Broad IgG repertoire in patients with chronic rhinosinusitis with nasal polyps regulates proinflammatory IgE responses

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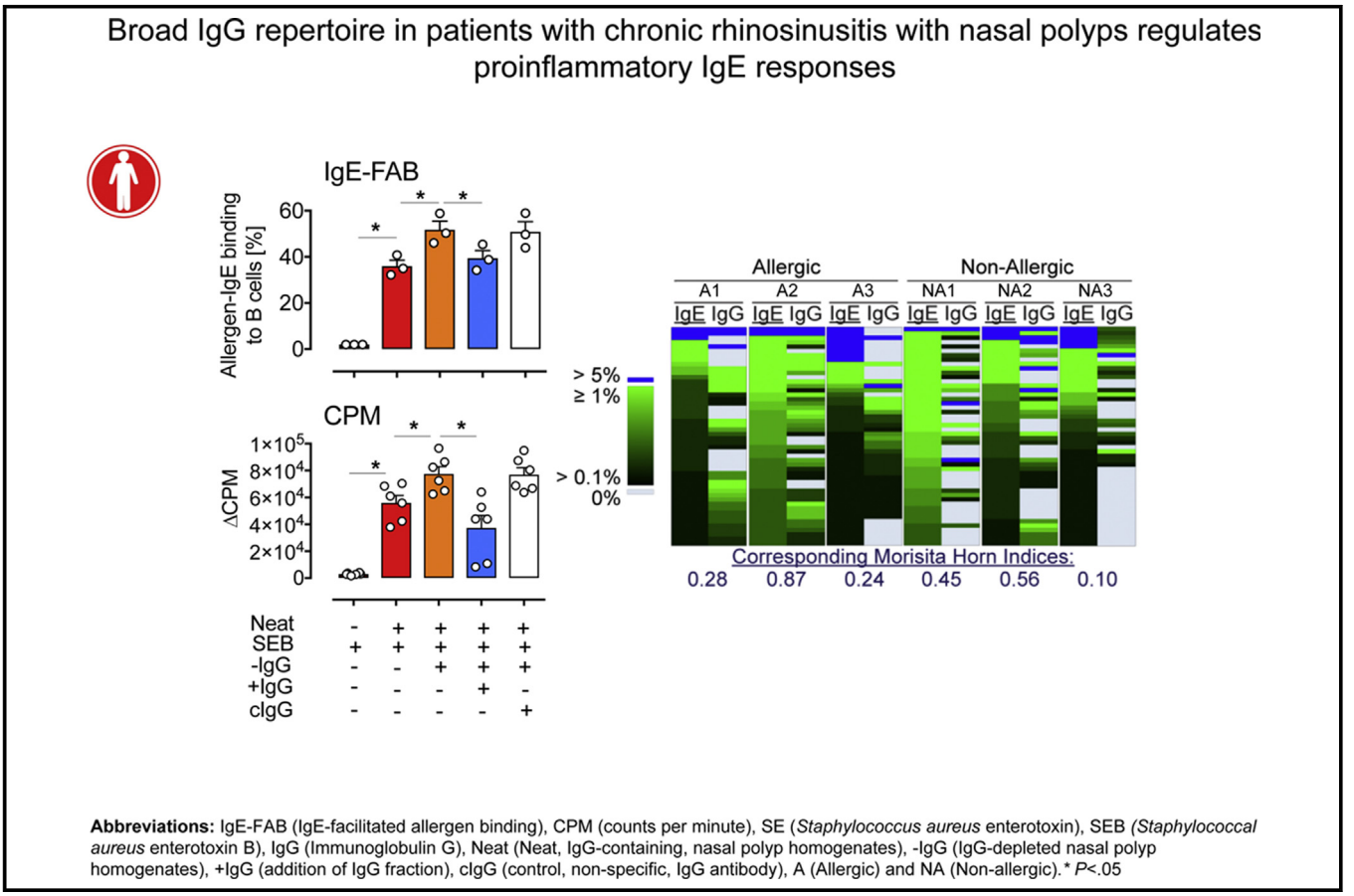
GRAPHICAL ABSTRACT



Abbreviations: IgE-FAB (IgE-facilitated allergen binding), CPM (counts per minute), SE (*Staphylococcus aureus* enterotoxin), SEB (*Staphylococcal aureus* enterotoxin B), IgG (Immunoglobulin G), Neat (Neat, IgG-containing, nasal polyp homogenates), -IgG (IgG-depleted nasal polyp homogenates), +IgG (addition of IgG fraction), cIgG (control, non-specific, IgG antibody), A (Allergic) and NA (Non-allergic).* *P*<.05

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Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is often characterized by local production of polyclonal IgE idiotypes. Although tissue IgE concentrations can be in the range of several thousand kilounits per liter, the regulatory mechanisms by which IgE-mediated inflammation is controlled in patients with nasal polyps are not well understood.

Objective: We sought to determine whether locally induced IgG antibodies in patients with nasal polyps can inhibit an IgE-mediated proallergic response.

Methods: Nasal polyp homogenates were collected from patients with grass pollen allergy with CRSwNP and nonallergic control subjects. IgE levels were measured using the Immuno Solid-phase Allergen Chip assay. IgE-containing nasal polyp homogenates with or without IgG depletion were evaluated for their capacity to promote IgE-facilitated allergen presentation, basophil activation, and histamine release. Local IgE and IgG repertoires were evaluated using Immunoglobulin 454 sequencing.

Results: We show that IgG plays a key role in controlling IgE-mediated inflammatory responses in patients with nasal polyps. Depletion of IgG from nasal homogenates resulted in an increase in CD23-mediated IgE-facilitated allergen binding to B cells but also enhanced FcεRI-mediated allergen-driven basophil activation and histamine release. A similar response was observed in relation to specific IgE antibodies to *Staphylococcus aureus* enterotoxins. The capacity of IgG in nasal polyps to limit IgE-mediated inflammation is based on the fact that IgG repertoires widely share the antigen targets with the IgE repertoires in both allergic and nonallergic subjects.

Conclusion: Polyclonal IgE idiotypes in patients with CRSwNP are functional, promote IgE-mediated proallergic inflammation, and are partially antagonized by corresponding IgG idiotypes. This is most likely due to the fact that IgE and IgG clonotypes are widely shared in patients with nasal polyps. (J Allergy Clin Immunol 2019;143:2086-94.)

