Title: Broad Immunglobulin G Repertoire in Chronic Rhinosinusitis with Nasal Polyps regulates pro-inflammatory IgE responses

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Key Messages

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

Capsule Summary

- Polyclonal IgE idiotypes in CRSwNP are functional, promote IgE-mediated inflammation and are partially antagonized by corresponding IgG-idiotypes.
- This mechanism is similar to the blocking antibodies induced by allergen-specific immunotherapy in the allergic subjects.

Key Words

IgG; antibody repertoire, IgE; Chronic rhinosinusitis; Nasal polyps; allergic rhinitis.

Abbreviations

CRSwNP, Chronic rhinosinusitis with nasal polyps; GPA, grass pollen allergies; Ig, Immunoglobulins; Th2, T helper 2 cells; SE-IgE, specific IgE antibodies to Staphylococcus aureus; IgE-FAB, IgE-Facilitated Allergen Binding Assay.
Acknowledgments: This work was supported by the Imperial College research funds.
We acknowledge Dr Kouser for her valuable feedback of the manuscript.

Disclosure of potential conflict of interest: M. H. Shamji serves as a consultant for Imperial College London and receives lecture fees from ALK-Abello, ASIT Biotech, Allergopharma, and UCB. S. R. Durham receives grant support from the Immune Tolerance Network, NIAID, ALK-Abello, Hørsholm Regeneron, and Biotech Tools and serves as a consultant from Anergis, Circassia, Biomay, Merck, Allergy Therapeutics, Med Update GmbH, and Food Standards. The rest of the authors declare that they have no relevant conflicts of interest.
ABSTRACT

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

Objective: We sought to determine whether locally induced IgG antibodies in the nasal polyps can inhibit IgE-mediated pro-allergic response.

Methods: Nasal polyp homogenates were collected from grass pollen allergics with CRSwNP and non-allergic controls. IgE levels were measured by ISAC. IgE-containing nasal polyp homogenates, with/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 by Immunoglobulin 454 sequencing.

Results: We show that IgG plays a key role in controlling IgE-mediated inflammatory responses in nasal polyps. Depletion of IgG from nasal homogenates resulted in an increase in CD23-mediated IgE-facilitated allergen binding to B cells (IgE-FAB), but also enhanced FceRI-mediated allergen driven basophil activation and histamine release. A similar response was observed in relation to specific IgE antibodies to Staphylococcus aureus (SE-IgE). 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 non-allergic subjects.

Conclusion: Polyclonal IgE idiotypes in CRSwNP are functional, promote IgE-mediated pro-allergic 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 nasal polyps.
INTRODUCTION

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 sufferers develop co-morbid asthma, aspirin-exacerbated respiratory disease, hyper-eosinophilia or Churg-Strauss syndrome. Recurrence of the disease after surgery is high albeit treatment with topical and oral glucocorticosteroid.\(^2\) It is reported in Europe that independent of their atopic status, 85% of nasal polyps are characterized by local polyclonal IgE production, abundant eosinophils and IL-4, IL-5, IL-13 producing T helper 2 cells.\(^3\)

Tissue IgE concentrations can be in the range of several thousand kU/L,\(^4\) and consist of functional polyclonal IgE-idiotypes\(^5\) formed locally.\(^6\) High tissue IgE concentrations are associated with asthma comorbidity\(^7\) and recurrence of disease.\(^8\) Anti-human monoclonal IgE antibody 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 \textit{Staphylococcus aureus} enterotoxins (SE) 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.\(^5,9\) SE has been shown to contribute to the formation of nasal polyps in both allergic and non-allergic patients.\(^4,10\) As a result, severe eosinophilic inflammation occurs as well as the production of IgE and IgG/IgG4 antibodies by B cells.\(^11\) Although several studies have reported elevated 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 pro-inflammatory response is controlled in the nasal polyp remains to be fully investigated.

