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

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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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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/Fc $\gamma$ RII 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 $\pm$ SEM, n=8 donors) of Fc $\gamma$ RII and Fc $\gamma$ RIIB on the basophils. **C and D,** Repercussion  
712 of Fc $\gamma$ RIIB blockade on the (C) expression of CD69 and (D) amount of IL-4 and IL-8  
713 secretion (mean $\pm$ SEM, n=8 donors). **E and F,** Repercussion of Fc $\gamma$ RIIA blockade on the  
714 (E) expression of CD69 and (F) amount of IL-4 and IL-8 secretion (mean $\pm$ 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.

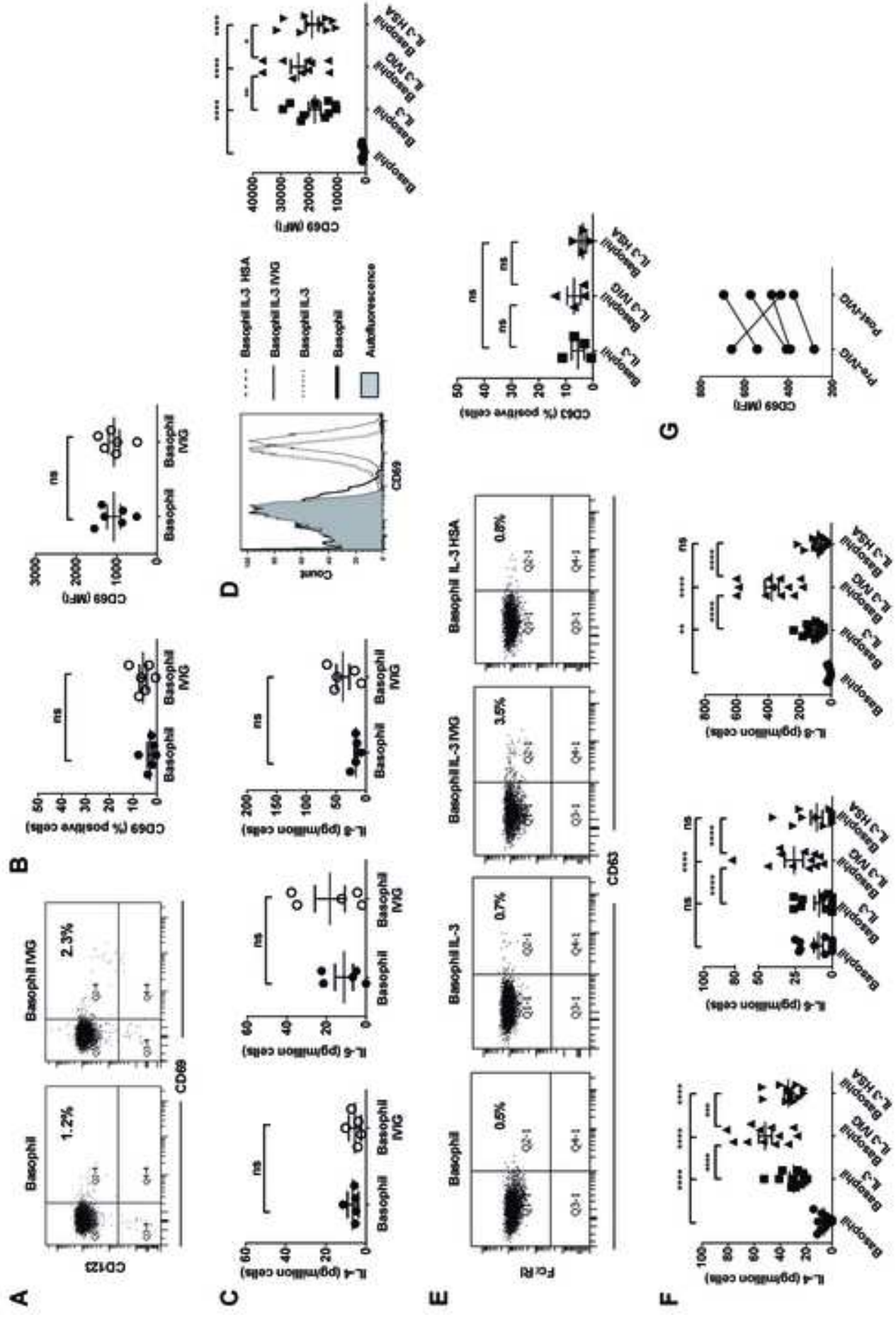
717

718 **FIG 6.** Inhibition of Syk pathway abrogates IVIG-induced activation of basophils. **A and**  
719 **B,** Representative histogram overlays and mean $\pm$ 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 $\pm$ SEM, n=5 donors). **E,** Syk inhibition  
723 abrogates IVIG-induced IL-4, and IL-8 secretion (mean $\pm$ 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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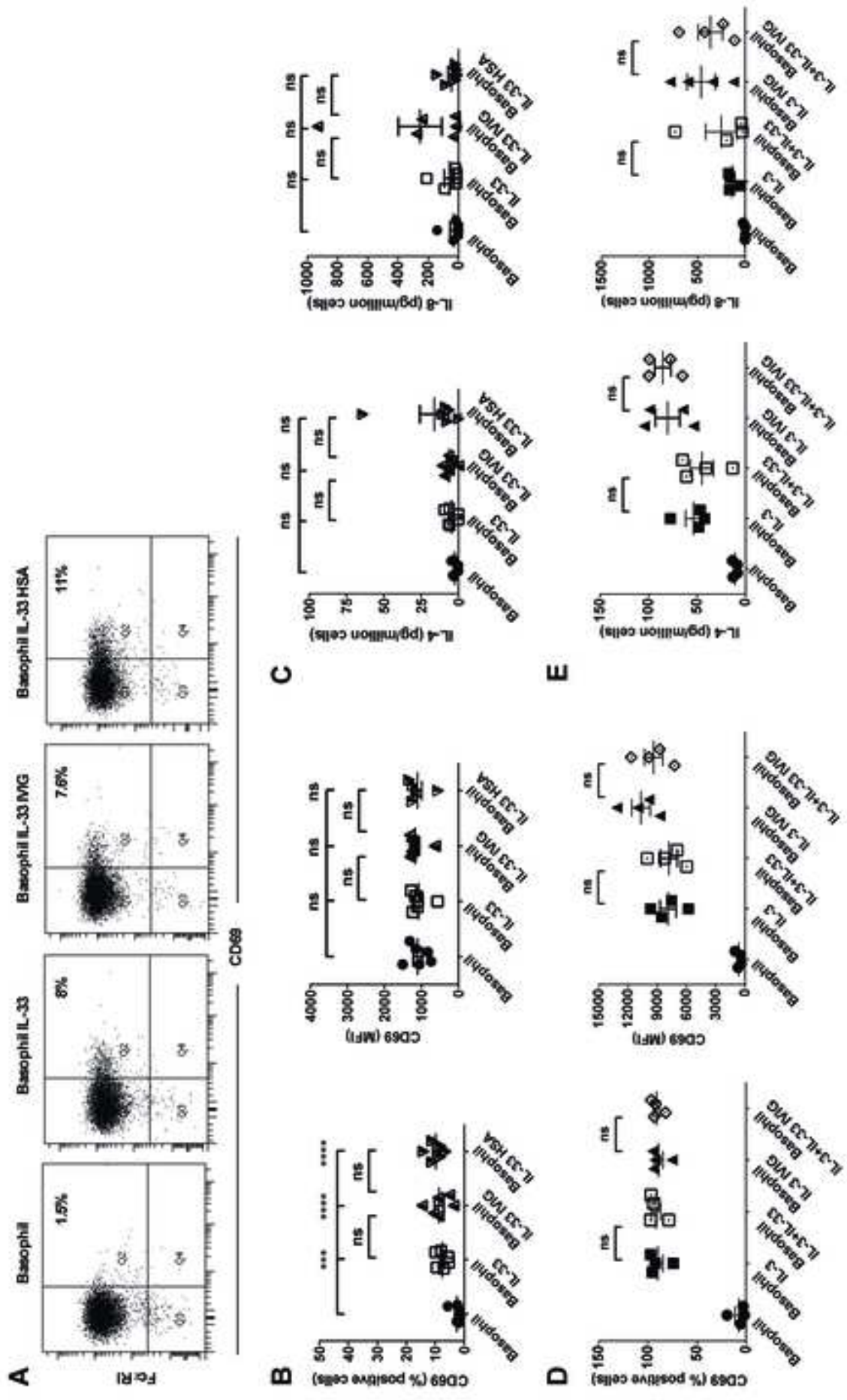