Key words: *IgG, antibody repertoire, IgE, chronic rhinosinusitis, nasal polyps, allergic rhinitis*

Abbreviations used
CRSwNP: Chronic rhinosinusitis with nasal polyps
CRTH2: Chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes
DAO: Diamine oxidase
GPA: Grass pollen allergy
IMGT: ImMunoGeneTics
NAC: Nonallergic control subject
SEB: Staphylococcal enterotoxin B
SE: *Staphylococcus aureus* enterotoxin
SE-IgE: Specific IgE antibodies to *Staphylococcus aureus* enterotoxins
sIgE: Allergen-specific IgE
VH: Variable heavy chain

Chronic rhinosinusitis with nasal polyps (CRSwNP) affects about 4% of the European population.[1] It is characterized by nasal obstruction, loss of smell, nasal secretions, and facial pressure. About 50% of these patients have comorbid asthma, aspirin-exacerbated respiratory disease, hypereosinophilia, or Churg-Strauss syndrome. Recurrence of the disease after surgery is high despite treatment with topical and oral glucocorticosteroids.[2] It is reported in Europe that, independent of atopic status, 85% of nasal polyps are characterized by local polyclonal IgE production, abundant eosinophils, and IL-4–, IL-5–, and IL-13–producing T_H2 cells.[3]

Tissue IgE concentrations can be in the range of several thousand kilounits per liter[4] and consist of functional polyclonal IgE idiotypes[5] formed locally.[6] High tissue IgE concentrations are associated with asthma comorbidity[7] and disease recurrence.[8] Anti-human IgE mAb treatment is effective in allergic patients with nasal polyps and asthma,[7] highlighting the contribution of local IgE to the pathogenesis of CRSwNP. Several recent studies have reported that the high total IgE concentrations in the nasal fluid are likely a result of local production of *Staphylococcus aureus* enterotoxins (SEs) that act as superantigens. In turn, SE superantigens can polyclonally activate T cells to amplify eosinophilic inflammation and B cells, resulting in the induction of polyclonal IgE.[4] This is often associated with the presence of SE-specific IgE antibodies.[5,9] SEs have been shown to contribute to the formation of nasal polyps in both allergic and nonallergic subjects.[4,10] As a result, severe eosinophilic inflammation occurs, as does production of IgE and IgG/IgG_4 antibodies by B cells.[11] Although several studies have reported increased quantitative levels of IgE in nasal polyps, their specificity and functional activity have not fully been determined. More importantly, the regulatory mechanisms by which the IgE-mediated proinflammatory response is controlled in patients with nasal polyps remain to be fully investigated.

First, we hypothesized that local specific IgE antibodies in homogenates from patients with CRSwNP are functional and promote a FcεRII-mediated proallergic response. Second, local SE-specific IgG antibodies are also induced and can inhibit FcεRII-mediated facilitated allergen presentation by B to T cells. Third, this IgG-associated inhibitory effect is due to a similar antibody repertoire of IgG- and IgE-producing B cells in the nasal polyps.

METHODS

Nasal polyp extraction and preparation of tissues

Patients with CRSwNP (see Table E1 in this article's Online Repository at www.jacionline.org), as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps criteria[12] based on symptoms and results of nasal endoscopy and computed tomography of the sinuses, were included. Subjects were excluded if they experienced an acute exacerbation of rhinosinusitis 2 weeks before inclusion from known immunodeficiency, cystic fibrosis, or fungal allergic sinus disease; if they used oral or nasal steroids in the 4 weeks preceding surgery; or if they used antihistamines before nasal polyp surgery. This study was approved by the ethics committee of Ghent University Hospital, and informed consent was obtained from all subjects before sample collection.

Subjects underwent a standardized allergen skin prick test and provided nasal polyp homogenates for specific IgE measurements; they were considered allergic on clinical grounds (positive skin prick test response or specific IgE level to inhalant allergens plus symptoms present). They were also registered as asthmatic based on symptoms and clinical diagnosis. Nasal polyp tissue was immediately collected during routine surgery.

Tissue specimens were weighed, and 1 mL of 0.9% NaCl solution was added per 0.1 g of tissue. The tissue was then homogenized with a mechanical homogenizer (B. Braun, Melsungen, Germany) at 1000 rpm for 5 minutes on

