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

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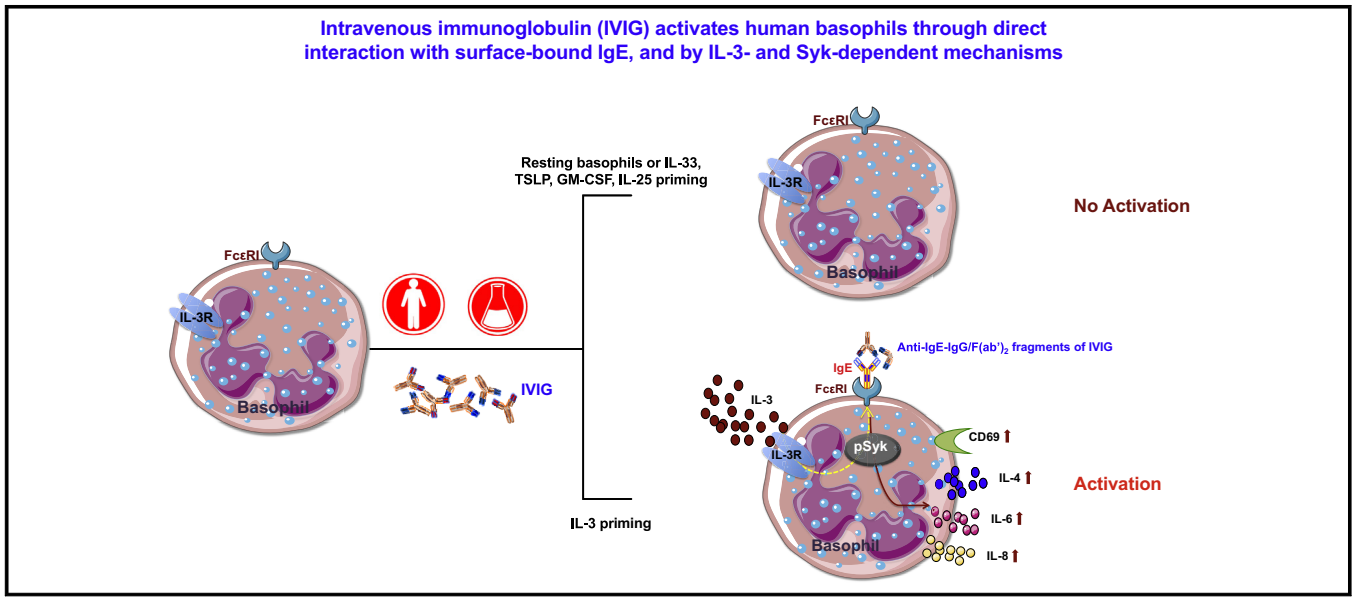
GRAPHICAL ABSTRACT



Background: Therapeutic normal IgG or intravenous immunoglobulin (IVIG) exerts anti-inflammatory effects through several mutually nonexclusive mechanisms. Recent data in mouse models of autoimmune disease suggest that IVIG induces IL-4 in basophils by enhancing IL-33 in SIGN-related 1–positive innate cells. However, translational insight on these data is lacking.

Objective: We sought to investigate the effect of IVIG on human basophil functions.

Methods: Isolated circulating basophils from healthy donors were cultured in the presence of IL-3, IL-33, GM-CSF, thymic stromal lymphopoietin, or IL-25. The effect of IVIG and F(ab')$_2$ and Fc IVIG fragments was examined based on expression of

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various surface molecules, phosphorylation of spleen tyrosine kinase, induction of cytokines, and histamine release. Basophil phenotypes were also analyzed from IVIG-treated patients with myopathy. Approaches, such as depletion of anti-IgE reactivity from IVIG, blocking antibodies, or inhibitors, were used to investigate the mechanisms.

Results: We report that IVIG directly induces activation of IL-3–primed human basophils, but IL-33 and other cytokines were dispensable for this effect. Activation of basophils by IVIG led to enhanced expression of CD69 and secretion of IL-4, IL-6, and IL-8. IVIG-treated patients with myopathy displayed enhanced expression of CD69 on basophils. The spleen tyrosine kinase pathway is implicated in these functions of IVIG and were mediated by F(ab')$_2$ fragments. Mechanistically, IVIG induced IL-4 in human basophils by interacting with basophil surface-bound IgE but independent of FcγRII, type II Fc receptors, C-type lectin receptors, and sialic acid–binding immunoglobulin-like lectins.

Conclusion: These results uncovered a pathway of promoting the T_H2 response by IVIG through direct interaction of IgG with human basophils. (J Allergy Clin Immunol 2019;144:524-35.)

Key words: *FcεRI, anti-IgE IgG, antisynthetase syndrome, polymyositis, dermatomyositis, dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin, dendritic cell immunoreceptor, FcγRIIB*

Abbreviations used

APC:	Allophycocyanin
DC:	Dendritic cell
DCIR:	Dendritic cell immunoreceptor
DC-SIGN:	Dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin
FcR:	Fc receptor
FITC:	Fluorescein isothiocyanate
HSA:	Human serum albumin
IL-33R:	IL-33 receptor
IVIG:	Intravenous immunoglobulin
PE:	Phycoerythrin
pSyk:	Phosphorylated Syk
qRT-PCR:	Quantitative RT-PCR
Siglec:	Sialic acid–binding immunoglobulin-like lectins
SIGN-R1:	SIGN-related 1
Syk:	Spleen tyrosine kinase
TSLP:	Thymic stromal lymphopoietin

Intravenous immunoglobulin (IVIG) is one of the widely used immunotherapeutic molecules for the treatment of diverse autoimmune and systemic inflammatory diseases.[1-4] High-dose (1-2 g/kg) IVIG therapy exerts anti-inflammatory effects through several mutually nonexclusive mechanisms, including inhibition of activation of innate immune cells, effector T cells (T_H1 and T_H17), and B cells; suppression of the complement pathway; neutralization of inflammatory cytokines and pathogenic antibodies; and expansion of regulatory T cells. These actions of IVIG implicate both Fc and F(ab')$_2$ fragments.[5,6]

Basophils are one of the rare granulocytes. They express various receptors to sense signals, including FcεRI, a high-affinity receptor for IgE, Toll-like receptors, and cytokine receptors, such as IL-3 receptor (CD123), IL-33 receptor (IL-33R), and thymic stromal lymphopoietin (TSLP) receptor. Activated basophils secrete several cytokines, including IL-4, IL-8, and IL-6, and regulate T_H2 polarization, immunoglobulin synthesis, and class-switching in B cells.[7,8]

Recent results from experimental models of systemic inflammatory and autoimmune diseases suggest that the anti-inflammatory effects of IVIG are mediated through basophils by using a 2-step process.[9] IL-33 produced by SIGN-related 1 (SIGN-R1)$^+$ innate cells on interaction with Fc–α(2,6)-sialic acid linkages activates basophils through IL-33R to induce IL-4. Basophil-derived IL-4 enhances expression of inhibitory FcγRIIB on effector macrophages,[9] thus adding to the previously known function of basophil-derived IL-4 in programing anti-inflammatory macrophages.[10] However, translational insight into these data is lacking. In particular, dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN; human orthologue of SIGN-R1)–positive human innate cells did not produce IL-33 when exposed to IVIG, indicating that the proposed pathway of basophil activation by IVIG does not apply to human subjects.[11] When patients are infused with high-dose IVIG, the IgG theoretically interacts with every component of the immune system. Therefore it is most likely that IVIG modulates human basophils through direct interaction rather than an indirect pathway of DC-SIGN–dependent IL-33.

In line with our proposition, we report that IVIG directly induces activation of human basophils and secretion of IL-4, IL-6, and IL-8 through interaction with basophil surface-bound IgE and through IL-3– and spleen tyrosine kinase (Syk)–dependent mechanisms. These functions of IVIG were mediated through F(ab')$_2$ fragments and were independent of IL-33, FcγRII, type II Fc receptors (FcRs), C-type lectin receptors, and sialic acid–binding immunoglobulin-like lectins (Siglecs). Basophils from IVIG-treated patients with myopathy also showed enhanced expression of the activation marker CD69. In the context of systemic autoimmune and inflammatory diseases, these results thus provide a unique pathway of promoting T_H2 responses by IVIG through direct interaction of IgG with human basophils.

METHODS

Preparations of IVIG

Sandoglobulin (CSL Behring, Bern, Switzerland) was dialyzed against a large volume of PBS 3 times, followed by RPMI-1640 at 4°C for 18 hours to remove the stabilizing agents.

F(ab')$_2$ fragments of IVIG were prepared by using pepsin digestion (2% wt/wt; Sigma-Aldrich, St Louis, Mo), followed by chromatography on a protein G Sepharose column (Pharmacia, Uppsala, Sweden). Fc fragments of IVIG were prepared by means of papain digestion (papain-coupled beads; Life Technologies, Grand Island, NY), followed by protein A Sepharose column chromatography and size-exclusion chromatography. End purification was performed by means of chromatography on an IgG-CH1 column (Life Technologies). The purity of F(ab')$_2$ and Fc fragments was confirmed by using SDS-PAGE.

Isolation and culture of basophils

Basophils were isolated from PBMCs of healthy donors' buffy bags (Centre Necker-Cabanel, EFS, Paris; INSERM-EFS ethical permission nos. 12/EFS/079 and 18/EFS/033) by using the Basophil Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and autoMACS (Miltenyi Biotec). The purity of basophils based on expression of FcεRI and CD123 was approximately 97%.

