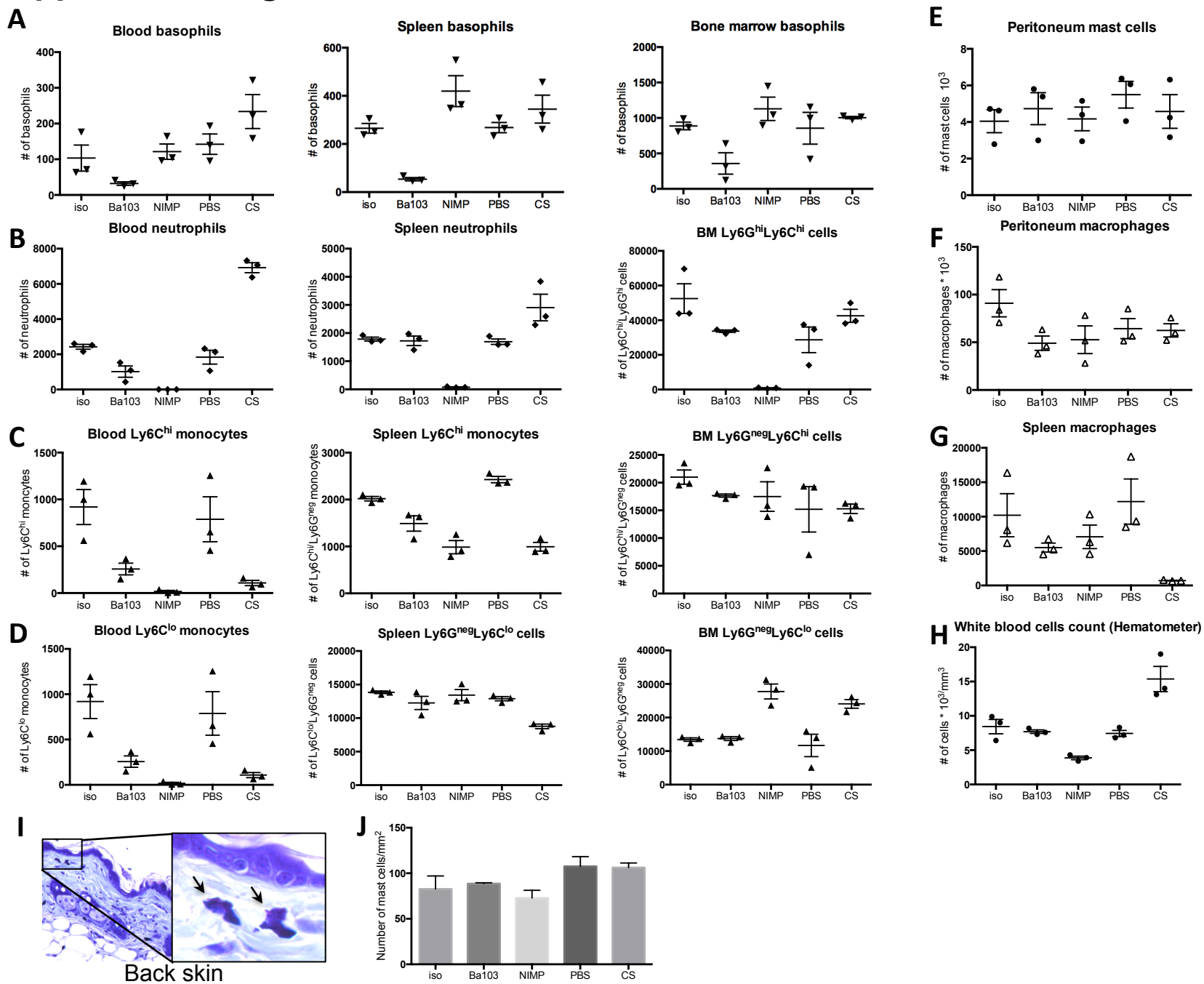
42
43 **Supplemental Figure 5. FcγR expression in FcγR-deficient mice.** Expression of **(A) FcγRIII,**
44 **(B) FcγRIV** and **(C) FcγRIIB** is represented as the Δ Geomean of FcγR-specific staining
45 compared to isotype control staining from blood leukocytes collected from untreated WT, FcγRI
46 ^{-/-}, FcγRIIB^{-/-}, FcγRIII^{-/-} and FcγRIV^{-/-} mice (n=4/group). Data are represented as mean +/- SEM.