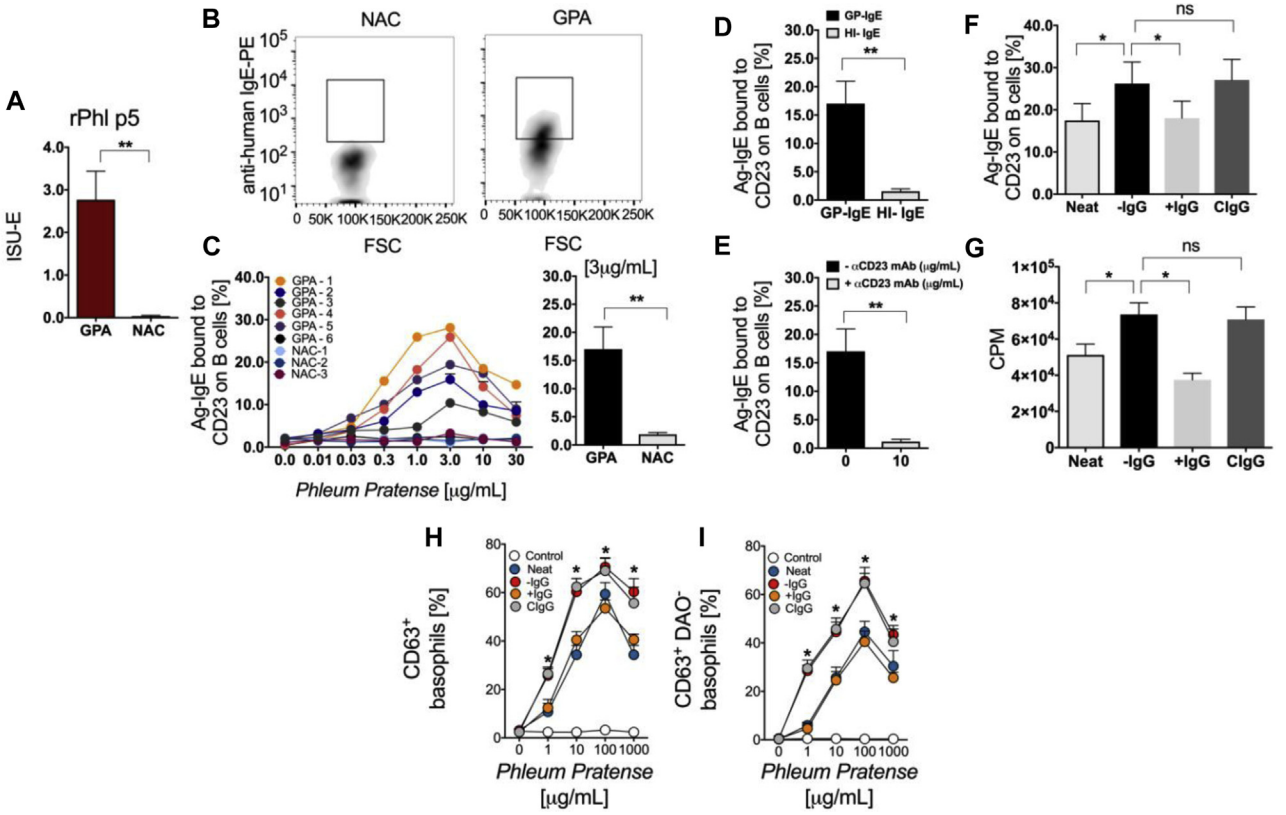


FIG 1. IgE in nasal polyps facilitates CD23-mediated allergen binding to B cells and allergen presentation. **A,** sIgE levels to *P pratense* in nasal polyp homogenates from patients with GPA (n = 6) and NACs (n = 6) were measured using the Immuno Solid-phase Allergen Chip *(ISAC)* assay. **B** and **C,** Quantification of allergen-IgE binding to B cells in IgE-containing nasal polyp homogenates (n = 6) and control homogenates from NACs (n = 6) with varying concentrations of *P pratense*. *FSC*, Forward scatter. **D,** Effect of heat inactivation on IgE-containing nasal polyp homogenates (n = 6) in the presence of 3 μg/mL allergen. **E,** CD23 dependency was determined by means of pretreatment of EBV-transformed B cells with anti-CD23 blocking antibody (0 or 10 μg/mL; n = 6). **F** and **G,** Effect of IgG depletion in nasal polyps on cooperative binding of allergen-IgE complexes binding to B cells (Fig 1, *F*) and IgE-facilitated allergen presentation by $CD19^+$ B cells to $CD4^+CD25^-$ T effector cells (Fig 1, *G*). **H** and **I,** Basophils obtained from patients with GPA were sensitized with neat IgE-containing, IgE-containing IgG-depleted *(−IgG)* nasal polyp homogenates (n = 6), IgG fraction *(+IgG)*, or nonspecific control IgG (*CIgG*; 1 mg/mL) in the presence of varying concentrations of *P pratense*. Basophils obtained from NACs were used as negative controls *(Control)*. All data are shown as means ± SEMs. **P* < .05 and ***P* < .01.

ice. After homogenization, the suspensions were centrifuged at 1500 rpm for 10 minutes at 4°C, and supernatants were collected. All supernatants were assayed for IgE by using the UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden).

Immunologic analysis

Methods for PBMC isolation, IgG depletion from nasal polyp homogenates, basophil activation and histamine release, and IgE-facilitated antigen binding and presentation are described in the Methods section in this article's Online Repository at www.jacionline.org.

Immunoglobulin 454 sequencing

IgE and IgG repertoire information was obtained by following the same scheme as described for IgA repertoires.[13] RNA was extracted and transcribed into cDNA with Superscript III (Invitrogen, Carlsbad, Calif) and random primers, according to the manufacturer's instructions. Immunoglobulin amplicons were generated by means of PCR with 30 cycles by using the following primers: variable heavy chain (VH; 5′-CGTATCGCCTCCCTCGCGCCATCAGGGCCTCAGTGAAGGTCTCCTGCAAG-3′) in combination with either Cγ for IgG (5′-CTATGCGCCTTGCCAGCCCGCTCAG[MID]GTTCCACGACACCGTCACC-3′) or Cε for IgE (5′CTATGCGCCTTGCCAGCCCGCTCAG[MID]AAGGGGAAGACGGATGGGCTCTG-3′) amplification. Gene-specific sequences are underlined, *MID* specifies a nucleotide sequence used in 10 variations to identify samples, and nonunderlined sequences represent adaptor sequences needed for emulsion PCR and 454 sequencing. PCR conditions were as follows: 95°C for 4 minutes; 25 times 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 35 seconds; and 72°C for 10 minutes. Amplicons were purified by means of gel electrophoresis, followed by gel extraction (QIAquick Gel Extraction kit; Qiagen, Hilden, Germany), and DNA concentrations were quantified by using the Quant-iT dsDNA HS Assay Kit (Invitrogen) measured with the Qubit Fluorometer (Invitrogen). Amplicons were prepared with the GS FLX Titanium SV emPCR Kit (Lib-A) for 454 pyrosequencing on the Genome Sequencer FLX system (Roche, Mannheim, Germany), according to the manufacturer's instructions.

Immunoglobulin sequence analysis

Immunoglobulin sequence analysis was performed as described, with some modifications.[13] In brief, sequences longer than 320 bp and comprising both primers were sorted according to their MID values. Sequences were further analyzed with ImMunoGeneTics (IMGT) HighV-QUEST