Cells (0.1×10^6 cells/well per 200 μL) were cultured in 96-well U-bottomed plate either alone in serum-free X-VIVO 15 medium, with IL-3

(100 ng/mL; ImmunoTools, Friesoythe, Germany), or with IL-3 plus IVIG (25 mg/mL) or human serum albumin (HSA; 10 mg/mL; LFB, Les Ulis, France) or $F(ab')_2$ fragments (16 mg/mL) or Fc fragments (9 mg/mL) for 24 hours to investigate the effect of IVIG on IL-3–primed basophils.

Cells were cultured with individual cytokines (IL-33, 1 ng/mL; GM-CSF, 10 ng/mL; IL-25, 10 ng/mL; or TSLP, 100 ng/mL; all from ImmunoTools) or cytokines plus IVIG for 24 hours to explore the effect of other cytokines on IVIG-mediated regulation of basophils. Also, basophils were sequentially stimulated with IL-3 and IL-33 for 1 hour each and cultured with IVIG or HSA for an additional 22 hours.

For blocking experiments, basophils were stimulated with IL-3 for 2 hours, followed by incubation with blocking mAbs to FcγRIIB (clone 2B6 $N_{297}D$, 10 μg/mL) or FcγRIIA (clone IV.3, 10 μg/mL) or isotype control mAbs for 1 hour and cultured with IVIG for an additional 21 hours.

Basophils were incubated with the Syk inhibitor R406 (5 μmol; InvivoGen, San Diego, Calif) or dimethyl sulfoxide for 1 hour and followed by stimulation with IL-3 for 2 hours. Cells were then cultured with IVIG for up to 24 hours to investigate the implication of the Syk pathway.

Basophils were analyzed for expression of various markers by flow cytometry (LSR II; BD Biosciences, San Jose, Calif) by using fluorochrome-conjugated mAbs. Syk phosphorylation was analyzed by using Cell Signaling Buffer Set A (Miltenyi Biotec). Data were analyzed with BD FACSDiva (BD Biosciences) and FlowJo (FlowJo, Ashland, Ore) software. Cell-free culture supernatants were used for analysis of histamine and cytokine levels.

Depletion of IgE-reactive IgG from IVIG

Plasma IgE (5.427 mg/mL) from a patient with secreted IgE myeloma was immobilized on a cyanogen bromide–activated Sepharose 4B (Sigma-Aldrich). IVIG was loaded (60 mg/mL) onto IgE Sepharose columns and incubated on a rotator at room temperature for 4 hours. The flow-through fraction was collected. After elution of column-bound IgG, flow-through IgG was again passed through the IgE Sepharose column 2 more times. IgG in the flow-through fraction was concentrated, and the concentration was determined by using a spectrophotometer (NanoDrop Technologies, Wilmington, Del).

IVIG depleted of anti-IgE reactivity (25 mg/mL) was added to IL-3–primed basophils (0.1×10^6 cells/well per 200 μL), as described earlier, for 24 hours.

Analysis of basophils from patients with myopathy

Heparinized blood from 7 patients with myopathy (45.71 ± 5.9 years old; 5 men; ethical approval from CPP-Ile-de-France VI, Groupe Hospitalier Pitié-Salpêtrière, Paris, France) was collected before and 2 to 5 days after IVIG treatment (2 g/kg). CD69 on basophils ($FcεRIα^+CD203c^+$) was analyzed by using flow cytometry. Because of low numbers, basophils were analyzed in only 5 patients (2 patients with antisynthetase syndrome and 1 each with polymyositis, immune-mediated necrotizing myopathy, or dermatomyositis).

Details on antibodies for flow cytometry and functional assays are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Measurement of cytokine and histamine levels

IL-4, IL-6 and IL-8 were analyzed in culture supernatants by means of ELISA (ELISA Ready-SET-Go; eBioscience Affymetrix, Santa Clara, Calif). Histamine levels were measured in culture supernatants by using the Histamine EIA Kit (Bertin Pharma, Montigny-le-Bretonneux, France).

RNA isolation and real-time quantitative RT-PCR

The RNeasy Micro Kit (Qiagen, Hilden, Germany) was used for RNA isolation from resting basophils or cells treated with IL-3 or IL-3 plus IVIG for 3 hours. Additionally, basophils were also treated with Syk inhibitor for 1 hour before stimulation with IL-3 plus IVIG. cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). Quantitative RT-PCR (qRT-PCR) was done by using the TaqMan Universal Master Mix II with UNG (Applied Biosystems, Foster City, Calif), and IL-4 expression was measured with TaqMan Gene Expression Assays (Applied Biosystems): Hs00174122_m1 (IL-4) and Hs02786624_g1 (glyceraldehyde-3-phosphate dehydrogenase).

Statistical analysis

Statistical analysis was performed with Prism 6 software (GraphPad Software, La Jolla, Calif). One-way ANOVA (with Tukey multiple comparison tests or Dunnett multiple comparison tests), and 2-way Mann-Whitney tests were used to determine statistical significance.

RESULTS

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

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

We then investigated whether IVIG modulates primed basophils, in particular IL-3, the major basophil-priming cytokine. We found that under IL-3 priming, IVIG significantly enhanced CD69, an activation marker of basophils (Fig 1, *D*). On the other hand, expression of CD13, CD62 ligand, CD123, and CD203c (see Fig E1 in this article's Online Repository at www.jacionline.org); expression of the degranulation-associated markers CD63 (Fig 1, *E*) and CD107a (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org); and histamine concentrations in supernatants (see Fig E2, *C*) were not significantly altered by IVIG.

Furthermore, IVIG significantly enhanced IL-4, IL-6, and IL-8 secretion by IL-3–primed basophils (Fig 1, *F*). qRT-PCR analysis also confirmed *Il4* induction by IVIG (see Fig E3 in this article's Online Repository at www.jacionline.org). Equimolar concentrations of HSA, used as a protein control for IVIG, did not significantly alter the expression of basophil markers and cytokine production, thus confirming that IVIG could directly induce activation of IL-3–primed basophils without leading to degranulation. Preliminary exploration in IVIG-treated patients with myopathy also confirmed enhancement of CD69 on the basophils of 4 of 5 patients analyzed (Fig 1, *G*).

IL-33 and other cytokines are dispensable for basophil activation by IVIG

Because IL-4 secretion by basophils in mice requires IL-33 stimulation after IVIG infusion,[9] we wondered whether IL-33 could, like IL-3, prime human basophils to be activated by using IVIG. Unlike IL-3 (Fig 1, *D-F*), only a marginal increase in expression of CD69 on basophils (Fig 2, *A* and *B*) or their cytokine production (Fig 2, *C*) was observed after IL-33 stimulation of basophils at a dose equivalent of that induced in IVIG-treated patients.[11,12] Despite enhancement of IL-33R expression by IL-3 (see Fig E4 in this article's Online Repository at www.jacionline.org), IL-33 when used in combination with IL-3 did not exert either a synergistic or additive effect on IVIG-induced basophil activation (Fig 2, *D* and *E*). Hence these results do not support a major role for IL-33 in priming human basophils toward IVIG responsiveness. Other cytokines, such as IL-25, TSLP, and GM-CSF, also had no significant effect on IVIG-induced basophil activation (see Fig E5 in this article's

