Title: Allergen-specific IgG+ memory B cells are temporally linked to IgE memory responses

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Disclosure of potential conflict of interest

I. Hoof, T. Stranzl, L. H. Christensen, C. Lundegaard, J. Ahrenfeldt, J. Holm, P. S. Andersen are employees of ALK-Abelló. M. H. Shamji serves as a consultant for Imperial College London and receives lecture fees from ALK-Abelló, ASIT Biotech, Allergopharma, and UCB. S. R. Durham receives grant support from the Immune Tolerance Network, NIAID, ALK-Abelló Hørsholm, Regeneron, and Biotech Tools and serves as a consultant from Anergis, Circassia, Biomay, Merck, Allergy

Therapeutics, Med Update GmbH, and Food Standards. All other authors declare that they have no relevant conflicts of interest.

Key Messages

- Life-long persistence of allergy is underscored by the existence of allergen-specific IgG+ memory B cells that are prone to isotype switching and secretion of IgE.
- The fixed composition of the IgE repertoire during the first year of SLIT treatment provides evidence to why long-term immunotherapy is not associated with any signs of disease progression.

Capsule Summary

Evidence from clinical trials with sublingual immunotherapy supports that immunological IgE memory responses originate from allergen-specific IgG+ B cells.

Key words

Immunoglobulin E, Sublingual Immunotherapy, grass pollen allergy, B cells, plasmablasts, memory B cells.

Abbreviations

GC, germinal center; IgE, Immunoglobulin E; IgG, Immunoglobulin G; IgG_E, IgG+ memory B cells; mab, monoclonal antibody; SHM, Somatic hypermutations; SLIT, Sublingual Allergen Immunotherapy; VH, heavy chain variable gene.

ABSTRACT

Background: Immunoglobulin E (IgE) are least abundant, tightly regulated and IgE producing B cells are rare. The cellular origin and evolution of IgE responses are poorly understood.

Objective: To investigate the cellular and clonal origin of IgE memory responses following mucosal allergen exposure by sublingual immunotherapy (SLIT).

Methods: In a randomized double-blind, placebo-controlled, time-course SLIT study, peripheral blood mononuclear cells (PBMCs) and nasal biopsies were collected from forty adults with seasonal allergic rhinitis at baseline, 4, 8, 16, 28 and 52 weeks. RNA was extracted from PBMCs, sorted B cells and nasal biopsies for VH repertoire sequencing. Moreover, monoclonal antibodies were derived from single B cell transcriptomes.

Results: Combining VH repertoire sequencing and single cell transcriptomics yielded direct evidence of a parallel boost of two clonally and functionally related B cell subsets of short-lived IgE+ plasmablasts and IgG+ memory B cells (termed IgG_E). Mucosal grass pollen allergen exposure by SLIT resulted in highly diverse IgE and IgG_E repertoires. These were extensively mutated and appeared relative stable as per heavy chain isotype, somatic hypermutations and clonal composition. Single IgG_E + memory B cell and IgE+ pre-plasmablast transcriptomes encoded antibodies that were specific for major grass pollen allergens and were able to elicit basophil activation at very low allergen concentrations.

Conclusion: For the first time, we have shown that upon mucosal allergen exposure, human IgE memory resides in allergen-specific IgG+ memory B cells. These rapidly switch isotype and expand into short-lived IgE+ plasmablasts and serve as a potential target for therapeutic intervention.