Figure No. 2

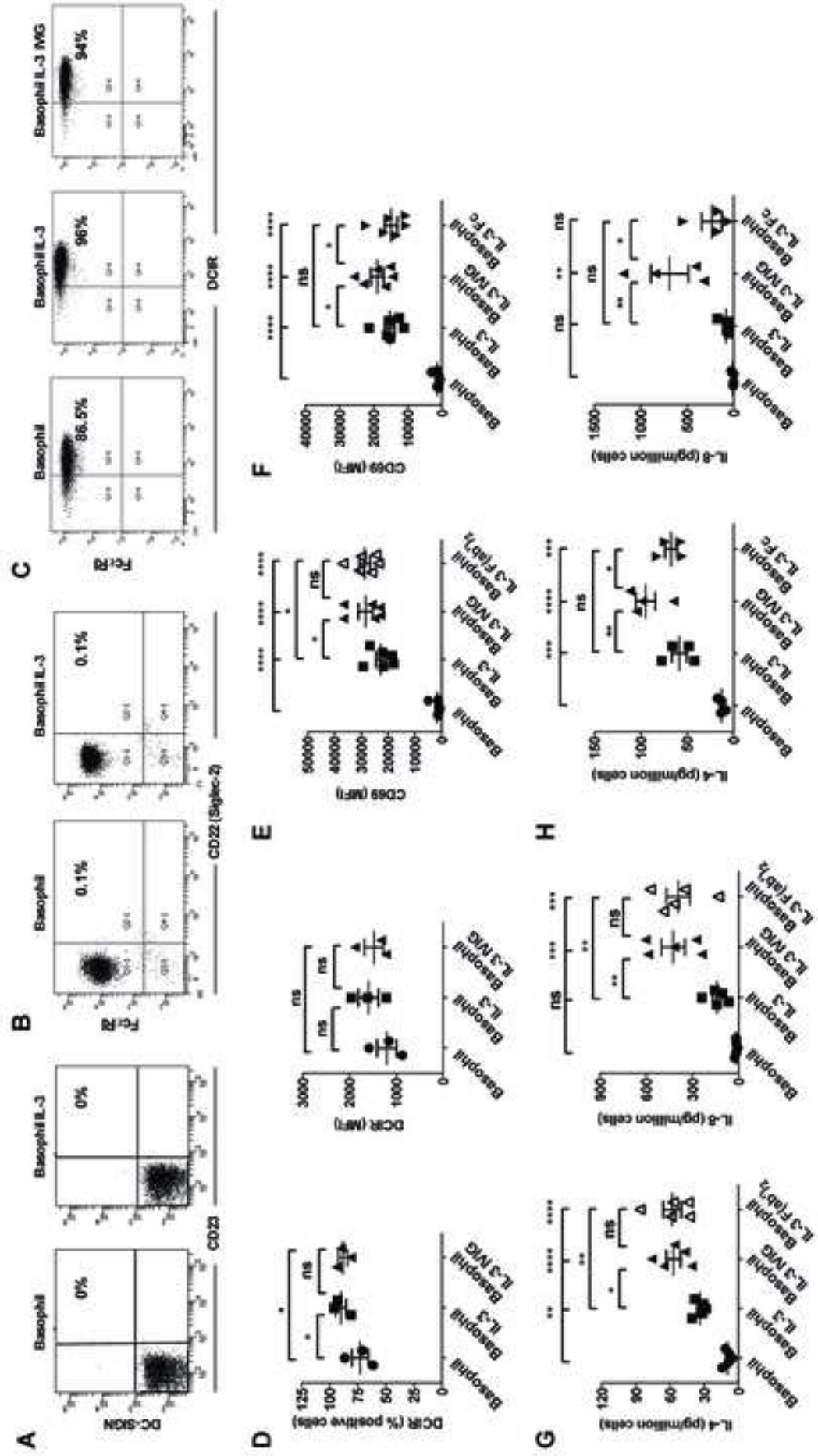
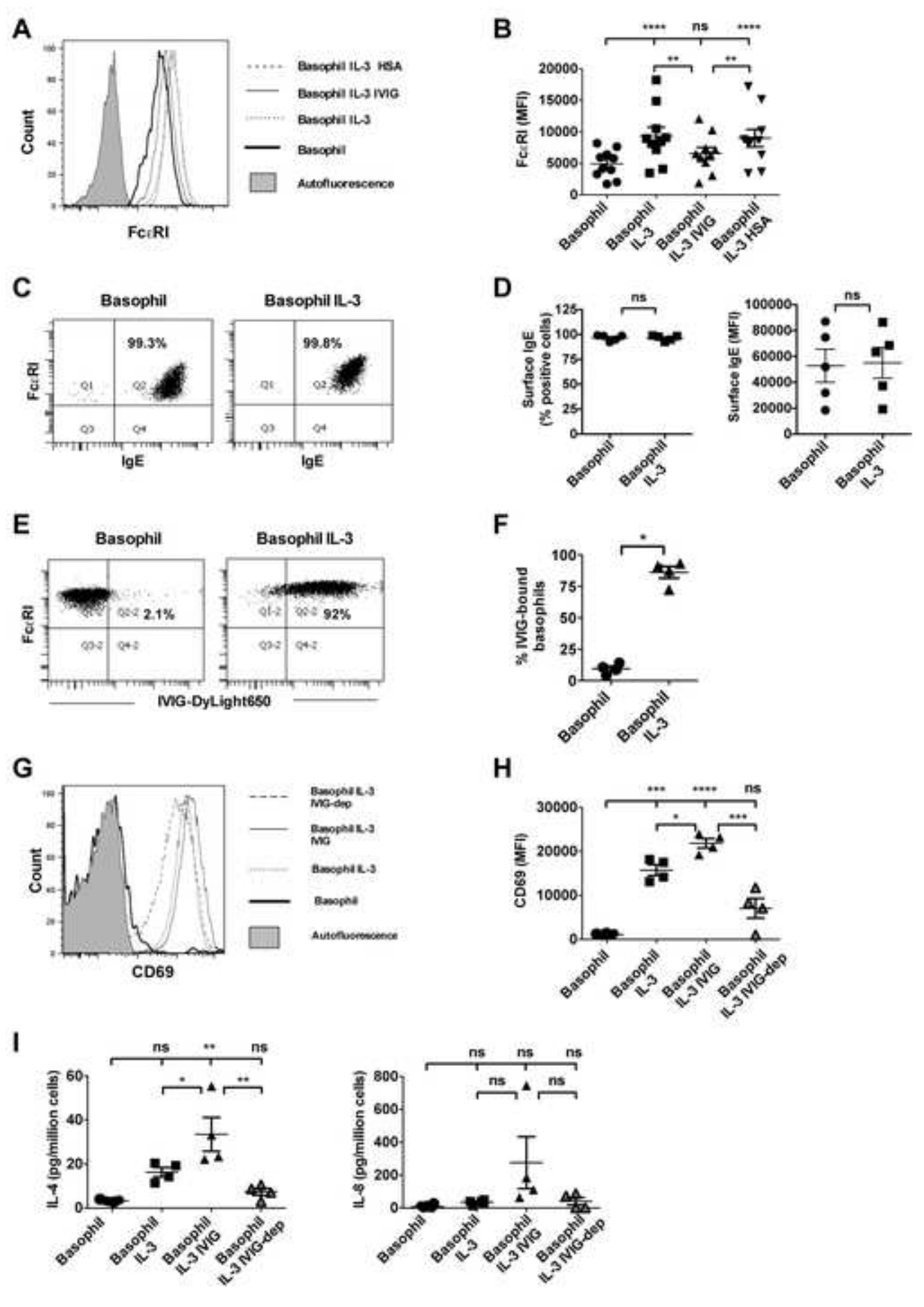