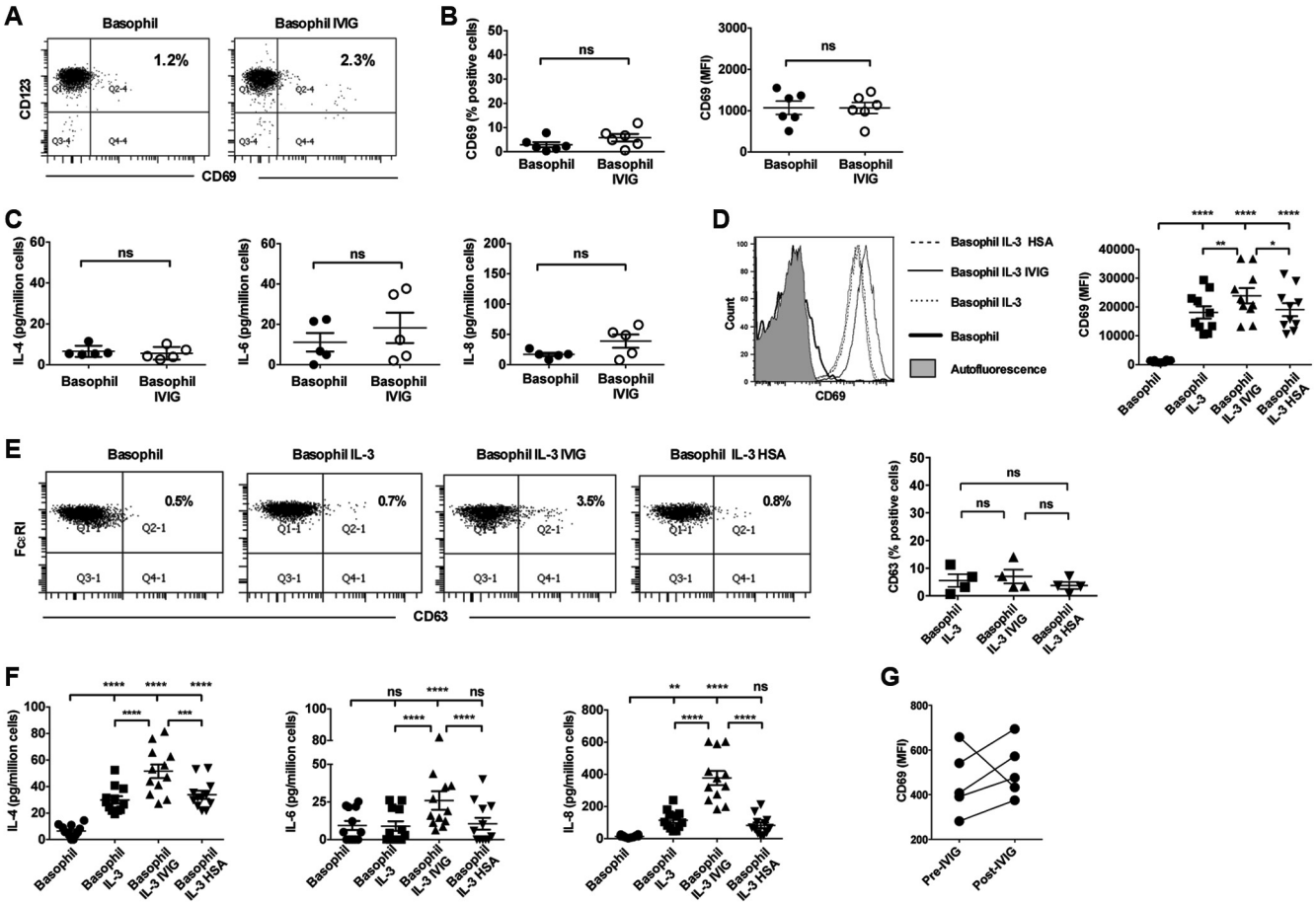

FIG 1. IVIG induces activation and cytokine secretion in IL-3–primed basophils. **A-C,** Isolated basophils from human circulation were cultured either alone or with IVIG. Fig 1, *A* and *B*, Representative dot plots and expression (percentage of positive cells and mean fluorescence intensity *[MFI]*) of CD69 on basophils (means ± SEMs, n = 6 donors). Fig 1, *C*, Amount of secretion of IL-4, IL-6, and IL-8 (means ± SEMs, n = 5 donors). *ns*, Not significant, 2-tailed Mann-Whitney test. **D-F,** Basophils were cultured either alone or with IL-3. IVIG or HSA was added after 2 hours of stimulation with IL-3. Fig 1, *D*, Representative histogram overlays and MFI of CD69 expression on basophils (means ± SEMs, n = 10 donors). Fig 1, *E*, Representative dot plots and percentages of basophils (means ± SEMs, n = 4 donors) positive for CD63. Fig 1, *F*, Effect of IVIG on secretion (in picograms per million cells) of IL-4, IL-6, and IL-8 (means ± SEMs, n = 12 donors) by IL-3–primed basophils. *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$. *ns*, Not significant, 1-way ANOVA with Tukey multiple comparison tests. **G,** Expression of CD69 on basophils of patients with myopathy before *(Pre-IVIG)* and after *(Post-IVIG)* IVIG therapy.

Online Repository at www.jacionline.org). Altogether, these results (Figs 1 and 2) indicate that IVIG induces IL-4 in human basophils, as had been described in a mouse model.[9] Unlike mice, however, IVIG appears to have a direct effect on human basophils, leading to IL-4 secretion as long as basophils were primed with IL-3.

IVIG induces basophil activation through F(ab')$_2$ fragments, whereas type II FcRs, C-type lectin receptors, and Siglecs are dispensable

We aimed to identify the receptors that mediate basophil activation. Recently, type II FcRs that include DC-SIGN and CD23, which interact with the Fc domain in the closed conformation, were reported to mediate the anti-inflammatory actions of IVIG.[13] However, human basophils were negative for CD23 and DC-SIGN,[14] thus ruling out their involvement in IVIG-induced basophil activation (Fig 3, *A*).

Because Fc–α(2,6)-sialic acid linkages could be recognized by various Siglecs, we investigated their implication in the cross-talk between IVIG and basophils. Siglec-2 (CD22) and Siglec-14 specifically recognize α(2,6)-sialic acid linkages. However, both resting and IL-3–primed basophils were negative for CD22 (Fig 3, *B*). In addition, basophils did not express Siglec-3, Siglec-5/14, Siglec-7, and Siglec-8 (see Fig E6 in this article's Online Repository at www.jacionline.org), which all possess some affinity for α(2,6)-sialic acid linkages. Siglec-10 was previously reported to be undetectable on basophils.[15]

Dendritic cell immunoreceptor (DCIR), a C-type lectin receptor, has been reported to recognize α(2,6)-sialic acid linkages of IgG.[16] Nearly 80% of steady-state and 95% of IL-3–primed basophils express DCIR, but IVIG did not alter this expression (Fig 3, *C* and *D*). Importantly, IVIG did not induce activation of resting basophils (Fig 1, *A-C*), despite these cells expressing DCIR, thus indirectly ruling out the role of DCIR in IVIG-induced basophil activation.

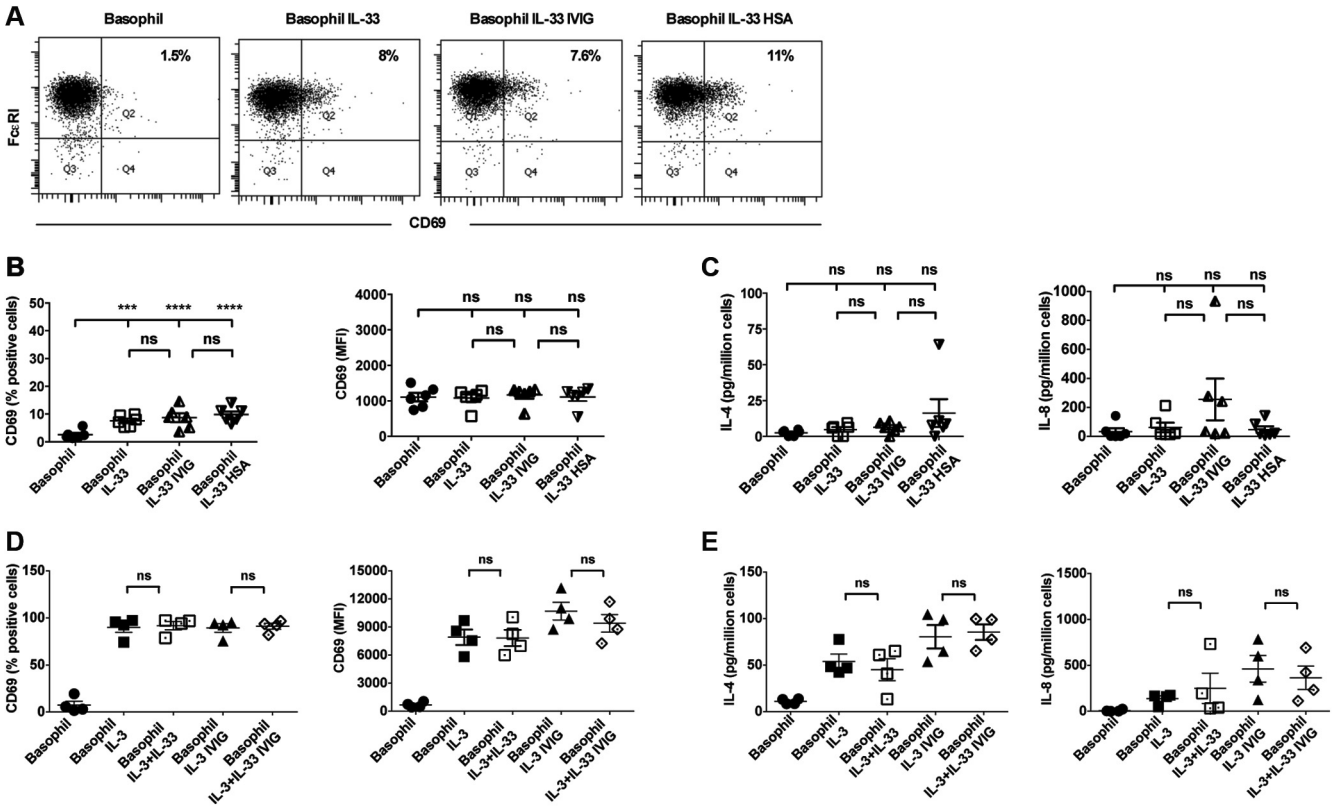

FIG 2. IL-33 is dispensable for activation of human basophils by IVIG. Basophils were cultured either alone or with IL-33. IVIG or HSA was added after 2 hours stimulation with IL-33. **A** and **B,** Representative dot plots and expression (percentage of positive cells and mean fluorescence intensity *[MFI]*) of CD69 on basophils (mean ± SEMs, n = 6 donors). **C,** Amount of secretion of IL-4 and IL-8 (mean ± SEM, n = 6 donors). **D** and **E,** Basophils were stimulated with IL-3 for 1 hour followed by IL-33 for an additional hour before culturing with IVIG or HSA. Fig 2, *D,* Expression (percentage of positive cells and MFI) of CD69 on basophils. Fig 2, *E,* Amount of secretion of IL-4 and IL-8 (means ± SEMs, n = 4 donors) ***$P < .001$ and ****$P < .0001$. *ns,* Not significant, 1-way ANOVA with Tukey multiple comparison tests.

The lack of involvement of known receptors for α(2,6)-sialic acid-linkages point toward a role for $F(ab')_2$ domain rather than Fc portion of IVIG on basophil activation. Accordingly, $F(ab')_2$ fragments of IVIG, but not Fc fragments, significantly enhanced CD69 (Fig 3, *E* and *F*) and production of both IL-4 and IL-8 (Fig 3, *G* and *H*).