INTRODUCTION

Allergic diseases are typically life-long and even in the absence of allergen exposure this phenomenon to occur requires some form of immunological memory. Current concepts on the cellular origin of IgE memory are primarily based on murine studies using various strains of transgenic mice.[1] It has been reported that IgG+ memory B cells are able to induce antigen-specific IgE memory responses when transferred into naïve hosts.[2,3] Although these studies do not exclude the possibility of long-lived IgE+ memory B cells, they confirm the importance of indirect isotype switching which leads to allergen-specific IgE responses. In contrast, one study reported a transfer of IgE memory responses by a subset of IgE+ B cells,[4] although it was later rectified to contain a mixed population of IgG+ and IgE+ B cells.[5] In general, studies have confirmed that IgE+ B cells have an impaired ability to enter germinal centers (GCs) leading to short-lived plasmablasts and absence of affinity maturation.[6,7] Similarly, IgE+ B cells are predisposed to differentiate into short-lived plasmablasts.[6,8] A more recent finding, using a murine model of peanut allergy, showed that allergen-specific IgG response precedes IgE response,[9] and expansion of allergen-specific IgG1+ memory B cells was accompanied by bone marrow reconstitution with IgE+ plasmablasts in mice re-challenged with allergen nine months after sensitization.[3] Taken together, mouse studies have provided convincing evidence for the role of IgG+ memory B cells in maintaining IgE memory responses. However, these findings have not yet been confirmed in allergic individuals. A recent study utilizing a validated and highly sensitive PCR-based methodology failed to identify IgE+ memory B cells in allergic patients,[9] and VH repertoire sequencing data are consistent with indirect switching to IgE from primarily IgG expressing B cells in humans.[10]

Moreover, observations from several clinical trials of grass pollen SLIT have shown an increase in IgE antibodies in serum that peaks in the first weeks of treatment followed by a gradual decline over time.[11–13] We therefore hypothesized that the transient increase in serum IgE during SLIT coincides with a clonal boost of migratory allergen-specific B cells in blood as previously demonstrated in a study of tetanus-toxoid vaccinations.[14] Here, we investigate the cellular and clonal origin of IgE memory responses using next generation sequencing (NGS) of total antibody heavy chain variable gene (VH) repertoires in combination with cell sorting techniques and single B cell transcriptomics.

METHODS

Clinical trial samples

The study (NCT02005627) was conducted at a single academic center, Imperial College London, and included recruitment of 40 adult patients with moderate to severe seasonal allergic rhinitis (see Repository Fig E1 for trial design and Table E1 for subject characteristics). The trial was a randomized double-blind, placebo-controlled, time course sublingual immunotherapy study (GRAZAX®, ALK-Abello Horsholm, Denmark). The trial protocol[15] and amendments were approved by the relevant ethics committees and institutional review boards. Written informed consent was obtained from all participants.

RNA extraction from PBMC, sorted cells and nasal biopsies

For the sampling time points Baseline, 4 weeks, 8 weeks, 16 weeks, 7 months and 12 months after SLIT treatment initiation, total RNA was purified from 20 million PBMCs and nasal biopsies using the RNeasy Mini kit (Qiagen) following the recommendations of the supplier. From sorted B cells, RNA was isolated using the RNeasy Mini kit if the sample contained more than 500,000 cells, otherwise the RNeasy Micro kit was used.

Immunoglobulin heavy chain sequencing and annotation

Amplification of the heavy chain V(D)J region, library preparation and high-throughput sequencing was performed by iRepertoire Inc (USA). The resulting sequences were trimmed and filtered for sequence quality, and paired-end reads were joined using PEAR v0.9.7.[16] Identical sequences were collapsed using fastx_collapser, a part of the FASTX Toolkit v0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Singleton sequences were discarded from further analysis. Isotype was assigned based

158 on the first 17 nucleotides of the constant region, and annotation of V family, J family,
159 CDR1, CDR2, and CDR3 was performed using migmap v0.9.8
160 (https://github.com/mikessh/migmap).[17] PCR cross-over events were removed by
161 discarding sequences that contributed with <5% to a given CDR3-defined clonotype.
162 VH sequences were clustered into clonal families using the DefineClones tool of the
163 Change-O package v0.3.9.[18] Sequences were assigned to the same clonal family if they
164 had the same V and J family annotation, if the CDR3 region had the same length and if
165 the sequence identity between the CDR3 regions was >90% on nucleotide level. An
166 IgE clonotype was defined as a clonal family that contains more than 50 IgE transcripts
167 at a given time point. A more sensitive transcription cut-off was chosen to define IgG_E
168 clonotypes: A clonal family was required to contain at least 10 IgE and at least 10 IgG
169 transcripts at any time point.
170
171 **B cell FACS sorting**
172 For B cell FACS sorting, PBMCs were stained with CD3 FITC, CD19 PE, IgD
173 PerCP/Cy5.5, CD38 PE-Cy7, CD138 APC and CD27 Pacific Blue. Live/Dead Fixable
174 Aqua Dead Cell Stain Kit (ThermoFisher, Waltham, MA, USA) was used to ensure
175 sorting of viable cells. Naïve B cells (CD19+, CD27-, IgD+), Memory B cells (Double
176 memory cells, CD19+CD27-IgD-; Classical memory, CD19+CD27+IgD-; and IgM
177 memory; CD19+,CD27+,IgD+) and plasmablasts (CD19+/low, CD27+, CD38+,
178 CD138-) populations were sorted into separate tubes. To prepare for single cell
179 transcriptomics, single memory B cells from patient D04 were gated as (IgE+, CD19+,
180 CD4-, CD8-) and sorted directly into 96-well PCR microtiter plates. Staining for
181 surface IgE appeared unspecific, likely reflecting surface-bound IgE complexes on non-
182 IgE memory B cells expressing the low affinity CD23 (FcεRII) receptor.