We firstly hypothesized that local specific IgE antibodies in homogenates from patients suffering from CRSwNP are functional and promote FcεRII-mediated pro-allergic response. Secondly, local SE-specific IgG antibodies are also induced and can inhibit FcεRII-mediated facilitated allergen presentation by B to T cells. Thirdly, 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 chronic rhinosinusitis with nasal polyps (CRSwNP, Table EI), defined by EPOS criteria\textsuperscript{12} based on symptoms and results of nasal endoscopy and computed tomography of the sinuses were included. Subjects were excluded if they suffered from an acute exacerbation of rhinosinusitis 2 weeks before inclusion, from known immunodeficiency, cystic fibrosis, fungal allergic sinus disease, or if they used oral or nasal steroids in the 4 weeks preceding surgery, or if they used antihistamines prior to nasal polyp surgery. This study was approved by the ethics committee of Ghent University hospital, 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 or specific IgE 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 tissue. The tissue was then homogenized with a mechanical homogenizer (B. Braun Melsungen, Germany) at 1000 rpm for 5 min on ice. After homogenization, the suspensions were centrifuged at 1500 rpm for 10 min at 4°C, and the supernatants were collected. All supernatants were assayed for IgE by the UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden).
Immunological analysis

Methods for PBMC isolation, IgG depletion from nasal polyp homogenates, basophil activation and histamine release, IgE-facilitated antigen binding and presentation are described in the Methods section in the Online Repository.

Immunoglobulin 454 Sequencing

IgE and IgG repertoire information was obtained following the same scheme as described for IgA repertoires.\textsuperscript{13} RNA was extracted and transcribed into cDNA with Superscript III (Invitrogen) and random primers according to the manufacturer’s instructions. Ig amplicons were generated by PCR with 30 cycles using the following primers:

- \text{VH} 5'-CGTATCGCCTCCCTCGCCATCAGGGCCTCAGTGAAGGTCTCCTGCAAG-3'
- \text{Cy} for IgG 5'-CTATGCGCCTTGCCAGCCCGCTCAG(MID)GTTCCACGACACCGTCACC-3'
- \text{Ce} for IgE 5'CTATGCGCCTTGCCAGCCCGCTCAG (MID) AAGGGGAAGCAGGATGGGCTCTG -3' amplification. Gene-specific sequences are underlined, MID specifies a nucleotide sequence used in ten variations to identify samples, non-underlined sequences represent adaptor sequences needed for emulsion PCR and 454 sequencing. PCR conditions were as follows: 95°C, 4 min; 25 × (94°C, 30 s; 62°C, 30 s; 72°C, 30 s; 72°C, 35 s); and 72°C, 10 min. Amplicons were purified by gel electrophoresis followed by gel extraction (QIAquick Gel Extraction kit; Qiagen) and DNA concentration was quantified by 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) according to the manufacturer’s instructions.
Immunoglobulin sequence analysis

Immunoglobulin sequence analysis was performed as described with some modifications. In brief, sequences longer than 320 bp and comprising both primers were sorted according to their MID. Sequences were further analyzed with ImMunoGeneTics (IMGT) HighV-QUEST (http://www.imgt.org). All sequences were compared against reference sequences from the IMGT database. Results obtained from IMGT 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 MHI, calculated as:

\[
MHI = \frac{2 \sum (a_n \times b_n)}{(d_a + d_b) \times aN \times bN} = \frac{\sum a_n^2}{aN^2} - \frac{\sum b_n^2}{bN^2}
\]

xN equals the total number of sequences in a given sample x, while xni presents the number of sequences of type i present in xN. The Shannon index, used to measure immunoglobulin repertoire diversity, was calculated with R studio (version 0.94.110; http://www.r-project.org/, http://rstudio.org/) using library “vegan” (command: diversity). The Shannon index equals \(- \sum p_i \times \ln p_i; p_i = n_i / N. N equals the number of sequences present in each analyzed IgE or IgG repertoire and n_i 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 using Mann-Whitney U test and within group comparisons were performed using Wilcoxon matched-pairs signed rank test. The statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., California, USA). $P < .05$ was considered to be significant.
RESULTS

IgE in nasal polyp homogenates from grass pollen allergics is functional and promote CD23-mediated pro-allergic response

To elucidate the regulatory mechanisms that control pro-inflammatory IgE-mediated responses in nasal polyps, we first examined the levels of allergen-specific IgE (sIgE) in grass pollen allergic (GPA) with CRSwNP sensitized to major *Phleum pratense* 5 (Phlp 5) allergen. sIgE in the nasal homogenate was elevated in GPA patients as compared to non-allergic (controls) (NAC) (*P* < .01; Fig 1, A).

