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

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ABSTRACT

BACKGROUND: Therapeutic normal immunoglobulin G or intravenous immunoglobulin (IVIG) exerts anti-inflammatory effects via several mutually nonexclusive mechanisms. Recent data in mouse models of autoimmune diseases suggest that IVIG induces IL-4 in basophils by enhancing IL-33 in SIGN-R1^{+} 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 the healthy donors were cultured in the presence of IL-3, IL-33, GM-CSF, TSLP or IL-25. The effect of IVIG, F(ab')$_2$ and Fc fragments of IVIG was examined on the expression of various surface molecules, phosphorylation of Syk, induction of cytokines, and histamine release. Phenotype of basophils was also analyzed from IVIG-treated myopathy patients. 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. The activation of basophils by IVIG led to enhanced expression of CD69 and secretion of IL-4, IL-6 and IL-8. IVIG-treated myopathy patients displayed enhanced expression of CD69 on the basophils. Syk pathway is implicated in these functions of IVIG and were mediated via 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 Siglecs.

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

Key Messages

- IVIG induces activation and secretion of IL-4, IL-6 and IL-8 in IL-3-primed human basophils but unlike mice IL-33 was dispensable
- IVIG induces human basophil activation via $F(ab')_2$ 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

Capsule summary

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

Key words

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

Abbreviations

DCIR: Dendritic cell immunoreceptor

DC-SIGN: dendritic cell-specific ICAM-3-grabbing nonintegrin

FcεRI: Fc epsilon type 1 receptor, high affinity IgE receptor

FcγR: Fc gamma receptor

FcγRIIA: Fc gamma type 2 receptor A

FcγRIIB: Fc gamma type 2 receptor B

HSA: Human serum albumin

IVIG: Intravenous immunoglobulin

SIGN-R1: SIGN- related 1

SYK: Spleen tyrosine kinase

INTRODUCTION

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 (1-2g/kg) IVIG therapy exerts anti-inflammatory effects by several mutually non-exclusive mechanisms including inhibition of the activation of innate immune cells, effector T (Th1, Th17) and B cells, suppression of 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 the 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 Th2 polarization, immunoglobulin synthesis and class-switch 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 via basophils by a two-step process.[9] IL-33 produced by SIGN-$R1^+$ innate cells upon interaction with Fc-α(2,6)-sialic acid linkages, activates basophils via IL-33R to induce IL-4. The basophil-derived IL-4 enhances the expression of inhibitory FcγRIIB on effector macrophages[9] thus adding onto the previously known function of basophil-derived IL-4 in programing anti-inflammatory macrophages.[10] However, translational insight on these data is lacking. In particular, 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 humans.[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 indirect pathway of DC-SIGN-dependent IL-33.

In line with our proposition, we report that IVIG directly induces the activation of human basophils and secretion of IL-4, IL-6 and IL-8 through interaction with basophil surface-bound IgE, and by IL-3- and Syk-dependent mechanisms. These functions of IVIG were mediated via $F(ab')_2$ fragments and were independent of IL-33, FcγRII, type II FcRs, C-type lectin receptors and Siglecs. Basophils from IVIG-treated myopathy patients also displayed enhanced expression of activation marker CD69. In the context of systemic autoimmune and inflammatory diseases, these results thus provide a unique pathway of promoting Th2 response by IVIG through direct interaction of IgG with human basophils.

METHODS

Preparations of IVIG

Sandoglobulin® (CSL Behring, Switzerland) was dialyzed against a large volume of PBS three 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 pepsin digestion (2% wt/wt; Sigma Aldrich) followed by chromatography on a protein G Sepharose column (Pharmacia). Fc fragments of IVIG were prepared by papain digestion (papain-coupled beads, Life Technologies) followed by protein A Sepharose column chromatography and size-exclusion chromatography. End purification was performed by chromatography on an IgG-CH1 column (Life Technologies). The purity of $F(ab')_2$ and Fc fragments were confirmed by SDS-PAGE.

Isolation and culture of basophils

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

To investigate the effect of IVIG on IL-3-primed basophils, cells ($0.1x10^6$/well/200 µL) were cultured in 96 well U-bottomed plate either alone in serum-free X-VIVO 15 medium; or with IL-3 (100 ng/mL, ImmunoTools); or with IL-3 plus IVIG (25 mg/mL) or human serum albumin (HSA, 10 mg/mL, LFB, France) or $F(ab')_2$ fragments (16 mg/mL) or Fc fragments (9 mg/mL) for 24 hours.

To explore the effect of other cytokines on IVIG-mediated regulation of 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. Also, basophils were sequentially stimulated with IL-3 and IL-33 for one hour each and cultured with IVIG or HSA for 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), FcγRIIA (Clone:IV.3; 10 μg/mL) or isotype control MAbs for 1 hour and cultured with IVIG for additional 21 hours.

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

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

Depletion of IgE-reactive IgG from IVIG

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

IVIG depleted of anti-IgE-reactivity (25 mg/mL) was added to IL-3-primed basophils ($0.1x10^6$/well/200 μL) as described earlier for 24 hours.

Analysis of basophils from myopathy patients

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

Antibodies for flow cytometry and functional assays

The details are provided in the supplementary file (in this article's Online Repository at www.jacionline.org)

Measurement of cytokines and histamine

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

RNA isolation and real-time quantitative RT-PCR

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

expression was measured using TaqMan Gene Expression Assays (Applied Biosystems™) #Hs00174122_m1 (IL-4), #Hs02786624_g1 (GAPDH).

Statistical analysis:

Statistical analysis was performed by Prism 6 GraphPad Software. One-way analysis of variance (with Tukey's multiple comparison tests or Dunnet's multiple comparison tests), and two-way Mann Whitney were used to determine the 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 phenotype or functions of resting basophils based on the 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, the expression of CD13, CD62L, CD123 and CD203c (Fig E1, in this article's Online Repository at www.jacionline.org), degranulation-associated markers CD63 (Fig 1, *E*) and CD107a (Fig E2, *A* and *B*), and histamine concentrations in the supernatants (Fig E2, *C*) were not significantly altered by IVIG.

Further, 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 (Fig E3). 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 myopathy patients also confirmed enhancement of CD69 on the basophils of four out of five patients analyzed (Fig 1, *G*).

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

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

CD69 on basophils (Fig 2, *A* and *B*) or their cytokine production (Fig 2, *C*) was observed following 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 (Fig E4), IL-33 when used in combination with IL-3 did not exert either synergistic or additive effect on IVIG-induced basophil activation (Fig 2, *D* and *E*). These results hence do not support a major role for IL-33 in priming human basophils towards IVIG responsiveness. Other cytokines like IL-25, TSLP and GM-CSF also had no significant effect on the IVIG-induced basophil activation (Fig E5). Altogether these results (Fig 1 and 2) indicate that IVIG induces IL-4 in human basophils, as had been described in 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 via F(ab')$_2$ fragments while type II FcRs, C-type lectin receptors and Siglecs are dispensable

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

As 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, -5/14, -7 and -8 (Fig E6), which all possess some affinity for (2,6)-sialic acid linkages. Siglec-10 was previously reported to be undetectable on basophils.[15]

DCIR, a C-type lectin receptor has been reported to recognize α(2,6)-sialic acid linkages of IgG.[16] Nearly 80% of the steady-state and 95% of the 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 the resting basophils (Fig 1, *A*-*C*) despite these cells express DCIR, thus indirectly ruling out the role of DCIR in IVIG-induced basophil activation.

