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1 **Intravenous immunoglobulin induces IL-4 in human basophils by signaling through**  
2 **surface-bound IgE**

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4 **Caroline Galeotti, MD, PhD<sup>1,2</sup>, Emmanuel Stephen-Victor, PhD<sup>1,\*</sup>, Anupama**  
5 **Karnam, MSc<sup>1,\*</sup>, Mrinmoy Das, PhD<sup>1</sup>, Laurent Gilardin, MD<sup>1,3</sup>, Mohan S Maddur,**  
6 **DVM, PhD<sup>1,4</sup>, Sandra Wymann, PhD<sup>5</sup>, Cédric Vonarburg, PhD<sup>5</sup>, Alain Chevailler,**  
7 **MD, PhD<sup>6</sup>, Jordan D Dimitrov, PhD<sup>1,4</sup>, Olivier Benveniste, MD<sup>3,7</sup>, Pierre Bruhns,**  
8 **PhD<sup>8,9</sup>, Srimi V Kaveri, DVM, PhD<sup>1,4</sup>, Jagadeesh Bayry, DVM, PhD<sup>1,4</sup>**

9  
10 <sup>1</sup>Institut National de la Santé et de la Recherche Médicale; Centre de Recherche des  
11 Cordeliers, Equipe-Immunopathologie et Immunointervention Thérapeutique; Sorbonne  
12 Université, Paris, F-75006, France

13 <sup>2</sup>Service de Rhumatologie Pédiatrique, Centre de Référence des Maladies Auto-  
14 Inflammatoires rares et des Amyloses, CHU de Bicêtre, le Kremlin Bicêtre, F-94270,  
15 France

16 <sup>3</sup>Département de Médecine Interne et Immunologie Clinique, Hôpital Pitié-Salpêtrière, AP-  
17 HP, Paris, F-75013, France.

18 <sup>4</sup>Université Paris Descartes, Sorbonne Paris Cité, Paris, F-75006, France

19 <sup>5</sup>Research Department, CSL Behring AG, 3014 Bern, Switzerland

20 <sup>6</sup>Laboratoire d'Immunologie et d'Allergologie; CHU d'Angers; Université d'Angers;  
21 INSERM Unité 1232; LabEx IGO "Immuno-Graft-Onco", Angers, F-49933, France

22 <sup>7</sup>Sorbonne Université, Institut National de la Santé et de la Recherche Médicale Unité 974,  
23 Paris, F-75013, France.

24 <sup>8</sup>Institut Pasteur, Department of Immunology, Unit of Antibodies in Therapy and  
25 Pathology, Paris, France

26 <sup>9</sup>INSERM Unité 1222, Paris, France

27 \* Equally contributed

28

29 **Correspondence to:** Jagadeesh Bayry, Institut National de la Santé et de la Recherche  
30 Médicale Unité 1138, Centre de Recherche des Cordeliers, 15 rue de l'Ecole de Médecine,  
31 Paris, F-75006, France. Tel: 00 33 1 44 27 82 03; Fax: 00 33 1 44 27 81 94  
32 E-mail: [jagadeesh.bayry@crc.jussieu.fr](mailto:jagadeesh.bayry@crc.jussieu.fr)

33

34

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46 **ABSTRACT**

47 **BACKGROUND:** Therapeutic normal immunoglobulin G or intravenous immunoglobulin  
48 (IVIG) exerts anti-inflammatory effects via several mutually nonexclusive mechanisms.  
49 Recent data in mouse models of autoimmune diseases suggest that IVIG induces IL-4 in  
50 basophils by enhancing IL-33 in SIGN-R1<sup>+</sup> innate cells. However, translational insight on  
51 these data is lacking.

52 **OBJECTIVE:** We sought to investigate the effect of IVIG on human basophil functions.

53 **METHODS:** Isolated circulating basophils from the healthy donors were cultured in the  
54 presence of IL-3, IL-33, GM-CSF, TSLP or IL-25. The effect of IVIG, F(ab')<sub>2</sub> and Fc  
55 fragments of IVIG was examined on the expression of various surface molecules,  
56 phosphorylation of Syk, induction of cytokines, and histamine release. Phenotype of  
57 basophils was also analyzed from IVIG-treated myopathy patients. Approaches such as  
58 depletion of anti-IgE-reactivity from IVIG, blocking antibodies or inhibitors were used to  
59 investigate the mechanisms.

60 **RESULTS:** We report that IVIG directly induces activation of IL-3-primed human  
61 basophils, but IL-33 and other cytokines were dispensable for this effect. The activation of  
62 basophils by IVIG led to enhanced expression of CD69 and secretion of IL-4, IL-6 and IL-  
63 8. IVIG-treated myopathy patients displayed enhanced expression of CD69 on the  
64 basophils. Syk pathway is implicated in these functions of IVIG and were mediated via  
65 F(ab')<sub>2</sub> fragments. Mechanistically, IVIG induced IL-4 in human basophils by interacting  
66 with basophil surface-bound IgE but independent of FcγRII, type II Fc receptors, C-type  
67 lectin receptors and Siglecs.

68 **CONCLUSION:** These results uncovered a pathway of promoting Th2 response by IVIG  
69 through direct interaction of IgG with human basophils.

70 **Key Messages**

- 71 • IVIG induces activation and secretion of IL-4, IL-6 and IL-8 in IL-3-primed human  
72 basophils but unlike mice IL-33 was dispensable
- 73 • IVIG induces human basophil activation via F(ab')<sub>2</sub> fragments but independent of  
74 FcγRII, C-type lectin receptors, type II Fc receptors and Siglecs
- 75 • Basophil activation by IVIG is mediated by a fraction of IgG that signals through  
76 basophil surface-bound IgE and the Syk pathway

77 **Capsule summary**

78 Therapeutic normal IgG (IVIG) activates human basophils through direct interaction with  
79 basophil surface-bound IgE, and by IL-3- and Syk-dependent mechanisms to promote Th2  
80 responses in the context of therapy of autoimmune diseases.

81

82 **Key words**

83 FcεRI, Anti-IgE IgG, Antisynthetase syndrome, Polymyositis, Dermatomyositis, DC-  
84 SIGN, DCIR, FcγRIIB

85 **Abbreviations**

86 DCIR: Dendritic cell immunoreceptor  
87 DC-SIGN: dendritic cell-specific ICAM-3-grabbing nonintegrin  
88 FcεRI: Fc epsilon type 1 receptor, high affinity IgE receptor  
89 FcγR: Fc gamma receptor  
90 FcγRIIA: Fc gamma type 2 receptor A  
91 FcγRIIB: Fc gamma type 2 receptor B  
92 HSA: Human serum albumin  
93 IVIG: Intravenous immunoglobulin  
94 SIGN-R1: SIGN- related 1  
95 SYK: Spleen tyrosine kinase

96 **INTRODUCTION**

97 Intravenous immunoglobulin (IVIG) is one of the widely used immunotherapeutic  
98 molecules for the treatment of diverse autoimmune and systemic inflammatory diseases.<sup>1-4</sup>  
99 High-dose (1-2g/kg) IVIG therapy exerts anti-inflammatory effects by several mutually  
100 non-exclusive mechanisms including inhibition of the activation of innate immune cells,  
101 effector T (Th1, Th17) and B cells, suppression of complement pathway, neutralization of  
102 inflammatory cytokines and pathogenic antibodies, and expansion of regulatory T cells.  
103 These actions of IVIG implicate both Fc- and F(ab')<sub>2</sub> fragments.<sup>5,6</sup>

104 Basophils are one of the rare granulocytes. They express various receptors to sense the  
105 signals including FcεRI, a high affinity receptor for IgE, toll-like receptors and cytokine  
106 receptors such as IL-3 receptor (CD123), IL-33 receptor (IL-33R) and thymic stromal  
107 lymphopoietin (TSLP) receptor. Activated basophils secrete several cytokines including  
108 IL-4, IL-8 and IL-6, and regulate Th2 polarization, immunoglobulin synthesis and class-  
109 switch in B cells.<sup>7,8</sup>

110 Recent results from experimental models of systemic inflammatory and autoimmune  
111 diseases suggest that the anti-inflammatory effects of IVIG are mediated via basophils by a  
112 two-step process.<sup>9</sup> IL-33 produced by SIGN-R1<sup>+</sup> innate cells upon interaction with Fc-  
113 α(2,6)-sialic acid linkages, activates basophils via IL-33R to induce IL-4. The basophil-  
114 derived IL-4 enhances the expression of inhibitory FcγRIIB on effector macrophages<sup>9</sup> thus  
115 adding onto the previously known function of basophil-derived IL-4 in programming anti-  
116 inflammatory macrophages.<sup>10</sup> However, translational insight on these data is lacking. In  
117 particular, DC-SIGN (human orthologue of SIGN-R1)-positive human innate cells did not  
118 produce IL-33 when exposed to IVIG indicating that the proposed pathway of basophil  
119 activation by IVIG does not apply to humans.<sup>11</sup> When patients are infused with high-dose

120 IVIG, the IgG theoretically interacts with every component of the immune system.  
121 Therefore, it is most likely that IVIG modulates human basophils through direct interaction  
122 rather than indirect pathway of DC-SIGN-dependent IL-33.

123 In line with our proposition, we report that IVIG directly induces the activation of human  
124 basophils and secretion of IL-4, IL-6 and IL-8 through interaction with basophil surface-  
125 bound IgE, and by IL-3- and Syk-dependent mechanisms. These functions of IVIG were  
126 mediated via F(ab')<sub>2</sub> fragments and were independent of IL-33, FcγRII, type II FcRs, C-  
127 type lectin receptors and Siglecs. Basophils from IVIG-treated myopathy patients also  
128 displayed enhanced expression of activation marker CD69. In the context of systemic  
129 autoimmune and inflammatory diseases, these results thus provide a unique pathway of  
130 promoting Th2 response by IVIG through direct interaction of IgG with human basophils.

131

132 **METHODS**

133 **Preparations of IVIG**

134 Sandoglobulin® (CSL Behring, Switzerland) was dialyzed against a large volume of PBS  
135 three times followed by RPMI-1640 at 4°C for 18 hours to remove the stabilizing agents.

136 F(ab')<sub>2</sub> fragments of IVIG were prepared by pepsin digestion (2% wt/wt; Sigma Aldrich)  
137 followed by chromatography on a protein G Sepharose column (Pharmacia). Fc fragments  
138 of IVIG were prepared by papain digestion (papain-coupled beads, Life Technologies)  
139 followed by protein A Sepharose column chromatography and size-exclusion  
140 chromatography. End purification was performed by chromatography on an IgG-CH1  
141 column (Life Technologies). The purity of F(ab')<sub>2</sub> and Fc fragments were confirmed by  
142 SDS-PAGE.

143 **Isolation and culture of basophils**

144 Basophils were isolated from the PBMC of healthy donors buffy bags (Centre Necker-  
145 Cabanel, EFS, Paris, INSERM-EFS ethical permission N°12/EFS/079 and N°18/EFS/033)  
146 by using basophil isolation kit II (Miltenyi Biotec) and autoMACS® (Miltenyi Biotec). The  
147 purity of basophils based on the expression of FcεRI and CD123 was ≈97%.

148 To investigate the effect of IVIG on IL-3-primed basophils, cells (0.1x10<sup>6</sup>/well/200 μL)  
149 were cultured in 96 well U-bottomed plate either alone in serum-free X-VIVO 15 medium;  
150 or with IL-3 (100 ng/mL, ImmunoTools); or with IL-3 plus IVIG (25 mg/mL) or human  
151 serum albumin (HSA, 10 mg/mL, LFB, France) or F(ab')<sub>2</sub> fragments (16 mg/mL) or Fc  
152 fragments (9 mg/mL) for 24 hours.

153 To explore the effect of other cytokines on IVIG-mediated regulation of basophils, cells  
154 were cultured with individual cytokines (IL-33:1 ng/mL, GM-CSF:10 ng/mL, IL-25:10

155 ng/mL or TSLP:100 ng/mL, all from ImmunoTools), or cytokines plus IVIG for 24 hours.  
156 Also, basophils were sequentially stimulated with IL-3 and IL-33 for one hour each and  
157 cultured with IVIG or HSA for additional 22 hours.

158 For blocking experiments, basophils were stimulated with IL-3 for 2 hours followed by  
159 incubation with blocking MAbs to Fc $\gamma$ RIIB (Clone:2B6 N<sub>297</sub>D; 10  $\mu$ g/mL), Fc $\gamma$ RIIA  
160 (Clone:IV.3; 10  $\mu$ g/mL) or isotype control MAbs for 1 hour and cultured with IVIG for  
161 additional 21 hours.

162 To investigate the implication of Syk pathway, basophils were stimulated with IL-3 for 2  
163 hours followed by incubation with Syk inhibitor, R406 (5  $\mu$ mol, InvivoGen) or DMSO for  
164 1 hour and cultured with IVIG for up to 24 hours.

165 Basophils were analyzed for the expression of various markers by flow cytometry (LSR II,  
166 BD Biosciences) using fluorochrome-conjugated MAbs. Phosphorylation of Syk was  
167 analyzed by using cell signaling buffer set A (Miltenyi Biotec). Data were analyzed by BD  
168 FACS DIVA (BD Biosciences) and Flowjo (FlowJo LLC). Cell-free culture supernatants  
169 were used for the analysis of histamine and cytokines.

#### 170 **Depletion of IgE-reactive IgG from IVIG**

171 Plasma IgE (5.427 mg/mL) from a patient with secreted IgE-myeloma was immobilized on  
172 a CNBr-activated Sepharose 4B (Sigma-Aldrich). IVIG was loaded (60 mg/mL) on to IgE  
173 Sepharose column and was incubated on a rotator at room temperature for 4 hours. The  
174 flow-through fraction was collected. Following elution of column-bound IgG, the flow-  
175 through IgG was again passed through the IgE Sepharose column for two more times. The  
176 IgG in the flow-through fraction was concentrated and the concentration was determined  
177 by spectrophotometer (NanoDrop Technologies).

178 IVIG depleted of anti-IgE-reactivity (25 mg/mL) was added to IL-3-primed basophils  
179 ( $0.1 \times 10^6$ /well/200  $\mu$ L) as described earlier for 24 hours.

#### 180 **Analysis of basophils from myopathy patients**

181 Heparinized blood from seven myopathy patients ( $45.71 \pm 5.9$  years; five men; ethical  
182 approval from CPP-Ile-de-France VI, Groupe Hospitalier Pitié-Salpêtrière, Paris) were  
183 collected before and 2-5 days post-IVIG treatment (2 g/kg). CD69 on the basophils  
184 ( $Fc\epsilon RI\alpha^+ CD203c^+$ ) was analyzed by flow cytometry. Due to low number, basophils were  
185 analyzed only in five patients (two patients with antisynthetase syndrome and one each  
186 with polymyositis, immune-mediated necrotizing myopathy or dermatomyositis).

#### 187 **Antibodies for flow cytometry and functional assays**

188 The details are provided in the supplementary file (in this article's Online Repository at  
189 [www.jacionline.org](http://www.jacionline.org))

#### 190 **Measurement of cytokines and histamine**

191 IL-4, IL-6 and IL-8 were analyzed in culture supernatants by ELISA (ELISA Ready-SET-  
192 Go, eBioscience Affymetrix). Histamine was measured in culture supernatants by  
193 histamine EIA kit (Bertin Pharma).

#### 194 **RNA isolation and real-time quantitative RT-PCR**

195 RNeasy Micro Kit (Qiagen) was used for RNA isolation from resting basophils, cells  
196 treated with IL-3 or IL-3 plus IVIG for three hours. Additionally, basophils were also  
197 treated with Syk inhibitor for one hour prior to stimulation with IL-3 plus IVIG. cDNA  
198 was synthesized using iScript<sup>TM</sup> cDNA synthesis kit (Bio-Rad). qRT-PCR was done using  
199 TaqMan<sup>TM</sup> Universal Master Mix II, with UNG (Applied Biosystems<sup>TM</sup>) and IL-4

200 expression was measured using TaqMan Gene Expression Assays (Applied Biosystems™)  
201 #Hs00174122\_m1 (IL-4), #Hs02786624\_g1 (GAPDH).

