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# Anti-IgE IgG autoantibodies isolated from therapeutic normal IgG intravenous immunoglobulin induce basophil activation

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3 **Anti-IgE IgG autoantibodies isolated from therapeutic normal IgG intravenous**  
4 **immunoglobulin induce basophil activation**

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24 **Keywords:** Basophils; anti-IgE IgG; IVIG; IL-3 ; CD69 ; IL-4 ; IL-8

28 Basophils are rare granulocytes. Despite represent only about 0.5% of the leukocytes,  
29 basophils have several important functions in physiology<sup>1,2</sup>. Although basophils lack the  
30 classical features of professional antigen presenting cells<sup>3-7</sup>, through the secretion of  
31 cytokines they orient the immune response by polarizing Th2 differentiation, and  
32 supporting B cell differentiation and class-switch. Basophils are also critical for mediating  
33 the protection against helminth infection<sup>1,2,8,9</sup>.

34

35 Basophils receive activation signals from diverse sources. It is well recognized that  
36 cytokines like IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), thymic  
37 stromal lymphopoietin (TSLP) and IL-33; various toll-like receptor ligands; and allergen-  
38 bound IgE provide activation signals for the basophils and induce the release of  
39 inflammatory mediators<sup>10-15</sup>. In addition, several reports also demonstrate the presence of  
40 anti-IgE autoantibodies that possess the capacity to induce basophil activation in patients  
41 with chronic spontaneous urticaria (CSU), atopic and non-atopic asthma and autoimmune  
42 diseases<sup>16-21</sup>. However, isolation and functional exploration of such anti-IgE IgG  
43 autoantibodies either from the healthy donors or from the patients are not attempted yet.  
44 By using pooled normal IgG preparation from the healthy donors, intravenous  
45 immunoglobulin G (IVIG)<sup>22</sup> that represents a complete repertoire of IgG of a normal  
46 individual, we attempted to address this outstanding question in the field.

47

48 Recently, we reported that at a concentration (25 mg/0.5 million cells/mL) corresponding  
49 to the level of IgG reached in the patients immediately following high-dose IVIG therapy,  
50 both IVIG and its F(ab')<sub>2</sub> fragments induce basophil activation<sup>23</sup>. Mechanistically, IVIG  
51 induces basophil activation by signaling through basophil surface-bound IgE in an IL-3  
52 and Syk-dependent mechanism<sup>23</sup>. We first performed dose-response experiments with

53 various concentrations of IVIG to comprehend if lower concentration of IVIG also induces  
54 basophil activation.

55

56 As a source of IVIG, Sandoglobulin® (CSL Behring, Switzerland) was used in the  
57 experiments. It was dialyzed several times against phosphate buffered saline followed by  
58 final dialysis in RPMI-1640 at 4°C for the removal of stabilizing agents. Peripheral blood  
59 basophils were isolated from the buffy bags of healthy donors obtained from Centre  
60 Necker-Cabanel, EFS, Paris (INSERM-EFS ethical permission N°18/EFS/033). The  
61 cellular fractions that contain peripheral blood mononuclear cells and basophils were  
62 collected by Ficoll density gradient centrifugation. From these fractions, basophils were  
63 isolated by basophil isolation kit II (Miltenyi Biotec) and autoMACS® (Miltenyi Biotec).  
64 The purity of basophils was 96-97%.

65

66 Basophils ( $0.1 \times 10^6$  cells/well/200  $\mu$ L) were distributed in 96 well U-bottomed plates. For  
67 the dose-response experiments with IVIG, basophils were cultured either alone in serum-  
68 free X-VIVO 15 medium; or with IL-3 (100 ng/mL, ImmunoTools); or with IL-3 plus  
69 three different doses of IVIG (25, 15 or 10 mg/mL) for 24 hours. IVIG was added  
70 following two hours of stimulation of basophils with IL-3. Basophils were analyzed for the  
71 expression of CD69 by flow cytometry (LSR II, BD Biosciences) using CD69-APC/Cy7  
72 MAb from BD Biosciences. Flow cytometry data were analyzed by BD FACS DIVA (BD  
73 Biosciences) and Flowjo (FlowJo LLC). Cell-free culture supernatants were used for the  
74 analyses of cytokines by ELISA (ELISA Ready-SET-Go, eBioscience Affymetrix).

75

76 As IVIG did not modify the activation status of either resting basophils or basophils  
77 primed with IL-33, TSLP, IL-25 or GM-CSF cytokines<sup>23</sup>, we used IL-3 priming

78 throughout the experiments. The dose-response experiments revealed that even at 10  
79 mg/mL concentration corresponding to the circulating IgG levels of healthy donors, IVIG  
80 could induce basophil activation as shown by the enhanced expression of CD69 and IL-4  
81 secretion (Figure 1a and b). These results suggest that occurrence of anti-IgE IgG  
82 autoantibodies is a common feature in the healthy population.