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

Classically, IL-3 has been known for its critical role in favoring basophil sensitization by IgE for augmented FcεRI-mediated signals and secretion of various inflammatory mediators.[17-19] Our data demonstrate that IL-3 priming is also a prerequisite for IVIG-induced basophil activation. IVIG significantly downregulated FcεRI on IL-3–primed basophils (Fig 4, *A* and *B*), suggesting that IVIG binding to FcεRI and/or to FcεRI-bound IgE triggered internalization of FcεRI. As expected, basophils displayed IgE on their surfaces (Fig 4, *C* and *D*), and IL-3 treatment dramatically licensed basophils to bind IVIG (Fig 4, *E* and *F*). However, incubation of basophils with additional IgE did not alter the intensity of basophil surface IgE, indicating that all FcεRI on the basophils is already saturated by IgE. These arguments point out that IVIG induces activation of basophils, possibly by means of signaling through basophil FcεRI-bound IgE rather than FcεRI. Importantly, depletion of anti-IgE reactivity within IVIG compromised the ability of IVIG to activate IL-3–primed basophils, as revealed by the poor increase in CD69 expression (Fig 4, *G* and *H*) and the abrogation of secretion of IL-4 and IL-8 (Fig 4, *I*).

Activating and inhibitory CD32/FcγRII are dispensable for regulation of basophil activation by IVIG

By interacting with the Fc domain of IgG, FcγRs influence activation of immune cells.[20] Human basophils mainly express FcγRIIA and FcγRIIB.[21] Although FcγRIIA is an activating receptor, signaling through FcγRIIB inhibits immune cell activation.[20] Therefore we wondered whether IVIG-induced basophil activation is regulated by FcγRII.

First, we analyzed the expression pattern of FcγRII on basophils. Although IL-3 enhanced expression of both FcγRIIA and FcγRIIB, a nonsignificant trend toward reduced expression of both the receptors was observed on IVIG stimulation (Fig 5, *A* and *B*). Thus unlike monocytes and B cells of patients with chronic inflammatory demyelinating polyneuropathy that showed enhanced FcγRIIB expression on IVIG therapy,[22] the ratio of intensity of expression of FcγRIIB to FcγRIIA remains

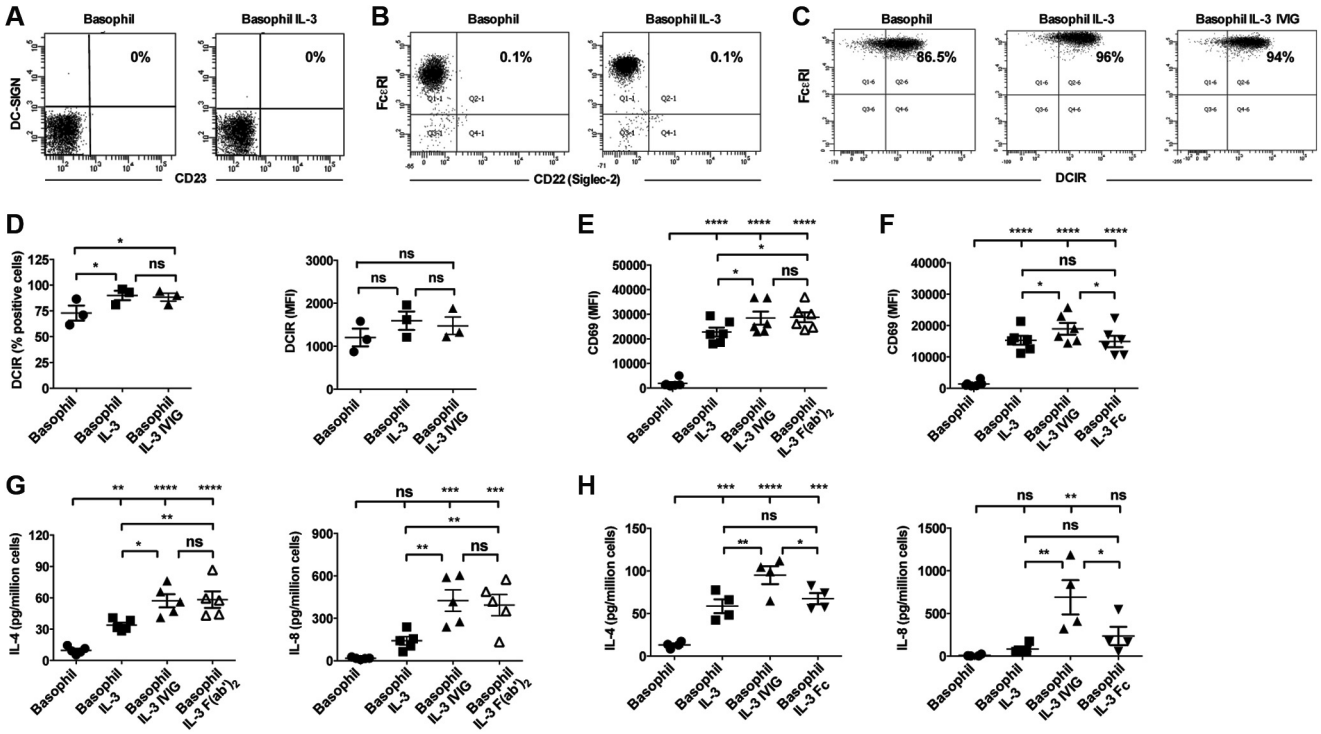

FIG 3. Expression of type II FcRs, Siglecs, and C-type lectin receptors on basophils and effects of $F(ab')_2$ and Fc fragments of IVIG on basophil activation. **A** and **B,** Representative dot plots of CD23, DC-SIGN, and CD22 expression on basophils. **C** and **D,** Representative dot plots and expression (percentage of positive cells and mean fluorescence intensity *[MFI]*) of DCIR on basophils (mean ± SEM, n = 3 donors). **E-H,** Basophils were cultured either alone or with IL-3 for 24 hours. IVIG or the $F(ab)_2$ or Fc fragment was added after 2 hours of stimulation with IL-3. Fig 3, *E* and *F*, Expression of CD69 (means ± SEMs, n = 6 donors). Fig 3, *G* and *H*, Amount of secretion of IL-4 and IL-8 (means ± SEMs, n = 4-5 donors). $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$. *ns*, Not significant, 1-way ANOVA with Tukey multiple comparison tests.

unchanged on IVIG-treated basophils. Our data are similar to those observed with splenic macrophages of IVIG-treated adults with immune thrombocytopenia.[23]

High-affinity rabbit anti-human IgE IgG was shown to negatively regulate IgE-induced activation of human basophils by coengaging FcγRIIB.[21] Hence we investigated whether FcγRIIB blockade would enhance basophil activation through IVIG. However, IVIG-induced activation of basophils was not significantly altered on FcγRIIB blockade (Fig 5, *C* and *D*).

Because FcγRIIA signaling induces activation of immune cells,[20] we explored whether IVIG-induced basophil activation implicates coengagement with this receptor. However, FcγRIIA blockade had no repercussions on IVIG-induced expression of CD69 and cytokines (Fig 5, *E* and *F*), demonstrating that FcγRII (activating or inhibitory) has no significant role in regulation of human basophil function by IVIG.

Syk pathway is critical for basophil activation by IVIG

FcεRI-mediated activation of human basophils *in vitro* requires both priming by IL-3 and the kinase Syk, which is recruited to the FcεRI signaling complex.[17-19] Noticeably, IL-3–mediated downstream signaling has also been reported to be Syk dependent.[24,25] Freshly isolated basophils showed basal phosphorylation of Syk (phosphorylated Syk [pSyk]). In line with the fact that IL-3 induces rapid phosphorylation of Syk, we found that IL-3 significantly enhanced pSyk. A treatment with IL-3 plus IVIG resulted in similar pSyk induction (Fig 6, *A* and *B*). Furthermore, inhibition of Syk with the inhibitor R406 abrogated IVIG-induced enhancement of CD69 (Fig 6, *C* and *D*) and production of IL-4 and IL-8 (Fig 6, *E*). qRT-PCR also confirmed abrogation of IVIG-induced *Il4* after Syk inhibition (see Fig E7 in this article's Online Repository at www.jacionline.org). Altogether, these data suggest that IVIG, because of its IgE reactivity, induces activation of IL-3–primed basophils by signaling through FcεRI-bound IgE.

DISCUSSION

Despite having pathogenic roles in various diseases,[8,26,27] recent evidence from mice also suggests that basophils are central to the anti-inflammatory effects of IVIG, thus providing an intriguing new function to these rare immune cells.[9] However, this proposed role of basophils in mediating the therapeutic benefits of IVIG could not be reproduced in another report.[28] It is important to note that both studies have used the anti-FcεRI mAb MAR-1 to deplete basophils, and this antibody has been reported to deplete FcεRI-positive dendritic cells (DCs) as well.[29,30] Also, compared with basophils from mice, human basophils display distinct features.[8,31,32] Therefore the effect of IVIG on basophil function is far from clear. Notably, data from human subject raise an alternative paradigm that IVIG might modulate basophil functions directly rather than the indirect IL-33–dependent pathway.[11]