202 **Statistical analysis:**

203 Statistical analysis was performed by Prism 6 GraphPad Software. One-way analysis of  
204 variance (with Tukey's multiple comparison tests or Dunnet's multiple comparison tests),  
205 and two-way Mann Whitney were used to determine the statistical significance.

206

207

208 **RESULTS**

209 **IVIG induces activation and cytokine secretion in IL-3-primed basophils**

210 We first probed the effect of IVIG on resting basophils. However, IVIG did not modify  
211 either phenotype or functions of resting basophils based on the analysis of CD69 (Fig 1, A  
212 and B) and secretion of IL-4, IL-6 and IL-8 (Fig 1, C) indicating that resting basophils are  
213 not the targets for IVIG.

214 We then investigated whether IVIG modulates primed basophils, in particular IL-3, the  
215 major basophil priming cytokine. We found that under IL-3-priming, IVIG significantly  
216 enhanced CD69, an activation marker of basophils (Fig 1, D). On the other hand, the  
217 expression of CD13, CD62L, CD123 and CD203c (Fig E1, in this article's Online  
218 Repository at [www.jacionline.org](http://www.jacionline.org)), degranulation-associated markers CD63 (Fig 1, E) and  
219 CD107a (Fig E2, A and B), and histamine concentrations in the supernatants (Fig E2, C)  
220 were not significantly altered by IVIG.

221 Further, IVIG significantly enhanced IL-4, IL-6 and IL-8 secretion by IL-3-primed  
222 basophils (Fig 1, F). qRT-PCR analysis also confirmed *il4* induction by IVIG (Fig E3).  
223 Equimolar concentrations of HSA, used as a protein control for IVIG did not significantly  
224 alter the expression of basophil markers and cytokine production, thus confirming that  
225 IVIG could directly induce activation of IL-3-primed basophils without leading to  
226 degranulation. Preliminary exploration in IVIG-treated myopathy patients also confirmed  
227 enhancement of CD69 on the basophils of four out of five patients analyzed (Fig 1, G).

228 **IL-33 and other cytokines are dispensable for the activation of basophils by IVIG**

229 Because IL-4 secretion by basophils in mouse requires an IL-33 stimulation following  
230 IVIG infusion,<sup>9</sup> we wondered if IL-33 could, like IL-3, prime human basophils to be  
231 activated by IVIG. Unlike IL-3 (Fig 1, D-F), only a marginal increase in the expression of

232 CD69 on basophils (Fig 2, *A* and *B*) or their cytokine production (Fig 2, *C*) was observed  
233 following IL-33 stimulation of basophils at a dose equivalent of that induced in IVIG-  
234 treated patients.<sup>11,12</sup> Despite enhancement of IL-33R expression by IL-3 (Fig E4), IL-33  
235 when used in combination with IL-3 did not exert either synergistic or additive effect on  
236 IVIG-induced basophil activation (Fig 2, *D* and *E*). These results hence do not support a  
237 major role for IL-33 in priming human basophils towards IVIG responsiveness. Other  
238 cytokines like IL-25, TSLP and GM-CSF also had no significant effect on the IVIG-  
239 induced basophil activation (Fig E5). Altogether these results (Fig 1 and 2) indicate that  
240 IVIG induces IL-4 in human basophils, as had been described in mouse model.<sup>9</sup> Unlike  
241 mice however, IVIG appears to have a direct effect on human basophils leading to IL-4  
242 secretion, as long as basophils were primed with IL-3.

243 **IVIG induces basophil activation via F(ab')<sub>2</sub> fragments while type II FcRs, C-type**  
244 **lectin receptors and Siglecs are dispensable**

245 We aimed at identifying the receptors that mediate basophil activation. Recently, “type II  
246 FcRs” that include DC-SIGN and CD23 that interact with Fc-domain in the closed  
247 conformation, were reported to mediate anti-inflammatory actions of IVIG.<sup>13</sup> But human  
248 basophils were negative for CD23 and DC-SIGN<sup>14</sup> thus ruling out their involvement in  
249 IVIG-induced basophil activation (Fig 3, *A*).

250 As Fc- $\alpha(2,6)$ -sialic acid linkages could be recognized by various Siglecs, we investigated  
251 their implication in the cross-talk between IVIG and basophils. Siglec-2 (CD22) and  
252 Siglec-14 specifically recognize  $\alpha(2,6)$ -sialic acid linkages. However, both resting and IL-  
253 3-primed basophils were negative for CD22 (Fig 3, *B*). In addition, basophils did not  
254 express Siglec-3, -5/14, -7 and -8 (Fig E6), which all possess some affinity for (2,6)-sialic  
255 acid linkages. Siglec-10 was previously reported to be undetectable on basophils.<sup>15</sup>

256 DCIR, a C-type lectin receptor has been reported to recognize  $\alpha(2,6)$ -sialic acid linkages of  
257 IgG.<sup>16</sup> Nearly 80% of the steady-state and 95% of the IL-3-primed basophils express  
258 DCIR, but IVIG did not alter this expression (Fig 3, *C* and *D*). Importantly, IVIG did not  
259 induce activation of the resting basophils (Fig 1, *A-C*) despite these cells express DCIR,  
260 thus indirectly ruling out the role of DCIR in IVIG-induced basophil activation.

261 The lack of involvement of known receptors for  $\alpha(2,6)$ -sialic acid-linkages point toward a  
262 role for F(ab')<sub>2</sub>-domain rather than Fc-portion of IVIG on basophil activation.  
263 Accordingly, F(ab')<sub>2</sub> fragments of IVIG but not Fc fragments significantly enhanced CD69  
264 (Fig 3, *E* and *F*) and the production of both IL-4 and IL-8 (Fig 3, *G* and *H*).

265 **Basophil activation by IVIG is mediated by a fraction of IgG that signals through**  
266 **basophil surface-bound IgE**

267 Classically, IL-3 has been known for its critical role in favouring basophil-sensitization by  
268 IgE for augmented Fc $\epsilon$ RI-mediated signals and secretion of various inflammatory  
269 mediators.<sup>17-19</sup> Our data demonstrates that IL-3-priming is also a pre-requisite for the  
270 IVIG-induced basophil activation. IVIG significantly down-regulated Fc $\epsilon$ RI on IL-3-  
271 primed basophils (Fig 4, *A* and *B*), suggesting that IVIG binding to Fc $\epsilon$ RI and/or to Fc $\epsilon$ RI-  
272 bound IgE triggered the internalization of Fc $\epsilon$ RI. As expected, basophils displayed IgE on  
273 their surface (Fig 4, *C* and *D*) and IL-3 treatment dramatically licensed basophils to bind  
274 IVIG (Fig 4, *E* and *F*). However, incubation of basophils with additional IgE, did not alter  
275 the intensity of basophil-surface IgE indicating that all Fc $\epsilon$ RI on the basophils are already  
276 saturated by IgE. These arguments point out that IVIG induces activation of basophils  
277 possibly via signalling through basophil Fc $\epsilon$ RI-bound IgE rather than Fc $\epsilon$ RI. Importantly,  
278 depletion of anti-IgE-reactivity within IVIG suppressed the ability of IVIG to activate IL-

279 3-primed basophils, revealed by the poor increase in CD69 expression (Fig 4, *G* and *H*),  
280 and the abrogation of secretion of IL-4 and IL-8 (Fig 4, *I*).

281 **Activating and inhibitory CD32/Fc $\gamma$ RII are dispensable for the regulation of basophil**  
282 **activation by IVIG**

283 By interacting with Fc-domain of IgG, Fc $\gamma$ Rs influence the activation of immune cells.<sup>20</sup>  
284 Human basophils mainly express Fc $\gamma$ RIIA and Fc $\gamma$ RIIB.<sup>21</sup> While Fc $\gamma$ RIIA is an activating  
285 receptor, signaling via Fc $\gamma$ RIIB inhibits activation of immune cells.<sup>20</sup> Therefore, we  
286 wondered whether IVIG-induced basophil activation is regulated by Fc $\gamma$ RII.

287 First, we analyzed the expression pattern of Fc $\gamma$ RII on basophils. IL-3 although enhanced  
288 the expression of both Fc $\gamma$ RIIA and Fc $\gamma$ RIIB, a non-significant trend towards reduced  
289 expression of both the receptors was observed upon IVIG stimulation (Fig 5, *A* and *B*).  
290 Thus, unlike monocytes and B cells of chronic inflammatory demyelinating  
291 polyneuropathy patients that showed enhanced Fc $\gamma$ RIIB expression upon IVIG therapy,<sup>22</sup>  
292 the ratio of intensity of expression of Fc $\gamma$ RIIB to Fc $\gamma$ RIIA remains unchanged on IVIG-  
293 treated basophils. Our data are similar to that observed with splenic macrophages of IVIG-  
294 treated adult immune thrombocytopenia patients.<sup>23</sup>

295 High-affinity Rabbit Anti-Human-IgE (RAHE) IgG was shown to negatively regulate IgE-  
296 induced activation of human basophils by co-engaging Fc $\gamma$ RIIB.<sup>21</sup> Hence, we asked  
297 whether Fc $\gamma$ RIIB blockade would enhance the activation of basophils by IVIG. However,  
298 IVIG-induced activation of basophils was not significantly altered upon Fc $\gamma$ RIIB blockade  
299 (Fig 5, *C* and *D*).

300 As Fc $\gamma$ RIIA signalling induces activation of immune cells,<sup>20</sup> we explored if IVIG-induced  
301 basophil activation implicates co-engagement with this receptor. But Fc $\gamma$ RIIA blockade  
302 had no repercussion on the IVIG-induced expression of CD69 and cytokines (Fig 5, *E* and

303 *F*), demonstrating that Fc $\gamma$ RII (activating or inhibitory) has no significant role in the  
304 regulation of human basophil function by IVIG.

305 **Syk pathway is critical for the basophil activation by IVIG**

306 Fc $\epsilon$ RI-mediated activation of human basophils in vitro requires both priming by IL-3 and  
307 the kinase Syk that is recruited to the Fc $\epsilon$ RI signalling complex.<sup>17-19</sup> Noticeably, IL-3-  
308 mediated down-stream signalling has also been reported to be Syk-dependent.<sup>24,25</sup> Freshly  
309 isolated basophils showed basal phosphorylation of Syk (pSyk). In line with the fact that  
310 IL-3 induces rapid phosphorylation of Syk, we found that IL-3 significantly enhanced  
311 pSyk. A treatment with IL3 plus IVIG resulted in similar pSyk induction (Fig 6, A and B).  
312 Further, inhibition of Syk, using inhibitor R406, abrogated IVIG-induced enhancement of  
313 CD69 (Fig 6, C and D) and production of IL-4 and IL-8 (Fig 6, E). qRT-PCR also  
314 confirmed abrogation of IVIG-induced *il4* following Syk inhibition (Fig E7). Altogether  
315 these data suggest that IVIG, due to IgE reactivity it contains, induces activation of IL-3-  
316 primed basophil by signalling through Fc $\epsilon$ RI-bound IgE.

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328 **DISCUSSION**

329 Despite having pathogenic roles in various diseases,<sup>8,26,27</sup> recent evidence from mouse also  
330 suggests that basophils are central to the anti-inflammatory effects of IVIG thus providing  
331 an intriguing new function to these rare immune cells.<sup>9</sup> However, this proposed role of  
332 basophils in mediating the therapeutic benefits of IVIG could not be reproduced in another  
333 report.<sup>28</sup> It is important to note that both studies have employed anti-FcεRI MAb MAR-1 to  
334 deplete the basophils and this antibody has been reported to deplete FcεRI-positive DCs as  
335 well.<sup>29,30</sup> Also, as compared to mouse, human basophils display distinct features.<sup>8,31,32</sup>  
336 Therefore, the effect of IVIG on basophil functions is far from clear. Notably, data from  
337 human raise an alternative paradigm that IVIG might modulate basophil functions directly  
338 rather than indirect IL-33-dependent pathway.<sup>11</sup>

339 Human basophils express receptors for various cytokines. In addition to IL-33, mainly  
340 produced by epithelial and endothelial cells, IL-3 secreted by activated T cells and mast  
341 cells is also known for inducing priming of basophils.<sup>17,33-36</sup> We sought to confirm whether  
342 human basophil priming by IL-33 at a dose equivalent of that induced by IVIG in patients  
343 with rheumatic and neurological autoimmune diseases<sup>11,12</sup> would stimulate IL-4 production  
344 as proposed from mouse studies. IL-33 indeed primed human basophils (based on the  
345 expression of CD69) and induced IL-4,<sup>37</sup> but the extent of priming was only marginal  
346 when compared to IL-3-mediated priming.<sup>17,19</sup> This marginal activation by IL-33 might be  
347 also due to the expression pattern of IL-33R as only 22.4±6.3% (n=8) basophils in steady-  
348 state express this receptor.

349 We investigated if IVIG could activate IL-33-primed basophils. However, IVIG did neither  
350 modify phenotype nor cytokine production in IL-33-primed basophils. In addition to IL-33,  
351 activated epithelial cells also release IL-25 and TSLP.<sup>38</sup> However, basophils were not  
352 sensitive for both these cytokines. A recent report also confirms that TSLP does not

353 activate human basophils.<sup>39</sup> GM-CSF on the other hand, significantly activated human  
354 basophils,<sup>40,41</sup> but the extent of activation was lesser than IL-3. Also, GM-CSF-priming  
355 had no consequence on IVIG-induced basophil activation.

356 Noticeably however, IL-3-priming licensed human basophils to undergo activation by  
357 IVIG. Rather than IL-33-mediated pathway of basophil IL-4 induction as suggested from  
358 the mouse studies, our data suggest an IL-3-mediated pathway of human basophil priming  
359 that enables them to directly respond to IVIG by secreting IL-4 (and other cytokines).  
360 Although IL-3 significantly enhanced the expression of IL-33R on the basophils, IL-33 did  
361 not potentiate IVIG-induced basophil activation when used in combination with IL-3.  
362 These data suggest that IL-3 is a major stimulator of basophil functions and could regulate  
363 basophil response to IL-33 (probably at higher concentrations as reported earlier<sup>37</sup>) by  
364 enhancing the IL-33R expression. In fact, under IL-3-stimulation conditions, CD69 and IL-  
365 33R were co-expressed on the basophils. However, this was not the case under IL-33-  
366 stimulation conditions, wherein only a minor population of basophils co-expressed CD69  
367 and IL-33R possibly because of marginal stimulation of basophils by IL-33 or IL-33R  
368 internalization. All our experiments in this report rely on *in vitro* stimulation system and  
369 hence it is important to prove these data in the context of systemic autoimmune and  
370 inflammatory diseases. Although data are preliminary, basophil activation also occurs *in*  
371 *vivo* in IVIG-treated myopathy patients. Further analyses of basophils in the inflamed  
372 tissues and secondary lymphoid organs should provide more insight on the regulation of  
373 basophil functions by IVIG.

374 Various studies reported that Fc $\gamma$ RIIB plays an important role in mediating the anti-  
375 inflammatory actions of IVIG. The enhanced expression of Fc $\gamma$ RIIB by IVIG has been  
376 proposed to increase the threshold level for the activation of innate cells by immune  
377 complexes.<sup>22,42-44</sup> However, the absolute requirement of Fc $\gamma$ RIIB in mediating anti-

378 inflammatory actions of IVIG could not be confirmed in other experimental models.<sup>45-48</sup>

379 Also, several effects of IVIG on human DCs, macrophages and CD4<sup>+</sup> T cells were

380 Fc $\gamma$ RIIB-independent.<sup>49-52</sup> Our current data on the basophils provide yet another evidence

381 for Fc $\gamma$ RII-independent action of IVIG on human cells.

