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

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

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

Recently, we reported that at a concentration (25 mg/0.5 million cells/mL) corresponding to the level of IgG reached in the patients immediately following high-dose IVIG therapy, both IVIG and its F(ab')$_2$ fragments induce basophil activation[23]. Mechanistically, IVIG induces basophil activation by signaling through basophil surface-bound IgE in an IL-3 and Syk-dependent mechanism[23]. We first performed dose-response experiments with

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

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

Basophils ($0.1x10_6$ cells/well/200 µL) were distributed in 96 well U-bottomed plates. For the dose-response experiments with IVIG, basophils were cultured either alone in serum-free X-VIVO 15 medium; or with IL-3 (100 ng/mL, ImmunoTools); or with IL-3 plus three different doses of IVIG (25, 15 or 10 mg/mL) for 24 hours. IVIG was added following two hours of stimulation of basophils with IL-3. Basophils were analyzed for the expression of CD69 by flow cytometry (LSR II, BD Biosciences) using CD69-APC/Cy7 MAb from BD Biosciences. Flow cytometry data were analyzed by BD FACS DIVA (BD Biosciences) and Flowjo (FlowJo LLC). Cell-free culture supernatants were used for the analyses of cytokines by ELISA (ELISA Ready-SET-Go, eBioscience Affymetrix).

As IVIG did not modify the activation status of either resting basophils or basophils primed with IL-33, TSLP, IL-25 or GM-CSF cytokines[23], we used IL-3 priming

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

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

These data prompted us to investigate anti-IgE IgG autoantibodies in IVIG. The ELISA performed with recombinant human IgE-kappa (AbD Serotec) revealed that both IVIG and its $F(ab')_2$ fragments recognize IgE in a dose-dependent manner (Figure 1e). Therefore, we next aimed at isolating the anti-IgE IgG autoantibodies from IVIG by affinity chromatography. IgE myeloma protein was coupled to a cyanogen bromide-activated sepharose 4B (Sigma-Aldrich). IVIG at a concentration of 60 mg/mL was added to the column and incubated at ambient temperature for 4 hours on a rotator. After draining the unbound IgG, column was washed several times with phosphate buffered saline. The anti-

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

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

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

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

using techniques like phage display technology should provide further insight on them[25]. Since anti-IgE autoantibodies induce IL-4 secretion in basophils, this might support Th2 and B cell differentiation. In the context of IVIG immunotherapy however, we propose that IL-4 induction in basophils might help in the suppression of effector Th1 and Th17 cells[22], and in the upregulation of inhibitory FcγRIIB on phagocytic cells to reduce inflammatory response to IgG-autoantigen immune complexes[26]. Thus, distinct natural autoantibodies diversify the action of IVIG on granulocytes[27].

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

JB designed the study. CG and AK performed the experiments. CG, AK, JD, SVK and JB analyzed the data. AC provided the essential tools. JB drafted the manuscript, and all the authors edited and approved the final version of the manuscript.