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 the 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 favouring basophil-sensitization by IgE for augmented FcεRI-mediated signals and secretion of various inflammatory mediators.[17-19] Our data demonstrates that IL-3-priming is also a pre-requisite for the IVIG-induced basophil activation. IVIG significantly down-regulated FcεRI on IL-3-primed basophils (Fig 4, *A* and *B*), suggesting that IVIG binding to FcεRI and/or to FcεRI-bound IgE triggered the internalization of FcεRI. As expected, basophils displayed IgE on their surface (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 are already saturated by IgE. These arguments point out that IVIG induces activation of basophils possibly via signalling through basophil FcεRI-bound IgE rather than FcεRI. Importantly, 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/FcγRII are dispensable for the regulation of basophil**
282 **activation by IVIG**

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

287 First, we analyzed the expression pattern of FcγRII on basophils. IL-3 although enhanced
288 the expression of both FcγRIIA and FcγRIIB, 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 FcγRIIB expression upon IVIG therapy,[22]
292 the ratio of intensity of expression of FcγRIIB to FcγRIIA 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.[23]

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

300 As FcγRIIA signalling induces activation of immune cells,[20] we explored if IVIG-induced
301 basophil activation implicates co-engagement with this receptor. But FcγRIIA blockade
302 had no repercussion on the IVIG-induced expression of CD69 and cytokines (Fig 5, *E* and

F), demonstrating that FcγRII (activating or inhibitory) has no significant role in the regulation of human basophil function by IVIG.

Syk pathway is critical for the basophil activation by IVIG

FcεRI-mediated activation of human basophils in vitro requires both priming by IL-3 and the kinase Syk that is recruited to the FcεRI signalling complex.[17-19] Noticeably, IL-3-mediated down-stream signalling has also been reported to be Syk-dependent.[24,25] Freshly isolated basophils showed basal phosphorylation of 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 IL3 plus IVIG resulted in similar pSyk induction (Fig 6, *A* and *B*). Further, inhibition of Syk, using 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* following Syk inhibition (Fig E7). Altogether these data suggest that IVIG, due to IgE reactivity it contains, induces activation of IL-3-primed basophil by signalling through FcεRI-bound IgE.

DISCUSSION

Despite having pathogenic roles in various diseases,[8,26,27] recent evidence from mouse 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 employed anti-FcεRI MAb MAR-1 to deplete the basophils and this antibody has been reported to deplete FcεRI-positive DCs as well.[29,30] Also, as compared to mouse, human basophils display distinct features.[8,31,32] Therefore, the effect of IVIG on basophil functions is far from clear. Notably, data from human raise an alternative paradigm that IVIG might modulate basophil functions directly rather than indirect IL-33-dependent pathway.[11]

Human basophils express receptors for various cytokines. In addition to IL-33, 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 of that induced by IVIG in patients with rheumatic and neurological autoimmune diseases[11,12] would stimulate IL-4 production as proposed from mouse studies. IL-33 indeed primed human basophils (based on the expression of CD69) and induced IL-4,[37] but the extent of priming was only marginal when compared to IL-3-mediated priming.[17,19] This marginal activation by IL-33 might be also due to the expression pattern of IL-33R as only 22.4±6.3% (n=8) basophils in steady-state express this receptor.

We investigated if IVIG could activate IL-33-primed basophils. However, IVIG did neither modify 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] GM-CSF on the other hand, significantly activated human basophils,[40,41] but the extent of activation was lesser 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 IL-33-mediated pathway of basophil IL-4 induction as suggested from the 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 the expression of IL-33R on the 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 functions and could regulate basophil response to IL-33 (probably at higher concentrations as reported earlier[37]) by enhancing the IL-33R expression In fact, under IL-3-stimulation conditions, CD69 and IL-33R were co-expressed on the basophils. However, this was not the case under IL-33-stimulation conditions, wherein only a minor population of basophils co-expressed CD69 and IL-33R possibly because of marginal stimulation of basophils by IL-33 or IL-33R internalization. All our experiments in this report relay 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 myopathy patients. Further analyses of basophils in the inflamed tissues and secondary lymphoid organs should provide more insight on the regulation of basophil functions by IVIG.

Various studies reported that FcγRIIB plays an important role in mediating the anti-inflammatory actions of IVIG. The enhanced expression of FcγRIIB by IVIG has been proposed to increase the threshold level for the activation of innate cells by immune complexes.[22,42-44] However, the absolute requirement of FcγRIIB in mediating 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 the basophils provide yet another evidence for FcγRII-independent action of IVIG on human cells.

Several targets and receptors have been identified for IVIG. In addition to the $F(ab')_2$-mediated recognition of various self-molecules like HLA, Fas, CD40, Siglecs, BAFF, 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 pattern of these receptors. In vitro-generated monocyte-derived DCs (equivalent of inflammatory DCs) express both DC-SIGN and DCIR while DCs ex vivo express mainly DCIR.[62] Although CD23 is expressed by B cells, macrophages and eosinophils, Siglec-2 is restricted to B cells. Human basophils, however, lack DC-SIGN, CD23 and Siglec-2. Despite positive for DCIR, resting basophils were not modified by IVIG, suggesting that DCIR is not sufficient (or predominant) in mediating basophil activation by IVIG. Also, other Siglecs that could recognize α(2,6)-sialic acid-linkages were absent on the 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 degranulation marker CD63.[63] It is possible that the anti-IgE content in IVIG is too low to activate fully 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 with type II FcRs. The sialylated or non-sialylated

glycans-mediated 'closed' vs 'open' conformation of Fc, switches engagement of Fc-domain towards type II or type I FcRs respectively.[66] Previous report showed that anti-IgE rabbit IgG inhibit basophil activation by co-engaging 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 chronic urticaria patients with anti-IgE or anti-FcεRI autoantibodies might be because of basophil-independent mechanisms. In fact, 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 following IL-3 as well as FcεRI-mediated activation.[17,24,25] Therefore, it is difficult to segregate the importance of IL-3-induced versus FcεRI-induced Syk activation. As IVIG could induce basophil activation only upon IL-3-priming suggests that IL-3-induced Syk phosphorylation is indispensable for basophil FcεRI-bound IgE-mediated activation by IVIG. Syk inhibitor R406 that is proposed for 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.

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

FIGURE LEGENDS

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

FIG 2. IL-33 is dispensable for the activation of human basophils by IVIG. Basophils were cultured either alone or with IL-33. IVIG or HSA were added following 2 hours stimulation with IL-33. **A** and **B,** Representative dot plots and expression (% positive cells and MFI) of CD69 on the basophils (mean±SEM, 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 one hour followed by IL-33 for additional hour before culturing with IVIG or HSA. (D) Expression (% positive cells and MFI) of CD69 on the basophils. (E) Amount of secretion of IL-4 and IL-8 (mean±SEM, n=4 donors) ***P<0.001; ****P<0.0001; ns, not 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 $F(ab')_2$- 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, $F(ab')_2$ 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