382 Several targets and receptors have been identified for IVIG. In addition to the F(ab')<sub>2</sub>-

383 mediated recognition of various self-molecules like HLA, Fas, CD40, Siglecs, BAFF,

384 immunoglobulins and others,<sup>53-59</sup> Fc- $\alpha$ (2,6)-sialic acid-linkages were reported to be

385 recognized by type II Fc receptors, Siglec-2 and DCIR.<sup>13,16,60,61</sup> However, human immune

386 cells display wide variations in the expression pattern of these receptors. In vitro-generated

387 monocyte-derived DCs (equivalent of inflammatory DCs) express both DC-SIGN and

388 DCIR while DCs ex vivo express mainly DCIR.<sup>62</sup> Although CD23 is expressed by B cells,

389 macrophages and eosinophils, Siglec-2 is restricted to B cells. Human basophils, however,

390 lack DC-SIGN, CD23 and Siglec-2. Despite positive for DCIR, resting basophils were not

391 modified by IVIG, suggesting that DCIR is not sufficient (or predominant) in mediating

392 basophil activation by IVIG. Also, other Siglecs that could recognize  $\alpha$ (2,6)-sialic acid-

393 linkages were absent on the basophils.

394 IVIG-induced activation of IL-3-primed human basophils did not lead to degranulation and

395 was distinct to the effect of anti-IgE antibodies identified in the asthmatic patients that

396 induced high expression of degranulation marker CD63.<sup>63</sup> It is possible that the anti-IgE

397 content in IVIG is too low to activate fully basophils to degranulate. Supporting this

398 assumption, antigens at low concentrations have been reported to induce Fc $\epsilon$ RI-mediated

399 activation of mast cells without causing degranulation.<sup>64,65</sup>

400 Glycosylation patterns of Fc-domains of IgG determine their engagement with classical

401 type I FcRs (that include Fc $\gamma$ Rs) or with type II FcRs. The sialylated or non-sialylated

402 glycans-mediated ‘closed’ vs ‘open’ conformation of Fc, switches engagement of Fc-  
403 domain towards type II or type I FcRs respectively.<sup>66</sup> Previous report showed that anti-IgE  
404 rabbit IgG inhibit basophil activation by co-engaging with FcγRIIB.<sup>21</sup> However, contrary  
405 to this, we observed activation of basophils by anti-IgE IgG present in IVIG. Also, FcγRII-  
406 blockade had no significant effect on IVIG-induced basophil activation. Based on all these  
407 arguments, we could infer that glycosylation content of Fc-domains of anti-IgE IgG in  
408 IVIG is enriched for sialylation that might have prevented engagement of Fc with FcγRII  
409 on basophils.

410 Basophils are implicated in the pathogenesis of chronic urticaria. The anti-IgE or anti-  
411 FcεRI autoantibodies in these patients trigger activation and degranulation of basophils.<sup>67</sup>  
412 IVIG is reported to be beneficial in such patients.<sup>68</sup> However, our preliminary data suggest  
413 that IVIG might not prevent degranulation of basophils and hence the efficacy of IVIG in  
414 chronic urticaria patients with anti-IgE or anti-FcεRI autoantibodies might be because of  
415 basophil-independent mechanisms. In fact, suppressive effect of IVIG on IgE production  
416 by B cells has been reported.<sup>69</sup>

417 Syk phosphorylation is one of the early signaling events in basophils following IL-3 as  
418 well as FcεRI-mediated activation.<sup>17,24,25</sup> Therefore, it is difficult to segregate the  
419 importance of IL-3-induced versus FcεRI-induced Syk activation. As IVIG could induce  
420 basophil activation only upon IL-3-priming suggests that IL-3-induced Syk  
421 phosphorylation is indispensable for basophil FcεRI-bound IgE-mediated activation by  
422 IVIG. Syk inhibitor R406 that is proposed for human pathologies<sup>70</sup> blocked IVIG-induced  
423 human basophil activation; thus it appears that both “classical” high-affinity IgE-induced  
424 degranulation events and IVIG’s anti-IgE activation (without degranulation) events use  
425 Syk for signal transduction.

426 To conclude, our report highlights a novel mechanism of activation of human basophils by  
427 IVIG and underlines discrepancies in the mechanisms of action of IVIG in humans and  
428 mice.

429

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437       **REFERENCES**

- 438       1.       Perez EE, Orange JS, Bonilla F, Chinen J, Chinn IK, Dorsey M, et al. Update  
439           on the use of immunoglobulin in human disease: A review of evidence. *J*  
440           *Allergy Clin Immunol.* 2017; 139:S1-S46.
- 441       2.       Gelfand EW. Intravenous immune globulin in autoimmune and inflammatory  
442           diseases. *N Engl J Med.* 2012; 367:2015-25.
- 443       3.       Gilardin L, Bayry J, Kaveri SV. Intravenous immunoglobulin as clinical  
444           immune-modulating therapy. *CMAJ.* 2015; 187:257-64.
- 445       4.       Lunemann JD, Nimmerjahn F, Dalakas MC. Intravenous immunoglobulin in  
446           neurology--mode of action and clinical efficacy. *Nat Rev Neurol* 2015; 11:80-9.
- 447       5.       Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG  
448           modulate the immune system? *Nat Rev Immunol* 2013; 13:176-89.
- 449       6.       Galeotti C, Kaveri SV, Bayry J. IVIG-mediated effector functions in  
450           autoimmune and inflammatory diseases. *Int Immunol* 2017; 29:491-8.
- 451       7.       Schroeder JT. Basophils: emerging roles in the pathogenesis of allergic disease.  
452           *Immunol Rev* 2011; 242:144-60.
- 453       8.       Karasuyama H, Miyake K, Yoshikawa S, Yamanishi Y. Multifaceted roles of  
454           basophils in health and disease. *J Allergy Clin Immunol* 2018; 142: 370-80.
- 455       9.       Anthony RM, Kobayashi T, Wermeling F, Ravetch JV. Intravenous  
456           gammaglobulin suppresses inflammation through a novel T(H)2 pathway.  
457           *Nature.* 2011; 475:110-3.
- 458       10.      Egawa M, Mukai K, Yoshikawa S, Iki M, Mukaida N, Kawano Y, et al.  
459           Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory  
460           M2 phenotype via basophil-derived interleukin-4. *Immunity* 2013; 38:570-80.

- 461 11. Sharma M, Schoindre Y, Hegde P, Saha C, Maddur MS, Stephen-Victor E, et  
462 al. Intravenous immunoglobulin-induced IL-33 is insufficient to mediate  
463 basophil expansion in autoimmune patients. *Sci Rep* 2014; 4:5672.
- 464 12. Maddur MS, Stephen-Victor E, Das M, Prakhar P, Sharma VK, Singh V, et al.  
465 Regulatory T cell frequency, but not plasma IL-33 levels, represents potential  
466 immunological biomarker to predict clinical response to intravenous  
467 immunoglobulin therapy. *J Neuroinflammation* 2017; 14: 58.
- 468 13. Fiebiger BM, Maamary J, Pincetic A, Ravetch JV. Protection in antibody- and  
469 T cell-mediated autoimmune diseases by antiinflammatory IgG Fcs requires  
470 type II FcRs. *Proc Natl Acad Sci U S A* 2015; 112:E2385-94.
- 471 14. Das M, Galeotti C, Stephen-Victor E, Karnam A, Kaveri SV, Bayry J. Human  
472 basophils may not undergo modulation by DC-SIGN and mannose receptor-  
473 targeting immunotherapies due to absence of receptors. *J Allergy Clin Immunol*  
474 2017; 139:1403-4.
- 475 15. Nutku E, Aizawa H, Lim L, Tashimoto H, Hudson S, Crocker P, et al.  
476 Expression of CD33-Related siglecs on human eosinophils, basophils and  
477 mast Cells. *J Allergy Clin Immunol* 2004; 113:S84
- 478 16. Massoud AH, Yona M, Xue D, Chouiali F, Alturaihi H, Ablona A, et al.  
479 Dendritic cell immunoreceptor: a novel receptor for intravenous  
480 immunoglobulin mediates induction of regulatory T cells. *J Allergy Clin*  
481 *Immunol* 2014; 133:853-63.
- 482 17. Voehringer D. Basophil modulation by cytokine instruction. *Eur J Immunol*  
483 2012; 42:2544-50.

- 484 18. Brunner T, Heusser CH, Dahinden CA. Human peripheral blood basophils  
485 primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E  
486 receptor stimulation. *J Exp Med* 1993; 177:605-11.
- 487 19. Yoshimura C, Yamaguchi M, Iikura M, Izumi S, Kudo K, Nagase H, et al.  
488 Activation markers of human basophils: CD69 expression is strongly and  
489 preferentially induced by IL-3. *J Allergy Clin Immunol* 2002; 109:817-23.
- 490 20. Nimmerjahn F, Ravetch JV. Fc $\gamma$  receptors as regulators of immune responses.  
491 *Nat Rev Immunol* 2008; 8:34-47.
- 492 21. Cassard L, Jonsson F, Arnaud S, Daeron M. Fc $\gamma$  receptors inhibit mouse and  
493 human basophil activation. *J Immunol* 2012; 189:2995-3006.
- 494 22. Tackenberg B, Jelcic I, Baerenwaldt A, Oertel WH, Sommer N, Nimmerjahn F,  
495 et al. Impaired inhibitory Fc $\gamma$  receptor IIB expression on B cells in chronic  
496 inflammatory demyelinating polyneuropathy. *Proc Natl Acad Sci U S A* 2009;  
497 106:4788-92.
- 498 23. Audia S, Santegoets K, Laarhoven AG, Vidarsson G, Facy O, Ortega-Deballon  
499 P, et al. Fc $\gamma$  receptor expression on splenic macrophages in adult immune  
500 thrombocytopenia. *Clin Exp Immunol* 2017; 188:275-82.
- 501 24. Hida S, Yamasaki S, Sakamoto Y, Takamoto M, Obata K, Takai T, et al. Fc  
502 receptor gamma-chain, a constitutive component of the IL-3 receptor, is  
503 required for IL-3-induced IL-4 production in basophils. *Nat Immunol*  
504 2009;10:214-22.
- 505 25. Siraganian RP, de Castro RO, Barbu EA, Zhang J. Mast cell signaling: the role  
506 of protein tyrosine kinase Syk, its activation and screening methods for new  
507 pathway participants. *FEBS Lett* 2010;584:4933-40.

- 508 26. Sharma M, Bayry J. Autoimmunity: Basophils in autoimmune and  
509 inflammatory diseases. *Nat Rev Rheumatol* 2015;11:129-31.
- 510 27. Pellefigues C, Dema B, Lamri Y, Saidoune F, Chavarot N, Lohéac C, et al.  
511 Prostaglandin D2 amplifies lupus disease through basophil accumulation in  
512 lymphoid organs. *Nat Commun* 2018; 9:725.
- 513 28. Campbell IK, Miescher S, Branch DR, Mott PJ, Lazarus AH, Han D, et al.  
514 Therapeutic effect of IVIG on inflammatory arthritis in mice is dependent on  
515 the Fc portion and independent of sialylation or basophils. *J Immunol*  
516 2014;192:5031-8.
- 517 29. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, et al.  
518 Inflammatory dendritic cells--not basophils--are necessary and sufficient for  
519 induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med*  
520 2010; 207:2097-111.
- 521 30. Phythian-Adams AT, Cook PC, Lundie RJ, Jones LH, Smith KA, Barr TA, et  
522 al. CD11c depletion severely disrupts Th2 induction and development in vivo. *J*  
523 *Exp Med* 2010;207:2089-96.
- 524 31. Stephen-Victor E, Das M, Sharma M, Galeotti C, Fohrer-Ting H, Sendid B, et  
525 al. Demystification of enigma on antigen-presenting cell features of human  
526 basophils: data from secondary lymphoid organs. *Haematologica* 2017; 102,  
527 e233-e37.
- 528 32. Miyake K, Shiozawa N, Nagao T, Yoshikawa S, Yamanishi Y, Karasuyama H.  
529 Trogocytosis of peptide-MHC class II complexes from dendritic cells confers  
530 antigen-presenting ability on basophils. *Proc Natl Acad Sci U S A* 2017; 114:  
531 1111-16.

- 532 33. Pecaric-Petkovic T, Didichenko SA, Kaempfer S, Spiegl N, Dahinden CA.  
533 Human basophils and eosinophils are the direct target leukocytes of the novel  
534 IL-1 family member IL-33. *Blood* 2009;113:1526-34.
- 535 34. Smithgall MD, Comeau MR, Yoon BR, Kaufman D, Armitage R, Smith DE.  
536 IL-33 amplifies both Th1- and Th2-type responses through its activity on  
537 human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol*  
538 2008; 20:1019-30.
- 539 35. Leyva-Castillo JM, Hener P, Michea P, Karasuyama H, Chan S, Soumelis V, et  
540 al. Skin thymic stromal lymphopoietin initiates Th2 responses through an  
541 orchestrated immune cascade. *Nat Commun* 2013;4:2847.
- 542 36. Sharma M, Das M, Stephen-Victor E, Galeotti C, Karnam A, Maddur MS, et al.  
543 Regulatory T cells induce activation rather than suppression of human  
544 basophils. *Sci Immunol* 2018; 3: eaan0829.
- 545 37. Suzukawa M, Iikura M, Koketsu R, Nagase H, Tamura C, Komiya A, et al. An  
546 IL-1 cytokine member, IL-33, induces human basophil activation via its ST2  
547 receptor. *J Immunol* 2008; 181: 5981-89.
- 548 38. Strickland DH, Upham JW, Holt PG. Epithelial-dendritic cell interactions in  
549 allergic disorders. *Curr Opin Immunol* 2010; 22: 789-94.
- 550 39. Salabert-Le Guen N, Hémond C, Delbove A, Poli C, Braudeau C, Fantou A, et  
551 al. Thymic stromal lymphopoietin does not activate human basophils. *J Allergy*  
552 *Clin Immunol* 2018; 141: 1476-79.
- 553 40. Tedeschi A, Salmaso C, Di Donato M, Lorini M, Miadonna A. Granulocyte-  
554 macrophage colony-stimulating factor and interleukin-3 cause basophil  
555 histamine release by a common pathway: downregulation by sodium.  
556 *Immunology* 1999; 96: 164-70.

- 557 41. Bischoff SC, de Weck AL, Dahinden CA. Interleukin 3 and  
558 granulocyte/macrophage-colony-stimulating factor render human basophils  
559 responsive to low concentrations of complement component C3a. *Proc Natl*  
560 *Acad Sci U S A* 1990; 87: 6813-17.
- 561 42. Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG  
562 mediated through the inhibitory Fc receptor. *Science* 2001; 291:484-6.
- 563 43. Bruhns P, Samuelsson A, Pollard JW, Ravetch JV. Colony-stimulating factor-1-  
564 dependent macrophages are responsible for IVIG protection in antibody-  
565 induced autoimmune disease. *Immunity* 2003;18:573-81.
- 566 44. Schwab I, Mihai S, Seeling M, Kasperkiewicz M, Ludwig RJ, Nimmerjahn F.  
567 Broad requirement for terminal sialic acid residues and Fc $\gamma$ RIIB for the  
568 preventive and therapeutic activity of intravenous immunoglobulins in vivo. *Eur*  
569 *J Immunol* 2014;44:1444-53.
- 570 45. Leontyev D, Katsman Y, Branch DR. Mouse background and IVIG dosage are  
571 critical in establishing the role of inhibitory Fc $\gamma$  receptor for the amelioration of  
572 experimental ITP. *Blood* 2012;119:5261-4.
- 573 46. Aloulou M, Ben Mkaddem S, Biarnes-Pelicot M, Boussetta T, Souchet H,  
574 Rossato E, et al. IgG1 and IVIg induce inhibitory ITAM signaling through  
575 Fc $\gamma$ RIII controlling inflammatory responses. *Blood* 2012;119:3084-96.
- 576 47. Othy S, Hegde P, Topcu S, Sharma M, Maddur MS, Lacroix-Desmazes S, et al.  
577 Intravenous gammaglobulin inhibits encephalitogenic potential of pathogenic T  
578 cells and interferes with their trafficking to the central nervous system,  
579 implicating sphingosine-1 phosphate receptor 1-mammalian target of rapamycin  
580 axis. *J Immunol* 2013;190:4535-41.