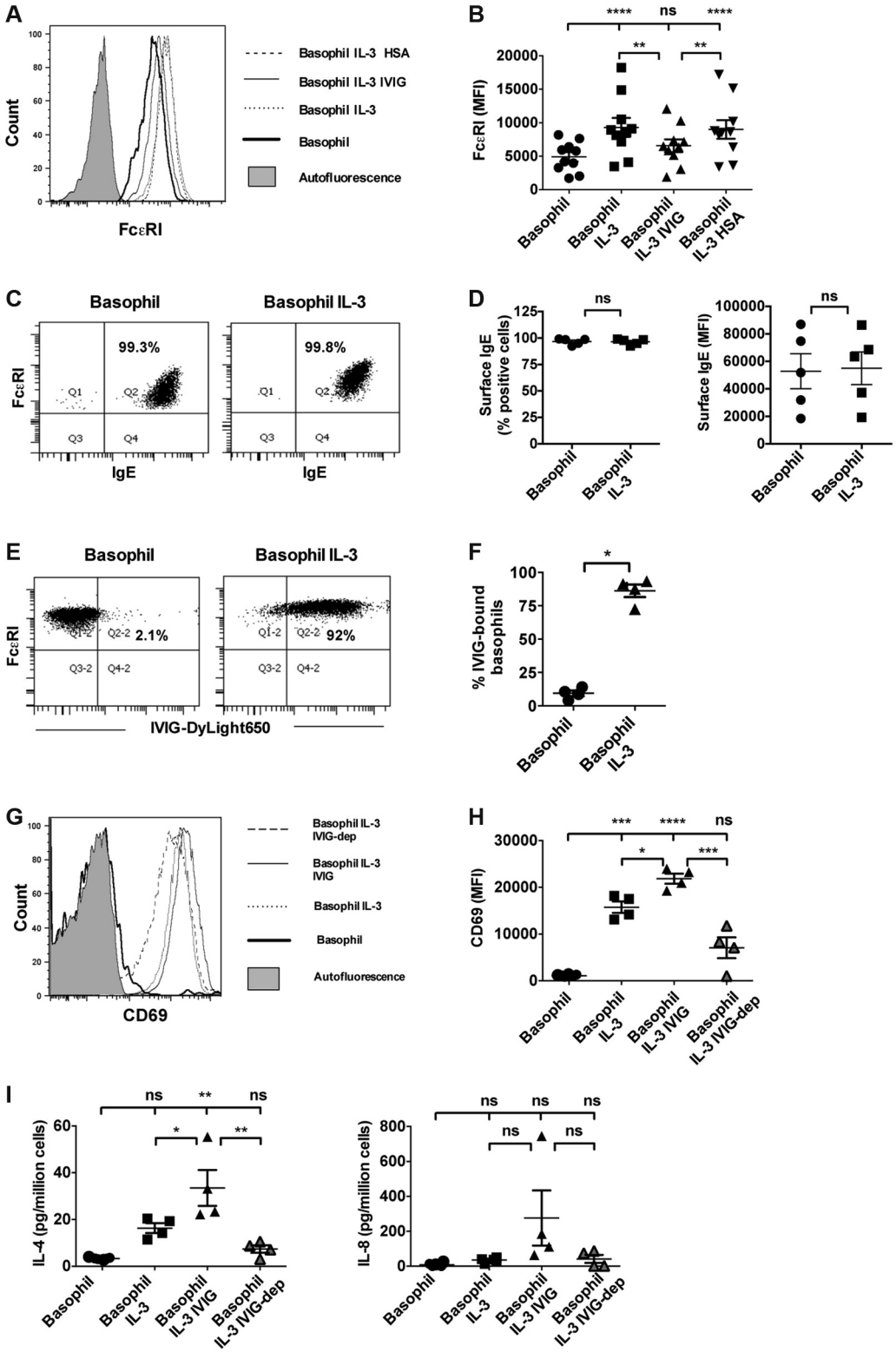

FIG 4. Basophil activation by IVIG is mediated by a fraction of IgG that signals through basophil FcεRI-bound IgE. **A** and **B,** Modulation of FcεRI expression (representative histogram overlays and mean fluorescence intensity (*MFI*); means ± SEMs, n = 10 donors) by IVIG in IL-3–primed basophils. **C,** Representative dot plots showing basophils positive for surface IgE. **D,** Percentage of basophils positive for surface IgE and its intensity (MFI; means ± SEMs, n = 5 donors). **E** and **F,** Percentage of basophils positive for IVIG binding (representative dot plots and means ± SEMs, n = 4 donors). **G-I,** Effect of anti-IgE reactivity–depleted IVIG on expression of CD69 (representative histogram overlays and MFI; means ± SEMs, n = 4 donors; Fig 4, *G* and *H*) and IL-4 and IL-8 secretion (means ± SEMs, n = 4 donors; Fig 4, *I*). **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. *ns*, Not significant, 2-tailed Mann-Whitney test or 1-way ANOVA with Tukey multiple comparison tests.

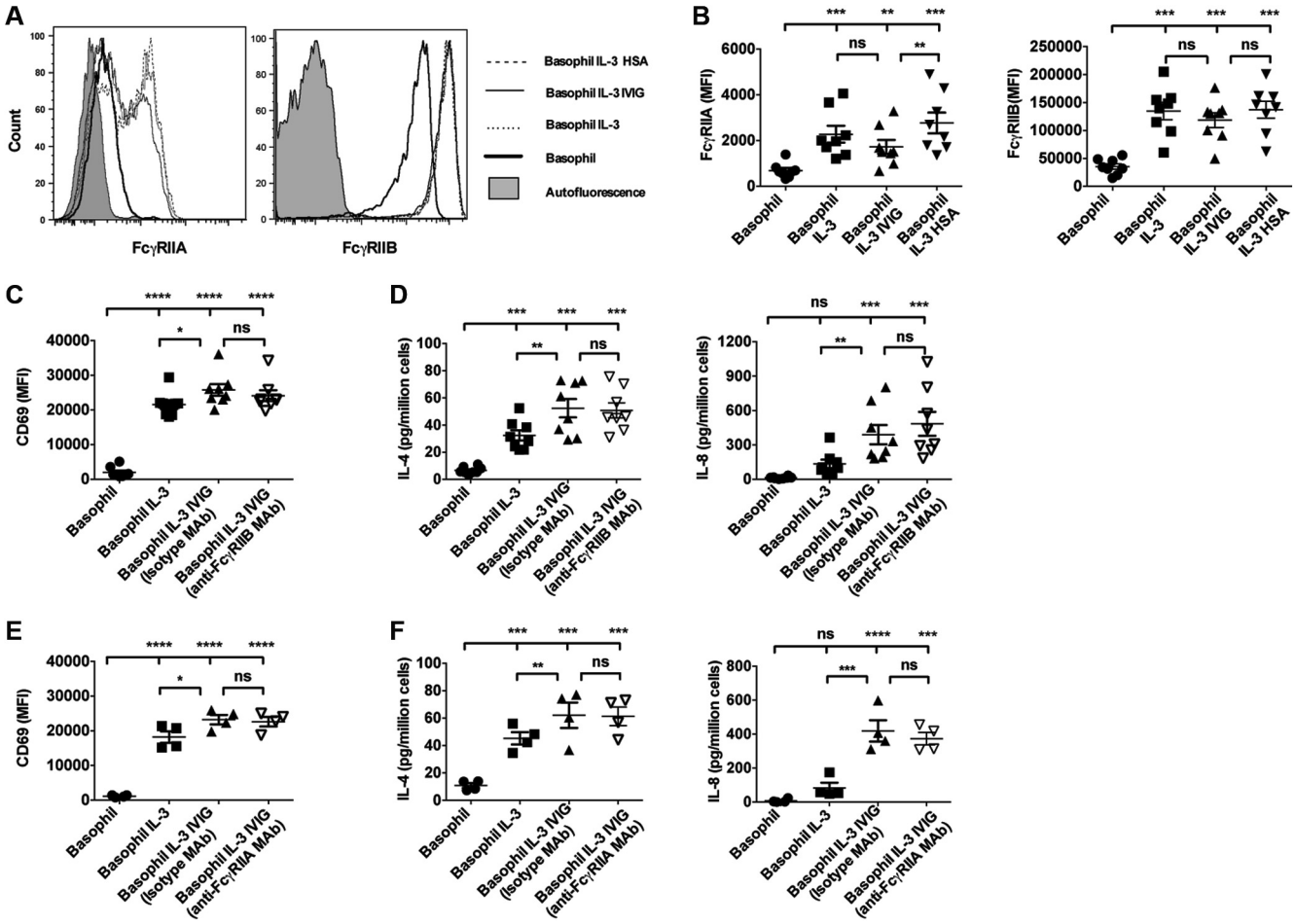

FIG 5. Activating and inhibitory CD32/FcγRII are dispensable for regulation of basophil activation by IVIG. Basophils were cultured either alone or with IL-3 for 24 hours. IVIG or HSA was added after 2 hours of stimulation with IL-3. **A** and **B,** Representative histogram overlays and mean fluorescence intensity *(MFI)* of expression (means ± SEMs, n = 8 donors) of FcγRIIA and FcγRIIB on basophils. **C** and **D,** Repercussion of FcγRIIB blockade on expression of CD69 (Fig 5, *C*) and amount of IL-4 and IL-8 secretion (Fig 5, *D*; means ± SEMs, n = 8 donors). **E** and **F,** Repercussion of FcγRIIA blockade on expression of CD69 (Fig 5, *E*) and amount of IL-4 and IL-8 secretion (Fig 5, *F*; means ± SEMs, n = 4 donors). *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$. *ns*, Not significant, 1-way ANOVA with Tukey multiple comparison tests.

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

We investigated whether IVIG could activate IL-33–primed basophils. However, IVIG modified neither phenotype nor cytokine production in IL-33–primed basophils. In addition to IL-33, activated epithelial cells also release IL-25 and TSLP.[38] However, basophils were not sensitive for both these cytokines. A recent report also confirms that TSLP does not activate human basophils.[39] On the other hand, GM-CSF significantly activated human basophils,[40,41] but the extent of activation was less than IL-3. Also, GM-CSF priming had no consequence on IVIG-induced basophil activation.