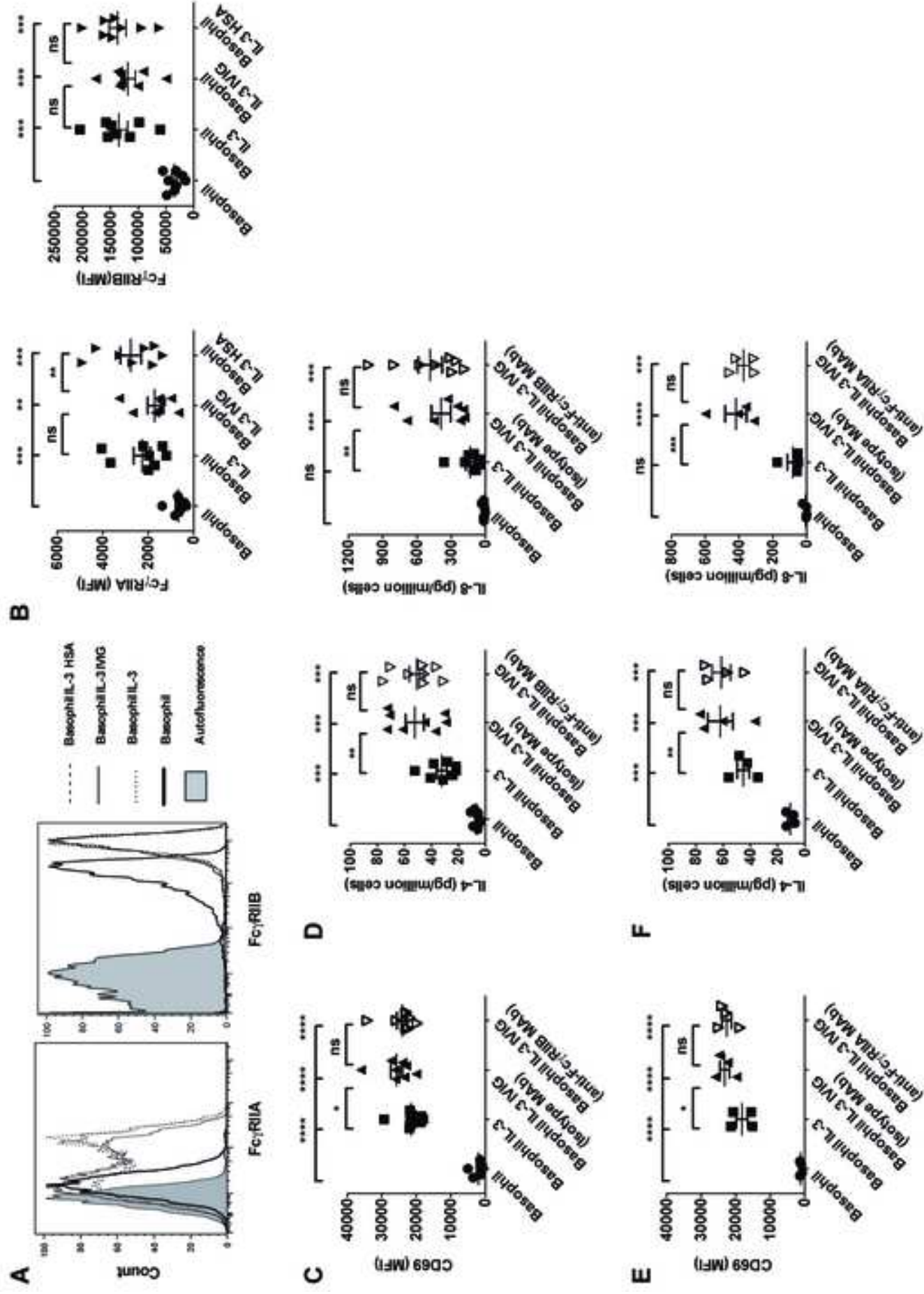
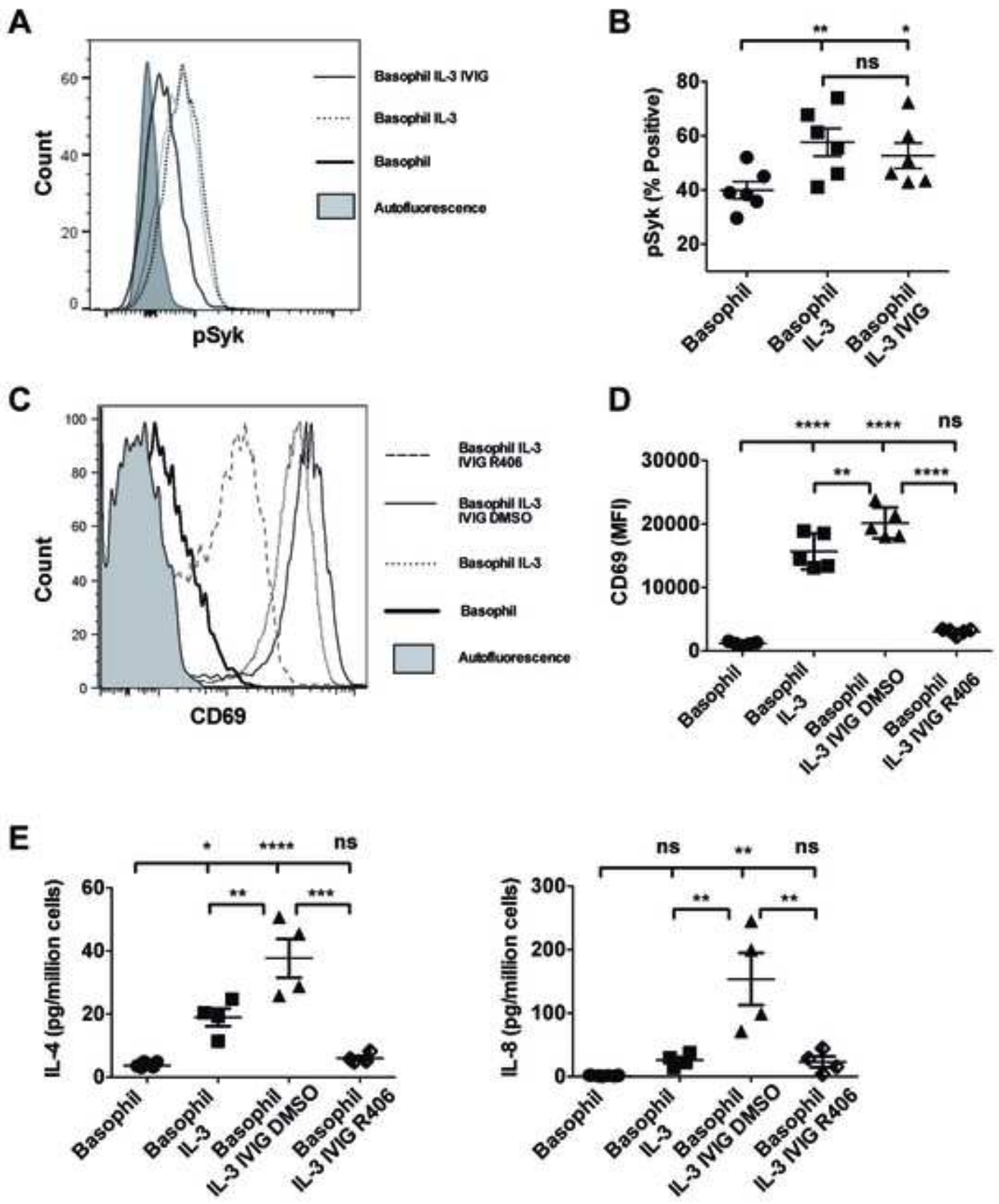