- 581 48. Crow AR, Lazarus AH. Mechanistic properties of intravenous immunoglobulin  
582 in murine immune thrombocytopenia: support for Fc $\gamma$ RIIB falls by the wayside.  
583 *Semin Hematol* 2016;53:S20-2.
- 584 49. Maddur MS, Vani J, Hegde P, Lacroix-Desmazes S, Kaveri SV, Bayry J.  
585 Inhibition of differentiation, amplification, and function of human TH17 cells  
586 by intravenous immunoglobulin. *J Allergy Clin Immunol* 2011;127:823-30.
- 587 50. Nagelkerke SQ, Dekkers G, Kustiawan I, van de Bovenkamp FS, Geissler J,  
588 Plomp R, et al. Inhibition of Fc $\gamma$ R-mediated phagocytosis by IVIg is  
589 independent of IgG-Fc sialylation and Fc $\gamma$ RIIb in human macrophages. *Blood*  
590 2014;124:3709-18.
- 591 51. Wiedeman AE, Santer DM, Yan W, Miescher S, Kasermann F, Elkon KB.  
592 Contrasting mechanisms of interferon- $\alpha$  inhibition by intravenous  
593 immunoglobulin after induction by immune complexes versus Toll-like receptor  
594 agonists. *Arthritis Rheum* 2013;65:2713-23.
- 595 52. Trinath J, Hegde P, Sharma M, Maddur MS, Rabin M, Vallat JM, et al.  
596 Intravenous immunoglobulin expands regulatory T cells via induction of  
597 cyclooxygenase-2-dependent prostaglandin E2 in human dendritic cells. *Blood*  
598 2013;122:1419-27.
- 599 53. Bayry J, Lacroix-Desmazes S, Donkova-Petrini V, Carbonneil C, Misra N,  
600 Lepelletier Y, et al. Natural antibodies sustain differentiation and maturation of  
601 human dendritic cells. *Proc Natl Acad Sci U S A* 2004;101:14210-5.
- 602 54. Blank M, Anafi L, Zandman-Goddard G, Krause I, Goldman S, Shalev E, et al.  
603 The efficacy of specific IVIG anti-idiotypic antibodies in antiphospholipid  
604 syndrome (APS): trophoblast invasiveness and APS animal model. *Int Immunol*  
605 2007;19:857-65.

- 606 55. Le Pottier L, Bendaoud B, Dueymes M, Daridon C, Youinou P, Shoenfeld Y, et  
607 al. BAFF, a new target for intravenous immunoglobulin in autoimmunity and  
608 cancer. *J Clin Immunol* 2007;27:257-65.
- 609 56. Prasad NK, Papoff G, Zeuner A, Bonnin E, Kazatchkine MD, Ruberti G, et al.  
610 Therapeutic preparations of normal polyspecific IgG (IVIg) induce apoptosis in  
611 human lymphocytes and monocytes: a novel mechanism of action of IVIg  
612 involving the Fas apoptotic pathway. *J Immunol* 1998;161:3781-90.
- 613 57. Shoenfeld Y, Rauova L, Gilburd B, Kvapil F, Goldberg I, Kopolovic J, et al.  
614 Efficacy of IVIG affinity-purified anti-double-stranded DNA anti-idiotypic  
615 antibodies in the treatment of an experimental murine model of systemic lupus  
616 erythematosus. *Int Immunol* 2002;14:1303-11.
- 617 58. von Gunten S, Vogel M, Schaub A, Stadler BM, Miescher S, Crocker PR, et al.  
618 Intravenous immunoglobulin preparations contain anti-Siglec-8 autoantibodies.  
619 *J Allergy Clin Immunol* 2007;119:1005-11.
- 620 59. Schneider C, Wicki S, Graeter S, Timcheva TM, Keller CW, Quast I, et al.  
621 IVIG regulates the survival of human but not mouse neutrophils. *Sci Rep*  
622 2017;7:1296.
- 623 60. Anthony RM, Wermeling F, Karlsson MC, Ravetch JV. Identification of a  
624 receptor required for the anti-inflammatory activity of IVIG. *Proc Natl Acad Sci*  
625 *U S A* 2008;105:19571-8.
- 626 61. Seite JF, Cornec D, Renaudineau Y, Youinou P, Mageed RA, Hillion S. IVIg  
627 modulates BCR signaling through CD22 and promotes apoptosis in mature  
628 human B lymphocytes. *Blood* 2010;116:1698-704.
- 629 62. Lundberg K, Albrekt AS, Nelissen I, Santegoets S, de Gruijl TD, Gibbs S, et al.  
630 Transcriptional profiling of human dendritic cell populations and models--

- 631 unique profiles of in vitro dendritic cells and implications on functionality and  
632 applicability. PLoS One 2013;8:e52875.
- 633 63. Chan YC, Ramadani F, Santos AF, Pillai P, Ohm-Laursen L, Harper CE, et al.  
634 Auto-anti-IgE: naturally occurring IgG anti-IgE antibodies may inhibit allergen-  
635 induced basophil activation. J Allergy Clin Immunol 2014;134:1394-401.
- 636 64. Gonzalez-Espinosa C, Odom S, Olivera A, Hobson JP, Martinez ME, Oliveira-  
637 Dos-Santos A, et al. Preferential signaling and induction of allergy-promoting  
638 lymphokines upon weak stimulation of the high affinity IgE receptor on mast  
639 cells. J Exp Med 2003;197:1453-65.
- 640 65. Grodzki AC, Moon KD, Berenstein EH, Siraganian RP. FcεRI-induced  
641 activation by low antigen concentrations results in nuclear signals in the  
642 absence of degranulation. Mol Immunol 2009;46:2539-47.
- 643 66. Pincetic A, Bournazos S, DiLillo DJ, Maamary J, Wang TT, Dahan R, et al.  
644 Type I and type II Fc receptors regulate innate and adaptive immunity. Nat  
645 Immunol 2014; 15: 707-16.
- 646 67. Kaplan AP, Greaves M. Pathogenesis of chronic urticaria. Clin Exp Allergy  
647 2009; 39: 777-87.
- 648 68. Amar SM, Harbeck RJ, Dreskin SC. Effect of Intravenous immunoglobulin in  
649 chronic urticaria with increased basophil CD203c expression. J Allergy Clin  
650 Immunol 2008; 121: S98.
- 651 69. Zhuang Q, Mazer B. Inhibition of IgE production in vitro by intact and  
652 fragmented intravenous immunoglobulin. J Allergy Clin Immunol 2001; 108:  
653 229-34.
- 654 70. Braselmann S, Taylor V, Zhao H, Wang S, Sylvain C, Baluom M, et al. R406,  
655 an orally available spleen tyrosine kinase inhibitor blocks Fc receptor signaling

656 and reduces immune complex-mediated inflammation. *J Pharmacol Exp Ther*  
657 2006; 319:998-1008.  
658

659 **FIGURE LEGENDS**

660 **FIG 1.** IVIG induces activation and cytokine secretion in IL-3-primed basophils. **A-C,**  
 661 Isolated basophils from the human circulation were cultured either alone or with IVIG. (A  
 662 and B) Representative dot plots and expression (% positive cells and mean fluorescence  
 663 intensity (MFI)) of CD69 on the basophils (mean±SEM, n=6 donors). (C) Amount of  
 664 secretion of IL-4, IL-6 and IL-8 (mean±SEM, n=5 donors). ns, not significant, two-tailed  
 665 Mann-Whitney test. **D-F,** Basophils were cultured either alone or with IL-3. IVIG or HSA  
 666 were added following 2 hours stimulation with IL-3. (D) Representative histogram  
 667 overlays and MFI of CD69 expression on the basophils (mean±SEM, n=10 donors), (E)  
 668 Representative dot plots and % of basophils (mean±SEM, n=4 donors) positive for CD63,  
 669 (F) Effect of IVIG on the secretion (pg/ml) of IL-4, IL-6 and IL-8 (mean±SEM, n=12  
 670 donors) by IL-3-primed basophils. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001; ns,  
 671 not significant, one-way ANOVA with Tukey's multiple comparison tests. **G,** Expression  
 672 of CD69 on the basophils of myopathy patients, before (Pre-IVIG) and Post-IVIG therapy.

673

674 **FIG 2.** IL-33 is dispensable for the activation of human basophils by IVIG. Basophils were  
 675 cultured either alone or with IL-33. IVIG or HSA were added following 2 hours  
 676 stimulation with IL-33. **A and B,** Representative dot plots and expression (% positive cells  
 677 and MFI) of CD69 on the basophils (mean±SEM, n=6 donors). **C,** Amount of secretion of  
 678 IL-4 and IL-8 (mean±SEM, n=6 donors). **D and E,** basophils were stimulated with IL-3 for  
 679 one hour followed by IL-33 for additional hour before culturing with IVIG or HSA. (D)  
 680 Expression (% positive cells and MFI) of CD69 on the basophils. (E) Amount of secretion  
 681 of IL-4 and IL-8 (mean±SEM, n=4 donors) \*\*\*\*P<0.001; \*\*\*\*\*P<0.0001; ns, not  
 682 significant, one-way ANOVA with Tukey's multiple comparison tests.

683

684 **FIG 3.** Expression of type II FcRs, Siglecs and C-type lectin receptors on basophils, and  
685 the effects of F(ab')<sub>2</sub>- and Fc-fragments of IVIG on basophil activation. **A and B,**  
686 Representative dot plots of CD23 and CD22 expression on the basophils. **C and D,**  
687 Representative dot plots and expression (% positive cells and MFI) of DCIR on the  
688 basophils (mean±SEM, n=3 donors). **E-H,** Basophils were cultured either alone or with IL-  
689 3 for 24 hours. IVIG, F(ab')<sub>2</sub> or Fc-fragments were added following 2 hours stimulation  
690 with IL-3. (E and F) The expression of CD69 (mean±SEM, n=6 donors). (G and H) The  
691 amount of secretion of IL-4, and IL-8 (mean±SEM, n=4-5 donors). \*P<0.05; \*\*P<0.01;  
692 \*\*\*P<0.001; \*\*\*\*P<0.001; ns, not significant, one-way ANOVA with Tukey's multiple  
693 comparison tests.

694

695 **FIG 4.** Basophil activation by IVIG is mediated by a fraction of IgG that signals through  
696 basophil FcεRI-bound IgE. **A and B,** Modulation of FcεRI expression (Representative  
697 histogram overlays and mean±SEM, n=10 donors) in IL-3-primed basophils by IVIG. **C,**  
698 Representative dot plots showing the basophils positive for surface IgE. **D,** Percentage of  
699 basophils positive for the surface IgE and its intensity (MFI) (mean±SEM, n=5 donors). **E**  
700 **and F,** Percentage of basophils positive for IVIG-binding (Representative dot plots and  
701 mean±SEM, n=4 donors). **G-I,** The effect of anti-IgE-reactivity-depleted IVIG on (G, H)  
702 the expression of CD69 (Representative histogram overlays and mean±SEM, n=4 donors)  
703 and (I) IL-4, and IL-8 secretion (mean±SEM, n=4 donors). \*P<0.05; \*\*P<0.01;  
704 \*\*\*P<0.001; \*\*\*\*P<0.0001; ns, not significant, two-tailed Mann-Whitney test or one-way  
705 ANOVA with Tukey's multiple comparison tests.

706

707 **FIG 5.** Activating and inhibitory CD32/FcγRII are dispensable for the regulation of  
708 basophil activation by IVIG. Basophils were cultured either alone or with IL-3 for 24  
709 hours. IVIG or HSA were added following 2 hours stimulation with IL-3. **A and B,**  
710 Representative histogram overlays and mean fluorescence intensity (MFI) of expression  
711 (mean±SEM, n=8 donors) of FcγRII and FcγRIIB on the basophils. **C and D,** Repercussion  
712 of FcγRIIB blockade on the (C) expression of CD69 and (D) amount of IL-4 and IL-8  
713 secretion (mean±SEM, n=8 donors). **E and F,** Repercussion of FcγRIIA blockade on the  
714 (E) expression of CD69 and (F) amount of IL-4 and IL-8 secretion (mean±SEM, n=4  
715 donors). \*P<0.05; \*\*P<0.001; \*\*\*P<0.001; \*\*\*\*P<0.0001; ns, not significant, one-way  
716 ANOVA with Tukey's multiple comparison tests.