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

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

Figure No. 1

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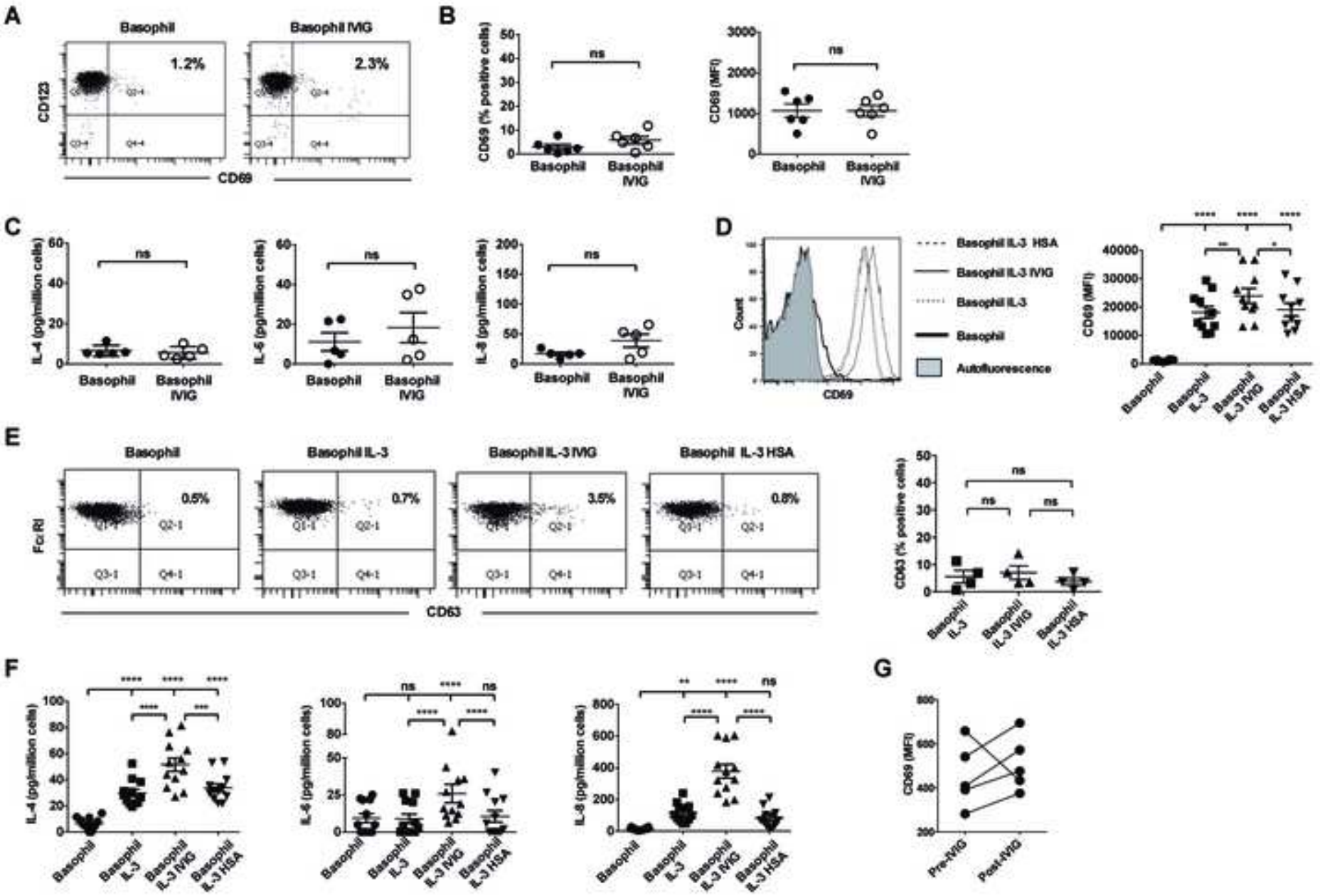

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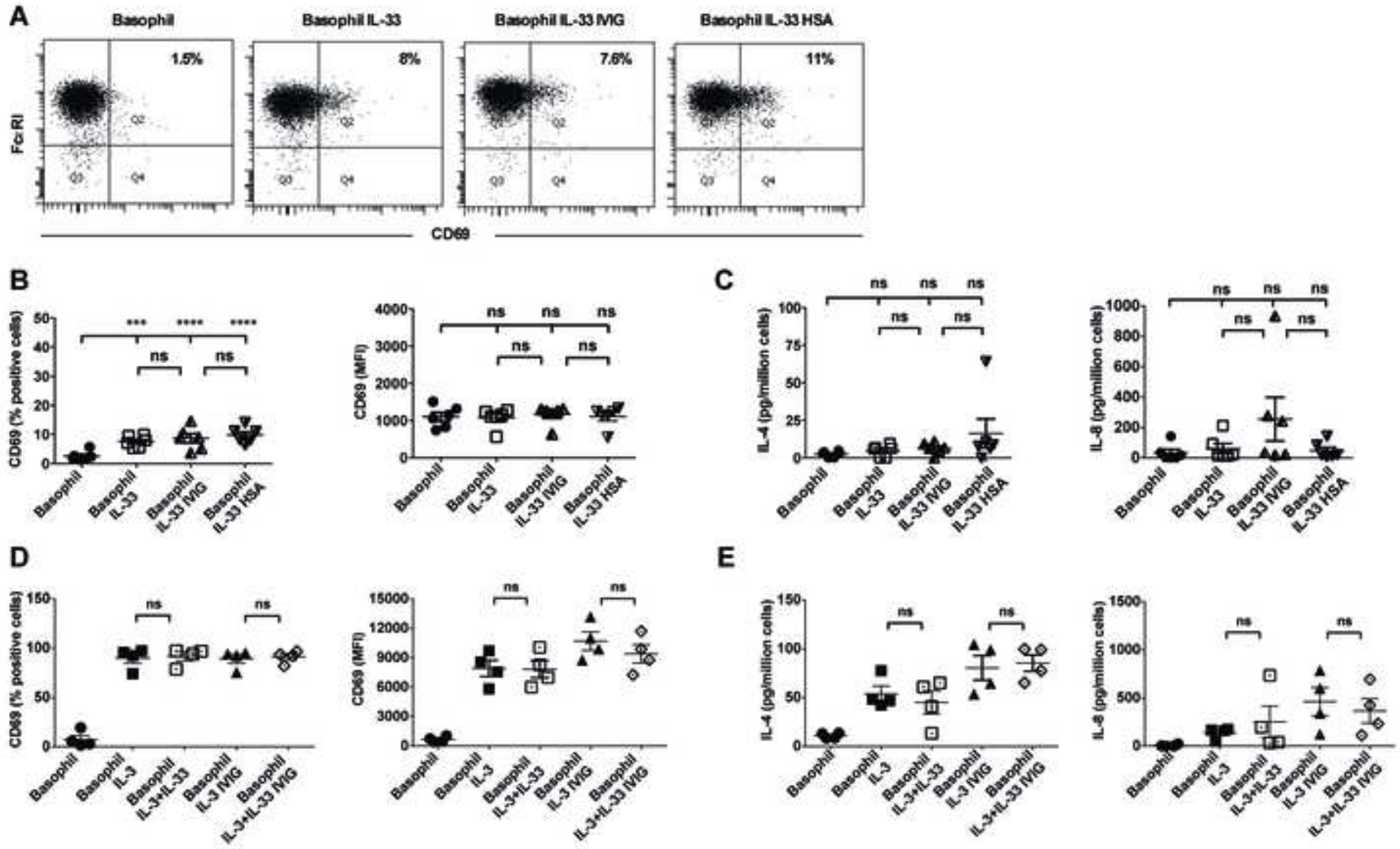

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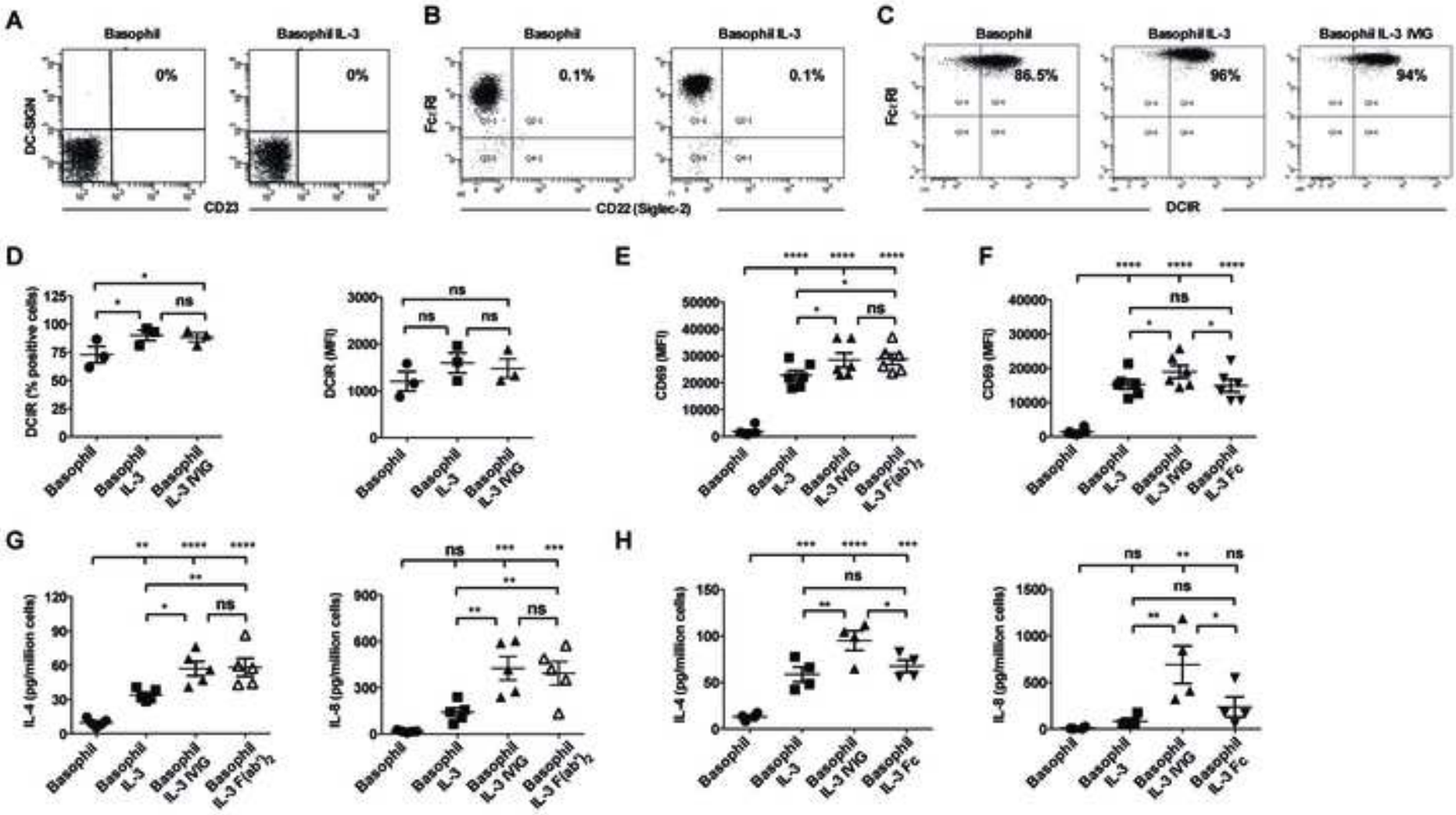