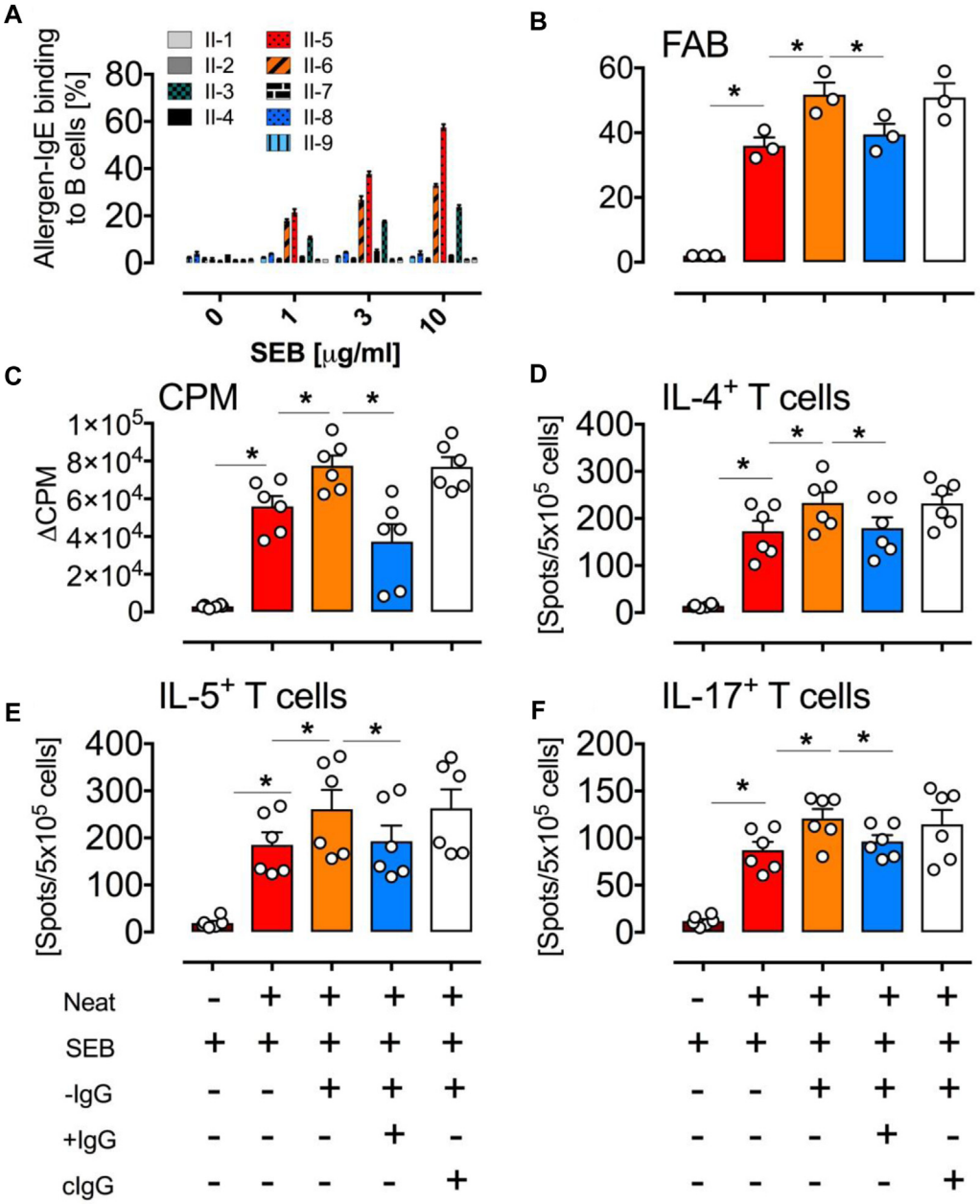


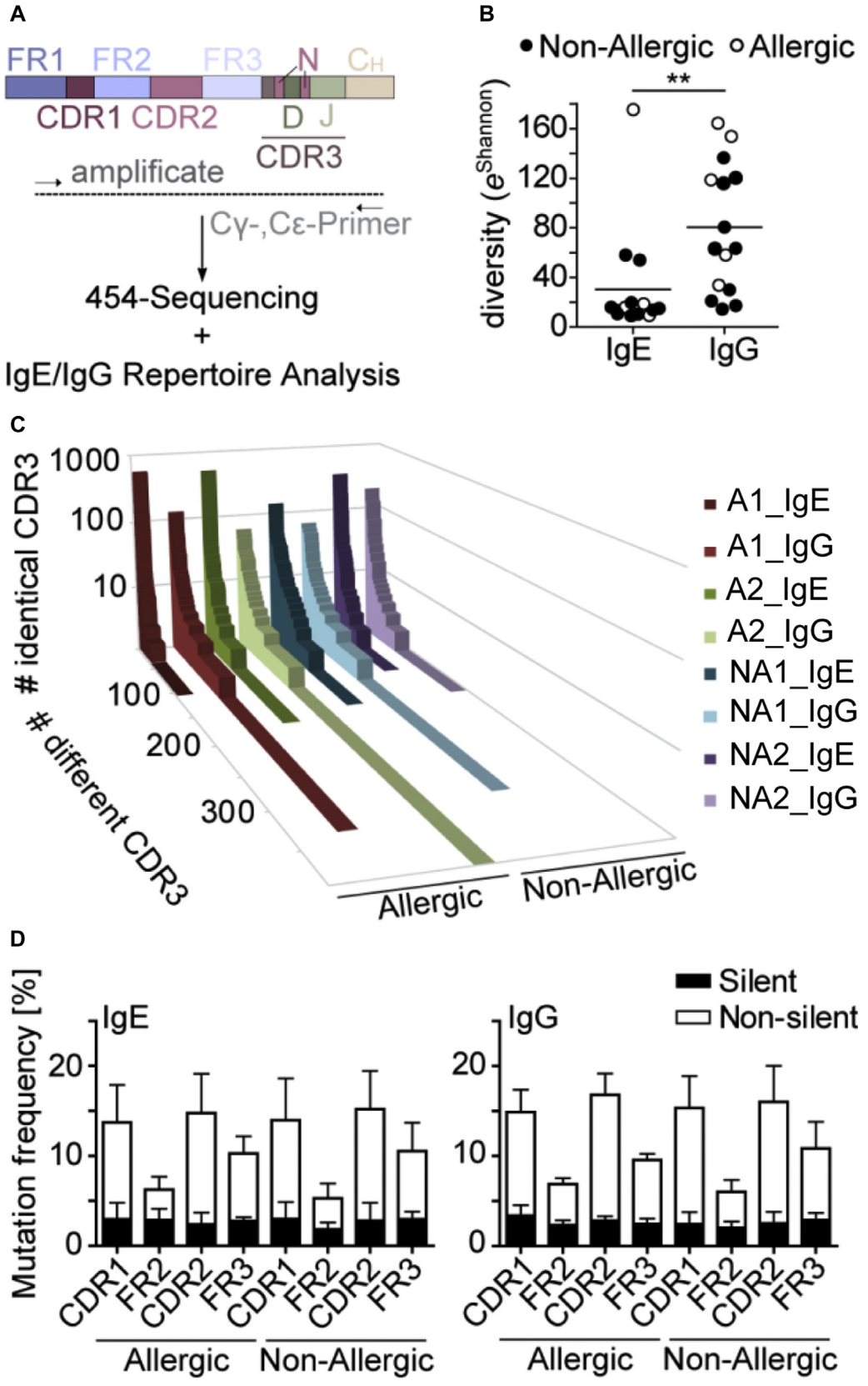
FIG 2. SE-IgE is functional and forms allergen-IgE complexes binding to CD23 on the surfaces of $CD19^+$ B cells. **A,** Binding of SE-IgE–containing nasal polyp homogenates (n = 9) was measured in the presence of a dose response of SEB. All data are shown as means ± SEMs. **B,** Effect of IgG depletion of nasal polyp homogenates on facilitated allergen binding. $CD19^+$ B cells were primed with SE-IgE, allergen-IgE complexes, and allergen-IgE complexes after IgG depletion from nasal polyp homogenates (n = 3). *FAB*, Facilitated allergen-binding assay. **C-F,** Effect of IgG depletion of nasal polyp homogenates on allergen-driven proliferative responses (Fig 2, *C*) and frequencies of $IL\text{-}4^+$ (Fig 2, *D*), $IL\text{-}5^+$ (Fig 2, *E*), and $IL\text{-}17^+$ (Fig 2, *F*) cells (all; n = 6). Data are shown as means ± SEMs. $^*P < .05$.

(http://www.imgt.rg/).[14,15] All sequences were compared against reference sequences from the IMGT database. Results obtained from the IMGT database were further analyzed with in-house–generated Excel and VBA scripts, and only productive sequences were used for downstream analysis.