Single cell transcriptomics

The assay used to capture whole mRNA transcripts is adapted from the Smart-seq2 protocol.[19,20] Briefly, mRNA was captured using poly-dT oligos and directly reverse-transcribed into full-length cDNA using the described template-switching LNA oligo.[19,21] Whole transcriptome cDNA was amplified by PCR. Quality and quantity of cDNA amplification were assessed by capillary electrophoresis using Fragment Analyzer (Advance Analytical) and fluorescent dsDNA intercalating-dye based assay (Picogreen, Invitrogen). Before sequencing, all libraries were purified using AMPure XP beads (0.9:1). Samples were sequenced on the Illumina sequencing platform, HiSeq2500 (Illumina). Libraries generating a total of 172 million uniquely mapped reads (median of ~1.8 million total uniquely filtered mapped reads per cell).

Single-cell RNA-seq data were mapped against the human hg19 reference genome and UCSC gene models using TopHat (v1.4.1., -library-type fr-unstranded). The single-cell RNA-seq data was integrated with the single cell data from Croote et al.[22] using the R library Seurat.[23]

Antibody expression and characterization

Recombinant IgE antibodies were transiently expressed in HEK293 suspension cultures (Freestyle 293, Thermo Fisher Scientific, Waltham, MA, USA). Expression plasmids were custom made at Genscript (Piscataway, NJ, USA). Recombinant IgE antibodies were screened for specificity by SPR (Biacore 3000, GE-Healthcare). Basophil activation assays were done as previously described.[24]

Statistical Analysis

208 P-values were calculated by a two-sample Wilcoxon test using the "R" open source
209 software.

RESULTS

Sublingual allergen immunotherapy activates two subsets of IgE+ and IgG+ B cells of common clonal origin

To evaluate the IgE repertoire development, antibody responses were investigated using NGS of VH repertoires amplified by PCR in 21 subjects at baseline and after 4 weeks of grass SLIT-tablet (for information about sequencing depth see Repository Table E2). A cluster and isotype assignment analysis identified 998 IgE clonotypes derived from the 4 weeks samples during SLIT. Twenty-two percent of these IgE clonotypes clustered together with a minor population of IgG transcripts indicating common clonal origin as shown in Fig 1, *A*. This defines a specific subset of the IgG repertoire we here call IgG_E and is likely to share antigen-specificity with the IgE repertoire.

The level of IgE transcripts per sample at baseline was low and increased after 4 weeks of SLIT in accordance with a boost of migrating IgE+ B cells (Fig 1, *B*). IgG_E transcripts also increased in response to SLIT although to a lower level than IgE (Fig 1, *B*). Similarly, transcripts of individual IgE clonotypes (Fig 1, *C*), identified in both baseline and week 4 samples, increased in response to SLIT, and the same transcriptional increase was observed for individual IgG_E clonotypes (Fig 1, *C*). Most of the IgE clonotypes shared between baseline and 4 weeks samples were already switched to IgE at baseline (Fig 1, *A* and Repository Fig E2) indicating a pre-commitment to the IgE linage prior to allergen exposure. The level of somatic hypermutations (SHM) in IgE repertoires was similar to that of IgG, IgG_E, and IgA (Fig E3, *A*) in agreement with sequential isotype switching from IgM to IgG and then IgE. Furthermore, the average level of SHM in the IgE and IgG_E repertoires did not increase, even within individual

IgE clonotypes (Fig E3, *B*), despite the daily high-dose administration of grass pollen tablet for 4 weeks. This indicates that switching from IgG to IgE happens without further affinity maturation.