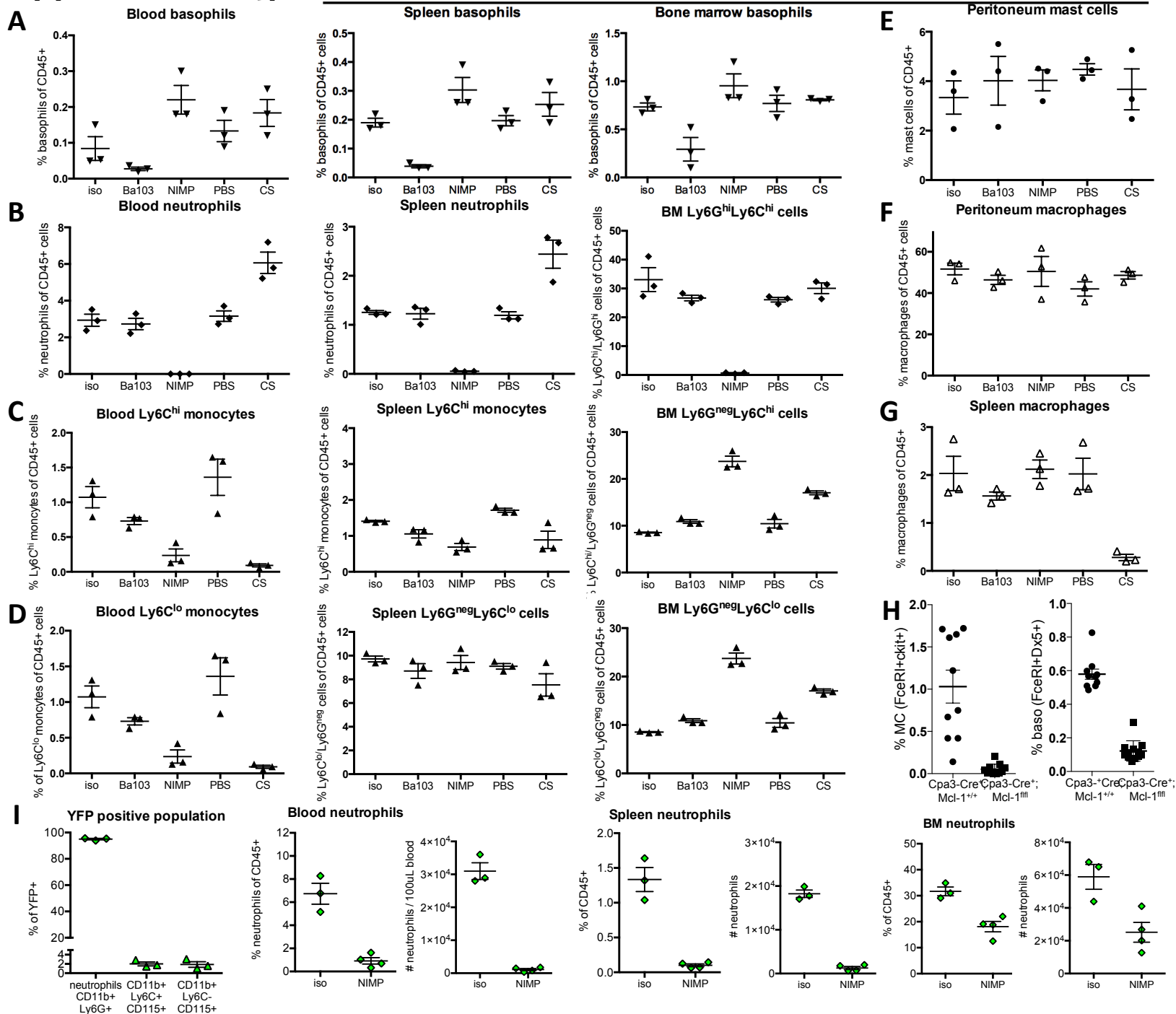
47

48 **Supplemental Figure 6. Blood leukocyte numbers in FcγR-deficient mice.** Leukocyte
49 populations were assessed using an ABC Vet automatic blood analyzer (Horiba ABX) from
50 blood collected from untreated WT, FcγRI^{-/-}, FcγRIIB^{-/-}, FcγRIII^{-/-} and FcγRIV^{-/-} mice
51 (n=4/group). “Granulocytes” represent mainly neutrophils (as judged by their size and
52 granularity). Data are represented as mean +/- SEM; each point represents one mouse.