Mutation frequencies were calculated as the number of mutations divided by the number of all nucleotides of the given framework or complementarity-determining regions. Repertoire similarity was expressed as the Morisita Horn index (MHI), which was calculated as follows:

$$\mathrm{MHI} = \frac{2\sum(\mathrm{an_i} \times \mathrm{bn_i})}{(\mathrm{da}+\mathrm{db}) \times \mathrm{aN} \times \mathrm{bN}} \quad \mathrm{da} = \frac{\sum \mathrm{an_i^2}}{\mathrm{aN^2}} \quad \mathrm{db} = \frac{\sum \mathrm{bn_i^2}}{\mathrm{bN^2}},$$

where *xN* equals the total number of sequences in a given sample *x*, and *xni* presents the number of sequences of type *i* present in *xN*. The Shannon index, which is used to measure immunoglobulin repertoire diversity, was calculated with R software (version 0.94.110; http://www.r-project.org/ and


A
FR1 FR2 FR3 N CH
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160
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40
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IgE
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identical CDR3
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A2_IgE
A2_IgG
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0
CDR1
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CDR2
FR3
Allergic
Non-Allergic

http://rstudio.org/) by using the library "vegan" (command: diversity). The Shannon index equals the following:

$$-\sum \mathrm{pi} \times \ln \mathrm{pi};\ \mathrm{pi} = \mathrm{ni}/\mathrm{N},$$

where N is the number of sequences present in each analyzed IgE or IgG repertoire and *ni* represents the number of a given sequence *i* in that repertoire, making *pi* the ratio of cluster *i* within the overall sequence set. We expressed repertoire diversity as the exponential Shannon index, which corresponds to a theoretical number of equally abundant species that would have the same Shannon index as the actual observed data set.

Statistics

Between-group comparisons were performed by using the Mann-Whitney *U* test, and within-group comparisons were performed by using the Wilcoxon matched-pairs signed-rank test. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). A P value of less than .05 was considered significant.

RESULTS

IgE in nasal polyp homogenates from patients with grass pollen allergy is functional and promotes CD23-mediated proallergic response

To elucidate the regulatory mechanisms that control proinflammatory IgE-mediated responses in nasal polyps, we first examined levels of allergen-specific IgE (sIgE) in patients with grass pollen allergy (GPA) and CRSwNP who were sensitized to the major *Phleum pratense* allergen Phl p 5. sIgE levels in nasal homogenates were increased in patients with GPA compared to those in nonallergic control subjects (NACs; $P < .01$; Fig 1, *A*).

To address the IgE-mediated T_H2 response, we used an *in vitro* functional biocellular assay that would assess the ability of sIgE to bind to an allergen and form cooperative allergen-IgE complexes that bind to low-affinity IgE receptor CD23 in B cells. Nasal polyp homogenates from 6 patients with GPA and 3 NACs were incubated with varying concentrations of *P pratense* allergen, and the resulting complexes were further incubated with a B-cell line homogenously expressing CD23.[16,17] Nasal polyp homogenates from patients with GPA resulted in a dose-dependent increase in allergen-IgE binding to CD23 B cells, which was optimal at 3 μg/mL allergen, compared to NACs ($P < .01$; Fig 1, *B* and *C*). No allergen binding to B cells was observed with nasal polyp homogenates from NACs (Fig 1, *B* and *C*). Heat inactivation of nasal polyp homogenates at 56°C for 30 minutes to denature IgE[17] resulted in a reduction in the capacity to form allergen-IgE complexes and subsequent binding to CD23 on B cells compared with non–heat-inactivated homogenates ($P < .01$; Fig 1, *D*). Furthermore, the cooperative allergen-IgE binding was inhibited by a neutralizing CD23 antibody ($P < .01$; Fig 1, *E*). Collectively, these results highlighted the importance of IgE present in nasal polyp homogenates to form cooperative complexes with allergen to bind to CD23 on B cells.

Depletion of IgG from the nasal homogenate of patients with GPA resulted in an increase in allergen-IgE binding to CD23 on B cells ($P < .05$), whereas the addition of nonspecific IgG antibody used as a nonspecific control did not inhibit this binding (Fig 1, *F*). Moreover, CD23-mediated IgE-facilitated allergen presentation by $CD19^+$ B cells to $CD4^+CD25^-$ effector T cells was enhanced by IgG depletion in nasal polyps ($P < .05$; Fig 1, *G*). Addition of the IgG fraction resulted in a similar magnitude of proliferation of $CD4^+CD25^-$ effector T cells when compared to neat, IgG-containing, nasal homogenate. Therefore these observations highlight the role of IgG as a regulator of IgE-mediated inflammatory response.

IgE in nasal polyp homogenates of patients with GPA induced basophil activation and histamine release

To determine the function of IgE in nasal polyp homogenates, we sensitized basophils with nasal polyp homogenates from patients with GPA (containing IgE). On stimulation with Phl p 5, proportions of $CD63^+$ chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes $(CRTH2)^+$ basophils were found to be increased in a dose-dependent manner (Fig 1, *H*). Furthermore, depletion of IgG from nasal homogenates resulted in an increase in numbers of $CD63^+CRTH2^+$ basophils (all $P < .05$; Fig 1, *H*).

To complement this observation, we investigated the effect of nasal polyp homogenates on the intracellular histamine level in basophils using diamine oxidase (DAO) labeled with phycoerythrin.[18] A significant increase in DAO^-CD63^+ basophils after allergen stimulation in patients with GPA was observed (all $P < .05$; Fig 1, *I*). Furthermore, depletion of IgG from nasal polyp homogenates resulted in an increase in the numbers of DAO^-CD63^+ basophils in patients with GPA but not in NACs, suggesting that IgG in nasal polyp homogenates negatively modulates IgE-mediated basophil activation and histamine release.

Regulatory roles of IgG antibodies on IgE-mediated inflammatory response

Recently, specific IgE antibodies to *Staphylococcus aureus* enterotoxins (SE-IgE) have been demonstrated within the tissues and sera of patients with nasal polyps.[10] Furthermore, SE-IgE in polyp tissue has been shown to be associated with asthma comorbidity and SE-IgE in sera of asthmatic patients with asthma severity.[19] Therefore we investigated SE-IgE bioactivity in our

FIG 3. IgG repertoires in nasal polyps are more complex than IgE repertoires. **A,** The heavy chain variable region 1 and part of the constant *(C)* region were amplified from cDNA. The sequenced amplificate contains framework region *(FR)* 2 and FR3 and complementarity-determining region *(CDR)* 1, CDR2, and CDR3. **B,** Exponent Shannon index was calculated for IgG and IgE sequence sets of allergic *(open circles)* and nonallergic *(solid circles)* subjects. *Horizontal lines* indicate means. $^{**}P < .01$. **C,** Abundance of each CDR3 sequence was plotted against the number of different CDR3 sequences obtained from each repertoire. Sequences were regarded as clonally related (identical; ie, belonging to the identical B-cell clone) when CDR3 sequences were identical in 95% of nucleotides. Two IgE and IgG repertoires of allergic and nonallergic subjects, respectively, are displayed. Each slide represents an IgE or IgG repertoire of 1 nasal polyp. **D,** The frequency of silent and nonsilent somatic mutations was determined for IgE and IgG repertoires of allergic and nonallergic subjects.