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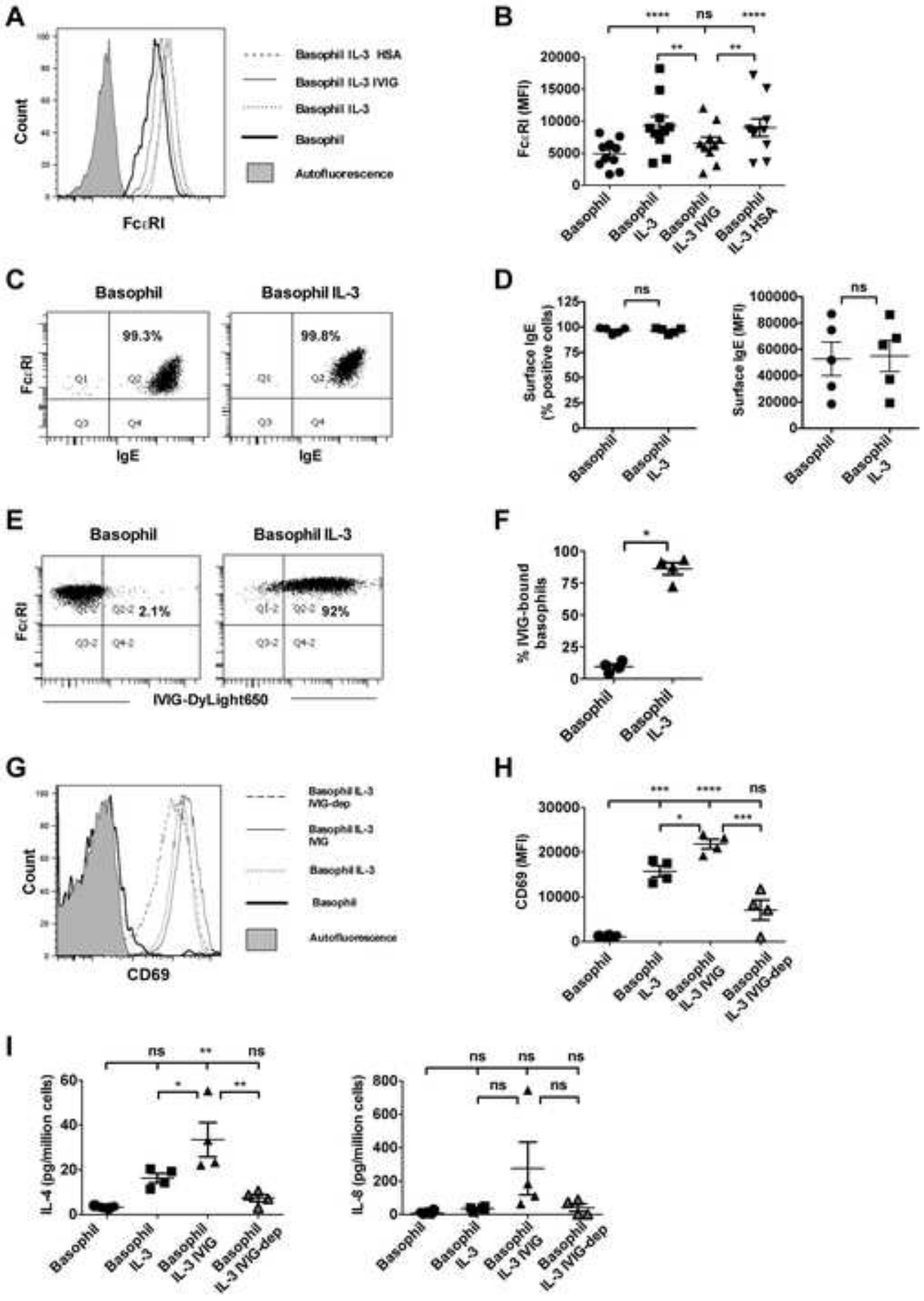

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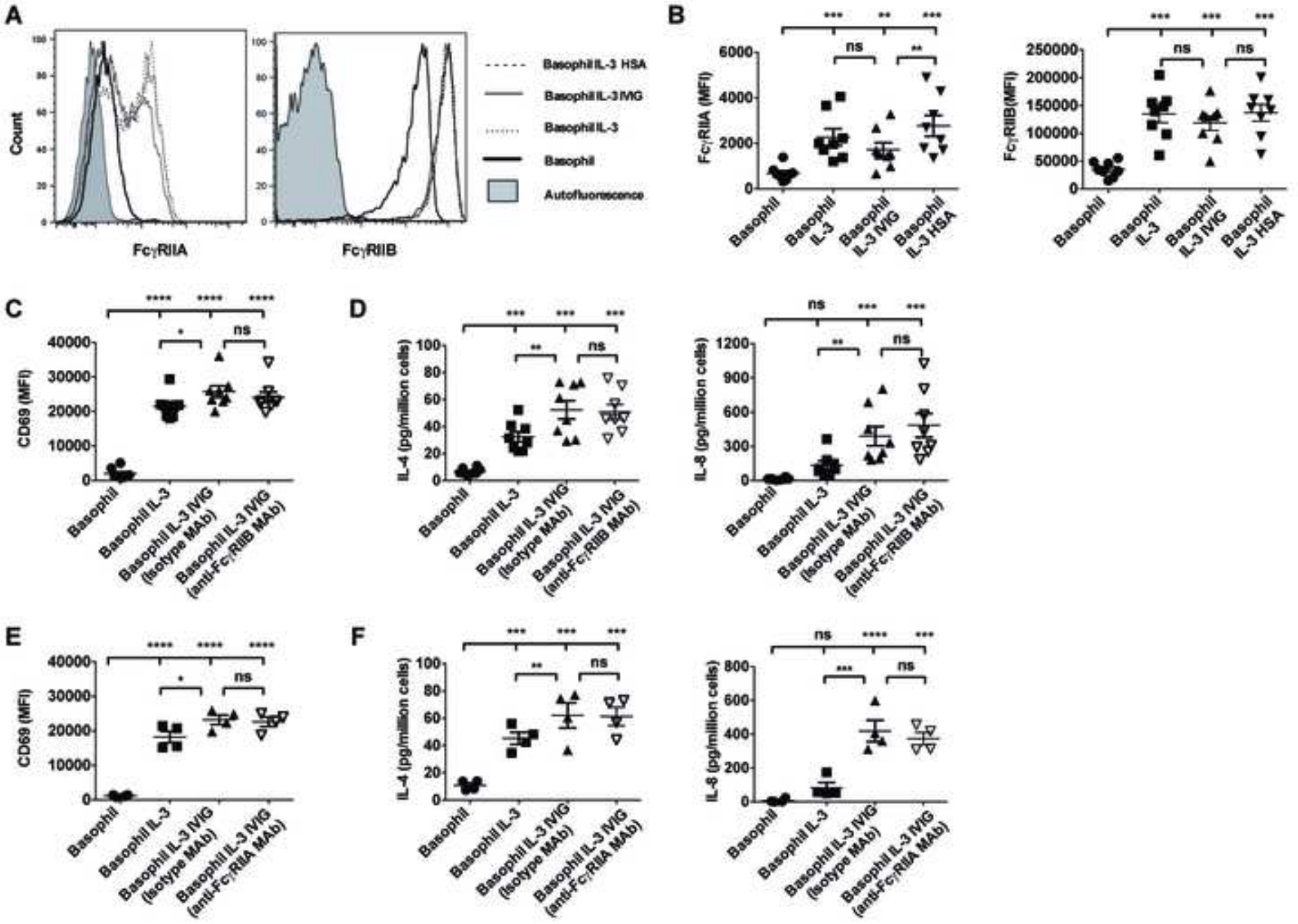