Stable composition of IgE repertoires during sublingual allergen immunotherapy

The effect of grass SLIT-tablet on IgE repertoire development during one year of treatment was investigated by analyzing longitudinal samples from three patients selected for high levels of IgE transcripts at week 4 (D04, D07, D16). VH transcripts for individual clonotypes tended to decline after 4 weeks of treatment indicating a reduced number of peripheral IgE+ B cells (Fig 2, *A*). Nonetheless, IgE repertoires remained diverse for over six months, and each time point had a substantial fraction of private as well as shared IgE clonotypes (Fig 2, *B*). The IgE isotype was conserved for most clonotypes throughout the six months of treatment. The IgE repertoire isolated at week 4 (i.e. at the peak of the serological IgE response) yielded the highest number of clonotypes which consistently constituted 51-52% of the repertoires at later time points (Fig 2, B, top row). This consistent re-sampling rate indicates a relatively fixed grass-tablet induced IgE repertoire. Sampling at later time points led to a progressively less efficient sampling of the IgE repertoire likely explained by a lower number of IgE producing B cells in the blood samples (i.e. contraction of the IgE repertoire) as evident by the gradual drop in IgE transcripts over time.

The parallel trajectories of the total levels of IgE and IgG_E VH transcripts further support simultaneous activation and co-evolution of two clonally related populations of IgE+ and IgG_E+ B cells (Fig 2, *C*). For both repertoires, SHM levels remained constant during treatment indicating no further affinity maturation (Fig 2, *D*). Thus, despite the daily exposure to allergen in the course of one year of SLIT, the cellular IgE memory

response, composed of proliferating IgE+ and IgG_E+ B cells, appeared relatively stable with no signs of isotype switching, clonal skewing or further mutagenesis.

Nasal and blood IgE repertoires are clonally related

To understand the relationship between antibody repertoires in blood and the nasal mucosa, VH repertoire sequencing was performed on nasal biopsies from 7 donors collected at baseline and after one year of treatment. Relative to blood, nasal biopsies contained a larger fraction of IgA transcripts compared to matching samples collected from blood (Fig 3, *A*).

IgE transcripts were present in all nasal biopsies except one, but at lower levels and comparable to blood baseline samples (Fig 3, *B*). In accordance, IgE repertoire diversity, i.e. the number of clonotypes per sample (Fig 3, *C*), and the transcript level of individual IgE clonotypes (Fig 3, *D*) were low and comparable to blood baseline values. There was a clear clonal relationship between blood and nasal IgE repertoires (Fig 3, *E*) which increased at week 4 during SLIT. Similar to the blood repertoire, nasal repertoires contained a significant and consistent fraction of IgG_E (Fig 3, *E* and Repository Fig E4). Thus, the IgE and IgG_E memory responses in blood, induced by oral allergen provocation, appeared closely associated to the quiescent nasal IgE repertoire.

The IgE memory response contains a transcriptionally heterogeneous population of memory B cells

To understand the cellular origin of the IgE repertoire in the periphery, VH repertoires were analyzed from sorted subsets of naïve B cells (CD20+, IgD+, CD38-), memory B cells (CD20+, IgD-, CD38-) and plasmablasts ($CD20^{low}$, IgD-, CD38+) collected at

week 4 (Fig 4, *A-B*). Naïve B cells were evenly distributed in a multitude of small clusters of IgM or IgD isotypes whereas plasmablasts were of relatively large sized clusters with all isotypes represented (except IgD) in accordance with a repertoire shaped by clonal expansion. Memory B cells contained a multitude of small clusters of all isotypes but were dominated by a few large IgE clonotypes (Fig 4, *B*).