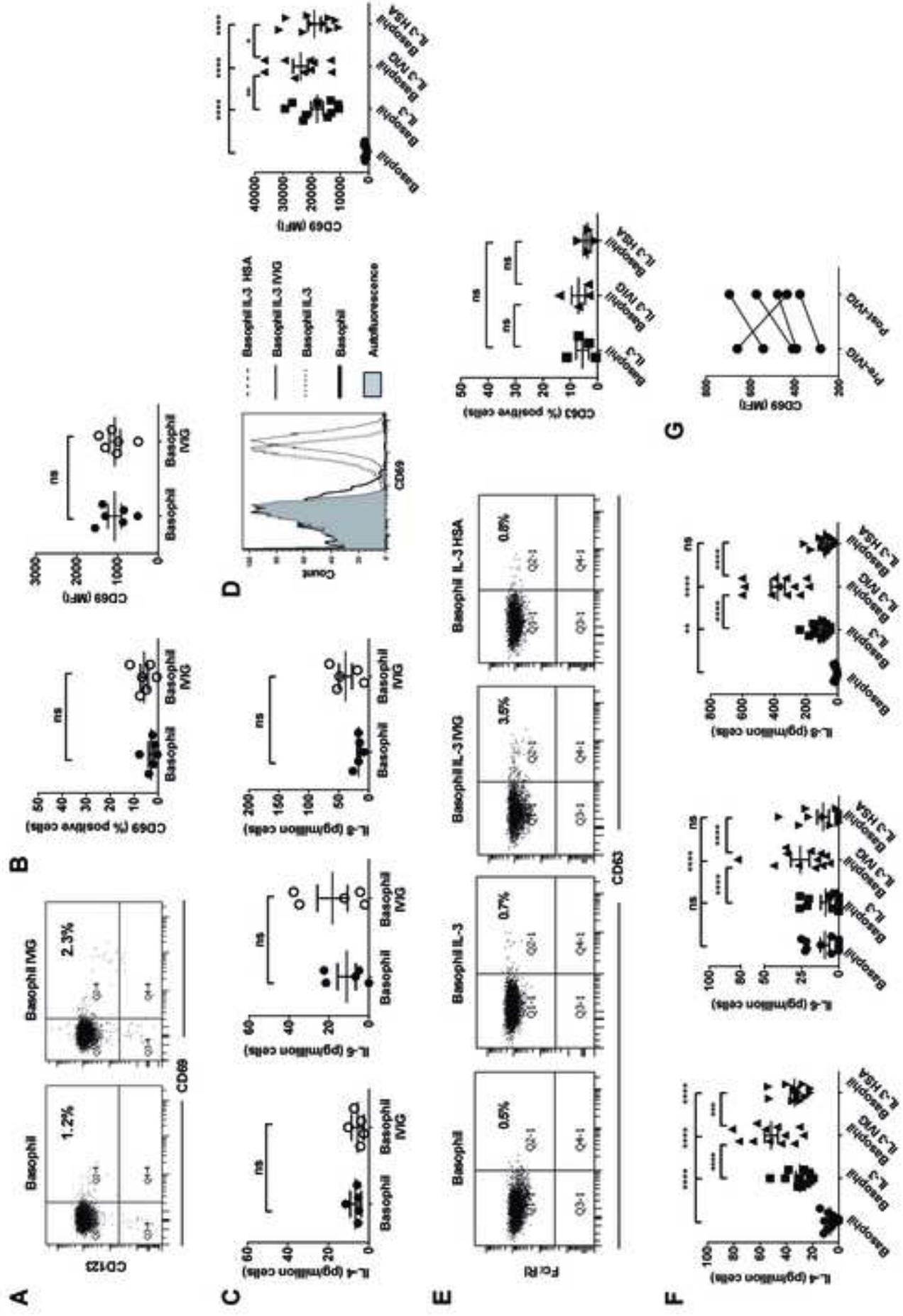
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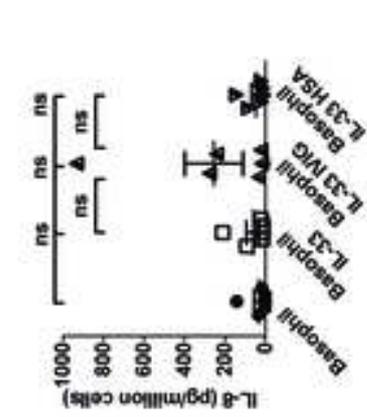
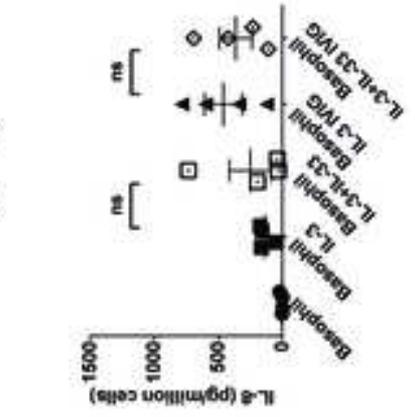
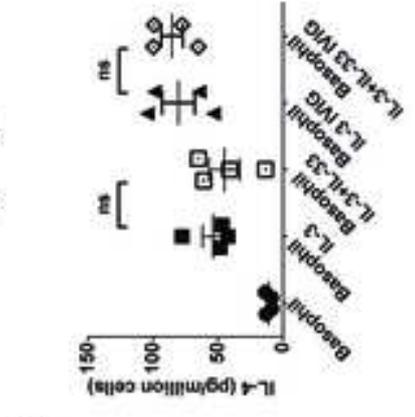
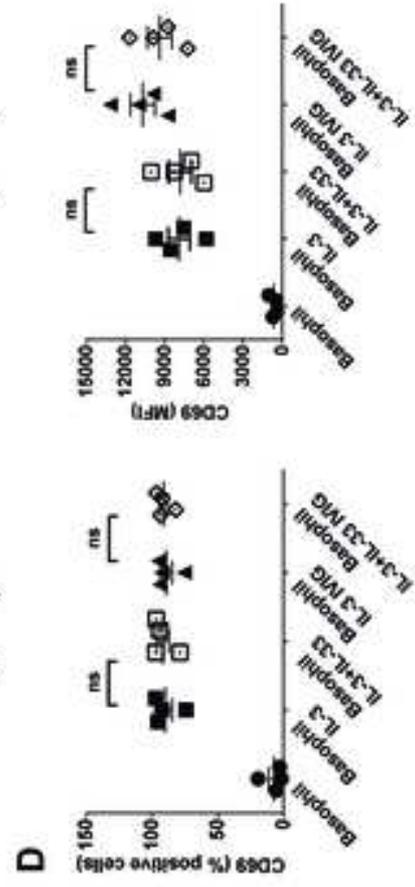
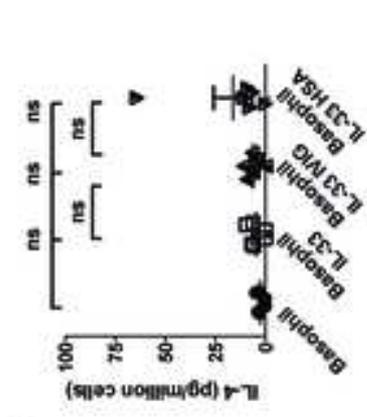
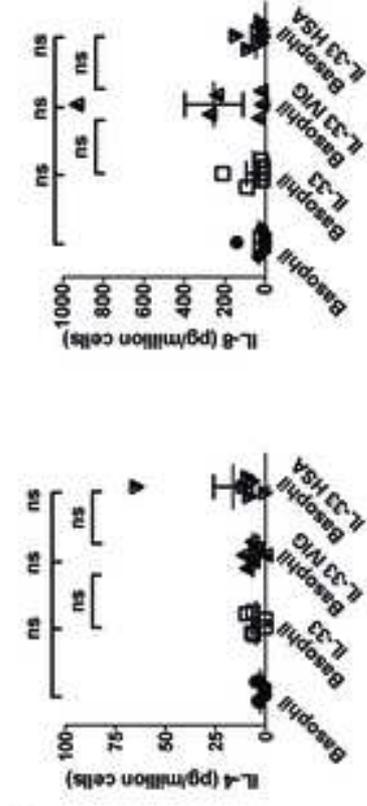
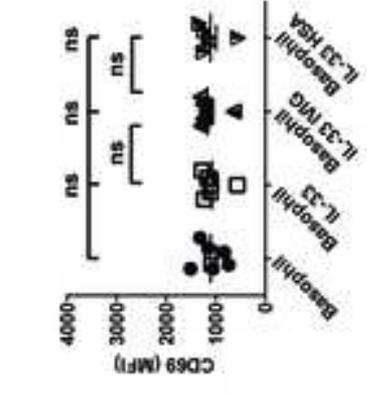
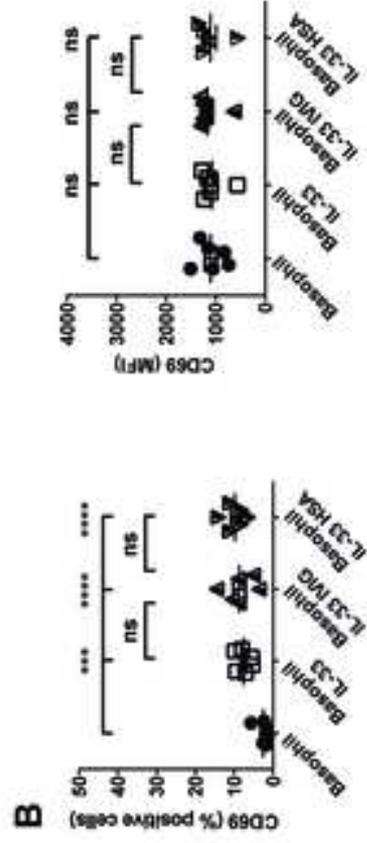
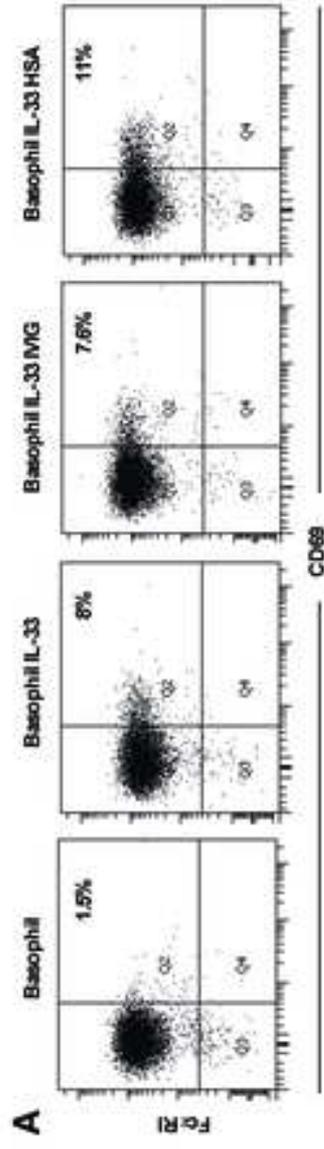
718 **FIG 6.** Inhibition of Syk pathway abrogates IVIG-induced activation of basophils. **A and**  
719 **B,** Representative histogram overlays and mean±SEM (n=6 donors) of phosphorylated Syk  
720 (pSyk) expression in basophils stimulated with IL-3 or IL-3 plus IVIG. **C and D,** The  
721 effect of Syk inhibition by R406 towards IVIG-induced expression of CD69  
722 (Representative histogram overlays and mean±SEM, n=5 donors). **E,** Syk inhibition  
723 abrogates IVIG-induced IL-4, and IL-8 secretion (mean±SEM, n=4 donors). \*P<0.05;  
724 \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001; ns, not significant, one-way ANOVA with  
725 Tukey's multiple comparison tests.