Noticeably, however, IL-3 priming licensed human basophils to undergo activation by IVIG. Rather than an IL-33–mediated pathway of basophil IL-4 induction, as suggested from mouse studies, our data suggest an IL-3–mediated pathway of human basophil priming that enables them to directly respond to IVIG by secreting IL-4 (and other cytokines). Although IL-3 significantly enhanced IL-33R expression on basophils, IL-33 did not potentiate IVIG-induced basophil activation when used in combination with IL-3. These data suggest that IL-3 is a major stimulator of basophil function and could regulate basophil response to IL-33 (probably at higher concentrations as reported earlier[37]) by enhancing IL-33R expression. In fact, under IL-3 stimulation conditions, CD69 and IL-33R were coexpressed on basophils. However, this was not the case under IL-33

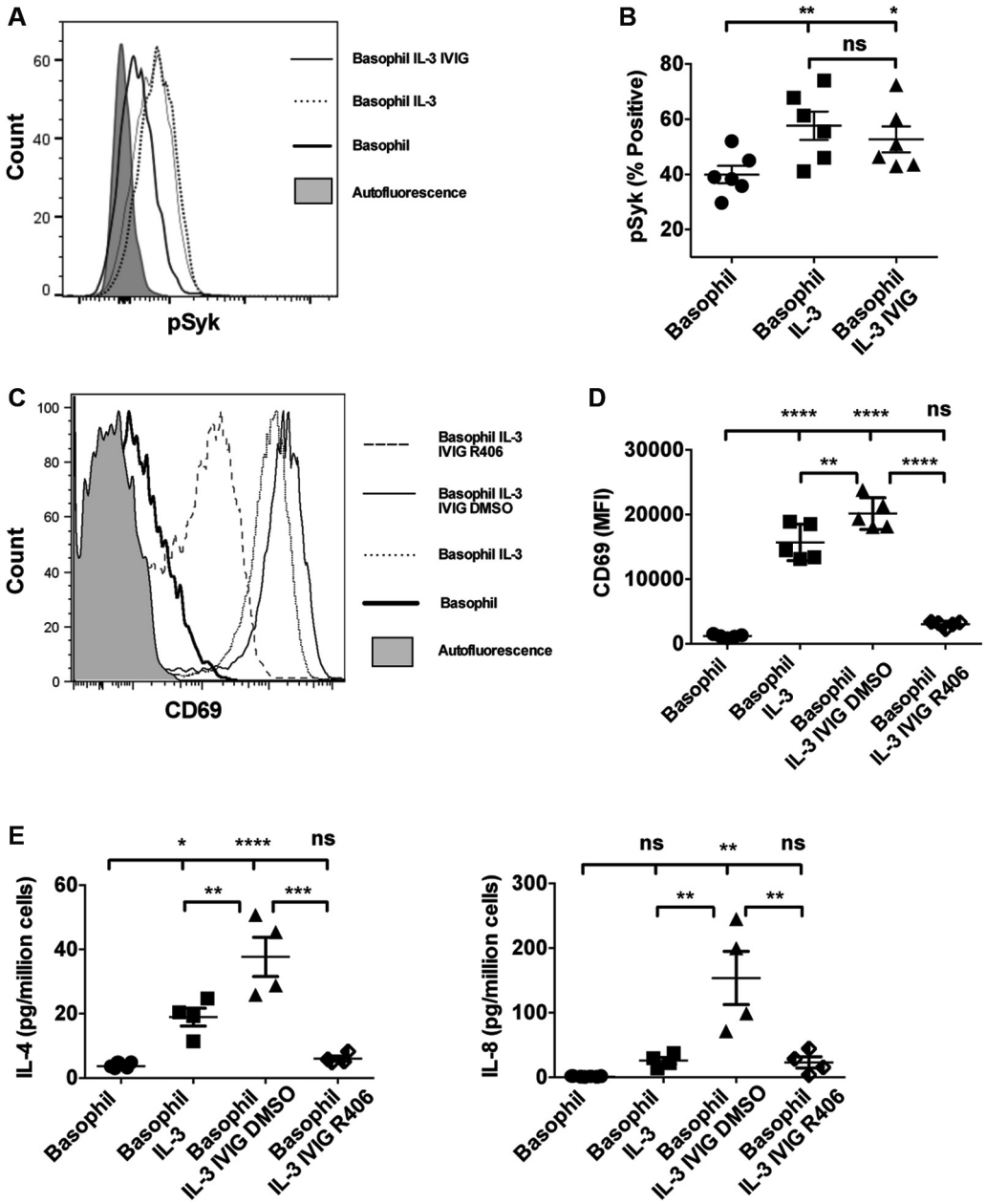

FIG 6. Inhibition of the Syk pathway abrogates IVIG-induced activation of basophils. **A** and **B,** Representative histogram overlays and means ± SEMs (n = 6 donors) of pSyk expression in basophils stimulated with IL-3 or IL-3 plus IVIG. **C** and **D,** Effect of Syk inhibition by R406 toward IVIG-induced expression of CD69 (representative histogram overlays and mean fluorescence intensity (*MFI*); means ± SEMs, n = 5 donors). *DMSO*, Dimethyl sulfoxide; *MFI*, mean fluorescence intensity. **E,** Syk inhibition abrogates IVIG-induced IL-4 and IL-8 secretion (means ± SEMs, n = 4 donors). *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$. *ns,* Not significant, 1-way ANOVA with Tukey multiple comparison tests.

stimulation conditions, wherein only a minor population of basophils coexpressed CD69 and IL-33R, possibly because of marginal stimulation of basophils by IL-33 or IL-33R internalization.

All our experiments in this report rely on *in vitro* stimulation system, and hence it is important to prove these data in the context of systemic autoimmune and inflammatory diseases. Although data are preliminary, basophil activation also occurs *in vivo* in IVIG-treated patients with myopathy. Further analyses of basophils in inflamed tissues and secondary lymphoid organs should provide more insight into regulation of basophil functions by IVIG.

Various studies have reported that FcγRIIB plays an important role in mediating the anti-inflammatory actions of IVIG. Enhanced expression of FcγRIIB by IVIG has been proposed to increase the threshold level for activation of innate cells by immune complexes.[22,42-44] However, the absolute requirement of FcγRIIB in mediating the anti-inflammatory actions of IVIG could not be confirmed in other experimental models.[45-48] Also, several effects of IVIG on human DCs, macrophages, and

$CD4^+$ T cells were FcγRIIB independent.[49-52] Our current data on basophils provide further evidence for FcγRII-independent action of IVIG on human immune cells.

Several targets and receptors have been identified for IVIG. In addition to the $F(ab')_2$-mediated recognition of various self-molecules, such as HLA, Fas, CD40, Siglecs, B cell–activating factor of the TNF family, immunoglobulins, and others,[53-59] Fc–α(2,6)-sialic acid linkages were reported to be recognized by type II Fc receptors, Siglec-2, and DCIR.[13,16,60,61] However, human immune cells display wide variations in the expression patterns of these receptors. *In vitro*–generated monocyte-derived DCs (equivalent of inflammatory DCs) express both DC-SIGN and DCIR, whereas DCs *ex vivo* express mainly DCIR.[62] Although CD23 is expressed by B cells, macrophages, and eosinophils, Siglec-2 is restricted to B cells. However, human basophils lack DC-SIGN, CD23, and Siglec-2. Despite being positive for DCIR, resting basophils were not modified by IVIG, suggesting that DCIR is not sufficient (or predominant) in mediating IVIG-induced basophil activation. Also, other Siglecs that could recognize α(2,6)-sialic acid linkages were absent on basophils.

IVIG-induced activation of IL-3–primed human basophils did not lead to degranulation and was distinct to the effect of anti-IgE antibodies identified in the asthmatic patients that induced high expression of the degranulation marker CD63.[63] It is possible that the anti-IgE content in IVIG is too low to fully activate basophils to degranulate. Supporting this assumption, antigens at low concentrations have been reported to induce FcεRI-mediated activation of mast cells without causing degranulation.[64,65]

Glycosylation patterns of Fc domains of IgG determine their engagement with classical type I FcRs (that include FcγRs) or type II FcRs. The sialylated or nonsialylated glycan–mediated "closed" versus "open" conformation of Fc switches engagement of the Fc domain toward type II or type I FcRs, respectively.[66] A previous report showed that anti-IgE rabbit IgG inhibit basophil activation by coengaging with FcγRIIB.[21] However, contrary to this, we observed activation of basophils by anti-IgE IgG present in IVIG. Also, FcγRII blockade had no significant effect on IVIG-induced basophil activation. Based on all these arguments, we could infer that glycosylation content of Fc domains of anti-IgE IgG in IVIG is enriched for sialylation that might have prevented engagement of Fc with FcγRII on basophils.

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

Syk phosphorylation is one of the early signaling events in basophils after IL-3– and FcεRI-mediated activation.[17,24,25] Therefore it is difficult to segregate the importance of IL-3–induced versus FcεRI-induced Syk activation. The fact that IVIG could induce basophil activation only on IL-3 priming suggests that IL-3–induced Syk phosphorylation is indispensable for basophil FcεRI-bound IgE-mediated activation by IVIG. The Syk inhibitor R406, which is proposed for use in human pathologies,[70] blocked IVIG-induced human basophil activation; thus it appears that both "classical" high-affinity IgE-induced degranulation events and IVIG's anti-IgE activation (without degranulation) events use Syk for signal transduction.

In conclusion, our report highlights a novel mechanism of activation of human basophils by IVIG and underlines discrepancies in the mechanisms of action of IVIG in human subjects and mice.