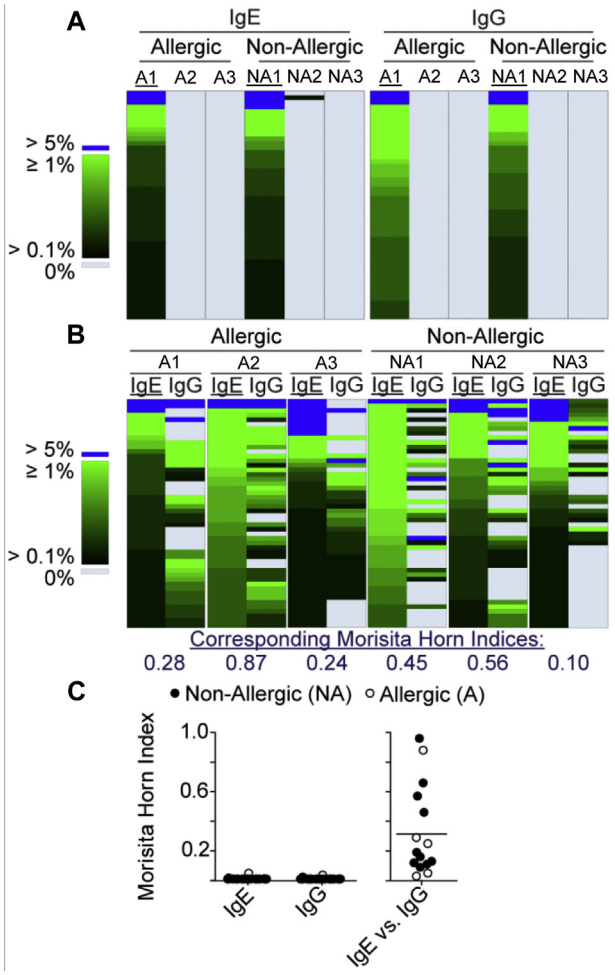

FIG 4. Nasal polyps generate highly clonally related IgE and IgG antibodies within the same subject but not between different subjects. **A,** IgE *(left)* or IgG *(right)* repertoires were compared across 3 representative allergic (A1 to A3) and nonallergic subjects (NA1 to NA3). Complementarity-determining region 3 *(CDR3)* sequences were clustered with 95% sequence identity, and their ratio within the overall repertoire was determined. All CDR3 sequences were sorted from most frequent to least frequent according to the underlined sample, and only the 50 most abundant ones were listed. Logarithmic transformation was used to generate a color gradient corresponding to lower ratios. Sequences with 5% or greater resemblance of the repertoire are represented as blue, green represents 5% or less resemblance, and gray represents absence in a given repertoire. **B,** IgE and IgG repertoires were compared in each nasal polyp. Heat maps from 3 representative allergic and nonallergic subjects show overlap between the 2 isotypes IgE and IgG. Morisita Horn indices quantify the detected overlap for each listed patient. **C,** *Left,* For allergic *(open circles)* and nonallergic *(solid circles)* subjects, IgE or IgG repertoires were compared between different subjects, and Morisita Horn indices were plotted for each pairwise comparison. *Right,* IgE and IgG repertoires overlap within the same subject. *Horizontal lines* indicate means.

patients, as well as the regulatory capacity of SE-IgG_4. Allergen-IgE complex binding to B cells in the presence of SE was determined by allergen-IgE complex binding to B cells preincubated with variable concentrations of SE in nasal polyp homogenates.

SE-IgE–containing nasal polyp homogenates in the presence of staphylococcal enterotoxin B (SEB) were able to form allergen-IgE complexes binding to B cells, which was enhanced when IgG was depleted. Binding of SEB-IgE complexes to B cells was dose dependent and optimal at 10 μg/mL SEB (Fig 2, *A*). Binding of allergen-IgE complexes to B cells in the absence of IgG was enhanced ($P < .05$), whereas a substantial inhibition of allergen-IgE complexes binding to B cells in the presence of IgG ($P < .05$; Fig 2, *B*) was observed. Furthermore, $CD19^+$ B cells were preincubated with allergen-IgE complexes and cultured with $CD4^+CD25^-$ T cells to determine the effect of nasal polyp homogenates in the absence of IgG in allergen presentation and cytokine production. Proliferation response was increased and further enhanced in the absence of IgG ($P < .05$; Fig 2, *C*). This was consistent with the T_H2 cytokine response after IgG depletion, as measured using FluoroSpot assay (Diaclone, Besancon, France). Allergen-driven IL-4^+CD4^+ ($P < .05$; Fig 2, *D*), IL-5^+CD4^+ ($P < .05$; Fig 2, *E*), and IL-17^+CD4^+ ($P < .05$; Fig 2, *F*) cytokine responses were enhanced in nasal polyp homogenates depleted of IgG. However, T_H2 cytokine response was reduced when IgG levels were replenished. These results demonstrate the functional role of IgE in nasal polyp homogenates to trigger facilitated allergen presentation, resulting in the induction of T_H2-mediated inflammation. Furthermore, we highlight the role of IgG in regulating this process.

Clonal relationship of IgG and IgE in nasal polyps

The functional capacity of IgG to modulate IgE-mediated responses prompted us to interrogate the clonal relationship of IgG and IgE in nasal polyps. Clonally related B cells share identical gene segment use and complementarity-determining region 3 sequences, both of which can be determined by using immunoglobulin repertoire analysis. To obtain immunoglobulin repertoire information, cDNA was prepared from nasal polyp RNA, and sequences encoding the variable region of the heavy chain (VH) of the VH1 gene family were amplified, representing the collection of VH1 gene family IgG- and IgE-secreting plasma cells in the respective samples (Fig 3, *A*). Sequences of the VH1 gene family were chosen because it covers a good share of the overall repertoire. The IgE repertoire was generally less diverse compared with the IgG repertoire in both allergic and nonallergic patients ($P < .01$; Fig 3, *B* and *C*). The frequency of somatic mutations in allergic and nonallergic subjects was similar for both IgE- and IgE-encoding sequences (Fig 3, *D*).

Comparing the immunoglobulin repertoire between samples from different patients, we found no evidence of shared VH1 sequences. In fact, different patients expressed highly individual IgE and IgG repertoires, even in patients A1 and A2, who were both allergic to grass pollen (Fig 4, *A*). However, when we compared IgE and IgG repertoires within each subject, we found that IgE and IgG clonotypes were widely shared (Fig 4, *B* and *C*). In fact, in many patients the majority of expanded IgE clonotypes were also represented in IgG repertoires. This indicates that the IgG and IgE repertoires in nasal polyps are characterized by abundant clonally related IgG- and IgE-secreting plasma cells. This observation offers a potential explanation for the striking capacity of IgG in nasal polyps to limit IgE-mediated immune responses by interfering with allergen potentially binding to cell-bound IgE.