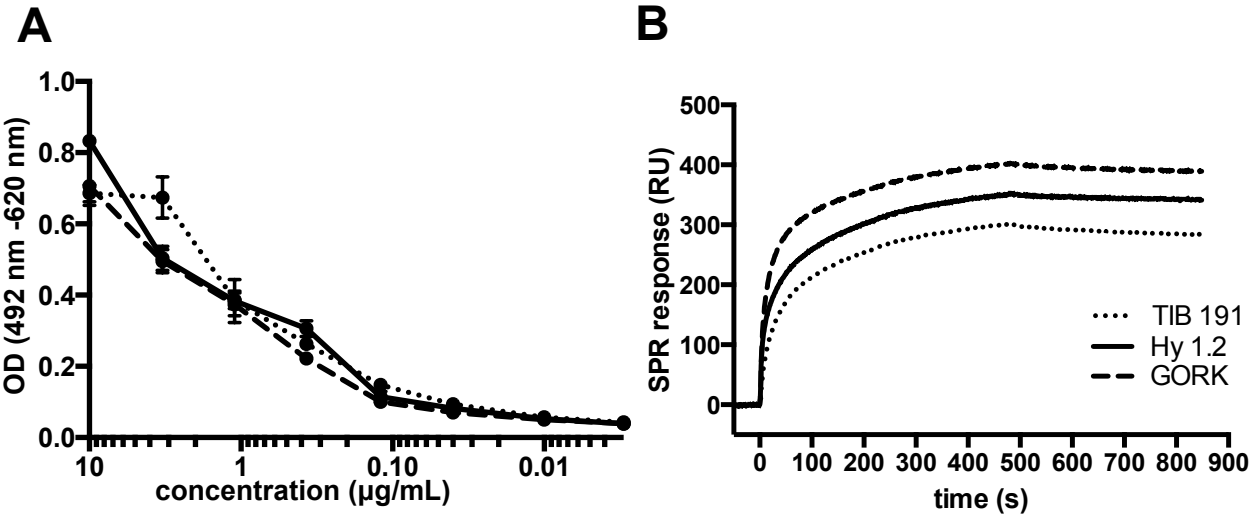
53
54 **Supplemental Figure 7. Mast cell degranulation after IgG1, IgG2a and IgG2b-induced PSA.**
55 WT mice were injected with IgE, IgG1, IgG2a, IgG2b anti-TNP mAbs or left untreated (n=3 for
56 all groups) and challenged with TNP-BSA. Mouse ear skin biopsies were collected 30 minutes
57 after TNP-BSA injection. Representation of a toluidine blue-stained ear skin sections with one
58 mast cell (indicated by an arrow) for one mouse of each group of mice.

59





Supplemental Figure 3

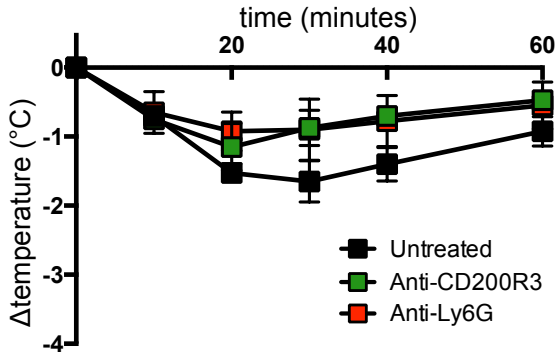


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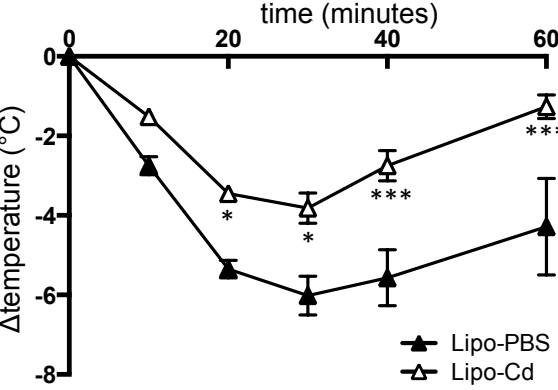
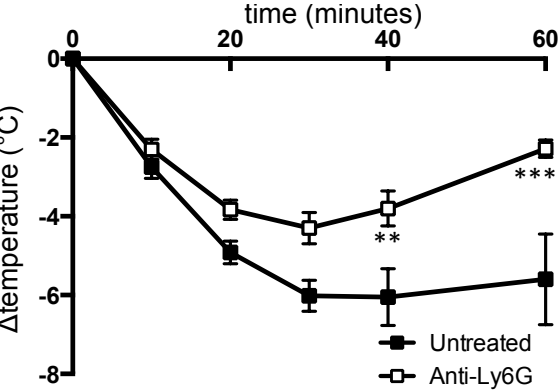
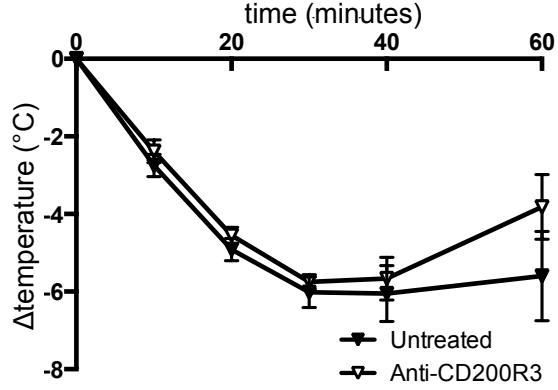
	k_{on} ($10^5 \text{M}^{-1} \text{s}^{-1}$)	k_{off} (10^{-4}s^{-1})	K_d (nM)
TIB 191 (IgG1)	0.97 (± 0.29)	2.27 (± 0.32)	2.34 (± 0.33)
Hy1.2 (IgG2a)	1.43 (± 0.43)	1.08 (± 0.30)	0.76 (± 0.31)
GORK (IgG2b)	2.15 (± 0.65)	1.19 (± 0.24)	0.55 (± 0.20)