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Key messages

- **IVIG induces activation and secretion of IL-4, IL-6, and IL-8 in IL-3–primed human basophils, but unlike in mice, IL-33 was dispensable.**
- **IVIG induces human basophil activation through $F(ab')_2$ fragments but independent of FcγRII, C-type lectin receptors, type II Fc receptors, and Siglecs.**
- **Basophil activation by IVIG is mediated by a fraction of IgG that signals through basophil surface-bound IgE and the Syk pathway.**

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METHODS

List of antibodies for flow cytometry and functional assays

CD63–phycoerythrin (PE; clone H5C6), CD13–allophycocyanin (APC; clone WM15), CD123-BV421 (clone 9F5), CD69-APC/Cy7 (clone FN50), CD209-APC (clone DCN46), CD22-PE (clone S-HCL-1), and CD62 ligand–fluorescein isothiocyanate (FITC; clone DREG-56) were from BD Biosciences. FcεRIα-FITC (clone CRA-1), Siglec-3–FITC (clone AC104.3E3), Siglec-5–FITC (clone 1A5), Siglec-7–FITC (clone REA214), Siglec-8–APC (clone 7C9), and anti-IgE–APC (clone MB10-5C4) mAbs were obtained from Miltenyi Biotec. CD203c-PE (clone NP4D6), CD23-PE (clone B3B4), CD107a-BV421 (clone H4A3), FcεRIα-BV510 (clone AER37 [CRA-1]), and DCIR-PE (clone 9E8) mAbs were from BioLegend (San Diego, Calif). Anti-IgE mAb (clone GE-1) was from Sigma-Aldrich. Unconjugated and FITC-labeled FcγRIIA mAb (clone IV.3) was purchased from Stem Cell Technologies (Vancouver, British Columbia, Canada). Human ST2/IL-33R–PE polyclonal goat IgG and isotype control mAbs for blocking experiments were from R&D Systems (Minneapolis, Minn). Anti-human pSyk (Tyr348; clone moch1ct) was from eBioscience. Anti-human FcγRIIB (clone 2B6 expressed as a human IgG1, kappa chimeric antibody bearing the $N_{297}D$ mutation) mAbs were coupled to Alexa Fluor 647 by using a Thermo Fisher Scientific kit, and IVIG was labeled with the Lightning-Link Rapid Dy-Light 650 Kit (Innova Biosciences, Cambridge, United Kingdom).

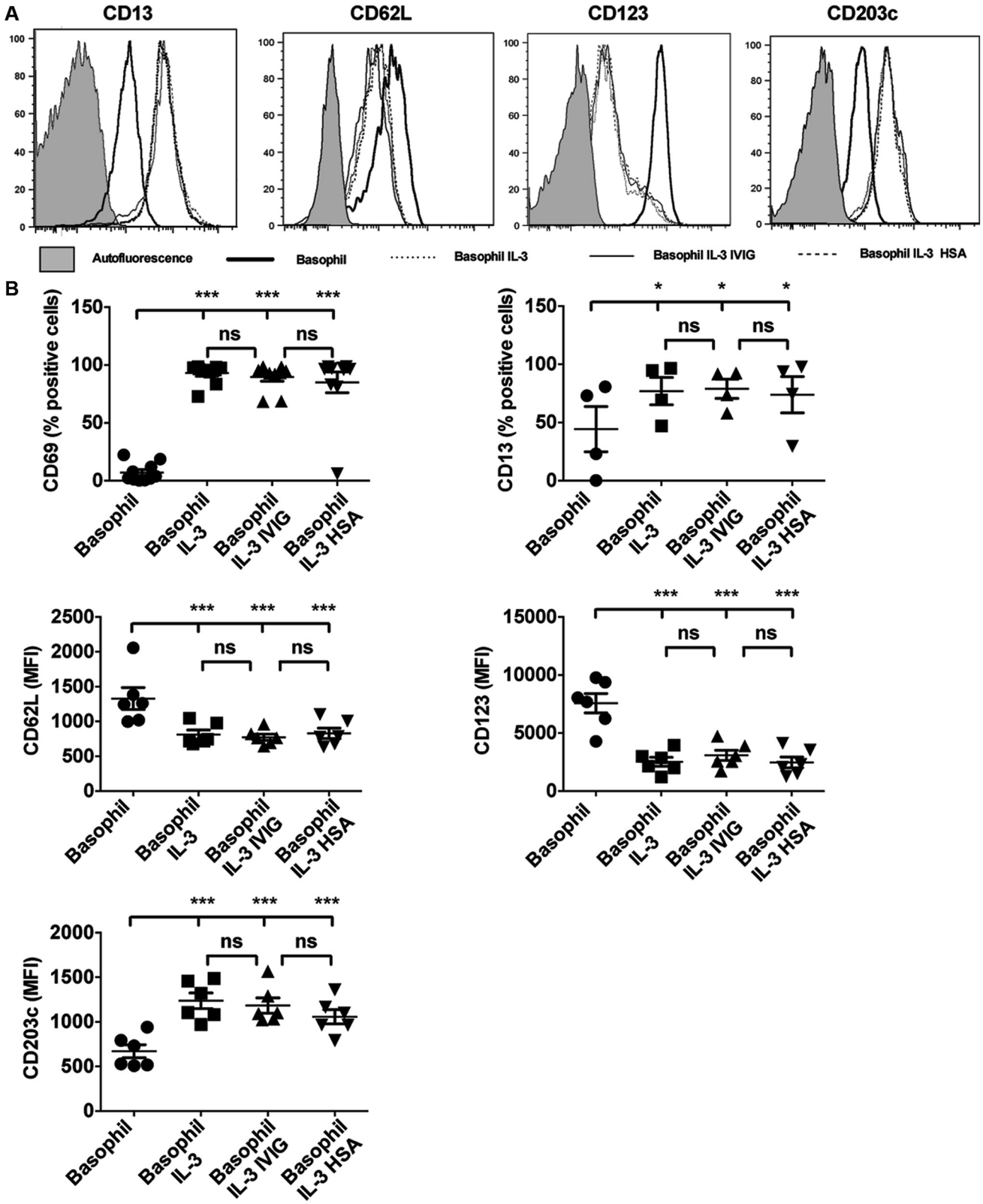

FIG E1. Effect of IVIG on expression of various surface markers in IL-3–primed basophils. **A** and **B,** Basophils were cultured either alone or with IL-3. IVIG or HSA was added after 2 hours of stimulation with IL-3. Representative histogram overlays (Fig 1, *A*) and expression (Fig 1, *B*; means ± SEMs, n = 4-12 donors) of CD69, CD13, (both in percentage of positive cells), CD62 ligand, CD123, and CD203c (all mean fluorescence intensity *[MFI]*) on basophils are shown. $*P < .05$ and $***P < .001$. *ns*, Not significant, 1-way ANOVA with Tukey multiple comparison tests.

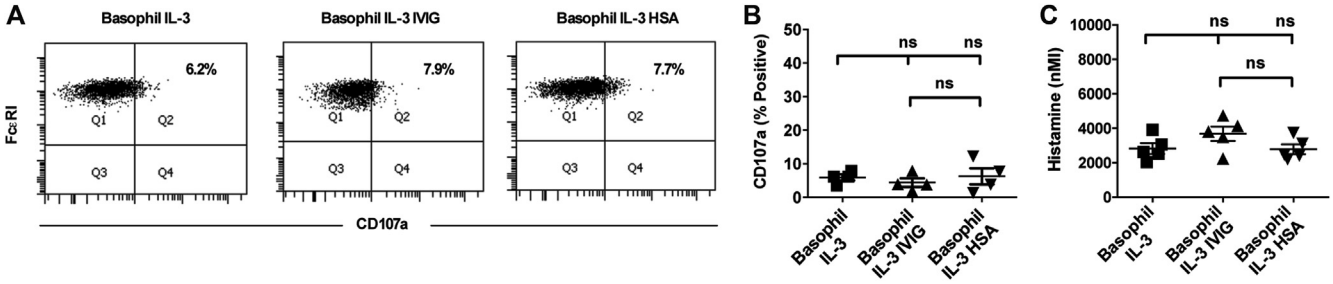

FIG E2. Activation of IL-3–primed basophils by IVIG is not associated with degranulation. **A** and **B,** Changes in CD107a expression. Representative plots and means ± SEMs of data from 4 independent donors. **C,** Amount of histamine in culture supernatants (means ± SEMs, n = 5 donors). *ns,* Not significant, 1-way ANOVA with Tukey multiple comparison tests.

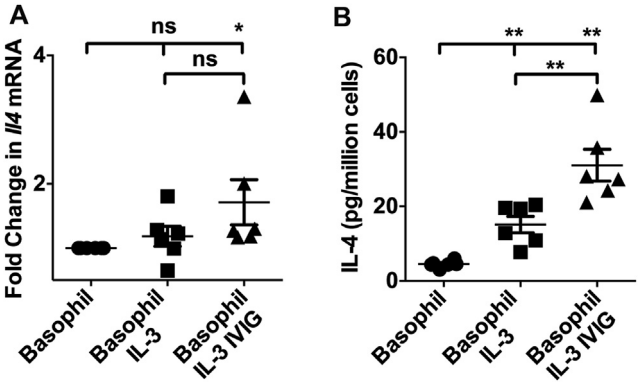

FIG E3. Real-time qRT-PCR analysis of *Il4* transcripts and amount of IL-4 secretion in resting basophils, cells treated with IL-3, or cell treated IL-3 plus IVIG for 3 hours. $*P < .05$ and $**P < .01$. *ns*, Not significant, 1-way ANOVA (with Dunnett [Fig E3, *A*] or Tukey [for Fig E3, *B*] multiple comparison tests).

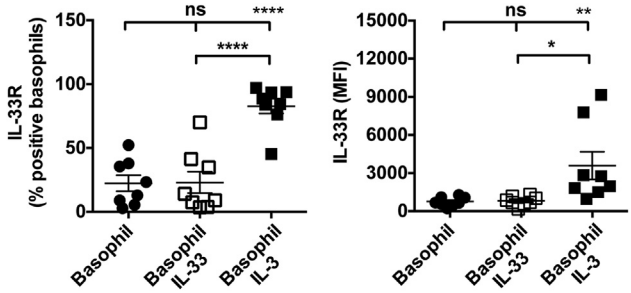

FIG E4. Expression of IL-33R (percentage of positive cells and mean fluorescence intensity *[MFI]*) on resting, IL-33–stimulated, or IL-3–stimulated basophils (means ± SEMs, n = 8 donors). *$P < .05$, **$P < .01$, and ****$P < .0001$. *ns*, Not significant, 1-way ANOVA with Tukey multiple comparison tests.