Figure No. 3









Galeotti et al

**Online Repository**

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

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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

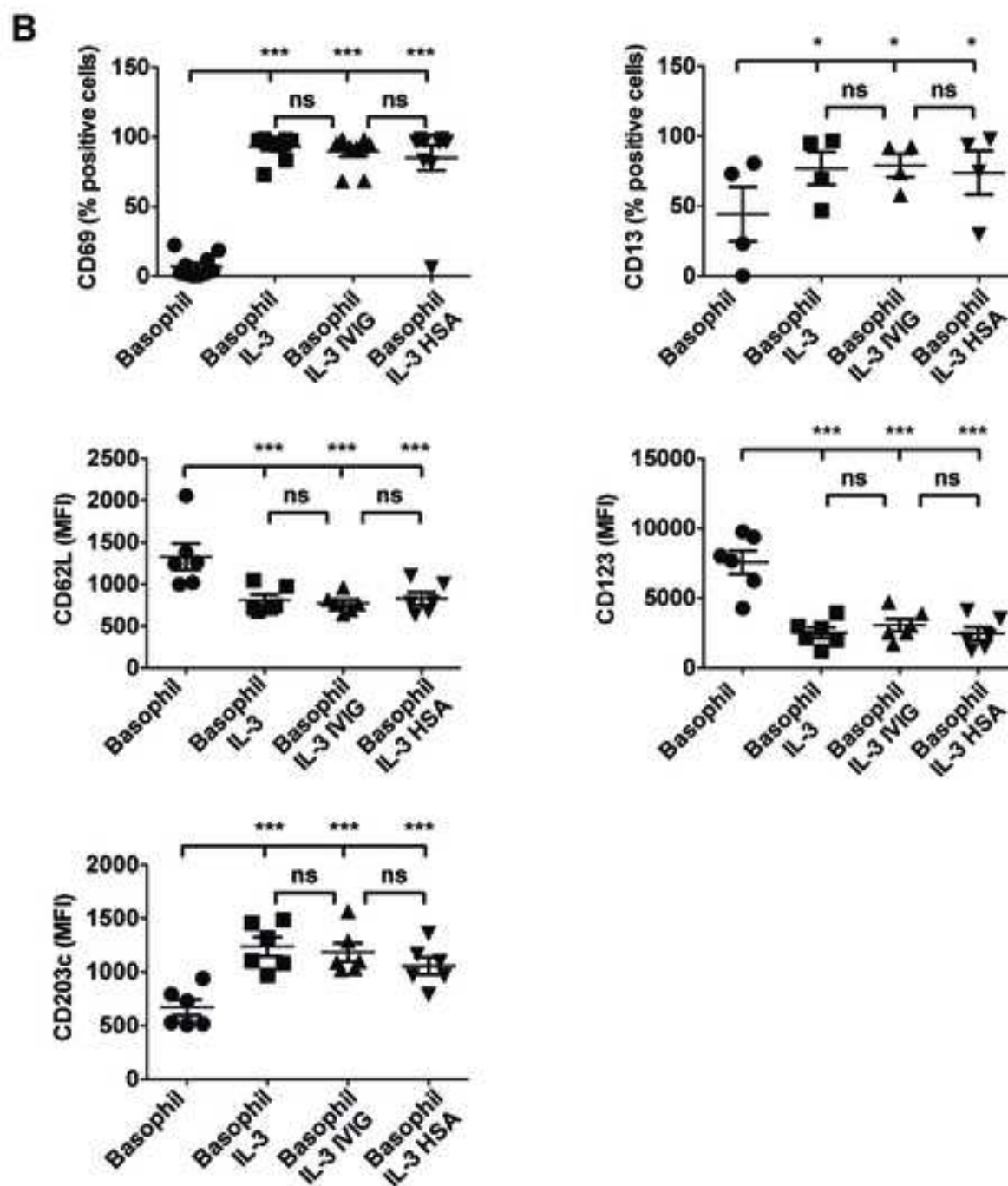
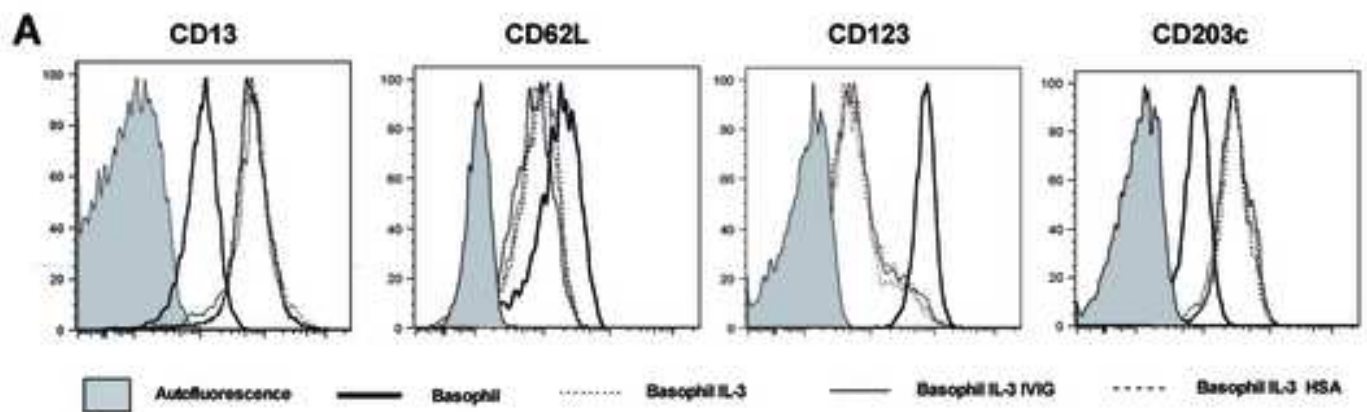
95 **FIG E5.** IL-25, TSLP and GM-CSF are dispensable for the activation of basophils by IVIG  
96 **A-C,** Basophils were cultured either alone or with (A) IL-25, (B) TSLP or (C) GM-CSF for  
97 24 hours. IVIG was added following 2 hours stimulation with respective cytokines. The  