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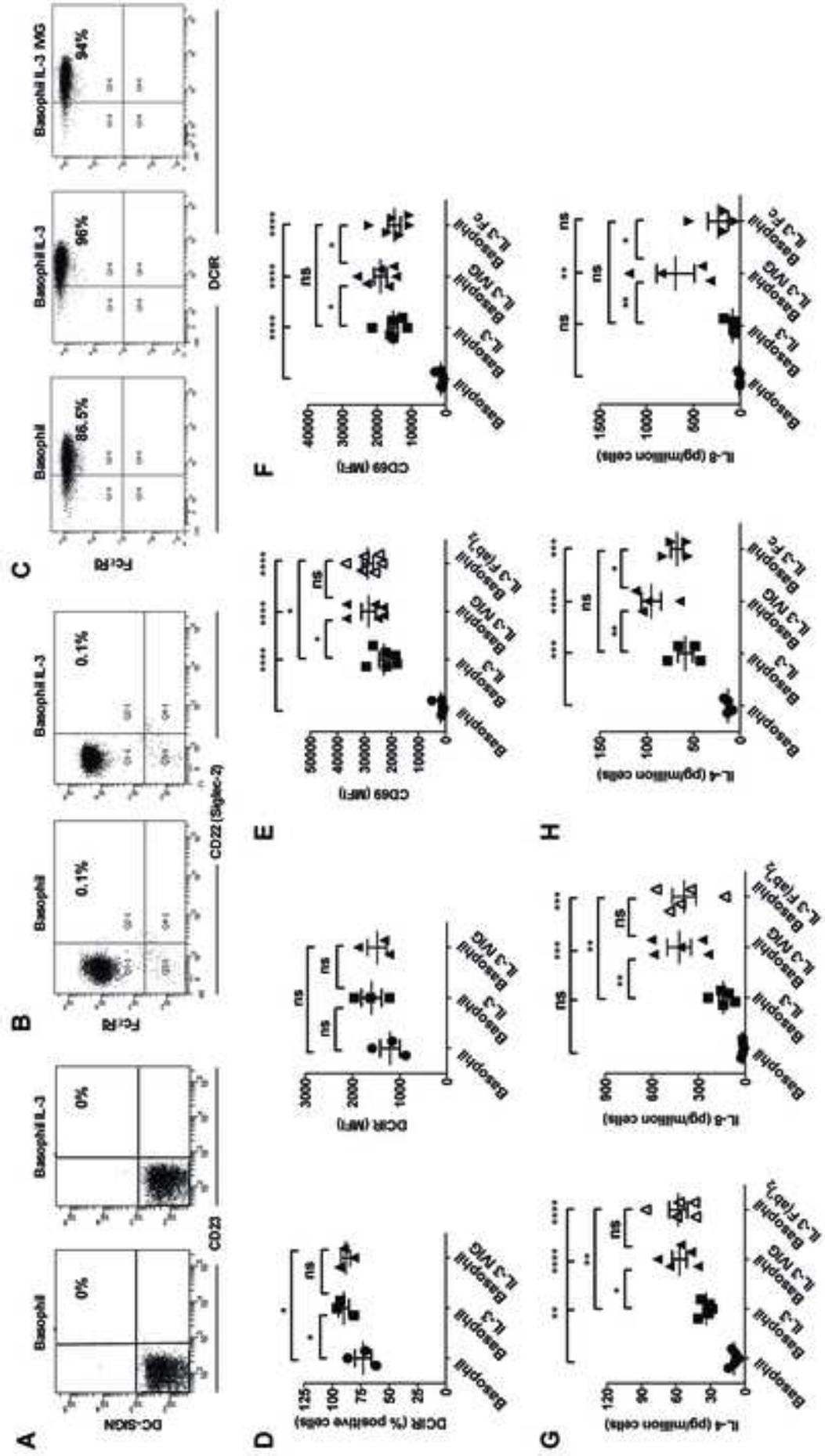
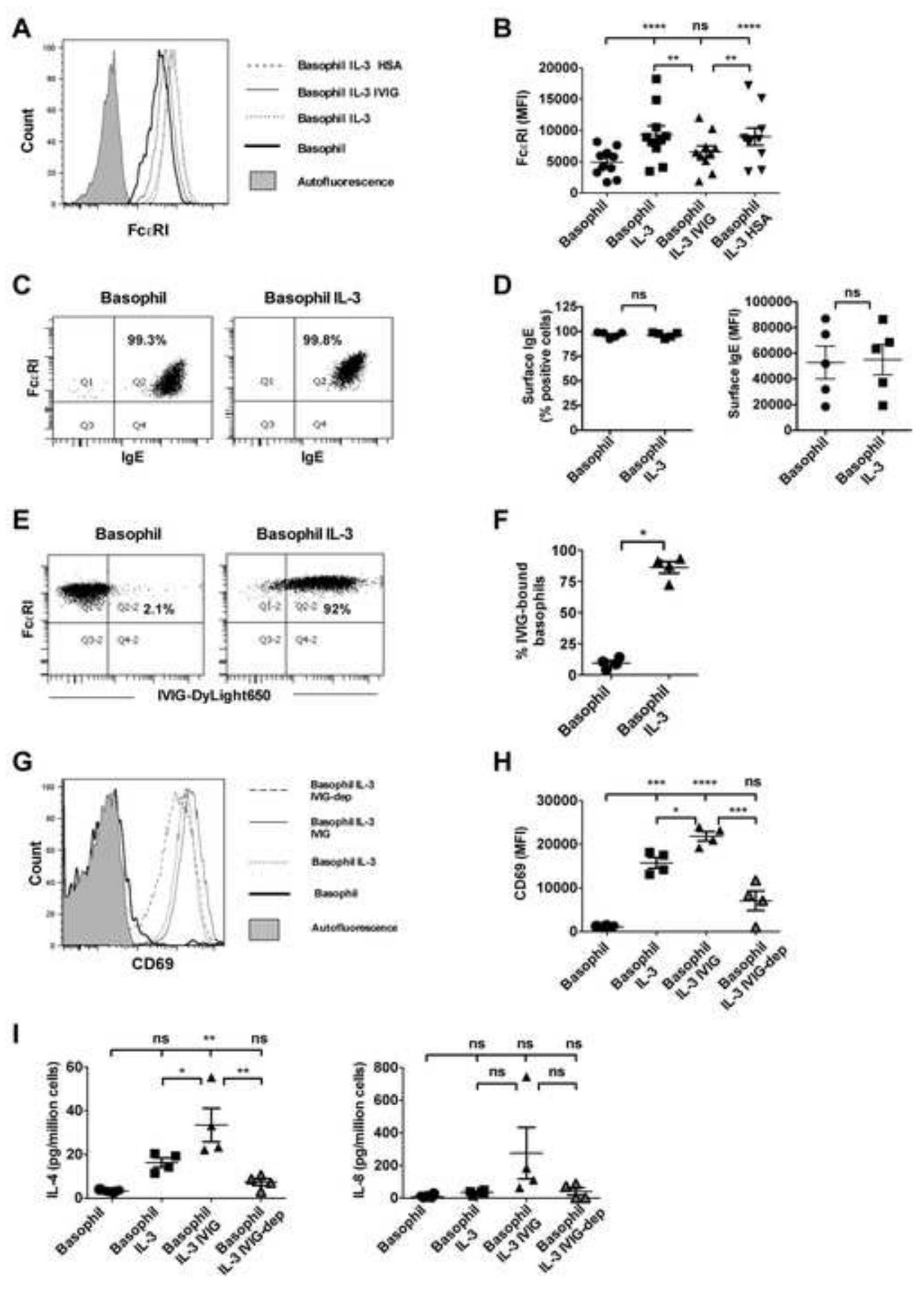
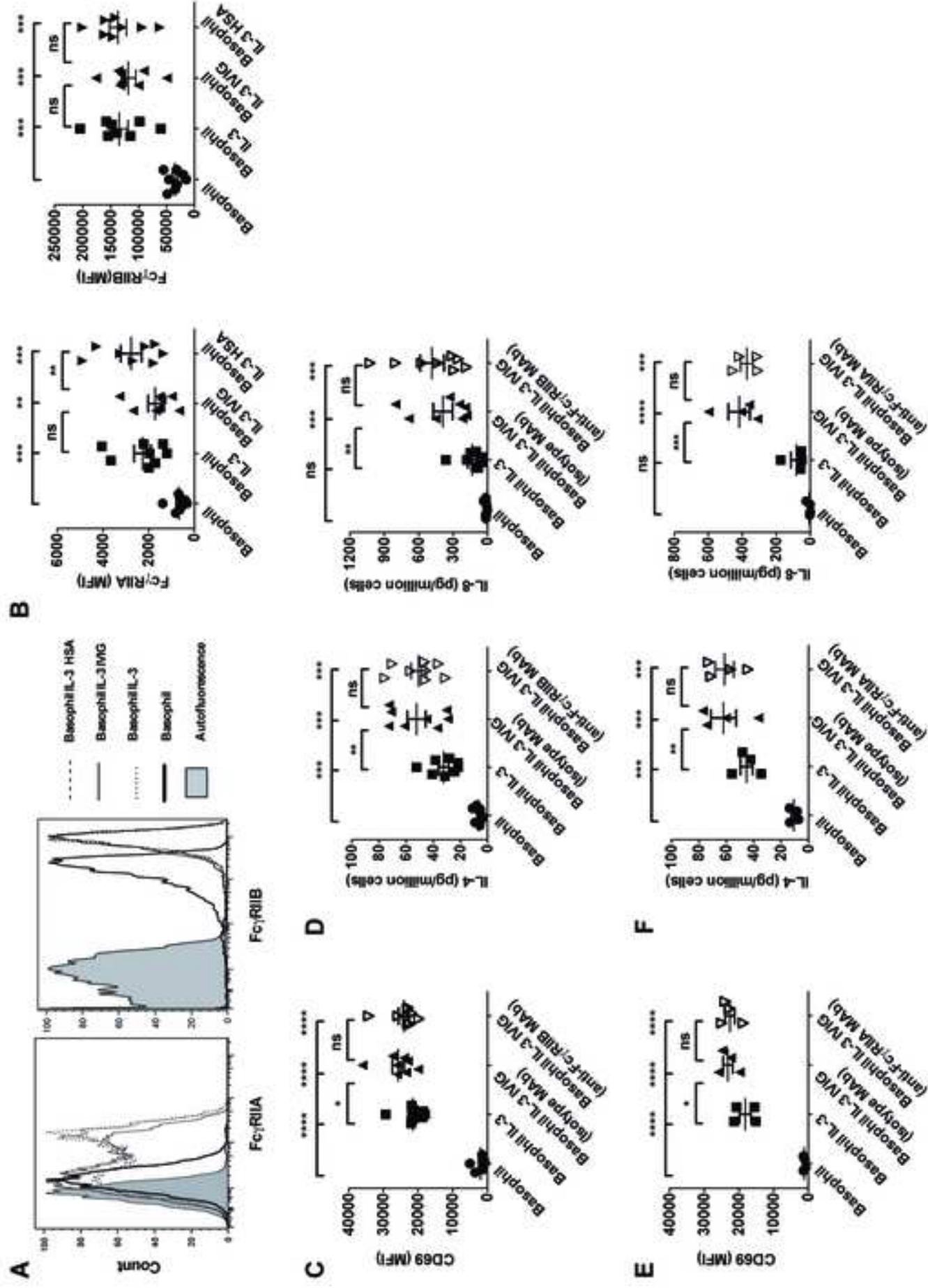
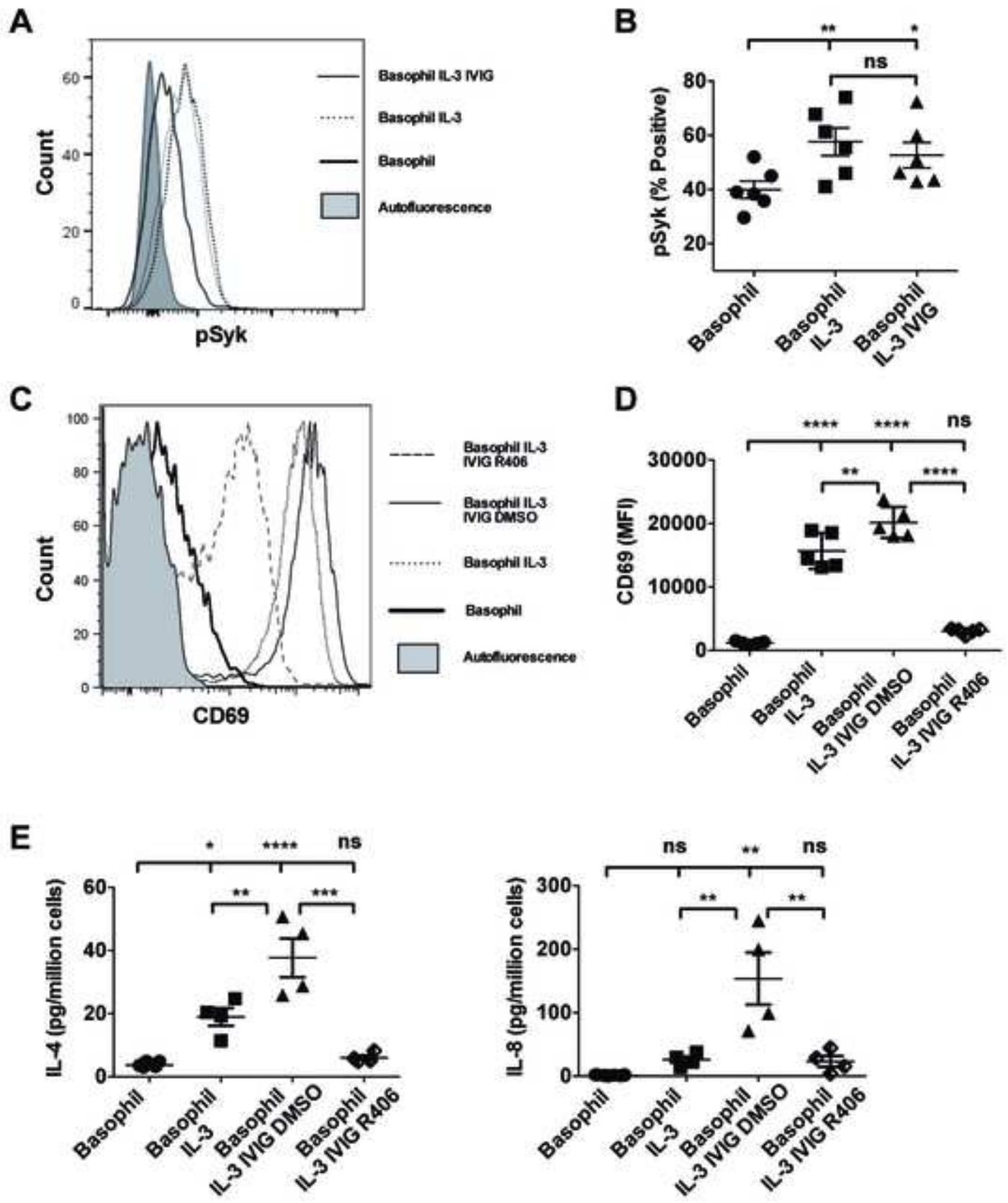


Figure No. 3







Galeotti et al

**Online Repository**

**Intravenous immunoglobulin induces IL-4 in human basophils by signaling through surface-bound IgE**

**Caroline Galeotti, MD, PhD<sup>1,2</sup>, Emmanuel Stephen-Victor, PhD<sup>1,\*</sup>, Anupama Karnam, MSc<sup>1,\*</sup>, Mrinmoy Das, PhD<sup>1</sup>, Laurent Gilardin, MD<sup>1,3</sup>, Mohan S Maddur, DVM, PhD<sup>1,4</sup>, Sandra Wymann, PhD<sup>5</sup>, Cédric Vonarburg, PhD<sup>5</sup>, Alain Chevailler, MD, PhD<sup>6</sup>, Jordan D Dimitrov, PhD<sup>1,4</sup>, Olivier Benveniste, MD<sup>3,7</sup>, Pierre Bruhns, PhD<sup>8,9</sup>, Srinivasa Kaveri, DVM, PhD<sup>1,4</sup>, Jagadeesh Bayry, DVM, PhD<sup>1,4</sup>**

<sup>1</sup>Institut National de la Santé et de la Recherche Médicale; Centre de Recherche des Cordeliers, Equipe- Immunopathologie et Immunointervention Thérapeutique; Sorbonne Université, Paris, F-75006, France

<sup>2</sup>Service de Rhumatologie Pédiatrique, Centre de Référence des Maladies Auto-Inflammatoires rares et des Amyloses, CHU de Bicêtre, le Kremlin Bicêtre, F-94270, France

<sup>3</sup> Département de Médecine Interne et Immunologie Clinique, Hôpital Pitié-Salpêtrière, AP-HP, Paris, F-75013, France.

<sup>4</sup>Université Paris Descartes, Sorbonne Paris Cité, Paris, F-75006, France

<sup>5</sup>Research Department, CSL Behring AG, 3014 Bern, Switzerland

<sup>6</sup>Laboratoire d'Immunologie et d'Allergologie; CHU d'Angers; Université d'Angers; INSERM Unité1232; LabEx IGO "Immuno-Graft-Onco", Angers, F-49933, France

<sup>7</sup>Sorbonne Université, Institut National de la Santé et de la Recherche Médicale Unité 974, Paris, F-75013, France.

<sup>8</sup>Institut Pasteur, Department of Immunology, Unit of Antibodies in Therapy and Pathology, Paris, France

<sup>9</sup>INSERM, Unité 1222, Paris, France

\* Equally contributed

29 **Correspondence to:** Jagadeesh Bayry, Institut National de la Santé et de la Recherche  
30 Médicale Unité 1138, Centre de Recherche des Cordeliers, 15 rue de l'Ecole de Médecine,  
31 Paris, F-75006, France. Tel: 00 33 1 44 27 82 03 ; Fax: 00 33 1 44 27 81 94  
32 E-mail: [jagadeesh.bayry@crc.jussieu.fr](mailto:jagadeesh.bayry@crc.jussieu.fr)

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34 **Total word count: 134**

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## 50 **METHODS**

### 51 **List of antibodies for flow cytometry and functional assays**

52 CD63-PE (Clone:H5C6), CD13-APC (Clone:WM15), CD123-BV421 (Clone:9F5), CD69-  
53 APC/Cy7 (Clone:FN50), CD209-APC (Clone:DCN46), CD22-PE (Clone:S-HCL-1) and  
54 CD62L-FITC (Clone:DREG-56) were from BD Biosciences. FcεRIα-FITC (Clone:CRA-1),  
55 SIGLEC3-FITC (Clone:AC104.3E3), SIGLEC5-FITC (Clone:1A5), SIGLEC7-FITC  
56 (Clone:REA214), SIGLEC8-APC (Clone:7C9), anti-IgE-APC (clone:MB10-5C4) MAbs  
57 were obtained from Miltenyi Biotec. CD203c-PE (Clone:NP4D6), CD23-PE (Clone:B3B4),  
58 CD107a-BV421 (Clone:H4A3), FcεRIα-BV510 (Clone:AER37 [CRA-1]) and DCIR-PE  
59 (Clone:9E8) MAbs were from BioLegend. Anti-IgE MAb (Clone:GE-1) was from Sigma  
60 Aldrich. Unconjugated and FITC-labelled FcγRIIA MAb (Clone:IV.3) was purchased from  
61 Stem Cells Technologies. Human ST2/IL-33R-PE polyclonal goat IgG and Isotype control  
62 MAbs for blocking experiments were from R&D Systems. Anti-human p-Syk (Tyr348)  
63 (clone: moch1ct) was from eBioscience. Anti-hFcγRIIB (Clone:2B6 variant N<sub>297</sub>D) MAbs  
64 were coupled to Alexa Fluor 647 by using ThermoFisher Scientific kit and IVIG was labelled  
65 with the Lightning-Link® Rapid DyLight® 650 kit (Innova Biosciences).

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72 **Supplementary Figure Legends**

73 **FIG E1.** Effect of IVIG on the expression of various surface markers in IL-3-primed  
74 basophils. **A and B,** Basophils were cultured either alone or with IL-3. IVIG or HSA were  
75 added following 2 hours stimulation with IL-3. (A) Representative histogram overlays and  
76 (B), expression (mean±SEM, n=4-12 donors) of CD69, CD13 (both in % positive cells),  
77 CD62L, CD123 and CD203c (all MFI) on the basophils. \*P<0.05; \*\*\*P<0.001; ns, not  
78 significant, one-way ANOVA with Tukey's multiple comparison tests.

79

80 **FIG E2.** Activation of IL-3-primed basophils by IVIG is not associated with degranulation. **A**  
81 **and B,** Changes in the expression of CD107a. Representative plots and mean±SEM of data  
82 from four independent donors. **C,** Amount of histamine in the culture supernatants  
83 (mean±SEM, n=5 donors). ns, not significant, one-way ANOVA with Tukey's multiple  
84 comparison tests.

85

86 **FIG E3. A and B.** Real-time quantitative RT-PCR analysis of *il4* transcripts and amount of  
87 IL-4 secretion in resting basophils, cells treated with IL-3 or IL-3 plus IVIG for three hours.  
88 \*P<0.05; \*\*P<0.05; ns, not significant, one-way ANOVA (with Dunnet's (for Panel A) or  
89 Tukey's (for Panel B) multiple comparison tests).

90

91 **FIG E4.** The expression of IL-33R (% positive cells and MFI) on resting, IL-33- or IL-3-  
92 stimulated basophils (mean±SEM, n=8 donors). \*P<0.05; \*\*P<0.01; \*\*\*\*P<0.0001; ns, not  
93 significant, one-way ANOVA with Tukey's multiple comparison tests.