Supplemental Figure 6

A IgG1-induced PSA in wt mice

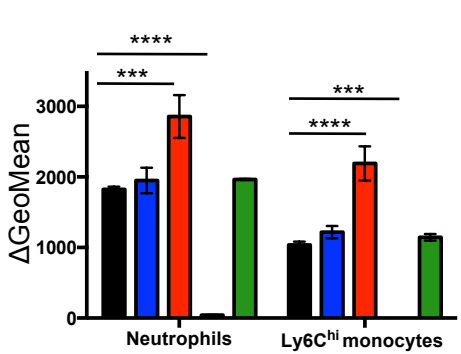


B IgG2a-induced PSA in wt Balb/c mice

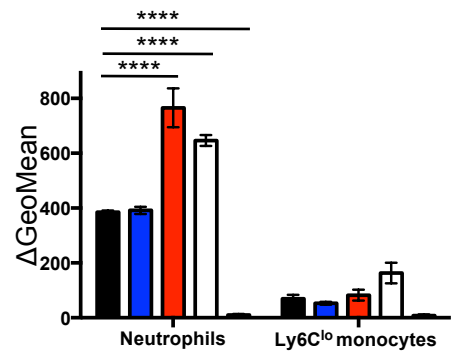


Supplemental Figure 5

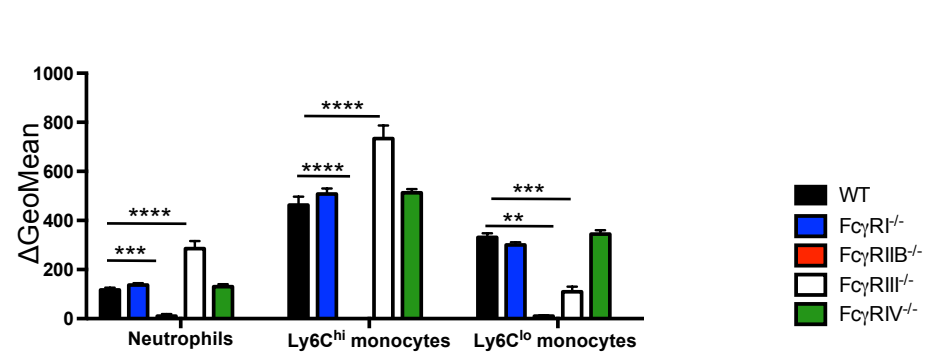
A FcγRIII expression



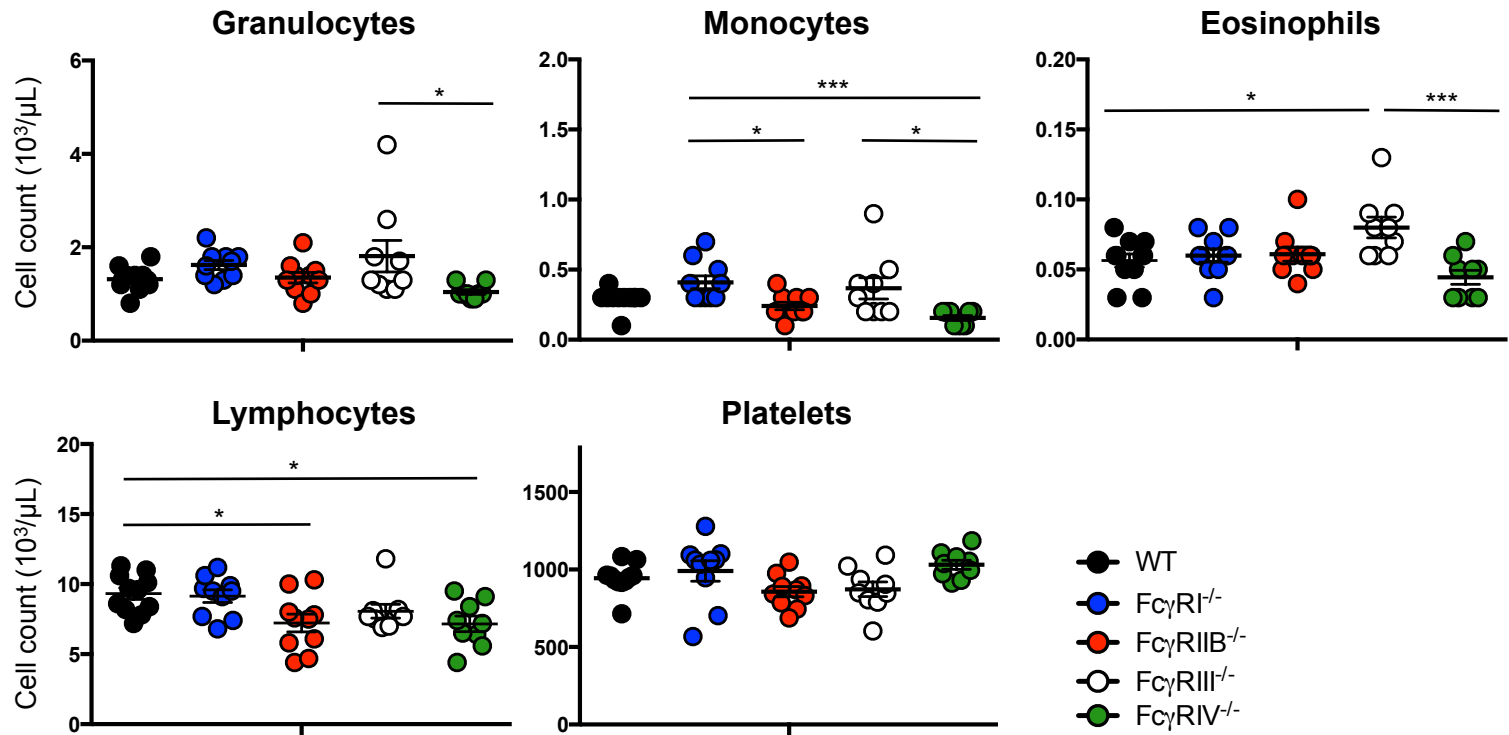