98 expression of 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.

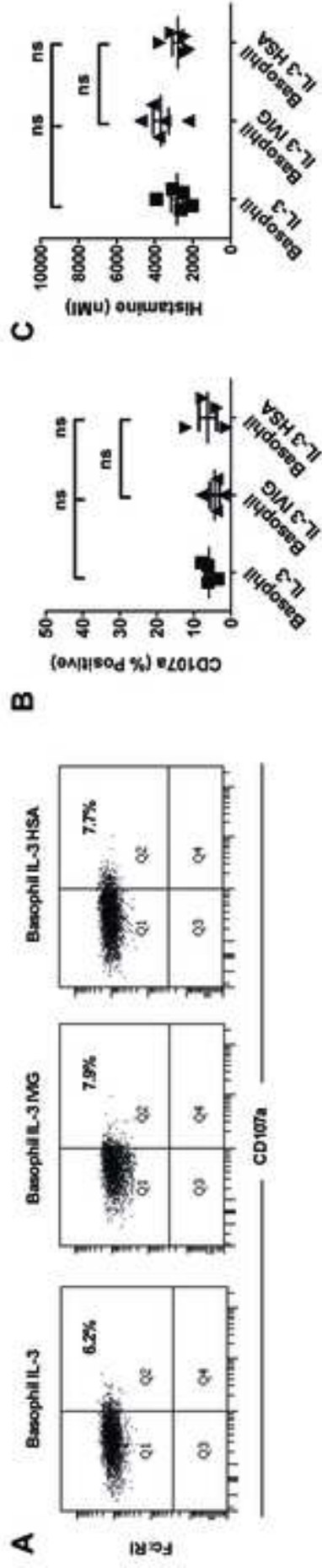
101

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.

104

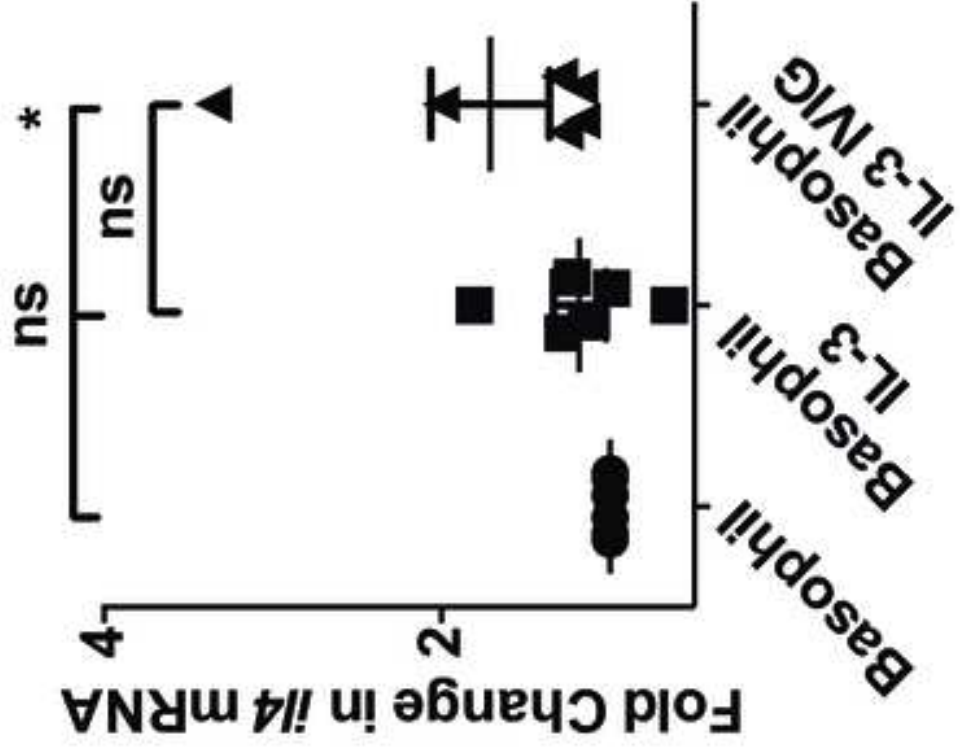
105 **FIG E7:** The effect of Syk inhibition towards IVIG-induced expression of *il4* transcripts  
106 (mean±SEM, n=5 donors). Basophils were stimulated with IL-3 plus IVIG for three hours.  
107 Additionally, cells were also treated with syk inhibitor R406 for one hour prior to stimulation  
108 with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