94

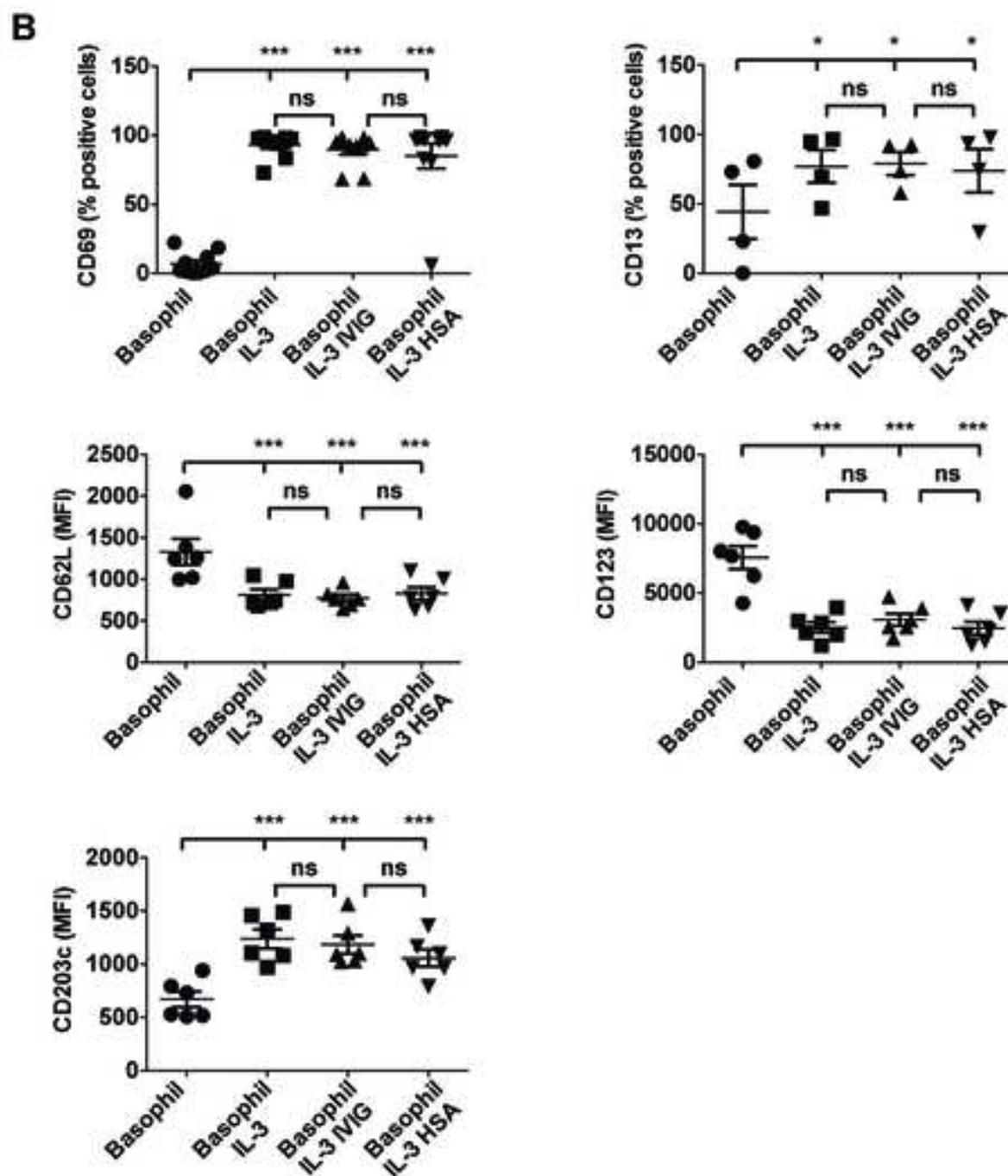
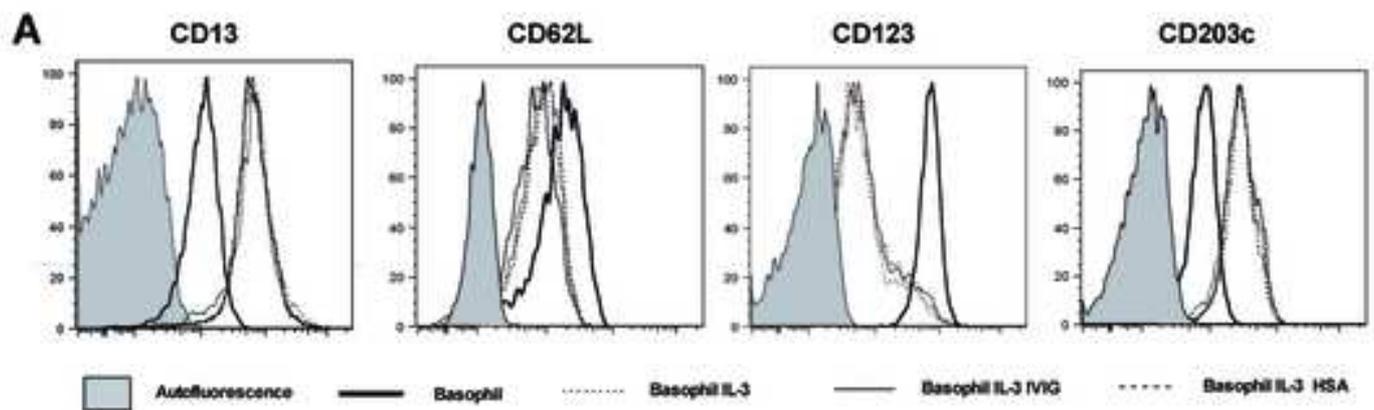
95 **FIG E5.** IL-25, TSLP and GM-CSF are dispensable for the activation of basophils by IVIG  
96 **A-C,** Basophils were cultured either alone or with (A) IL-25, (B) TSLP or (C) GM-CSF for  
97 24 hours. IVIG was added following 2 hours stimulation with respective cytokines. The  
98 expression of CD69 (% positive cells or MFI) and the amount of secretion of IL-4  
99 (mean±SEM, n=5 donors) are presented. \*P<0.05; \*\*P<0.01; ns, not significant, one-way  
100 ANOVA with Tukey's multiple comparison tests.

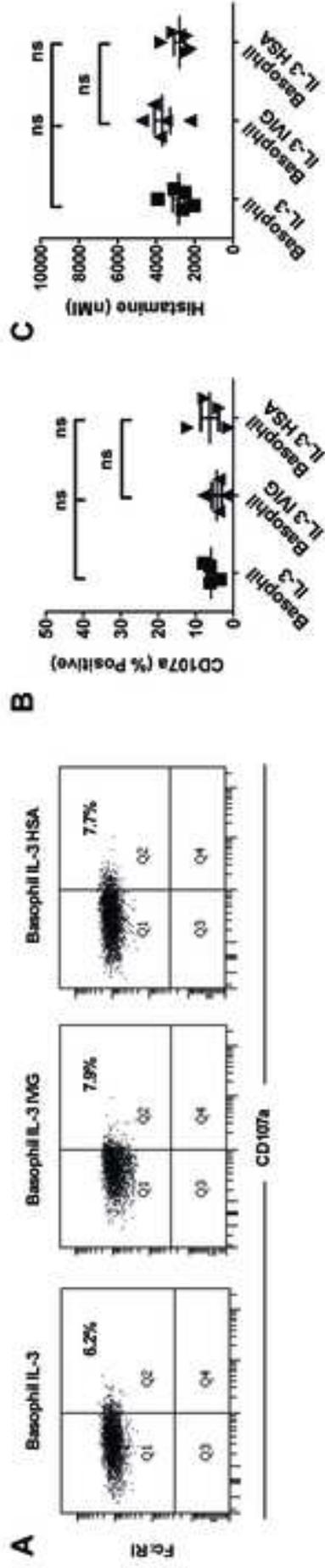
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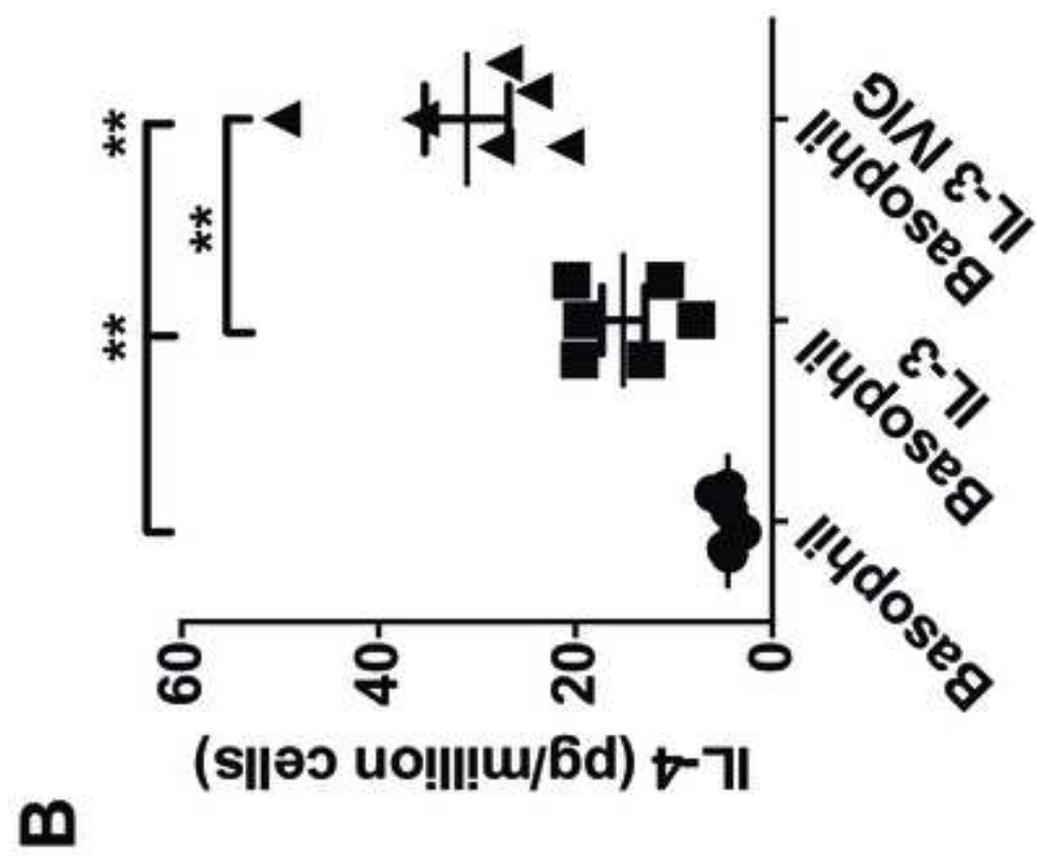
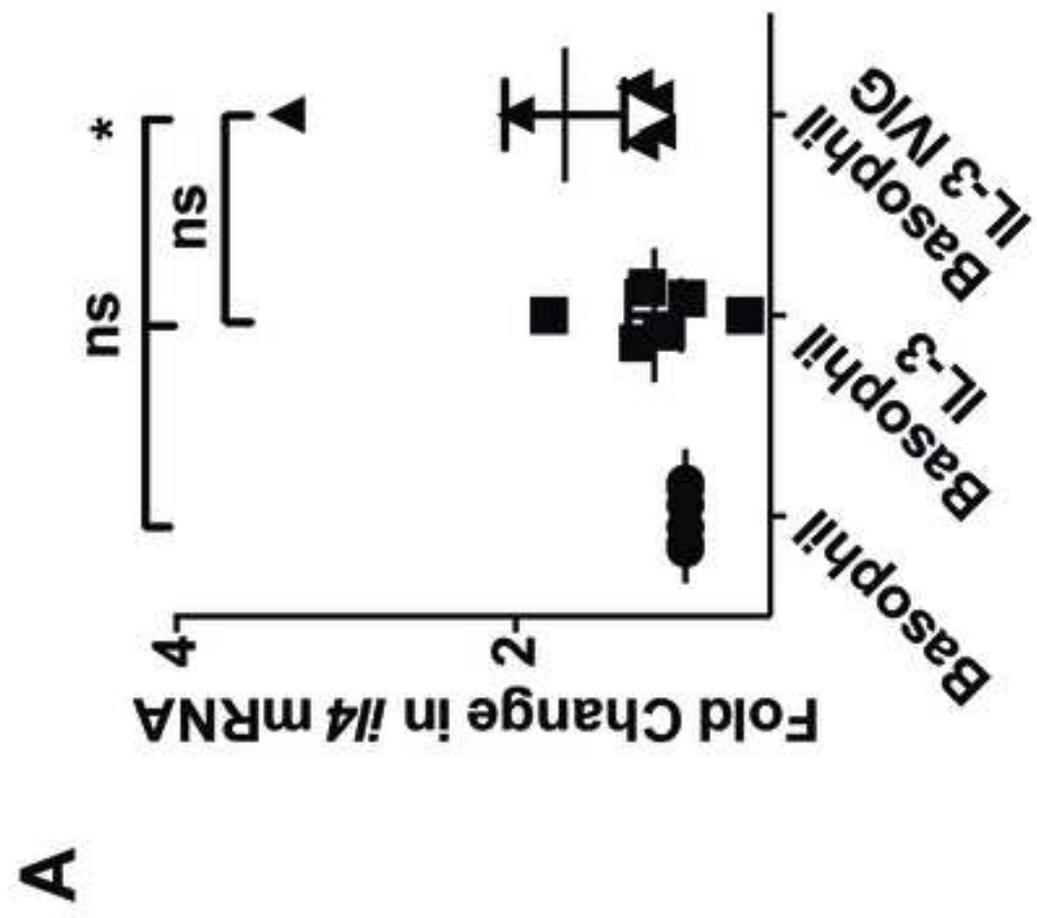
102 **FIG E6. A-B,** The expression of (A) Siglec-3 and Siglec-5/14; (B) Siglec-7 and Siglec-8 on  
103 resting and IL-3-primed basophils.