B FcγRIV expression



C FcγRIIB expression



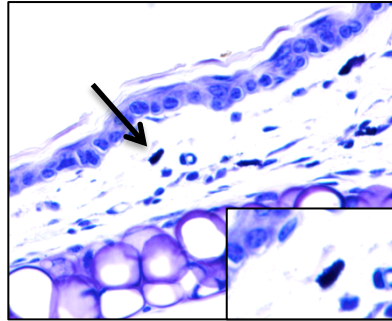
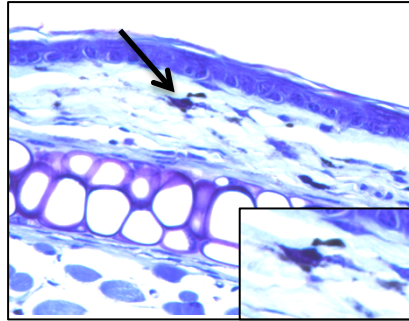
Supplemental Figure 6



Supplemental Figure 7

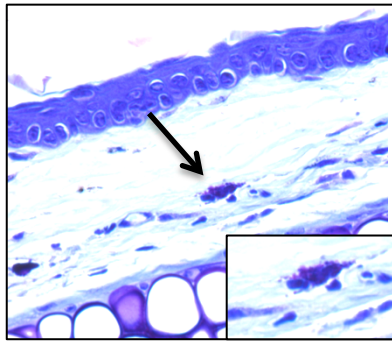
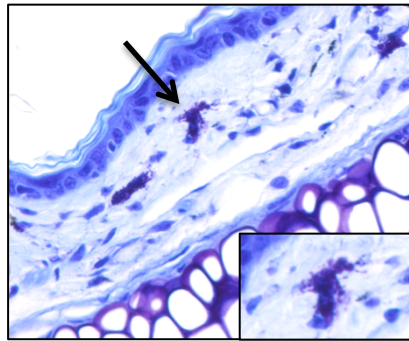
Untreated

TNP-BSA



IgE-PSA

IgG1-PSA



IgG2a-PSA

IgG2b-PSA