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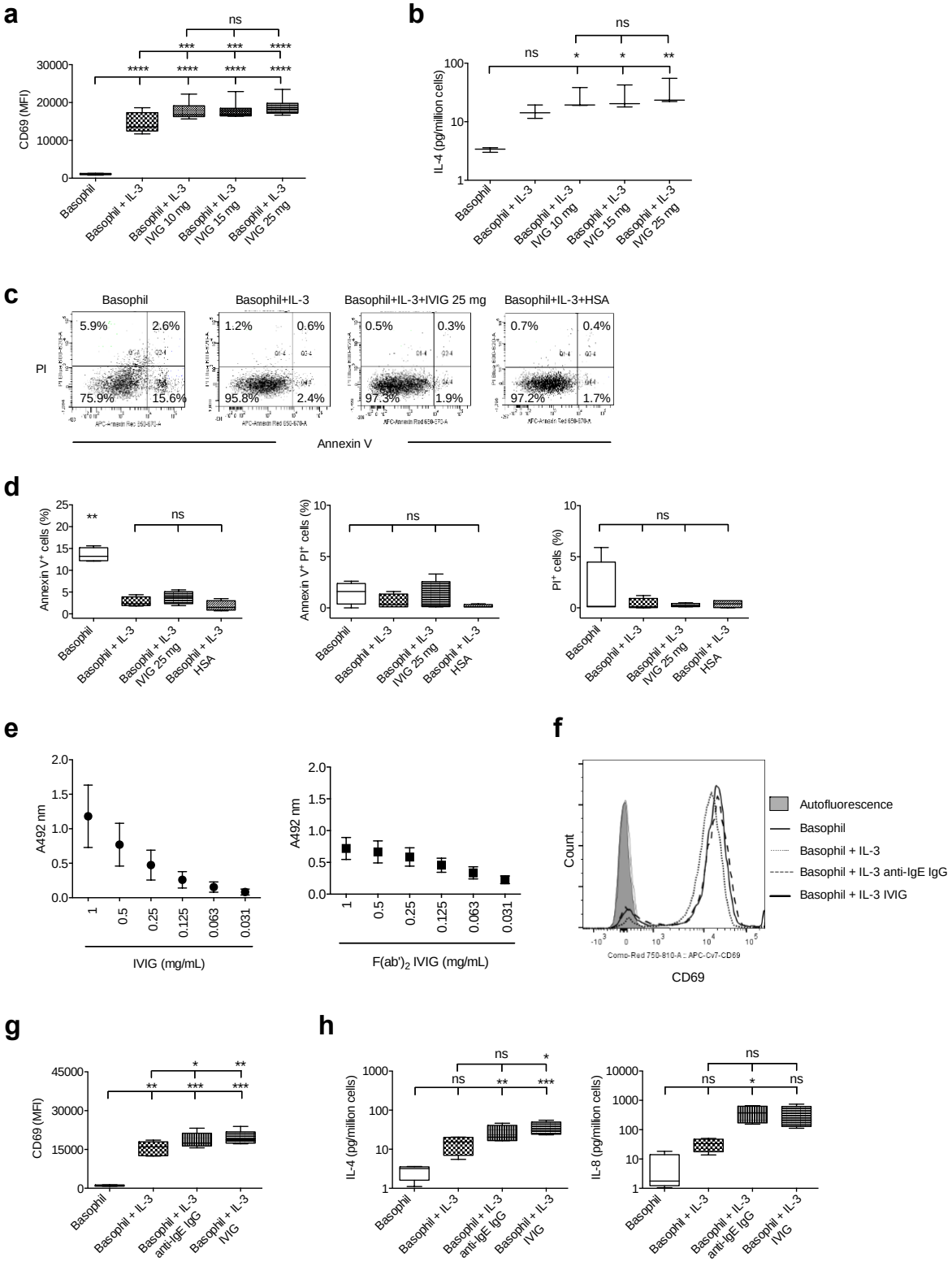
FIGURE LEGEND

Figure 1. Anti-IgE IgG autoantibodies isolated from IVIG induce basophil activation. **(a, b)** Basophils isolated from the healthy donors were cultured either alone ($0.1x10^6$ cells/well/200 μL) or with IL-3 (100 ng/mL). Three different concentrations of IVIG (25, 15 or 10 mg/ml) were added to the cells following 2 hours stimulation with IL-3. After 24 hours, the expression of CD69 (MFI, median fluorescence intensity, n=7 donors) and the amounts of IL-4 (n=3 donors) in the culture supernatants were analyzed. **(c, d)** The viability of basophils as analyzed by Annexin V and PI staining. Representative dot plots (c) and data from four experiments (d) using different donors are presented. HSA, human serum albumin used at 10 mg/mL concentration. **(e)** Recognition of IgE by IVIG (left panel) or $(Fab')_2$ fragments (right panel). Data (mean ± SEM) are from the two experiments. **(f-g)** Basophils were cultured either alone or with IL-3 (100 ng/mL). IVIG (25 mg/mL) or anti-IgE IgG (1.5 mg/mL) were added to the cells following 2 hours stimulation with IL-3. After 24 hours, the expression (MFI, median fluorescence intensity) of CD69 was analyzed. Representative histogram overlays (e) and data from 5 donors (f) are presented. **(h)** The amounts (pg) of IL-4 and IL-8 in the culture supernatants of above experiments. Data from 4 donors are presented. All data are plotted using box & whiskers plot, wherein whiskers denote minimum and maximum values and the dividing line in the box symbolises the median. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, not significant, one-way ANOVA with Tukey's multiple comparison tests.


a
CD69 (MFI)
Basophil
Basophil + IL-3
Basophil + IL-3 IVIG 10 mg
Basophil + IL-3 IVIG 15 mg
Basophil + IL-3 IVIG 25 mg
b
IL-4 (pg/milion cells)
c
Basophil
Basophil+IL-3
Basophil+IL-3+IVIG 25 mg
Basophil+IL-3+HSA
PI
Annexin V
d
Annexin V+ cells (%)
Annexin V+ PI+ cells (%)
PI+ cells (%)
Basophil + IL-3 HSA
e
A492 nm
IVIG (mg/mL)
F(ab')2 IVIG (mg/mL)
f
Count
CD69
Autofluorescence
Basophil
Basophil + IL-3
Basophil + IL-3 anti-IgE IgG
Basophil + IL-3 IVIG
g
CD69 (MFI)
Basophil + IL-3 anti-IgE IgG
Basophil + IL-3 IVIG
h
IL-4 (pg/milion cells)
IL-8 (pg/milion cells)