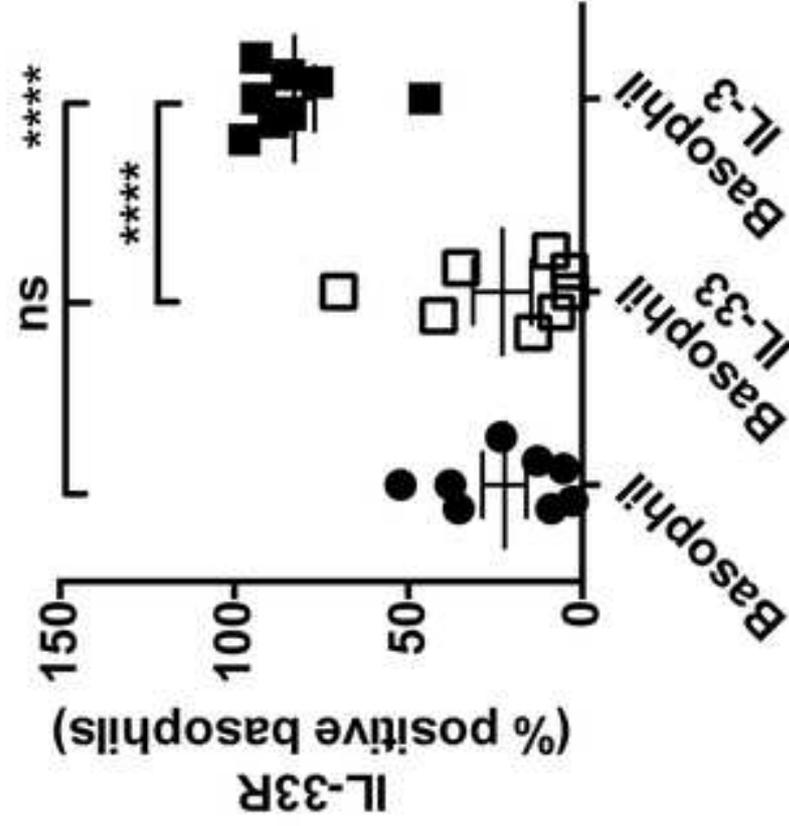
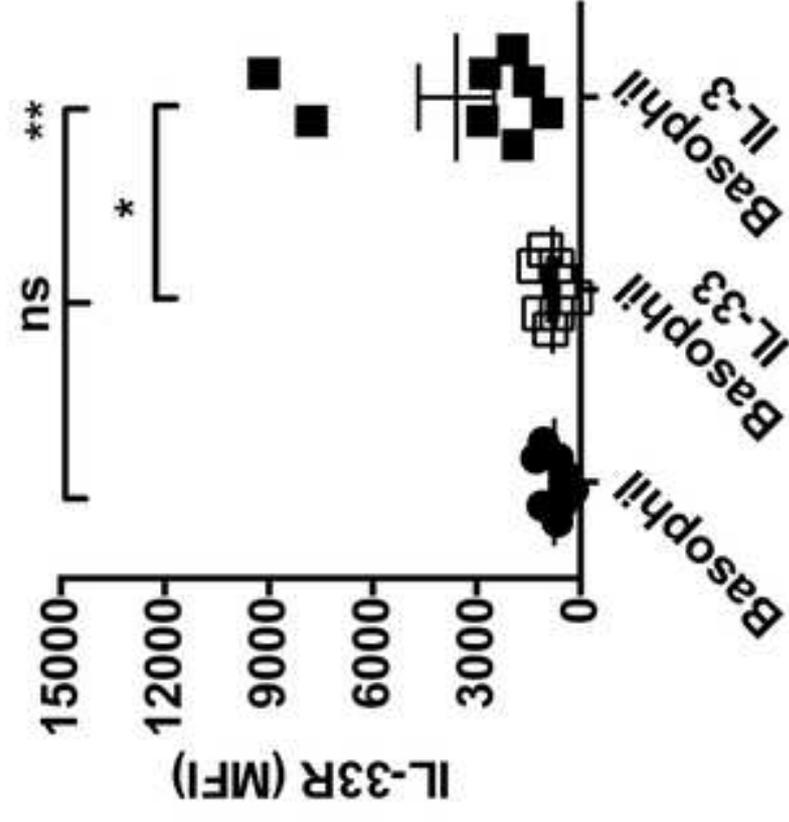
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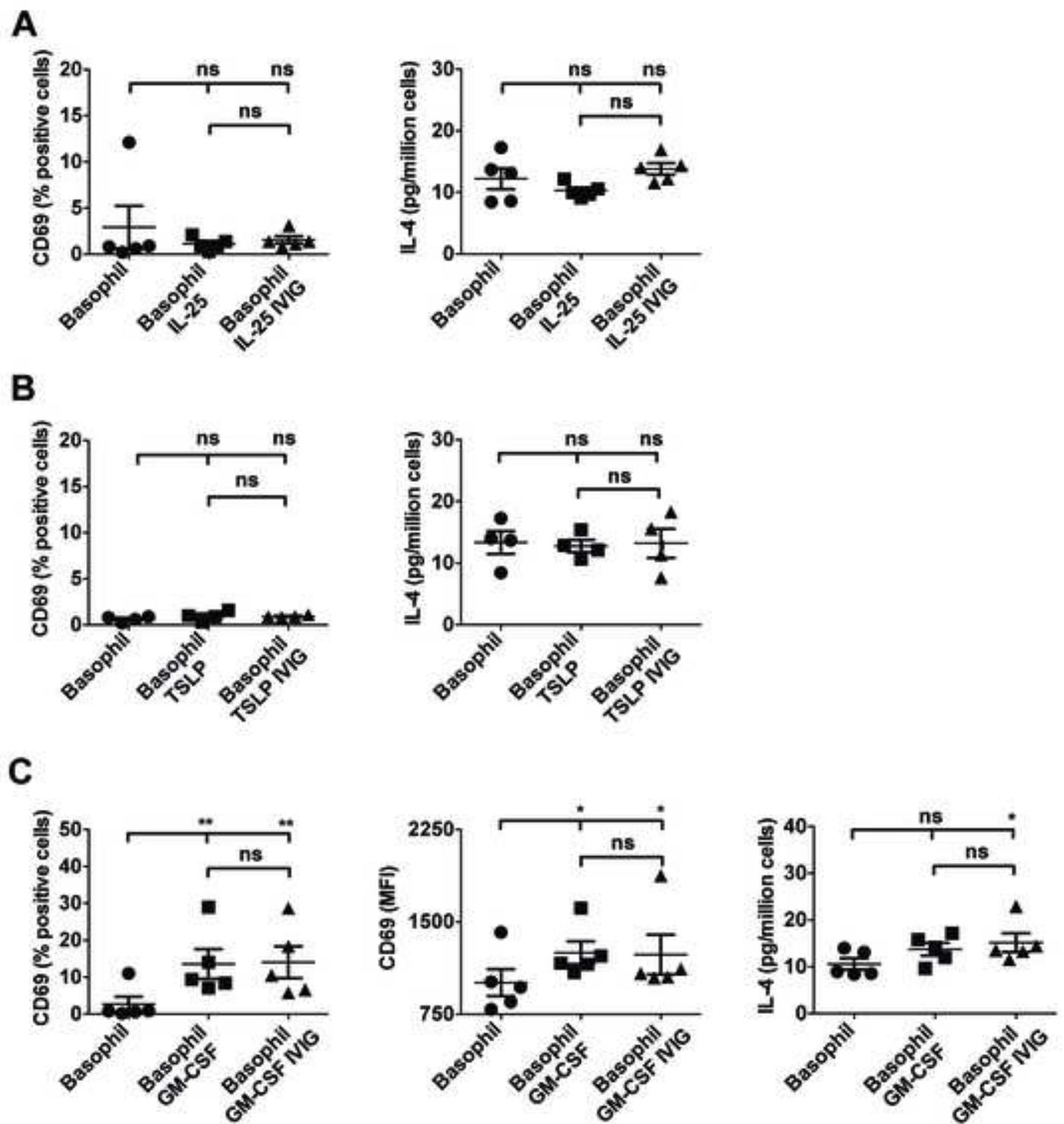
105 **FIG E7:** The effect of Syk inhibition towards IVIG-induced expression of *il4* transcripts  
106 (mean±SEM, n=5 donors). Basophils were stimulated with IL-3 plus IVIG for three hours.  
107 Additionally, cells were also treated with syk inhibitor R406 for one hour prior to stimulation  
108 with IL-3 plus IVIG. \*P<0.05; two-tailed Mann-Whitney test.



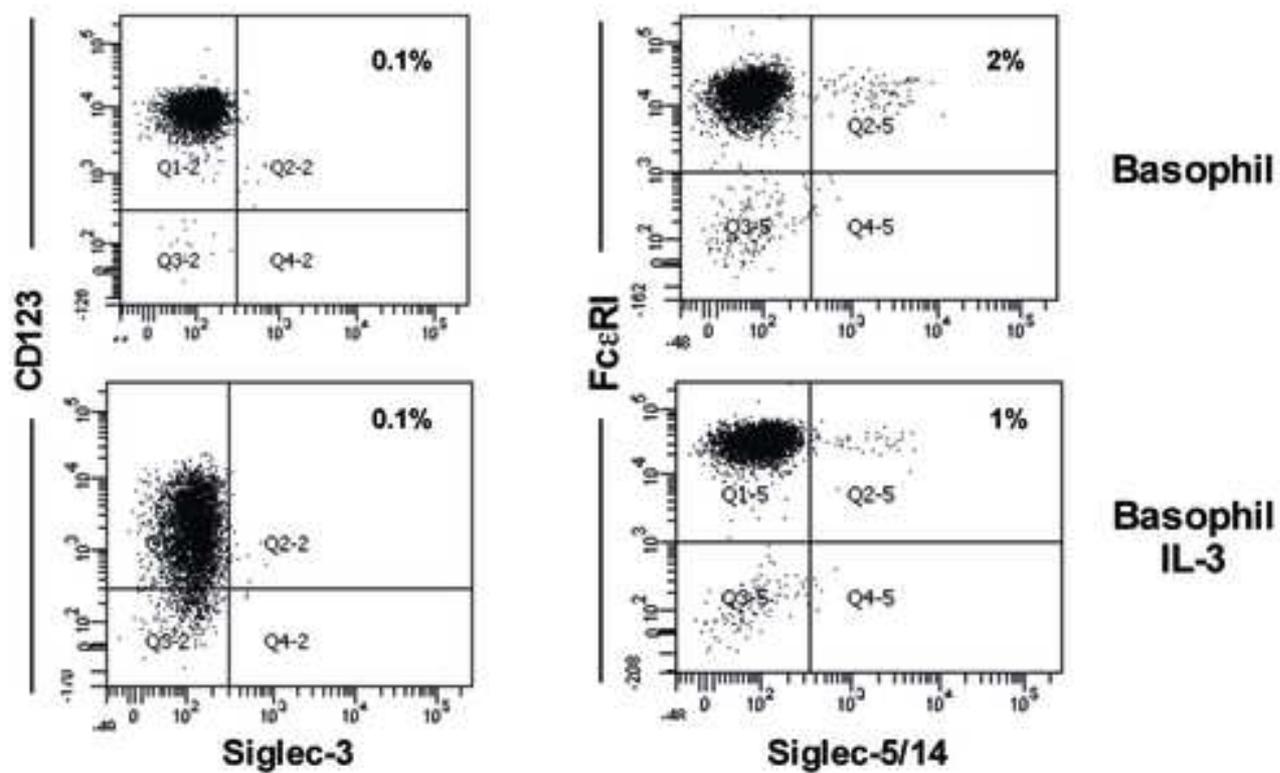




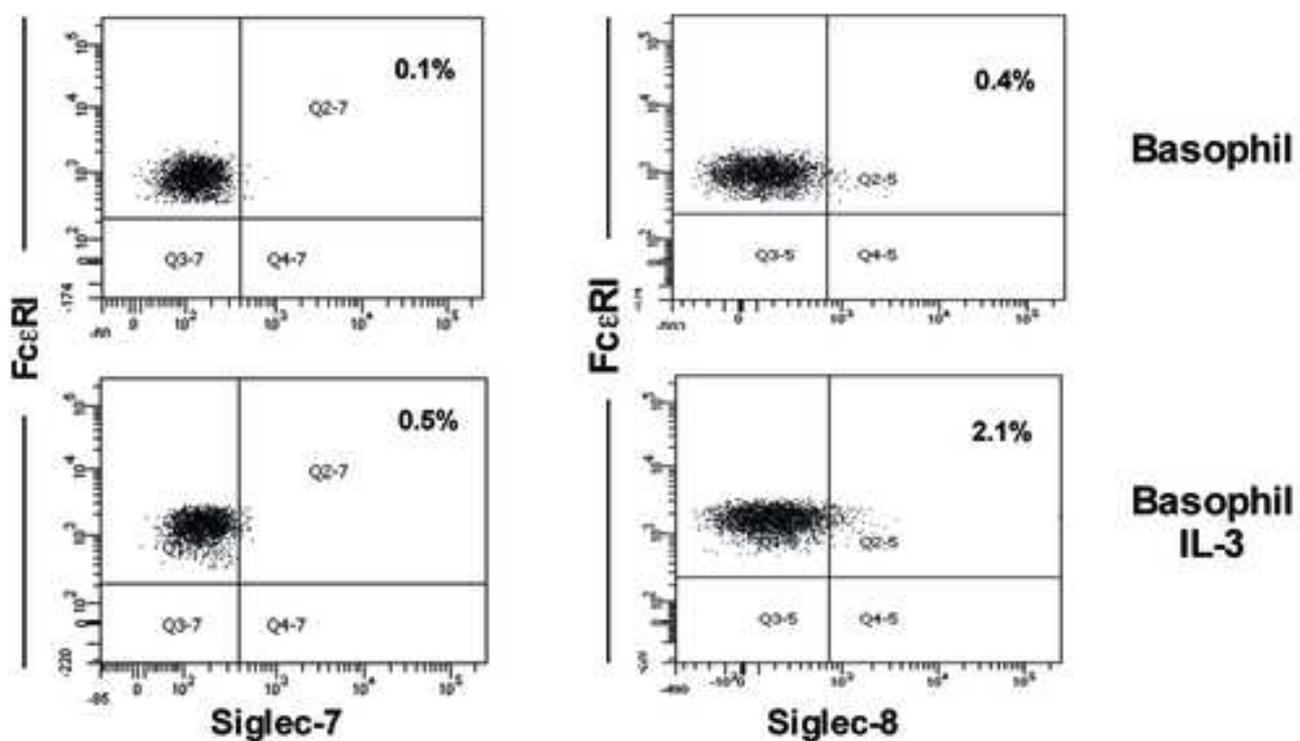


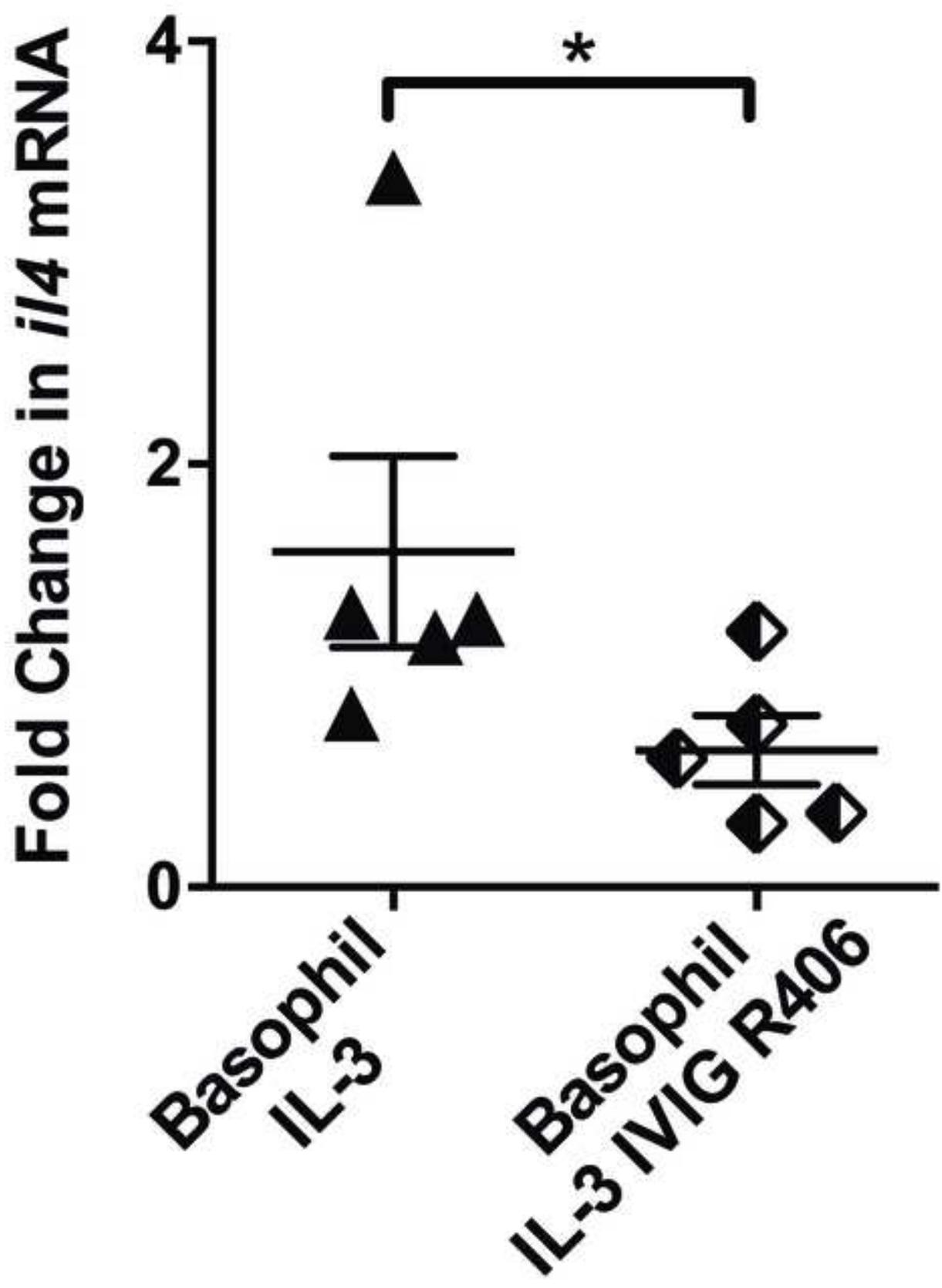


**A**



**B**





# Intravenous immunoglobulin (IVIg) activates human basophils through direct interaction with surface-bound IgE, and by IL-3- and Syk-dependent mechanisms