Transcriptomic profiling of single grass-specific IgG+ memory B cells and IgE+ pre-plasmablasts.

To address the observed transcriptional heterogeneity in the memory B cell compartment, we performed indexed single cell FACS sorting followed by single-cell transcriptomic profiling of single memory B cells of subject D04 week 4 sample (experimental flow outlined in Fig 5, *A*).

Transcriptomic data were integrated with a reference dataset of 973 single cells prepared from CD19+ B cells[22] and clustered by tSNE analysis of normalized gene expression counts (Fig 5, *B*). Most of the sorted memory B cells (85/93) had the expected naïve/memory phenotype. However, 8 cells clustered as plasmablasts indicating phenotypic heterogeneity within the population of sorted memory B cells. The expression of signature genes of the memory/naïve (*MS4A1* and *IRF8*) and plasmablast (*PRDM1* and *IRF4*) populations was consistent with previous reports[22] (Repository Fig E5). In accordance with the negative selection for CD38 expression in the FACS sorting protocol, the 8 cells with a plasmablast phenotype differed from the reference population in CD38 expression (Fig 5, *C*). Two transcriptomes contained productive IgE transcripts, and they both clustered with the plasmablast-like subset of cells. Thus, it appears that the CD38- CD27+ pre-plasmablast population is enriched in IgE+ cells explaining the large number of IgE transcripts in sorted memory B cells.

For 64 single cell transcriptomes, it was possible to retrieve the full sequence of cognate pairs of antibody heavy and light chain variable region genes. These cognate pairs were aligned to the total antibody VH repertoires (Fig 5, *D*) which allowed for selection of 11 antibody sequences based on clonal relationship to IgE and/or IgG clonotypes. An additional 5 antibodies were selected based on the presence of sterile germline transcripts of the IGHE locus (GLTε)[22] indicative of active involvement in TH2 inflammatory response.[25] Fourteen of the 16 antibodies bound to grass extract and were mostly specific for major allergens (Table 1).

Antibodies were of high affinity and able to trigger basophil activation at very low concentration when combined (Fig 5, *E*). Eleven transcriptomes of the memory/naïve phenotype encoded allergen-specific antibodies of the IgG1, IgG2 and IgG4 isotypes which in 7 cases were co-expressed as IgE according to VH repertoire sequencing thereby demonstrating a direct link between ongoing IgE memory response and allergen-specific IgG memory B cells. Interestingly, most of the selected B cell transcriptomes contained CD23 transcripts (15/16) and 10/12 of the GLTε+ transcriptomes encoded allergen-specific antibodies. Further, the co-existence of clonally related IgG_E+ memory B cells and IgE+ plasmablasts agrees with the difference in transcriptional levels (IgE>>IgG_E; Fig 1, *B*) and the slower kinetics in synthesis of allergen specific IgG relative to IgE in the early phases of SLIT (Repository Fig E6).

DISCUSSION

We demonstrate for the first time that the serological increase in allergen-specific IgE following mucosal allergen exposure was accompanied by a cellular boost of IgE producing plasmablasts in blood. We observed high levels of IgE transcripts in sorted populations of plasmablasts (CD19+, CD20low, CD27+ CD38+) and identified single allergen-specific B cells with a CD38 negative pre-plasmablast phenotype in sorted memory B cells (CD19+, CD20+, CD27+ CD38-). Stimulation of human B cells *ex vivo* shows the emergence of plasmablast-like IgE+ B cells (Blimp-1+, IgE^{high}, CD38) from PBMCs of allergic patients after 5-7 days of co-culture with allergen[26] and IgE+ B cells ($CFSE^{low}CD19^{mid}$, $CD27^{high}$) from tonsils after co-stimulation with IL-4 and anti-CD40 antibody.[8] Both of these phenotypes were compatible with IgE+ pre-plasmablast and plasmablast phenotypes. The simultaneous drop in serum titers and IgE transcripts suggests that the human IgE+ plasmablasts are short-lived. Together with the absence of accumulation of SHMs, this parallels observations in mice, where IgE+ plasma cells were short-lived and showed reduced affinity maturation, presumably due to a transient and incomplete GC phase.[6]