83

84 We then investigated if the induction of basophil activation by IVIG was due to non-  
85 specific cell activation caused by cell death. Therefore, we measured the viability of cells  
86 by Annexin V staining. Basophils were cultured in the medium alone or with IL-3 or IL-3  
87 plus IVIG (25 mg/mL) or IL-3 plus human serum albumin (HSA, 10 mg/mL, Laboratoire  
88 Français du fractionnement et des Biotechnologies, France) for 24 hours. HSA was used an  
89 irrelevant protein control for IVIG. The cells were stained with Annexin V (APC-Annexin  
90 V, BD Biosciences) and propidium iodide (PI, Invitrogen™). We indeed confirm that  
91 basophil activation induced by IVIG was not because of non-specific cell activation caused  
92 by cell death. In fact, viability of cells as analyzed by Annexin V-PI staining was similar  
93 between IL-3-treated, IVIG-treated and HSA-treated cells (Figure 1c and d).

94

95 These data prompted us to investigate anti-IgE IgG autoantibodies in IVIG. The ELISA  
96 performed with recombinant human IgE-kappa (AbD Serotec) revealed that both IVIG and  
97 its F(ab')<sub>2</sub> fragments recognize IgE in a dose-dependent manner (Figure 1e). Therefore, we  
98 next aimed at isolating the anti-IgE IgG autoantibodies from IVIG by affinity  
99 chromatography. IgE myeloma protein was coupled to a cyanogen bromide-activated  
100 sepharose 4B (Sigma-Aldrich). IVIG at a concentration of 60 mg/mL was added to the  
101 column and incubated at ambient temperature for 4 hours on a rotator. After draining the  
102 unbound IgG, column was washed several times with phosphate buffered saline. The anti-

103 IgE IgG autoantibodies were eluted by using 3M potassium thiocyanate, dialyzed against  
104 phosphate buffered saline and concentrated. IgG concentration was measured by  
105 spectrophotometer (NanoDrop Technologies).

106

107 Based on the amount of eluted IgG, we estimate that anti-IgE IgG represent nearly 0.3-  
108 0.5% of total IgG (IVIG). We then investigated if these isolated anti-IgE IgG are  
109 functional. IL-3-primed basophils from healthy individuals were stimulated with either  
110 IVIG (25 mg/0.5x10<sup>6</sup> cells/mL) or isolated anti-IgE IgG (1.5 mg/0.5x10<sup>6</sup>/mL) for 24 hours.  
111 This concentration of anti-IgE IgG was selected considering the levels of IgG and the  
112 number of basophils in the circulation of healthy individuals. We found that isolated anti-  
113 IgE IgG were able to induce basophil activation similar to that of IVIG even at 16-times  
114 lesser concentrations: both induced similar level of CD69 expression (Figure 1f and g), and  
115 secretion of IL-4 and IL-8 (Figure 1h).

116

117 The site of recognition of anti-IgE IgG autoantibodies from IVIG is not known. As we  
118 used IgE myeloma protein for the isolation of anti-IgE IgG, and yet these isolated IgG  
119 induced activation of basophils from various donors carrying distinct IgE molecules imply  
120 that anti-IgE IgG do not signal basophils by interacting with Fab-region of basophil-bound  
121 IgE. It is likely that these antibodies might recognize either Cε2 or Cε4 domains of IgE but  
122 not Cε3 domain (which is binding site of FcεRI and hence inaccessible for the recognition  
123 by anti-IgE IgG) similar to the previous report<sup>24</sup>; although we do not rule out the  
124 recognition of Cε1 domain as well.

125

126 The precise function of anti-IgE IgG autoantibodies in healthy individuals is not clear at  
127 this stage. Isolation and characterization of such anti-IgE IgG monoclonal antibodies by

128 using techniques like phage display technology should provide further insight on them<sup>25</sup>.  
129 Since anti-IgE autoantibodies induce IL-4 secretion in basophils, this might support Th2  
130 and B cell differentiation. In the context of IVIG immunotherapy however, we propose that  
131 IL-4 induction in basophils might help in the suppression of effector Th1 and Th17 cells<sup>22</sup>,  
132 and in the upregulation of inhibitory Fc $\gamma$ RIIB on phagocytic cells to reduce inflammatory  
133 response to IgG-autoantigen immune complexes<sup>26</sup>. Thus, distinct natural autoantibodies  
134 diversify the action of IVIG on granulocytes<sup>27</sup>.  
135

136

137 **CONFLICT OF INTEREST**

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140

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150

151

152 **AUTHOR CONTRIBUTIONS**

153 JB designed the study. CG and AK performed the experiments. CG, AK, JD, SVK and JB

154 analyzed the data. AC provided the essential tools. JB drafted the manuscript, and all the

155 authors edited and approved the final version of the manuscript.