In order to address the IgE-mediated Th2 response, we used an *in vitro* functional bio-cellular assay that would assess the ability of sIgE to bind to an allergen and form co-operative allergen-IgE complexes that bind to the low affinity IgE receptor, CD23. Nasal polyp homogenates from six GPA and three NAC were incubated with varying concentrations of *Phleum pratense* allergen and the resulting complexes were further incubated with a B cell line homogenously expressing CD23. Nasal polyp homogenates from GPA resulted in a dose-dependent increase in allergen-IgE binding to CD23 B cells which was optimal at 3 µg/mL of allergen, compared to NAC (*P* < .01; Fig 1, B-C). No allergen binding to B cells was observed with nasal polyp homogenate from NAC (Fig 1, B-C). Heat-inactivation of nasal polyp homogenates at 56°C for 30min to denature IgE (Heat-IgE) resulted in a reduction in the capacity to form allergen-IgE complexes and subsequent binding to CD23 on B-cells as compared to non-heat-inactivated homogenates (GP-IgE) (*P* < .01; Fig 1, D). Furthermore, the co-operative allergen-IgE binding was inhibited by neutralizing CD23 antibody (*P* < .01; Fig 1, E). Collectively, these results highlighted the
importance of IgE present in nasal polyp homogenate s to form co-operative complexes with allergen to bind to CD23 on B cells.

Depletion of IgG from the nasal homogenate of GPA resulted in an increase in allergen-IgE binding to CD23 on B cells \((P < .05)\) while the addition of non-specific control IgG (ClgG) used as a non-specific control did not inhibit this binding (Fig 1, \(F\)). Moreover, CD23-mediated IgE-facilitated allergen presentation by CD19\(^+\) B cells to CD4\(^+\)CD25\(^-\) T effector cells was enhanced by IgG depletion in nasal polyps \((P < .05; \text{Fig 1, } G)\). Addition of the IgG fraction resulted in a similar magnitude of proliferation of CD4\(^+\)CD25\(^-\) T effector cells, when compared to neat nasal homogenate. These observations therefore highlight the role of IgG as a regulator to IgE-mediated inflammatory response.

**IgE in nasal polyp homogenates of grass pollen allergic patients induced basophil activation and histamine release**

In order to determine the function of IgE in nasal polyp homogenates, we sensitized basophils with the nasal polyp homogenates from GPA (containing IgE). Upon stimulation with Phlp 5, the proportions of CD63\(^+\)CRTh2\(^+\) basophils were found to be increased in a dose-dependent manner (Fig 1, \(H\)). Further to this, depletion of IgG from the nasal homogenates resulted in an increase in CD63\(^+\)CRTh2\(^+\) basophils (all, \(P < .05; \text{Fig 1, } H)\). To complement this observation, we investigated the effect of nasal polyp homogenates on the intracellular histamine level in basophils using diamine oxidase (DAO) labelled with Phycoerythrin (PE).\(^{18}\) A significant increase in DAO\(\cdot\)CD63\(^+\) basophils following allergen stimulation in GPA was observed (all, \(P < .05; \text{Fig 1, } I)\). Furthermore, depletion of IgG from nasal homogenates resulted in an
increase in DAO+CD63+ basophils in GPA but not in NAC, suggesting that IgG in nasal polyp homogenate negatively modulates IgE-mediated basophil activation.

**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 tissue as well as in serum of patients with nasal polyps. Furthermore, SE-IgE in polyp tissue has been shown to be associated with asthma comorbidity, and SE-IgE in serum of asthma patients with asthma severity.