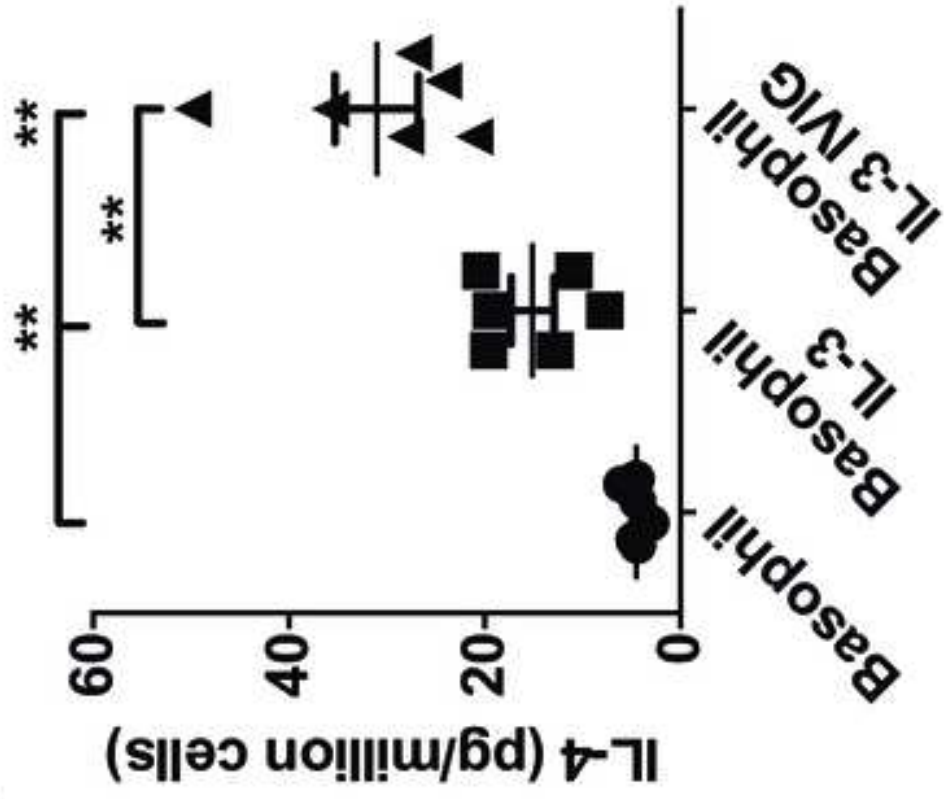


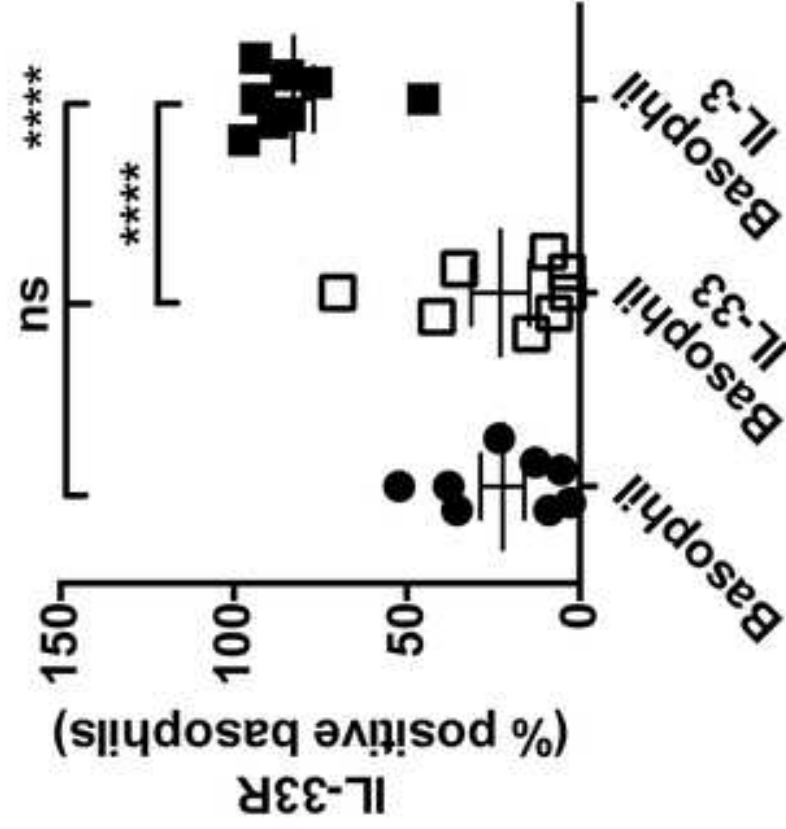
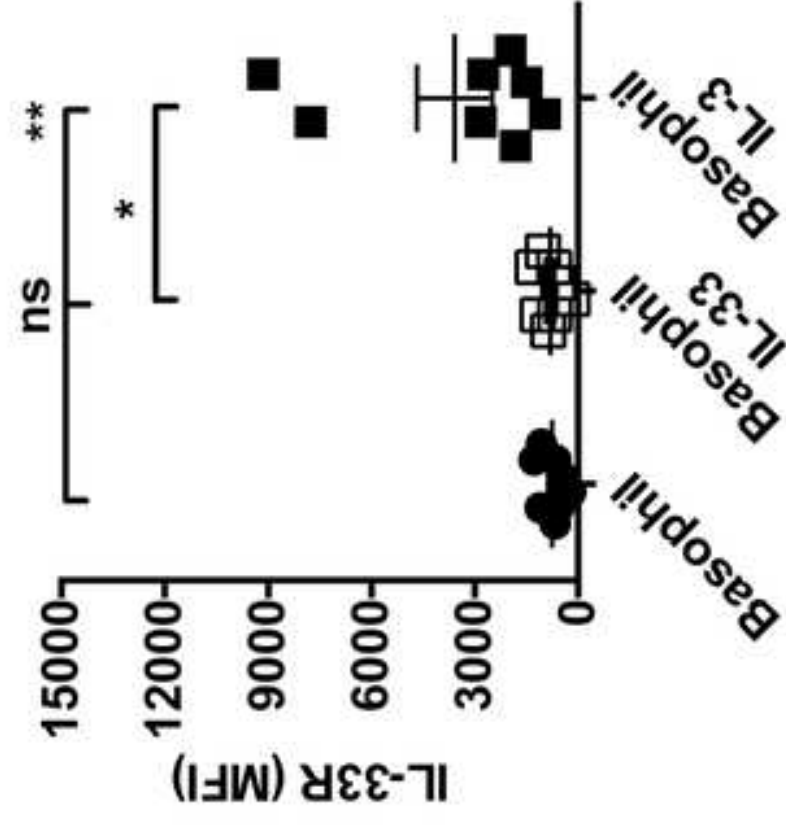