Such extra-follicular formation of IgE memory responses[27] could explain why allergic diseases progress slowly, in particular in adulthood, due to a slowly evolving IgE repertoire. Longitudinal studies with samples taken years apart have demonstrated that IgE repertoires in allergic subjects are oligoclonal and persist over time.[28] Similarly, it has been demonstrated that IgE repertoires sampled in two birch pollen seasons are overlapping.[29] We also observed such persistence of the IgE repertoire demonstrated by the limited clonal evolution, by the overlap between SLIT induced blood IgE repertoires and nasal repertoires taken 11 months apart, and by the absence of further isotype switching of the IgE repertoire. Considering the daily exposure to high doses

of allergen for one year, this implies that allergen-exposure, as such, is not the main cause for diversification of the IgE repertoire.

We isolated single IgG+ memory B cells at the peak of the IgE memory response that encoded antibodies specific for the major grass allergens and belonged to clonotypes simultaneously expressing IgE. Considering the high fraction of allergen-specific antibodies, at least 14 out of 67 cognate VH/VL pairs, in this population of memory B cells selected solely on phenotypic markers, the observed co-expression of CD23 and GLTε appears as a potential marker for memory B cells involved in IgE responses. Both markers are known to be under STAT-6 control and induced by IL-4.[30,31] It remains to be determined if these “TH2-polarized” memory B cells are present in a "quiescent" state or the result of the daily exposure to allergen during SLIT. Further, the high frequency of allergen-specific B cells reported here contrasts all previous studies in allergic patients, which typically report very low prevalence of allergen-specific B cells.[22,32–36] The high level of IgE transcripts (15%) in the PBMC fraction of subject D04 was in accordance with the observation that 10 of the top 100 clonotypes in the plasmablast sorted fraction were IgE producing.

Are those IgG_E+ memory B cells the source of the IgE+ plasmablast response, and hence the provenance of IgE memory? Several observations support this notion: i) Single cell transcriptomic analysis showed an equal representation of IgG_E+ memory B cells and IgE+ plasmablasts and the absence of IgE+ memory B cells. ii) Most (9/11) of the allergen-specific IgG_E+ memory B cells contained GLTε transcripts pointing to recent exposure to T_H2 cytokines, such as IL-4, and thereby active involvement in the ongoing allergic inflammation. iii) The upregulation of IgG_E+ memory B cells coincided with the increase in specific IgE titers and was misaligned with the much

later increase in allergen-specific serum IgG, and hence not associated with a concurrent IgG_E+ plasmablast response. iv) IgE transcript levels were consistently higher than levels for IgG_E supporting that it is preferentially isotype-switched IgE+ B cells that leave the memory state and differentiate into plasmablasts. v) The similar and constant levels of SHM in IgE and IgG_E repertoires, even within clonotypes, indicate isotype switching outside germinal centers and hence absence of affinity maturation. Thus, allergen-specific IgG+ memory B cells, capable of rapid extra-follicular isotype switching to IgE, are likely the progenitors of the IgE-secreting plasmablasts forming the serological IgE memory response at the site of inflammation, as recently proposed by Gould and colleagues.[1] Moreover, the observation of high ratios of allergen-specific to total IgE and low levels of IgG in the nasal mucosa of rhinitis patients corroborates these findings.[37]

One important question remains: Do long lived IgE+ memory B cells exist? The low levels of IgE transcripts at baseline in blood and nasal samples could represent a rare population of long-lived IgE+ memory B cells giving rise to the subsequent IgE plasmablast response whereas long-lived plasma-cells are not likely to be found in blood.[38] Considering the simultaneous presence of IgG_E transcripts in these baseline samples, such IgE transcripts could also be the result of homeostatic self-renewal of IgG_E+ memory B cells turning into IgE+ plasmablasts by microbial products or bystander T cell help.[39] Further, such allergen-specific plasma cells in allergic subjects have previously been identified,[8,22] and B cell cultures from allergic, but not from healthy donors, expressed IgE by T cell bystander activation suggesting differences in the state of activation of memory B cells in these donors.[40] In further support, mouse

studies suggest that lifelong food allergy is the consequence of recurrent activation of memory B cells leading to relatively short-lived plasma cells.[3]