We therefore investigated SE-IgE bioactivity in our patients as well as the regulatory capacity of SE-IgG4. Allergen-IgE complexes binding to B cells in the presence of SE was determined by allergen-IgE complexes binding to B cells pre-incubated 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 IgE-SE to B cells was dose-dependent and optimal at 10 µg/mL of SE (Fig 2, A). The binding of allergen-IgE complexes to B cells in the absence of IgG was enhanced ($P < .05$); while a substantial inhibition of allergen-IgE complexes binding to B cells in the presence of IgG ($P < .05$; Fig 2, B) was observed. Furthermore, to determine the effect of nasal polyp homogenates in the absence of IgG in allergen presentation and cytokine production, CD19+ B cells were pre-incubated with allergen-IgE complexes and cultured with CD4+CD25− T cells. Proliferation response was increased and further enhanced in the absence of IgG ($P < .05$; Fig 2, C). This was consistent with the Th2 cytokine response following IgG depletion as measured by FluoroSpot assay.
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, Th2 cytokine response was reduced when level of IgG was replenished. These results demonstrate the functional role of IgE in nasal polyp homogenates to trigger facilitated allergen presentation, resulting in the induction of Th2-mediated inflammation. Further to this, 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 usage and complementary determining region 3 (CDR3) sequences both of which can be determined by Ig repertoire analysis. To obtain Ig repertoire information, cDNA was prepared from nasal polyps RNA and variable heavy chain sequences 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 VH1 gene family was chosen due to the fact that is covers a good share of the overall repertoire. The IgE repertoire was generally less diverse compared to the IgG repertoire in both allergic and non-allergic patients (P < .01; Fig 3, B-C). The frequency of somatic mutations in allergic and non-allergic patients was similar for both IgE and IgE-encoding sequences (Fig 3, D).

Comparing the Ig 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 2 and 3 who were both allergic to grass pollen (Fig 4, A). However, when we compared IgE and IgG repertoires within each individual, we found that IgE and IgG clonotypes were widely shared (Fig 4, B-C). In fact, in many patients the majority of expanded IgE clonotypes was also represented in IgG repertoires. This indicates that the IgG and IgE repertoire in nasal polyps is 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 have functional capacity to elicit CD23-mediated pro-allergic T cell responses. IgE in the nasal homogenate trigger basophil activation and histamine release at a single cell level. Moreover, we also report that depletion of IgG antibody highlights their immunomodulatory role in controlling 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 number of evidences linking local IgE production to inflammation in CRSwNP can be seen in the literature. Previous studies illustrated that specific and total IgE concentration are elevated in nasal polyps compared to serum or non-polyp mucosa illustrating an association between increased total IgE level and eosinophilic inflammation.\(^4\) Further studies from two independent groups also illustrated elevated specific IgE in sinus tissue of CRSwNP patients\(^{20}\) and higher IgE concentration in sinus mucosa of CRSwNP patients.\(^{21}\) Our data are in line with these previous finding whereby we observed an increase in sIgE of grass pollen allergic patients with CRSwNP to Phlp 5, when compared to healthy controls.