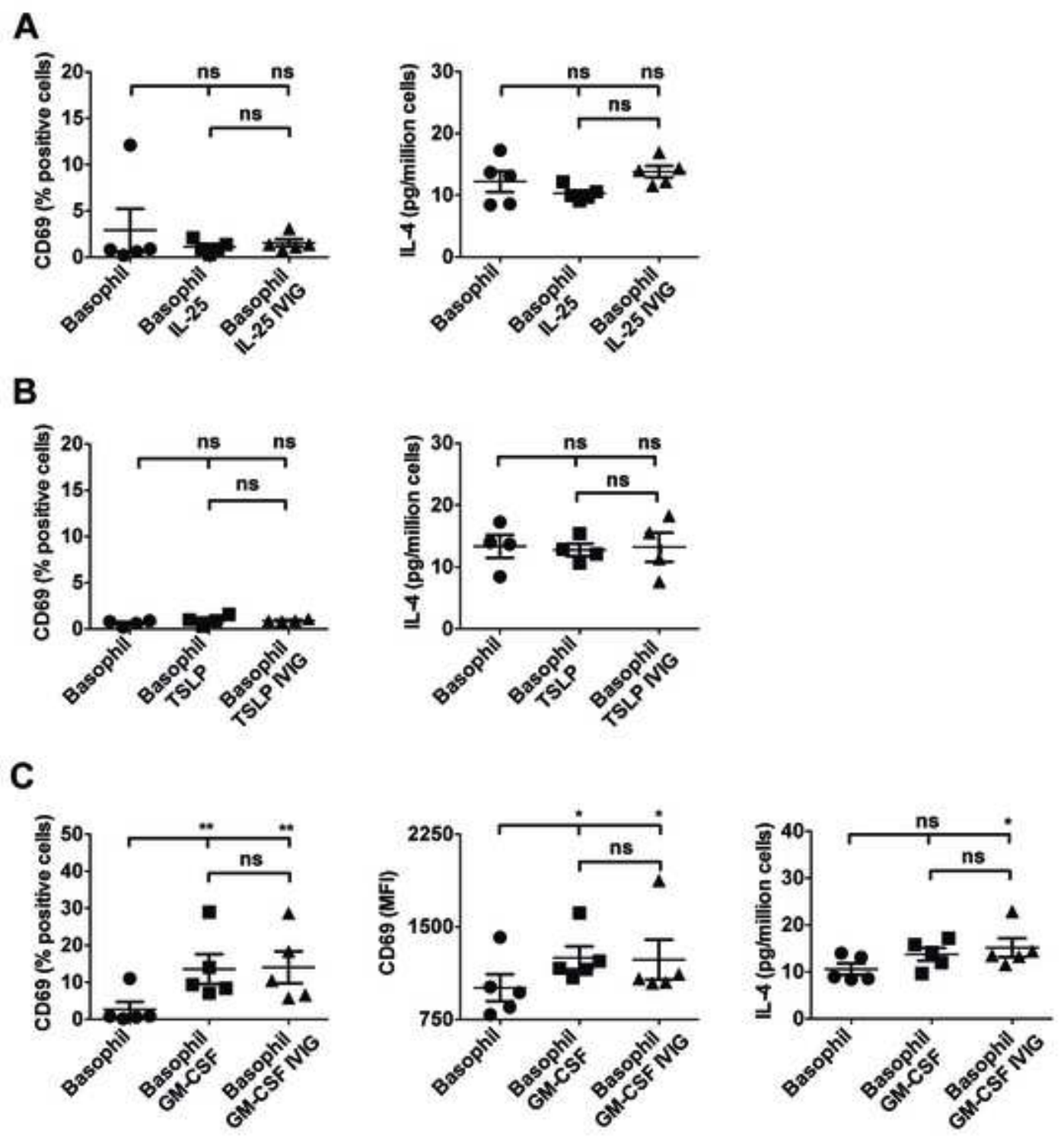
**A**



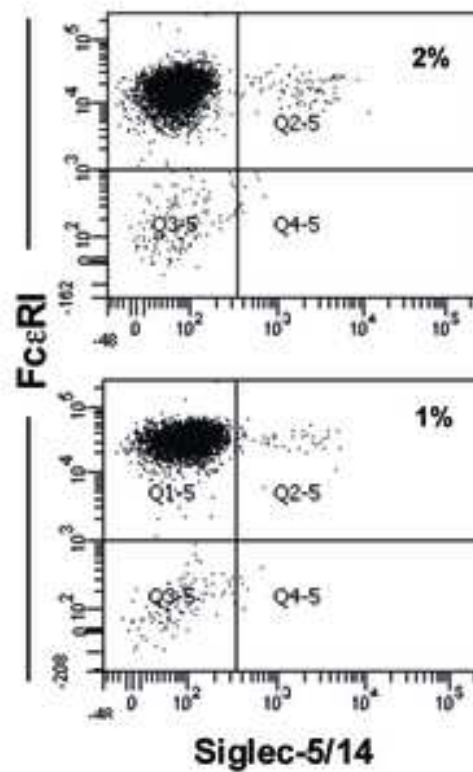
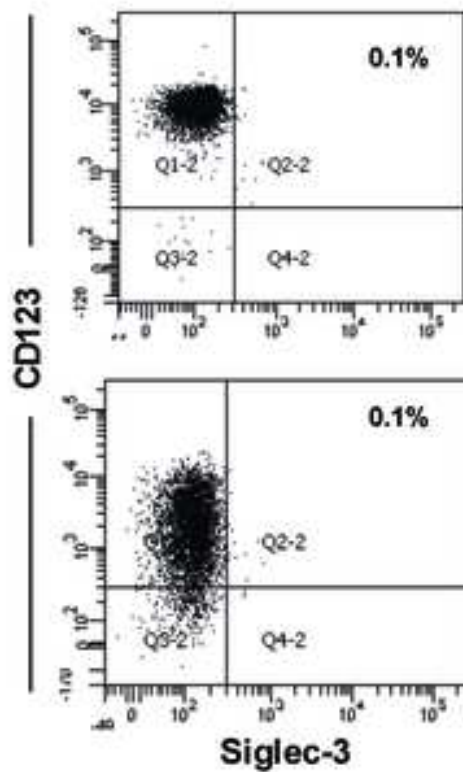
**B**