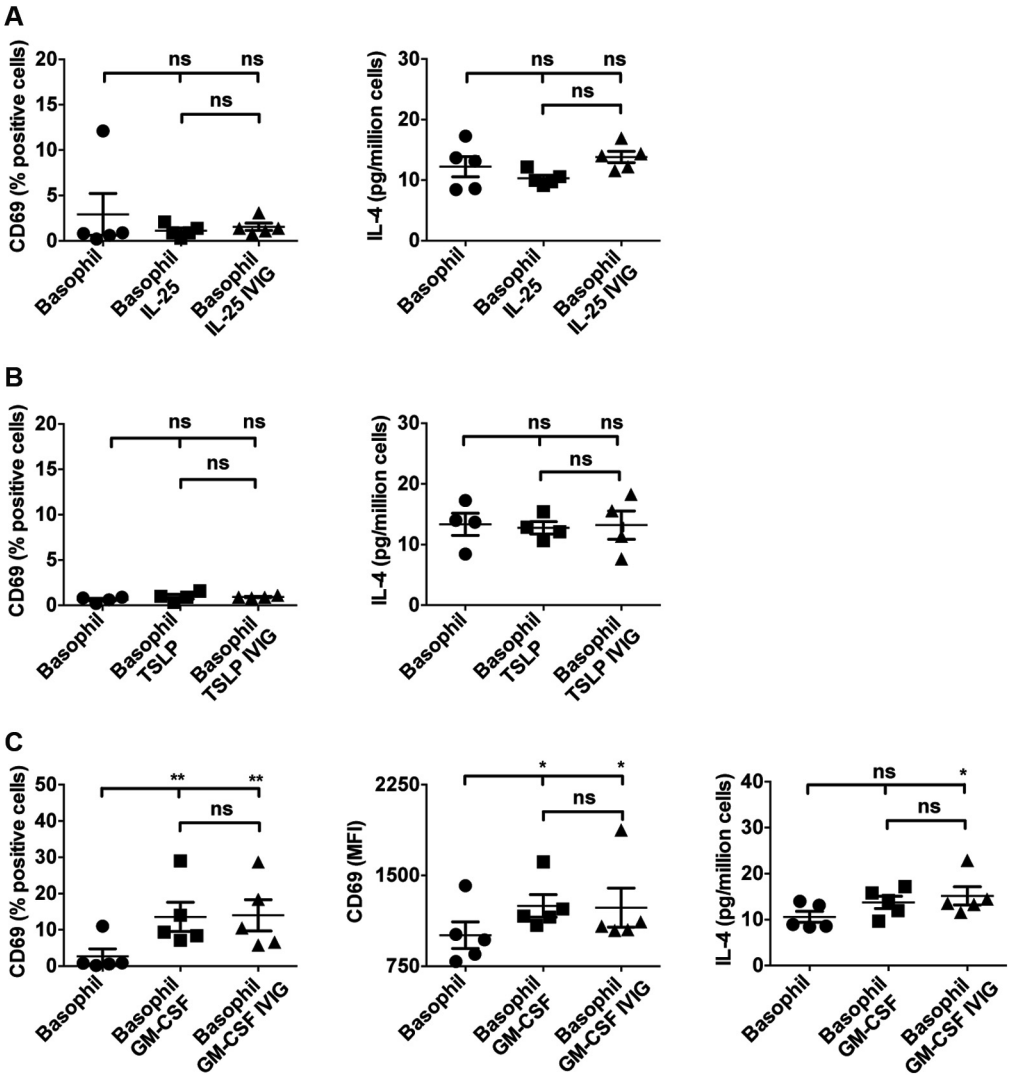

FIG E5. IL-25, TSLP, and GM-CSF are dispensable for activation of basophils by IVIG. Basophils were cultured either alone or with IL-25 **(A)**, TSLP **(B)**, or GM-CSF **(C)** for 24 hours. IVIG was added after 2 hours of stimulation with respective cytokines. Expression of CD69 (percentage of positive cells or mean fluorescence intensity *[MFI]*) and amount of secretion of IL-4 (means ± SEMs, n = 5 donors) are presented. $*P < .05$ and $**P < .01$. *ns*, Not significant, 1-way ANOVA with Tukey multiple comparison tests.

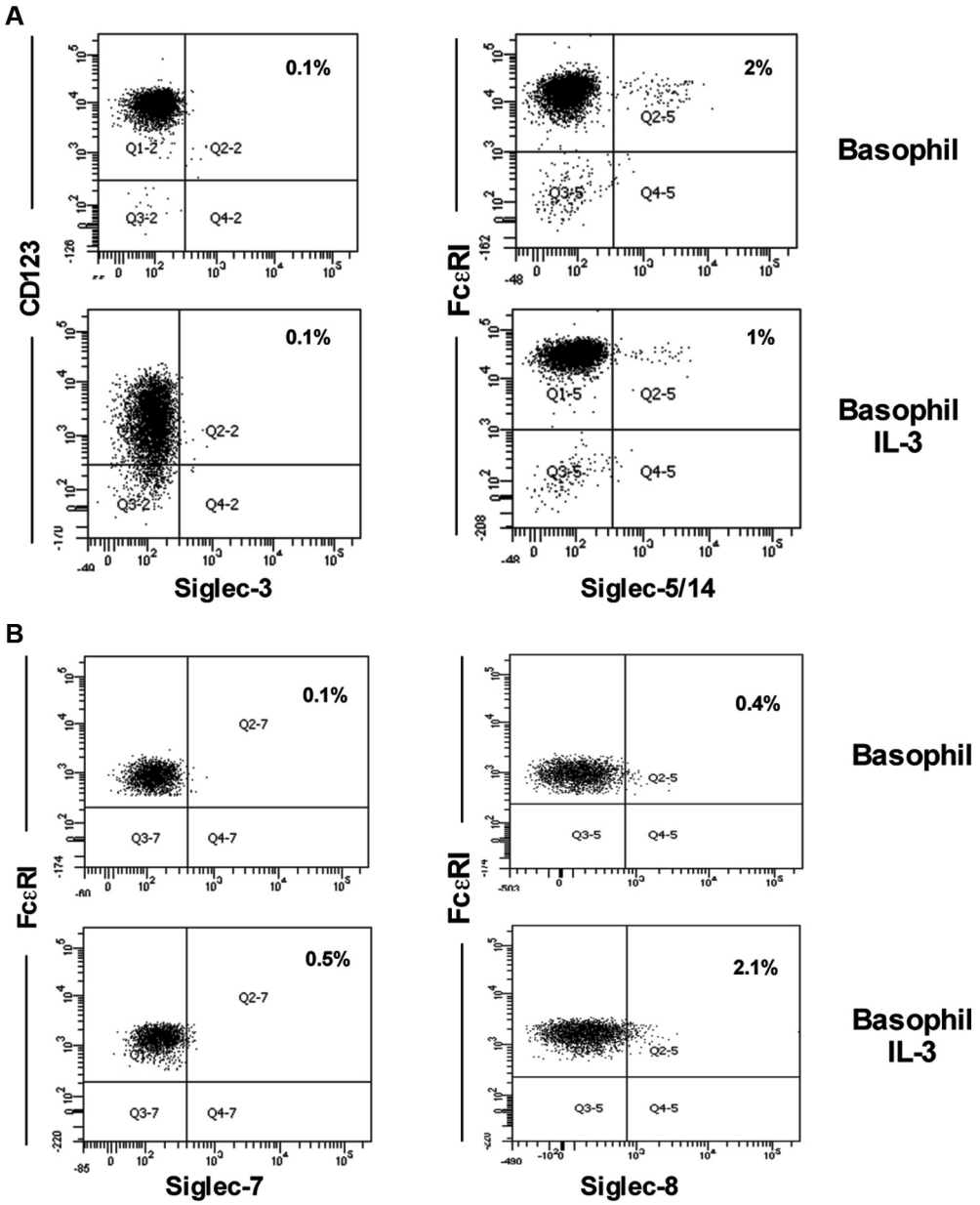

FIG E6. Expression of Siglec-3 and Siglec-5/14 **(A)** and Siglec-7 and Siglec-8 **(B)** on resting and IL-3–primed basophils.

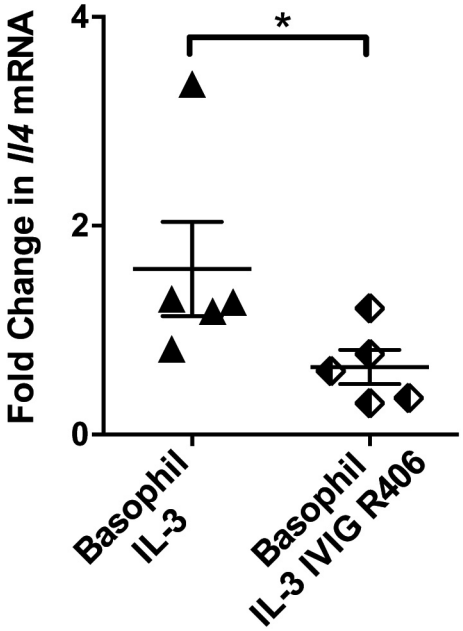

FIG E7. Effect of Syk inhibition toward IVIG-induced expression of *Il4* transcripts (means ± SEMs, n = 5 donors). Basophils were stimulated with IL-3 plus IVIG for 3 hours. Additionally, cells were also treated with the Syk inhibitor R406 for 1 hour before stimulation with IL-3 plus IVIG. $*P < .05$, 2-tailed Mann-Whitney test.