DISCUSSION

For the first time, we have demonstrated that IgE present in nasal polyps has the functional capacity to elicit CD23-mediated

proallergic T-cell responses. IgE in the nasal homogenate triggers basophil activation and histamine release at a single-cell level. Moreover, we also report that depletion of IgG antibodies highlights their immunomodulatory role in controlling the IgE-mediated type 2 inflammatory response in nasal polyps. This is most likely due to the fact that IgE and IgG clonotypes are widely shared in nasal polyps.

A growing amount of evidence linking local IgE production to inflammation in patients with CRSwNP can be seen in the literature. Previous studies showed that specific and total IgE concentrations are increased in nasal polyps compared with those in serum or nonpolyp mucosa, illustrating an association between increased total IgE level and eosinophilic inflammation.[4] Further studies from 2 independent groups also showed increased specific IgE levels in sinus tissue of patients with CRSwNP[20] and greater IgE concentrations in the sinus mucosa of patients with CRSwNP.[21] Our data are in line with these previous finding by which we observed an increase in sIgE levels of patients with GPA with CRSwNP to Phl p 5 when compared with healthy control subjects.

Local production of IgE through plasma cells in the mucosa has been previously described.[22] In addition to this, local IgE production has been shown to be polyclonal and functional.[5] Although limited, the functionality of local IgE in nasal polyp tissue homogenates has been described in a previous study to mediate basophil degranulation after grass pollen stimulation.[5] Furthermore, though IgE specificity has not been investigated further, the increase in sIgE levels observed in this study is likely a result of a polyclonal rather than monoclonal response. Future studies to investigate the expansion of non–allergen-specific immunoglobulins in addition to the specific response will allow confirmation of this.

Here we assessed the functionality of local IgE in nasal polyp homogenates of patients with CRSwNP in greater detail. We confirmed that sIgE antibodies present in nasal polyps are functional and have the ability to form cooperative allergen-IgE complexes, which bind to FcεRII (CD23) on the surfaces of B cells and trigger facilitated allergen presentation, resulting in the perpetuation of T_H2 inflammation. We also showed that IgE present in nasal polyps has the capacity to induce basophil activation and histamine release, as represented by expression of basophil surface marker CD63 and the intracellular histamine marker DAO. There are no studies that have described the functionality of local IgE in nasal polyp homogenates of patients with CRSwNP to this extent. Mast cells infiltrate nasal polyp tissue more than basophils, and it has also been shown in a separate study that mast cell activation is a sensitive alternative assay to the Basophil Activation Test.[23] However, assessing FcεRI-mediated responses in basophils rather than mast cells is a well-established method, with various reports validating the reliability of this assay.[18,24,25] We had limited nasal polyp homogenates, which made it difficult to also assess mast cell activation and histamine release. After these observations, future studies could explore the effect of IgE in nasal polyps in mediating FcεRI responses in tissue-resident mast cells. However, because this was a proof-of-concept study, the findings in this study would need to be validated in a bigger clinical study involving a larger sample size.

Previous studies have reported the use of IgE-facilitated allergen-binding assay as a validated tool to assess the functionality of inhibitory IgG_4, which is dependent on affinity, avidity, and antibody clonality.[16,17,23] The concept of allergen-IgE complex formation has been shown with grass pollen,[16,17,26] birch pollen,[27] cat allergen, and peanut allergen.[28-30] This phenomenon can occur with any allergenic protein. In the case of allergen immunotherapy, which results in the induction of IgE and allergen-neutralizing IgG_4,[16,26,31] IgG_4 antibodies compete with IgE and prevent basophil activation[18] and CD23-mediated facilitated allergen binding to B cells.[17,26,27] IgG_4 depletion results in the recovery of allergen-IgE binding to B cells.[26,32] In this study we also observed the regulatory role of IgG antibody in nasal polyp homogenates of patients with CRSwNP. IgG-regulated FcεRII (CD23) mediated IgE-facilitated allergen presentation by B to T cells. Moreover, IgG also controls SE-IgE responses in a similar manner. It is well accepted that in the case of allergen immunotherapy, allergen-specific IgG antibody responses, in particular IgG_4 responses, are induced.[26] These blocking antibodies can directly inhibit IgE-dependent histamine release and presentation of antigen to T cells.[33] Therefore the observations seen in this study by which IgG blocking antibodies can interfere with allergen-IgE interaction in nasal polyps of allergic patients is similar to what would be observed after allergen immunotherapy treatment. Our results showed that SE-IgE containing nasal polyp homogenates were able to form allergen-IgE complexes binding to B cells, when SEB was present. When IgG was depleted from nasal polyp homogenates, allergen-IgE complexes binding to B cells was significantly enhanced. We were also able to show that when IgG antibodies were depleted, T-cell proliferation was significantly higher. We believe that IgG regulates IgE-facilitated allergen presentation by eliciting its blocking activity and competing with IgE. This phenomenon is dependent on specificity, affinity, avidity, and clonality.

The regulatory role of IgG in nasal polyp homogenates could imply its therapeutic potential. For example, SE-IgG antibodies can act as a therapeutic tool for patients in whom SEs are involved in the clinical manifestation of disease severity. Furthermore, our findings agree with the role of IgG_4 after allergen immunotherapy. The concept of passive immunotherapy was introduced by Robert Cooke in 1935 when serum from ragweed immunotherapy–treated patients had blocking activity for skin reactivity.[34] More recently, Orengo et al[35] also demonstrated that a single dose of subcutaneous Fel d 1–specific IgG_4 mAb (REGN1908/9, 600 mg) was able to inhibit total nasal symptom scores after cat allergen challenge in patients with cat allergy, highlighting the therapeutic potential of IgG_4.[35] Therefore it is likely, although it needs to be proved, that SE-IgG_4 with blocking capacity can potentially be used therapeutically. Future studies that specifically investigate IgG response in nasal polyps would definitely be beneficial to further validate this proof-of-concept study.