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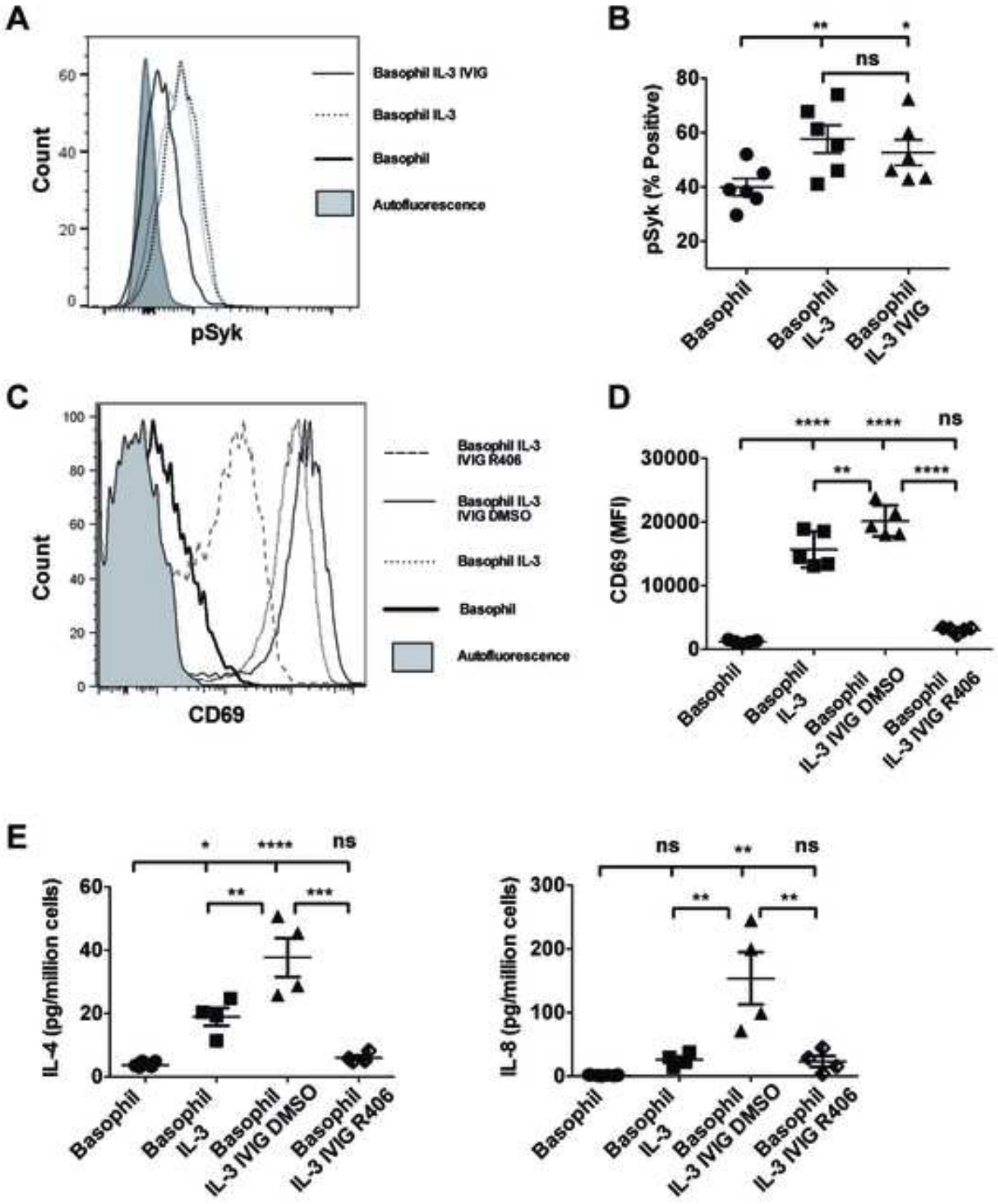

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Intravenous immunoglobulin induces IL-4 in human basophils by signaling through surface-bound IgE

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METHODS

List of antibodies for flow cytometry and functional assays

CD63-PE (Clone:H5C6), CD13-APC (Clone:WM15), CD123-BV421 (Clone:9F5), CD69-APC/Cy7 (Clone:FN50), CD209-APC (Clone:DCN46), CD22-PE (Clone:S-HCL-1) and CD62L-FITC (Clone:DREG-56) were from BD Biosciences. FcεRIα-FITC (Clone:CRA-1), SIGLEC3-FITC (Clone:AC104.3E3), SIGLEC5-FITC (Clone:1A5), SIGLEC7-FITC (Clone:REA214), SIGLEC8-APC (Clone:7C9), 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 (CloneAER37 [CRA-1]) and DCIR-PE (Clone:9E8) MAbs were from BioLegend. Anti-IgE MAb (Clone:GE-1) was from Sigma Aldrich. Unconjugated and FITC-labelled FcγRIIA MAb (Clone:IV.3) was purchased from Stem Cells Technologies. Human ST2/IL-33R-PE polyclonal goat IgG and Isotype control MAbs for blocking experiments were from R&D Systems. Anti-human p-Syk (Tyr348) (clone: moch1ct) was from eBioscience. Anti-hFcγRIIB (Clone:2B6 variant $N_{297}D$) MAbs were coupled to Alexa Fluor 647 by using ThermoFisher Scientific kit and IVIG was labelled with the Lightning-Link® Rapid DyLight® 650 kit (Innova Biosciences).

Supplementary Figure Legends

FIG E1. Effect of IVIG on the 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 were added following 2 hours stimulation with IL-3. (A) Representative histogram overlays and (B), expression (mean±SEM, n=4-12 donors) of CD69, CD13 (both in % positive cells), CD62L, CD123 and CD203c (all MFI) on the basophils. *P<0.05; ***P<0.001; ns, not significant, one-way ANOVA with Tukey's multiple comparison tests.