IgE class switching can occur directly from IgM to IgE or from sequential rearrangements via IgG1, IgG2 or IgG4.[7,10,41] The intermittent IgG phase allows for affinity maturation and was proposed as the mechanism involved in the production of affinity-matured IgE antibodies in memory responses.[7,42] Prior studies using deep sequencing of human IgE repertoires show that IgE VH genes are most closely related to clonal lineages of IgG, particularly IgG1, and share extensive patterns of hypermutation with this isotype.[10,33] In agreement, peanut allergen-specific antibodies isolated from antigen-specific B cells were in most cases derived from class-switched cells expressing IgG.[35] We found direct evidence of indirect switching by identifying single IgG1+, IgG2+ and IgG4+ memory B cells expressing allergen-specific antibodies which were simultaneously expressed as clonal variants in the IgE repertoire. Since IgE clonal families often were of the same lineage as IgG, showed no clonal relationship to IgM and contained SHM at levels comparable to IgG, we conclude that direct switching from IgM to IgE has an insignificant role in allergen-specific IgE memory responses. Our previous work showed indirect evidence, switching from all IgG subclasses and less from IgM,[43] and we now prove the inferred antibody production. Similar to others,[10,33,35] a limitation of the current study is that it cannot formally exclude the existence of IgE+ memory B cells given the limited sampling depth of single-cell transcriptomics. Second, the daily exposure of high allergen doses might lead to different cellular dynamics than the daily low-level exposure during a pollen season. Thus, IgE memory responses induced by sublingual application of SLIT tablets might not fully represent a memory response to natural allergen exposure considering that the end-result of SLIT is clinical tolerance.

Our findings have several clinical implications. The relatively fixed composition of the IgE repertoire during the first year of SLIT demonstrates why long-term immunotherapy is not associated with any signs of disease progression, such as *de novo* sensitizations. Moreover, we demonstrate that antigen exposure *per se* is not a driving factor for IgE repertoire diversification. Finally, the existence of a distinct population of allergen-specific IgG+ memory B cells, prone to isotype switching and IgE secretion, can explain the life-long persistence of allergy and is an obvious new target for therapeutic intervention.

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585 **Figure captions**
586
587 **Figure 1. Early clonal development of the IgE memory response at baseline and**
588 **after 4 weeks during grass SLIT. (A)** Transcript level and isotype distribution of the
589 100 most frequent IgG_E clonotypes. Each vertical bar in the large panels indicates the
590 transcriptional level of individual clonotypes and is colored according to isotype
591 distribution. Data are sorted according to clonotype transcript levels at week 4.
592 Compressed panels below show the isotype distribution within each clonotype.
593 Horizontal placement indicates clonal relationship between time points. **(B)** Total levels
594 of IgE and IgG_E transcripts of individual donors before and during treatment. **(C)**
595 Transcript levels of individual IgE clonotypes identified at both baseline and week 4
596 and for the whole set of identified IgG_E clonotypes. **(D)** Number of IgE and IgG_E
597 clonotypes identified per donor.
598
599 **Figure 2. Longitudinal development of the IgE memory response during one year**
600 **of grass SLIT. (A)** Transcript level and isotype distribution of all IgE clonotypes from
601 3 donors at multiple time points, as indicated. Clonotypes are sorted according to
602 transcript levels at week 4. Horizontal placement indicates clonal relationship between
603 time points. **(B)** Proportion of IgE clonotypes that are shared between a reference time
604 point (full dark purple pie) and all other time points during six months of SLIT. In each
605 row a different time point serves as reference and arrows indicate the direction of
606 comparison. Dark purple coloring denotes the proportion of shared IgE clonotypes and
607 pie size is log-proportional to the IgE repertoire size at the given time point. Clonotypes
608 from 3 donors were pooled. **(C)** Longitudinal analysis of the total transcript levels of
609 IgE and IgG_E. Each line represents the response of a single subject. **(D)** The average
610 frequency of somatic hypermutations for IgE clonotypes that were identified at a

minimum of 3 time points (upper panel) and, in the case of IgG_E, of all clonotypes belonging to the IgG_E repertoires at each time point (lower panel). Each dot represents the average nonsynonymous mutation rate among transcripts contained in any particular clonotype.