Immunoglobulin repertoire analyses were performed to further understand the regulatory mechanism of IgG antibodies. Although shared IgG and IgE repertoires have been studied extensively in mice, providing mechanistic insights, less is known about the relationship of IgG and IgE repertoires in human subjects.[36] In our study we showed that IgE repertoires were generally less diverse compared with IgG repertoires in both allergic and nonallergic subjects, whereas the frequency of somatic mutations in allergic and nonallergic subjects was similar for both IgE- and IgG-encoding sequences. It has been previously shown that high IgE titers were observed in nasal polyp

homogenates, despite unchanged levels in serum, compared with control samples.[11] This observation suggests that IgE is produced locally in nasal polyps. Additionally, immunohistology studies have demonstrated a large plasma cell population in nasal polyps, supporting the idea of local IgE- and IgG-producing plasma cells in nasal polyps. Therefore these observations offer a potential explanation for the striking capacity of IgG in nasal polyps to limit IgE-mediated immune responses. It is noteworthy, however, that future studies to confirm and allow the quantification of IgG- and IgE-producing plasma cells in nasal polyps are required to further validate this concept. The IgE repertoire was matched by clonally related IgG antibodies and IgG functionally inhibited IgE, indicating a regulatory function of local IgG in IgE-mediated perturbation of allergic responses.

In summary, the current study suggests that local IgE found in nasal polyp homogenates of allergic patients with CRSwNP are functional and regulated by local IgG antibodies. The capacity of IgG to regulate the IgE-mediated inflammatory response is due to the fact that IgG repertoires widely share targets with IgE repertoires.

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Key messages

- **Polyclonal IgE idiotypes in patients with CRSwNP are functional and promote IgE-mediated inflammation.**
- **IgE idiotypes are partially antagonized by the corresponding IgG idiotypes.**

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METHODS

PBMC isolation

Heparinized blood diluted 1:1 with RPMI 1640 (Invitrogen) was layered onto 30% Ficoll-Paque Plus (GE Healthcare, Fairfield, Conn) density gradient and centrifuged for 25 minutes at 2200 rpm at room temperature. The PBMC layer was collected, washed, and resuspended in RPMI 1640. Cell viability was greater than 97%, as determined by using trypan blue exclusion.

IgG depletion from nasal polyp homogenates

IgG depletion from nasal polyp homogenates was carried out with Proteus Protein G Antibody Purification Mini-Kits (Bio-Rad, Oxford, United Kingdom), according to the manufacturer's protocol. The protein G spin column was equilibrated with 650 μL of binding buffer A (pH 7.4) and centrifuged at 4400 rpm for 1 minute. Sera and nasal fluid samples were diluted 1:1 with binding buffer before applying the protein G spin column and centrifuged at 2600 rpm for 10 minutes. The column was washed 3 times with 650 μL of binding buffer to remove any contaminants. The mini plugs were eluted twice in separate Proteus spin columns by adding 65 μL of neutralizing buffer (pH 9.0) and 500 μL of elution buffer (pH 2.0) on top of the protein G mini plug and centrifuged at 4400 rpm for 1 minute. IgG-depleted samples and eluted bound IgG antibodies were collected separately and measured for IgG_4 levels by using the ImmunoCAP100 system (Phadia, Uppsala, Sweden), according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Eluted purified IgG antibodies were added back into the IgG-depleted samples, where experiments involve the use of −IgG and +IgG conditions. Polyclonal IgG antibody was used as a control (Thermo Fisher, Paisley, United Kingdom).

Basophil activation and histamine release

Effect of IgE-containing and IgE-containing IgG-depleted nasal polyp homogenates from allergic subjects on basophil activation and histamine release using DAO was assessed using flow cytometry. *P pratense* allergen extract (0, 10, and 100 ng/mL) was incubated with nasal polyp homogenates and isolated basophils for 15 minutes at 37°C. Enriched basophils were obtained using the EasySep Human Basophil Isolation Kit (Stemcell Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's instructions. Cells were immunostained with anti-human CD3, CD303, CD294 (CRTH2), CD203c, CD63 and CD107a (all from BD Biosciences, San Jose, Calif). Samples were centrifuged (5 minutes at 200 *g*), and supernatants were discarded. Cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences). Cells were stained with fluorochrome (V500)–labeled DAO (BD Biosciences) for 30 minutes at 4°C, washed, and resuspended in 450 μL of ice-cold fixative solution (BD Biosciences) before acquisition on a BD FACSCanto II flow cytometer. Analyses were performed with BD FACSDiva software (version 6.1.1; BD Biosciences).

IgE-facilitated antigen binding

The EBV-transformed B-cell line expressing high levels of CD23 was maintained and characterized, as previously described.[E1,E2] Allergic or nonatopic IgE containing nasal polyp homogenates was preincubated with different concentrations of *P pratense* at 37°C in a 5% CO_2 atmosphere for 1 hour to form allergen-IgE complexes, as previously described.[E2] In parallel, various concentrations of SEB were preincubated with *P pratense* at 37°C in a 5% CO_2 atmosphere for 1 hour. Nasal polyp homogenates containing high IgE concentrations were also heat inactivated at 56°C for 30 minutes. EBV-transformed B cells (1×10^5/5 μL) were added to the allergen–IgE mixture and incubated for a further 1 hour at 4°C. Cells were washed, followed by addition of polyclonal human anti-IgE phycoerythrin-labeled antibody (1:50 dilution for 45 minutes), to detect bound complexes by using the BD FACSCanto II flow cytometer and analyzed with FACSDiva software. B cells were gated by using forward scatter/side scatter parameters, and a positive marker was set by using cells incubated with indicator serum only. Unlabeled anti-human CD23 mAb (500 mg/L, clone MHM6) was used as a blocking antibody for CD23-dependency experiments (DakoCytomation, Cambridge, United Kingdom).

IgE-facilitated antigen presentation

T cells were enriched from PBMCs by using magnetic isolation (Stemcell Technologies), according to the manufacturer's instructions. In brief, 5×10^7 cells were incubated with isolation cocktail for 5 minutes at room temperature, followed by addition of 40 μL/mL EasySep RapidSpheres (Stemcell Technologies), and adjusted to a final volume of 2.5 mL by using MACS buffer. Sera from allergic patients (20 μL) containing high levels of *P pratense*–specific IgE levels of greater than 30 kU/L were preincubated with 0, 0.1, 1, and 10 μg/mL allergen (5 μL) at 37°C for 1 hour to form allergen–IgE complexes. Then autologous B cells enriched from PBMCs from allergic patients (irradiated at 6000 rads) were added to the allergen-complexed serum and incubated for 18 hours before coculture with $CD4^+CD25^-$ T cells for 7 days. Proliferation of T cells was determined based on tritiated thymidine incorporation. The frequency of *P pratense*–specific IL-4^+CD4^+ and IL-5^+CD4^+ and IL-17^+CD4^+ T cells was measured by using the FluoroSpot assay.

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TABLE E1. Patients' demographics

	Allergic patients with CRSwNP (n = 9)	Nonallergic patients with CRSwNP (n = 9)
Sex (female/male)	3/6	9/0
Age (y), mean ± SEM	38.11 ± 3.71	52.2 ± 4.10
Asthma	1/9	7/9
AERD	0/9	2/9

AERD, Aspirin-exacerbated respiratory disease.