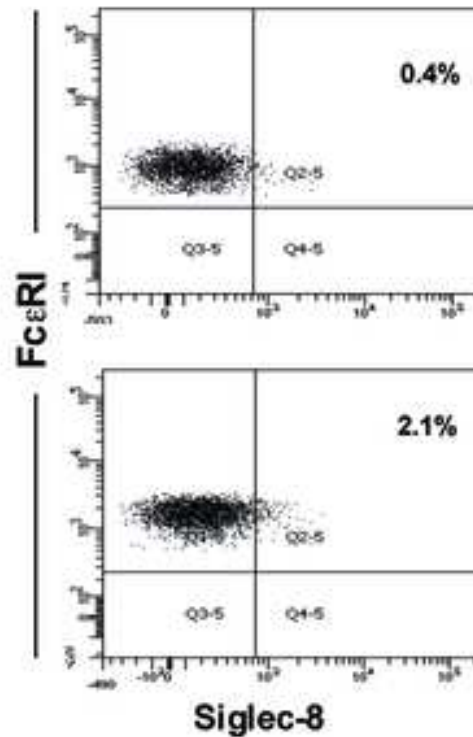
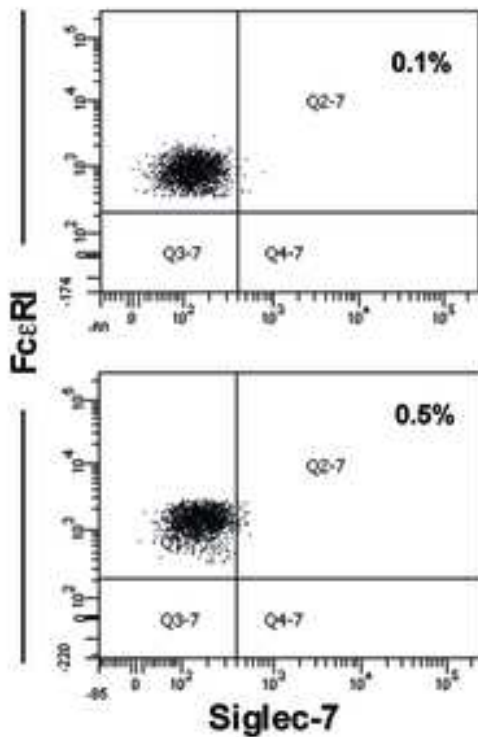
**A**



**Basophil**

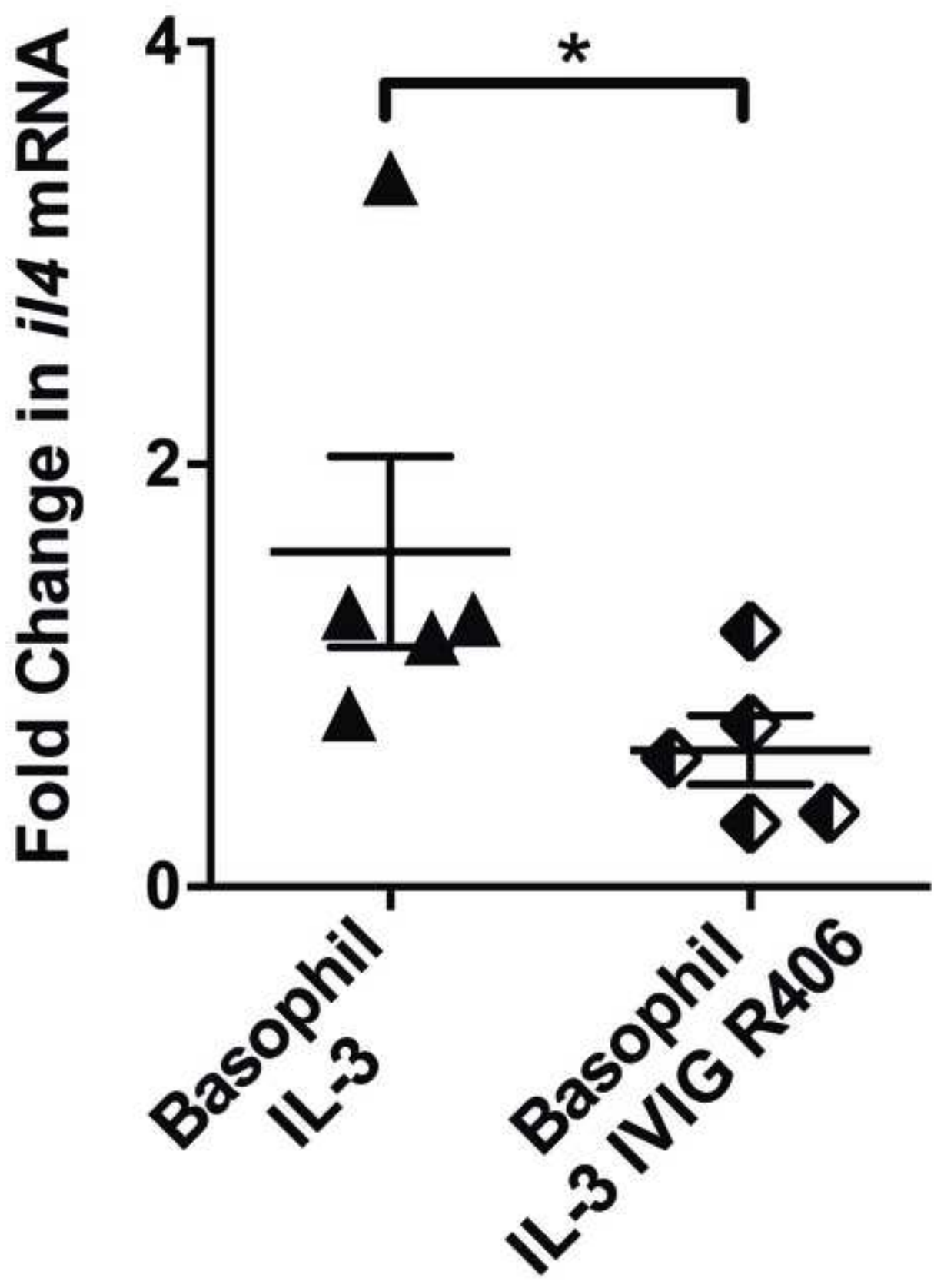
**Basophil  
IL-3**

**B**



**Basophil**

**Basophil  
IL-3**



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