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

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

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

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

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

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

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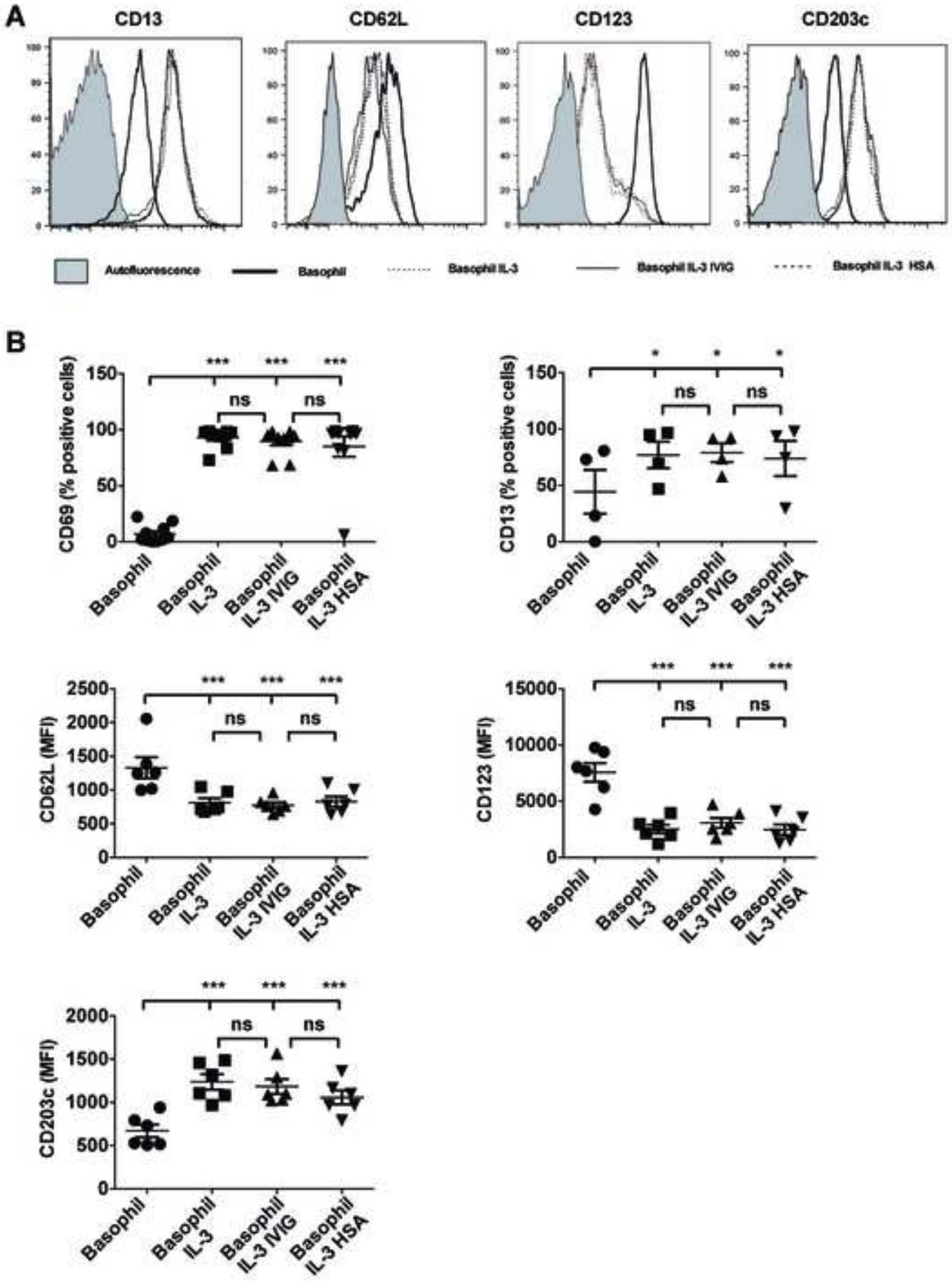