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## FIGURE LEGEND

238 **Figure 1. Anti-IgE IgG autoantibodies isolated from IVIG induce basophil activation.**

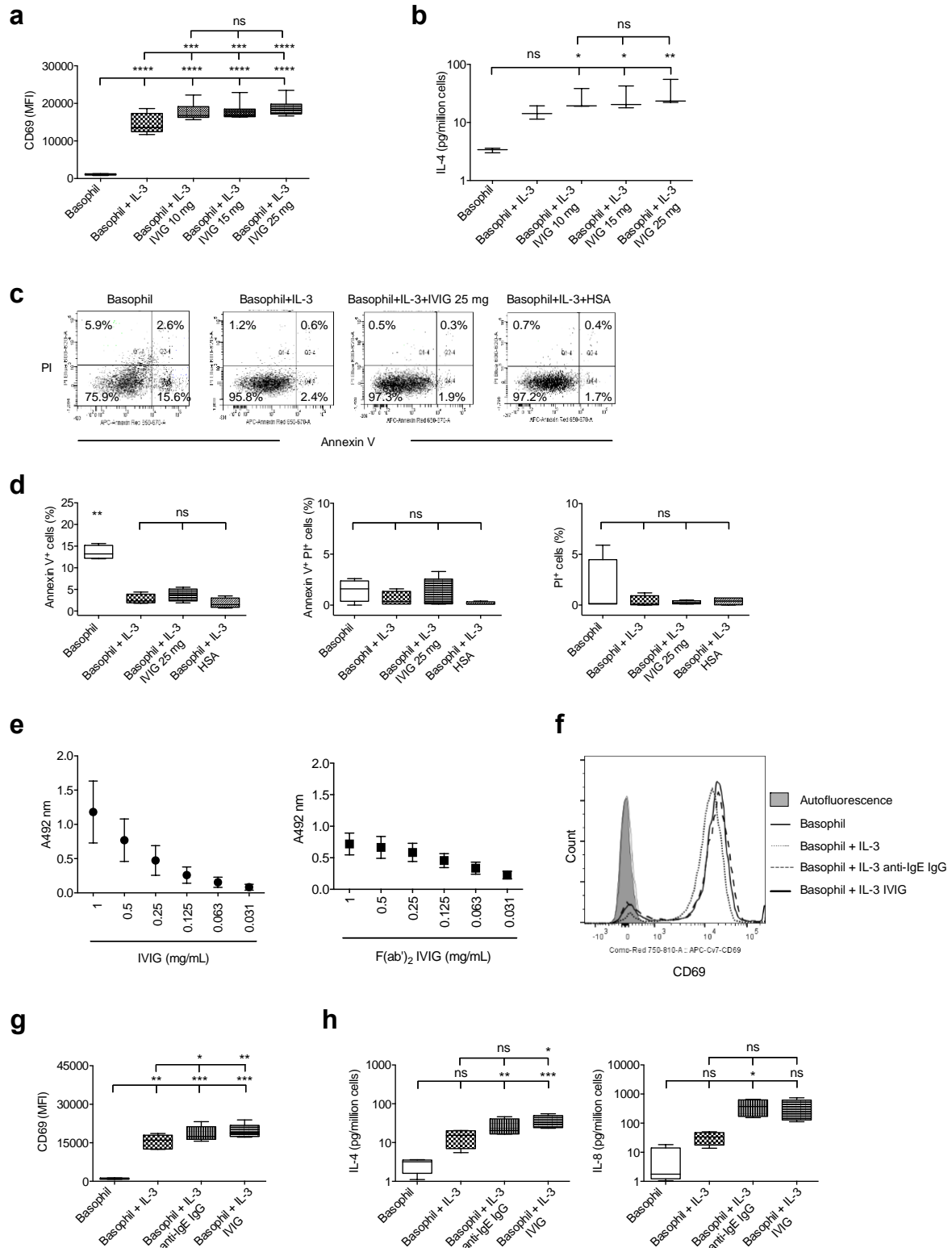
239 **(a, b)** Basophils isolated from the healthy donors were cultured either alone ( $0.1 \times 10^6$   
240 cells/well/200  $\mu$ L) or with IL-3 (100 ng/mL). Three different concentrations of IVIG (25,  
241 15 or 10 mg/ml) were added to the cells following 2 hours stimulation with IL-3. After 24  
242 hours, the expression of CD69 (MFI, median fluorescence intensity, n=7 donors) and the  
243 amounts of IL-4 (n=3 donors) in the culture supernatants were analyzed. **(c, d)** The  
244 viability of basophils as analyzed by Annexin V and PI staining. Representative dot plots  
245 (c) and data from four experiments (d) using different donors are presented. HSA, human  
246 serum albumin used at 10 mg/mL concentration. **(e)** Recognition of IgE by IVIG (left  
247 panel) or (Fab')<sub>2</sub> fragments (right panel). Data (mean  $\pm$  SEM) are from the two  
248 experiments. **(f-g)** Basophils were cultured either alone or with IL-3 (100 ng/mL). IVIG  
249 (25 mg/mL) or anti-IgE IgG (1.5 mg/mL) were added to the cells following 2 hours  
250 stimulation with IL-3. After 24 hours, the expression (MFI, median fluorescence intensity)  
251 of CD69 was analyzed. Representative histogram overlays (e) and data from 5 donors (f)  
252 are presented. **(h)** The amounts (pg) of IL-4 and IL-8 in the culture supernatants of above  
253 experiments. Data from 4 donors are presented. All data are plotted using box & whiskers  
254 plot, wherein whiskers denote minimum and maximum values and the dividing line in the  
255 box symbolises the median. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001; ns, not  
256 significant, one-way ANOVA with Tukey's multiple comparison tests.

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