The local production of IgE through plasma cells in the mucosa has been previously described.\(^{22}\) In addition to this, local IgE production has been illustrated to be polyclonal and functional.\(^5\) Though limited, the functionality of local IgE in nasal polyp tissue homogenates have been described in a previous study to mediate basophil degranulation following grass pollen stimulation.\(^5\) Whilst the specificity of the IgE have not been further investigated, the increase in allergen-specific IgE observed in
this study is likely as a result of polyclonal response, rather than monoclonal. Future studies to investigate the expansion of non-allergen specific immunoglobulins in addition to the specific response will allow the confirmation of this. Here, we assessed the functionality of local IgE in nasal polyp homogenates of CRsWNP in greater detail. We have confirmed that allergen-specific IgE antibodies present in the nasal polyps are functional and have the ability to form co-operative allergen-IgE complexes, which bind to FcεRII (CD23) on the surface of B cells and trigger facilitated allergen presentation resulting in the perpetuation of Th2 inflammation. We also showed that IgE present in the nasal polyps has the capacity to induce basophil activation and histamine release, as represented by expression of basophil surface markers (CD63 and CD203c) and intracellular histamine marker DAO. There are no studies that have described the functionality of local IgE in nasal polyp homogenates of CRsWNP to this extent. Mast cells infiltrate nasal polyp tissue more than basophils and it has also previously been shown in a separate study that mast cell activation is a sensitive alternative assay to basophil activation test (BAT). 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. We had limited nasal polyp homogenates that made it difficult to also assess mast cell activation and histamine release. Following these observations, future studies could explore the effect of IgE in nasal polyps in mediating FcεRI responses in tissue-resident mast cells. However, as 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-FAB as a validated tool to assess functionality of inhibitory IgG₄, which is dependent on affinity, avidity and antibody clonality.¹⁶-¹⁷, ²³ The concept of allergen-IgE complex formation has been shown with grass pollen¹⁶-¹⁷,²⁶, birch pollen²⁷, cat allergen and peanut allergen²⁸-³⁰. This phenomenon can occur with any allergenic protein. In the case of allergen immunotherapy which results in induction of IgE as well as allergen neutralizing IgG₄, these compete with IgE and prevent basophil activation¹⁸ and CD23-mediated facilitated allergen binding to B cells.¹⁷, ²⁶-²⁷ Depletion of IgG₄ result in the recovery of allergen-IgE binding to B cells²⁶, ³². In this study, we also observed the regulatory role of IgG antibody in nasal polyp homogenates of CRSwNP. IgG regulated FceRII (CD23) mediated IgE-facilitated allergen presentation by B to T cells. Moreover, IgG also controls specific IgE antibodies to Staphylococcus aureus superantigens (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 IgG₄, is induced.²⁶ These blocking antibodies can directly inhibit IgE-dependent histamine release and presentation of antigen to T cells.³³ The observations seen in this study whereby IgG blocking antibodies can interfere with allergen-IgE interaction in nasal polyps of allergic subjects is therefore similar of what would be observed following allergen immunotherapy (AIT) treatment. Our results illustrated 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 the nasal polyp homogenates, the allergen-IgE complexes binding to B cells was significantly enhanced. We were also able to illustrate that when IgG antibodies were depleted, proliferation of T cells 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 the 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. Further to this, our findings agree with the role of IgG4 following 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. More recently, Orengo and colleagues also demonstrated that a single dose of subcutaneous Fel d 1-specific IgG₄ monoclonal antibodies (REGN1908/9, 600mg) was able to inhibit total nasal symptom scores after cat allergen challenge in cat allergic patients, highlighting the therapeutic potential of IgG₄. It is likely, although it needs to be proven, that SE-IgG₄ with blocking capacity can therefore 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. Though shared IgG and IgE repertoire have been studied extensively in mice providing mechanistic insights, less is known about the relationship of IgG and IgE repertoire in human. In our study, we illustrated that IgE repertoires were generally less diverse compared to IgG repertoires in both allergic and non-allergic patients while the frequency of somatic mutations in allergic and non-allergic patients was similar for both IgE and IgG encoding sequences. It has been previously shown that high IgE titre was observed in nasal polyp homogenates, despite unchanged level in serum, as compared to control samples.
This observation suggests that IgE is produced locally in the nasal polyps. Additionally, immunohistology studies have demonstrated large plasma cell population in nasal polyps, supporting the idea of local IgE- and IgG- producing plasma cells in nasal polyps. These observations therefore offer a potential explanation for the striking capacity of IgG in nasal polyps to limit IgE-mediated immune responses. It is noteworthy 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 a 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 suggest that local IgE found in the nasal polyp homogenates of CRSwNP allergic patients are functional and are regulated by local IgG antibodies. The capacity of IgG to regulate IgE-mediated inflammatory response is due to the fact that IgG repertoire widely shares the targets with IgE repertoire.
FIGURE LEGENDS