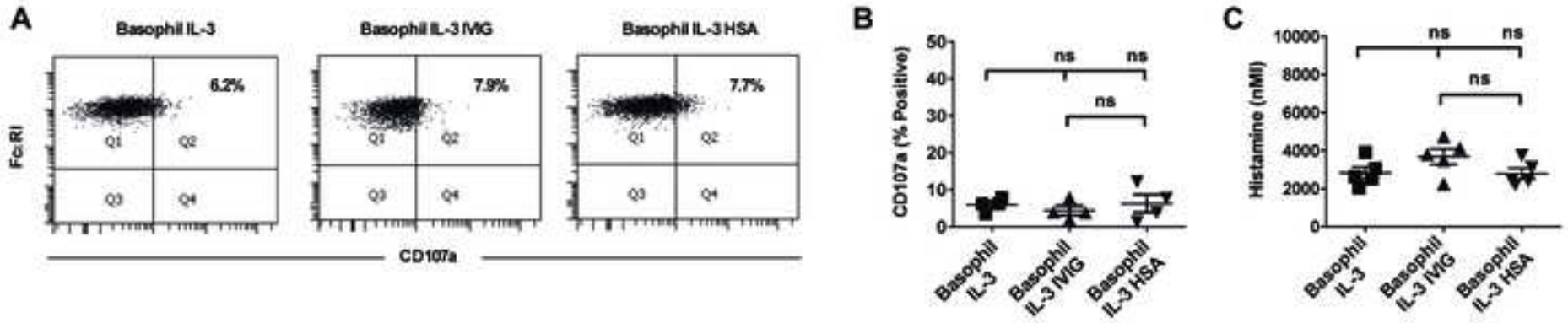

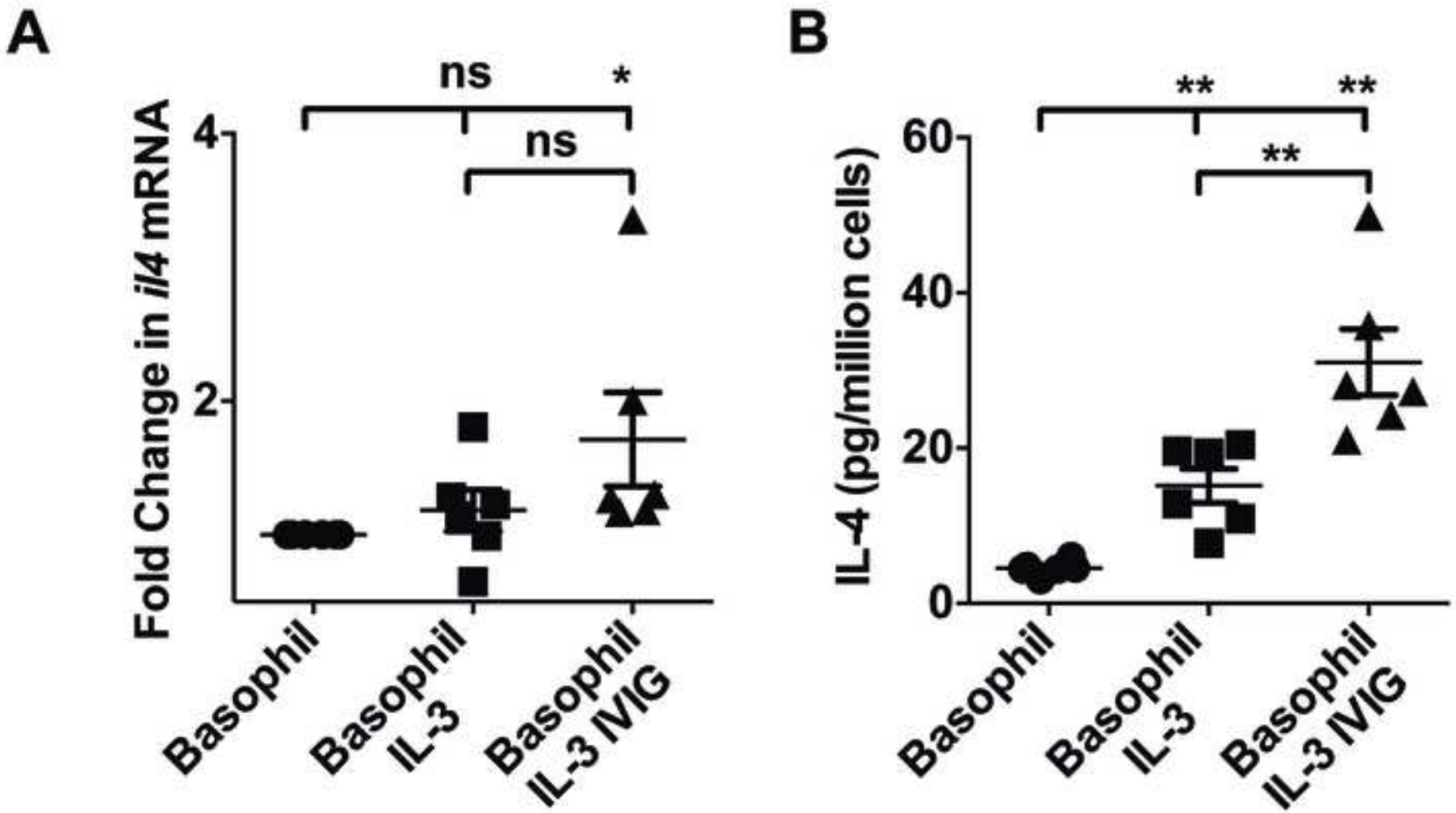

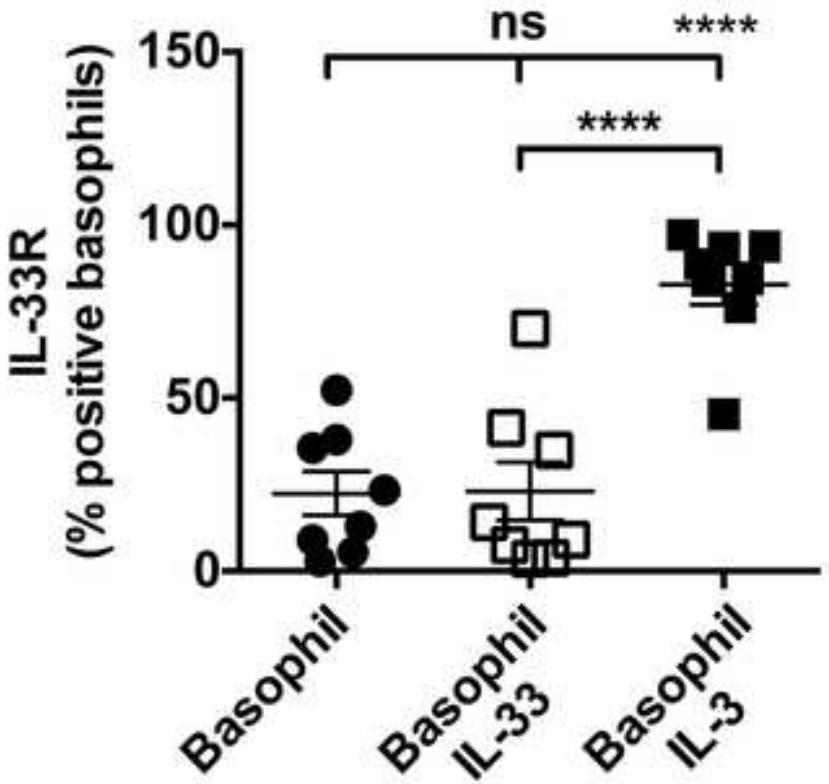

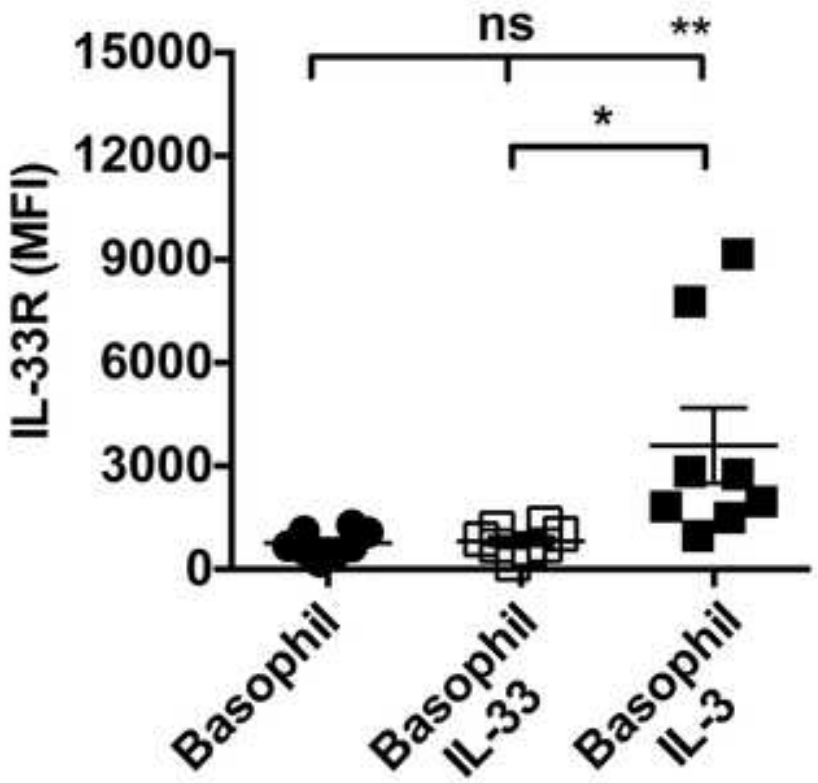

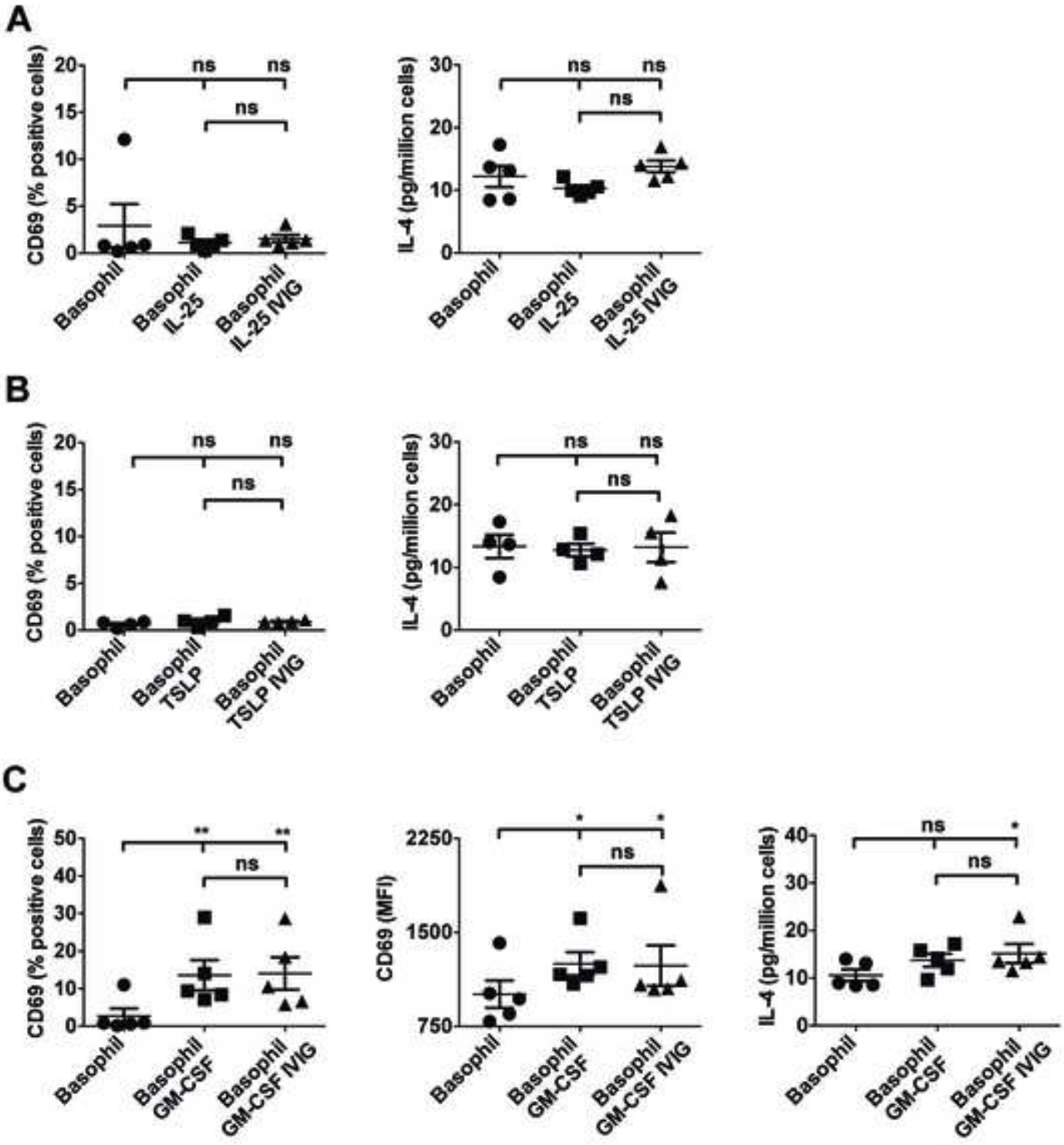