Figure 3. The IgE repertoire of the nasal mucosa from 7 grass patients at baseline and after 12 months of grass SLIT-tablet treatment. (A) Isotype distribution profiles of pooled nasal antibody repertoires from the 7 donors. The corresponding data from pooled blood PBMC repertoires of these seven donors at baseline is shown for comparison (left column). **(B)** Transcript levels of total IgE of individual donors in nasal biopsies and blood. **(C)** Number of IgE clonotypes per nasal sample in comparison to matched blood baseline from the same donors. **(D)** Transcript levels of individual IgE clonotypes in nasal biopsies and blood (at baseline) as indicated. **(E)** Pooled analysis of the overlap of IgE repertoire from the seven selected donors. Overlap in IgE clonotype usage (dark colors) of the nasal repertoires and the blood at baseline (upper row) or at the blood IgE peak point of 4 weeks into SLIT (lower row). The total number of identified IgE clonotypes at each time point is indicated in brackets.

Figure 4. Cellular phenotypes of IgE memory B cell responses. (A) FACS sorting of PBMCs of patient D04 collected at week 4. Cells were bulk sorted using phenotypic markers for naïve B cells, plasmablasts and memory B cells as indicated. **(B)** VH repertoire sequencing of the FACS sorted populations. Waterfall plot of the transcriptional levels and isotype distribution for individual clonotypes. Only the 100 most frequent clonotypes are shown for each sample of sorted cells.

Figure 5. Single cell transcriptomic profiling of memory B cells sorted from donor D04 at week 4 during SLIT. (A) Single cell sequencing and analysis workflow. **(B)** A pooled tSNE analysis combining the 93 single cell transcriptomes of subject D04 (black dots) with a reference data set of 973 B cell transcriptomes (grey dots)).[22] The two transcriptomes with productive and IgE rearrangements are labeled in purple and marked by red arrows, remaining grass-specific antibodies are labeled in orange. **(C)** Transcription levels of the plasmablast marker CD38 in all transcriptomes belonging to the plasma cell cluster in the tSNE analysis in comparison to the CD38 levels in plasma cells of the reference data set. **(D)** Transcript levels and isotype distribution in VH antibody repertoires, at different time points, of individual VH genes identified by both bulk repertoire sequencing and single cell transcriptomics. **(E)** Allergen-induced activation of basophils passively coated with 3 different mixtures of purified monoclonal antibodies (mab) according to specificity. (Blue line: mix of 6 Phl p 5 specific mabs. Red line: mix of 6 Phl p 6 specific mabs. Green line: mix of all 11 mabs). Overlapping grey lines show the activity of the individual 11 mabs.

Table 1 Summary of data on monoclonal antibody (MAB) clones.

	MAB03	MAB07	MAB13	MAB17	MAB36	MAB38	MAB40	MAB41	MAB44	MAB46	MAB58	MAB77	MAB78	MAB83	MAB93	MAB94
Single cell transcriptome																
Isotype	IgG4	IgG2	IgG1	IgE	IgG1	IgG2	IgG1	IgG1	IgG1	IgG2	IgG1	IgG1	IgG1	IgE	IgG1	IgG4
CD23 transcript count	426	347	2438	475	842	1	826	262	1275	3179	1477	494	956	0	928	780
Sterile GLTe	-	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Phenotype	MEM	MEM	MEM	PB	PB	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	PB	MEM	MEM
Bulk sequencing																
Isotypes	IgE	IgE	IgE	IgE	-	IgE	-	-	IgE/IgG	-	IgE/IgG	IgE/IgG	-	IgE/IgG	IgG	IgE/IgA
Specificity																
Phl p extract	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
Phl p 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Phl p 5	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+	-
Phl p 6	-	-	+	-	+	-	-	-	-	+	-	+	-	+	-	-

Allergen-specific clones are shaded in grey. Phl p: Phleum pratense, Ig: Immunoglobulin