Figure 1. IgE in nasal polyps facilitates CD23-mediated allergen binding to B cells and allergen presentation. (A) Levels of sIgE to Phlp in nasal polyp homogenates from GPA (n=6) and NAC (n=6) were measured by Immunosolid allergen Chip Assay (ISAC). (B-C) Quantification of allergen-IgE binding to B cells in IgE-containing nasal polyp homogenates (n=6) and control homogenates from NAC (n=6) with varying concentrations of Phlp. (D) Effect of heat inactivation on IgE-containing nasal polyps homogenates (n=6) in the presence of 3 µg/mL of allergen. (E) CD23-dependency was determined by pre-treatment of EBV-transformed B cells with anti-CD23 blocking antibody (0 or 10 µg/mL) (n=6). Effect of IgG depletion in nasal polyps on (F) Co-operative binding of allergen-IgE complexes binding to B cells and (G) IgE-facilitated allergen presentation by CD19+ B cells to CD4+CD25- T effector cells. (H-I) Basophils obtained from GPA were sensitized with neat IgE-containing, IgE-containing IgG depleted (-IgG) nasal polyp homogenates (n=6), IgG fraction (+IgG) or non-specific control IgG (CIgG; 1 mg/mL) in the presence of varying concentrations of Phlp. Basophils obtained from NAC were used as a negative control (Control). All data are shown as mean ± SEM. * P < .05, ** P < .01.

Figure 2. SE-specific IgE (SE-IgE) are functional and form allergen-IgE complexes binding to CD23 on surface 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 mean ± SEM. (B) Effect of IgG depletion of nasal polyps homogenates on facilitated allergen presentation and pro-allergic inflammatory cytokine release. CD19+ B cells were primed with SE-IgE, Ag-IgE
complexes and Ag-IgE complexes following IgG depletion from nasal polyp homogenate (n=3). Allergen-driven proliferative responses (C), frequency of IL-4+ (D), IL-5+ (E) and IL-17+ (F) cells were measured (all; n=6). Data are shown as mean ± SEM. * P < .05.

Figure 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 regions 2, 3 (FR2, 3) and complementarity determining regions 1-3 (CDR1-3). (B) Exponent Shannon index was calculated for IgG and IgE sequence sets of allergic (open circles) and non-allergic (closed circles) patients. Horizontal lines indicate mean. ** 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, i.e. belonging to the identical B cell clone) when CDR3 sequences were identical in 95% of nucleotides. Two IgE and IgG repertoires of allergic and non-allergic patients respectively are displayed. Each slide represents an IgE or IgG repertoire of one nasal polyp. (D) The frequency of silent and non-silent somatic mutations was determined for IgE and IgG repertoires of allergic and non-allergic patients.

Figure 4. Nasal polyps generate highly clonally related IgE and IgG antibodies within the same individual but not between different individuals. (A) IgE (left) or IgG (right) repertoires were compared across three representative allergic and non-allergic patients. 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% resemblance of the repertoire are represented as blue, green represents ≤5% resemblance whilst grey represents absence in a given repertoire. (B) IgE and IgG repertoires were compared in each nasal polyp. Heatmaps from three representative allergic and non-allergic patients illustrate overlap between the two isotypes IgE and IgG. Morisita Horn indices (MHI) quantify the detected overlap for each listed patient. (C) Left: For allergic (open circles) and non-allergic (closed circles) patients IgE or IgG repertoires were compared between different individuals and MHI was plotted for each pairwise comparison. Right: IgE and IgG repertoire overlap within the same individual. Horizontal lines indicate mean.
References


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PBMC Isolation

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

IgG depletion from nasal polyp homogenates

IgG depletion from nasal polyp homogenates was carried out using Proteus Protein G Antibody Purification mini-kits (Bio-rad, Oxford) as per manufacturer’s protocol. The protein G spin column was equilibrated with 650 µL of binding buffer A (pH 7.4), centrifuged at 4400 rpm for 1 minute. Sera and nasal fluid samples were diluted 1:1 with binding buffer before applying to the protein G spin column and centrifuged at 2600 rpm for 10 minutes. The column was washed three 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 neutralising buffer (pH 9.0) and 500 µL of elution buffer (pH 2.0) on top of the protein G mini plug, centrifuged at 4400 rpm for 1 minute. The IgG-depleted samples and the eluted bound IgG antibodies were collected separately and were measured for levels of IgG₄ using ImmunoCAP100 system (Phadia, Uppsala, Sweden) according to 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 (ThermoFisher, Paisley, UK).