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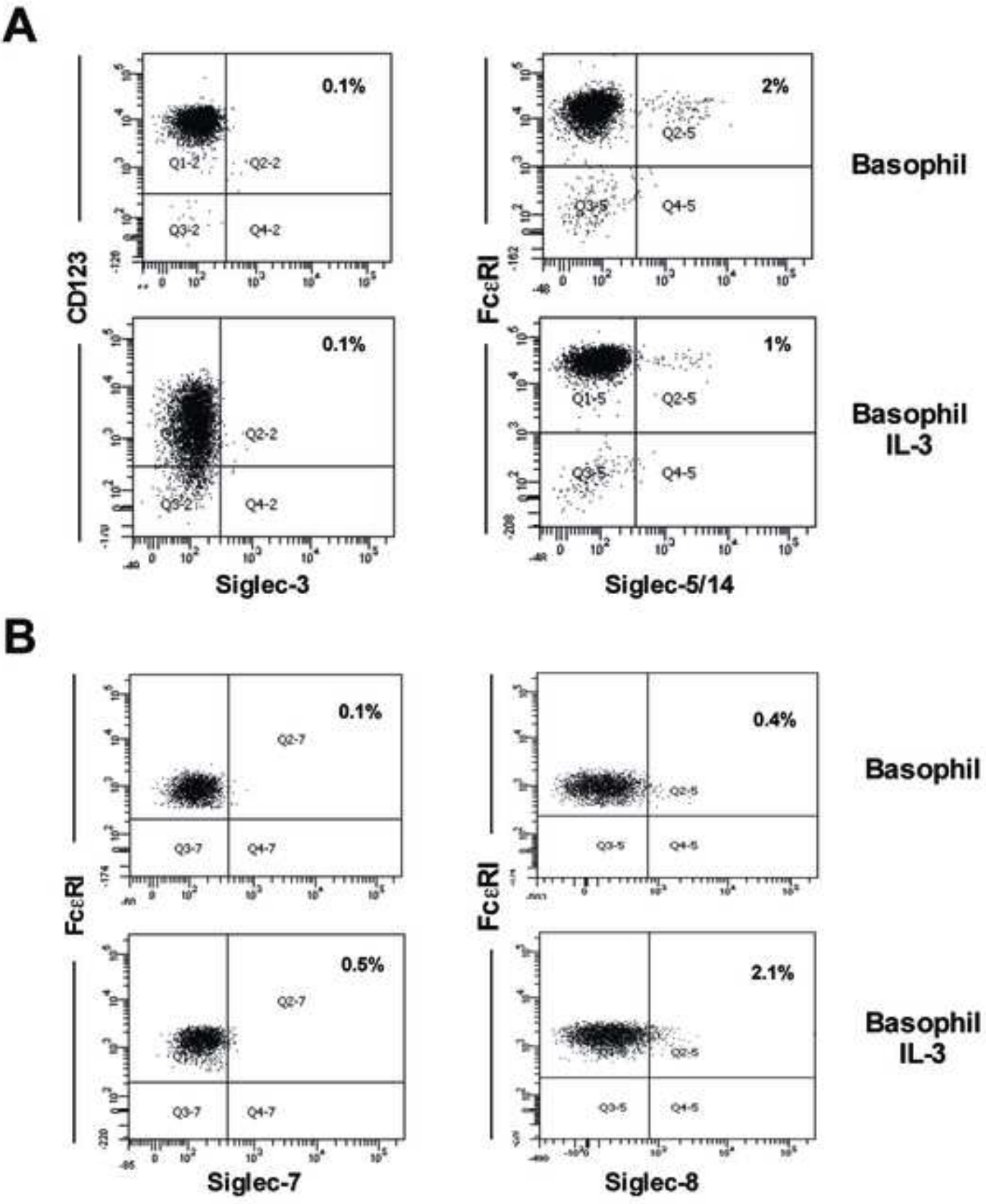

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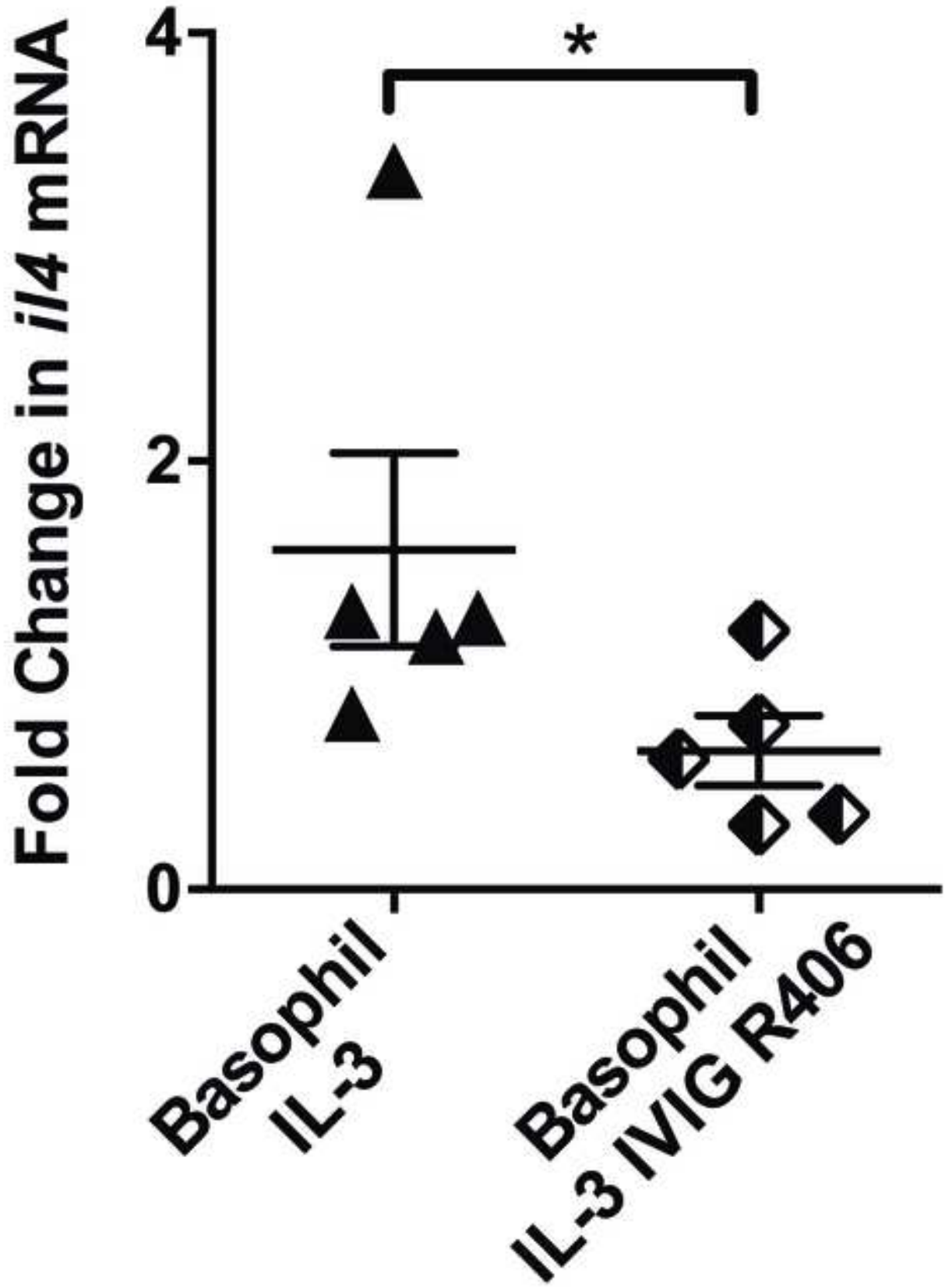

Graphical Abstract

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