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 diamine oxidase was assessed by flow cytometry. Phleum pretense allergen extract (0, 10 and 100 ng/mL) was incubated with nasal polyp homogenates for 15 min at 37°C. Cells were immunostained with
anti-human CD3, CD303, CD294 (CRTh2), CD203c, CD63 and CD107a (all BD Biosciences, San Jose, CA). Red blood cells were lysed with BD lysing solution (BD Biosciences, San Jose, CA) for 10 min at room temperature in the dark, samples were centrifuged (5min, 200g) and the supernatants discarded. Cells were fixed and permeabilised with BD Cytofix/Cytoperm™ (BD Biosciences, San Jose, CA). Cells were stained with fluorochrome (V500)-labelled DAO (BD Biosciences, San Jose, CA) for 30 min at 4ºC, washed and re-suspended in 450µL ice-cold fixative solution (BD Biosciences, San Jose, CA) prior to acquisition on the BD FACSCanto II flow cytometer. Analyses were performed using BD FACS Diva V6.1.1 software (BD Biosciences).

**IgE-Facilitated Antigen Binding**

The EBV-transformed B cell line expressing high levels of CD23 was maintained and characterised as previously described1, 2. Allergic or non-atopic IgE containing nasal polyp homogenates were pre-incubated with different concentrations of *P. pratense* at 37°C, 5% CO₂ for 1 h to form allergen-IgE complexes as described2. In parallel, various concentration of Staphylococcal Enterotoxin B (SEB) was pre-incubated with *P. pratense* at 37°C, 5% CO₂ for 1 h. Nasal polyps homogenates containing high levels of IgE concentrations were also heat-inactivated at 56ºC for 30 minutes. EBV-transformed B cells (1x10⁵/5µL and were added to allergen–IgE mixture and incubated for further 1 h at 4°C. Cells were washed followed by the addition of polyclonal human anti-IgE PE-labelled antibody (1:50 dilution for 45 min) to detect bound complexes by BD FACS Canto II flow cytometer and analysed using FACS Diva software. B cells were gated using forward scatter/side scatter parameters and a positive marker was set using cells incubated with indicator serum only. Unlabelled anti-human CD23 monoclonal antibody (500 mg/L; Clone MHM6) was used as blocking antibody for CD23-dependency experiments (Dakocytomation, Cambridge, UK).
T cells were enriched from PBMCs using magnetic isolation (Stemcell Technologies) as per manufacturer’s instructions. In brief, 5x10^7 cells were incubated with isolation cocktail for 5 min at room temperature, followed by addition of 40 µL/mL of rapidspheres, and adjusted to a final volume of 2.5 mL using MACS buffer. Serum from allergic subjects (20 µL) containing high levels of *P. pratense* specific IgE>30KU/L was pre-incubated with 0, 0.1, 1 and 10 µg/mL allergen (5 µL) at 37 °C for 1 h 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 hrs prior to co-culture with CD4⁺CD25⁻ T cells for 7 days. Proliferation of T cells was determined by tritiated thymidine (³H) incorporation. Frequency of *P. pratense*-specific IL-4⁺CD4⁺ and IL-5⁺CD4⁺ IL-17⁺CD4⁺ T cells was measured by FluoroSpot assay (Diaclone, Besancon, France).

References

Title: Broad Immunoglobulin G Repertoire in Chronic Rhinosinusitis with Nasal Polyps regulates pro-inflammatory IgE responses

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Table E1: Patient Demographics

<table>
<thead>
<tr>
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<th>Allergic CRSwNP (n=9)</th>
<th>Non-Allergic CRSwNP (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F:M)</td>
<td>3:6</td>
<td>9:0</td>
</tr>
<tr>
<td>Age (y), mean (±SEM)</td>
<td>38.11 (3.71)</td>
<td>52.2 (4.10)</td>
</tr>
<tr>
<td>Asthma</td>
<td>1/9</td>
<td>7/9</td>
</tr>
<tr>
<td>AERD</td>
<td>0/9</td>
<td>2/9</td>
</tr>
</tbody>
</table>

F = Female, M = Male, AERD = Aspirin-Exacerbated Respiratory Disease, Y = year, SEM = Standard error of the mean