1 **Title: Allergen-specific IgG+ memory B cells are temporally linked to IgE**
2 **memory responses**
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43

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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.

75 **ABSTRACT**

76 **Background:** Immunoglobulin E (IgE) are least abundant, tightly regulated and IgE
77 producing B cells are rare. The cellular origin and evolution of IgE responses are poorly
78 understood.
79 **Objective:** To investigate the cellular and clonal origin of IgE memory responses
80 following mucosal allergen exposure by sublingual immunotherapy (SLIT).
81 **Methods:** In a randomized double-blind, placebo-controlled, time-course SLIT study,
82 peripheral blood mononuclear cells (PBMCs) and nasal biopsies were collected from
83 forty adults with seasonal allergic rhinitis at baseline, 4, 8, 16, 28 and 52 weeks. RNA
84 was extracted from PBMCs, sorted B cells and nasal biopsies for VH repertoire
85 sequencing. Moreover, monoclonal antibodies were derived from single B cell
86 transcriptomes.
87 **Results:** Combining VH repertoire sequencing and single cell transcriptomics yielded
88 direct evidence of a parallel boost of two clonally and functionally related B cell subsets
89 of short-lived IgE+ plasmablasts and IgG+ memory B cells (termed IgG_E). Mucosal
90 grass pollen allergen exposure by SLIT resulted in highly diverse IgE and IgG_E
91 repertoires. These were extensively mutated and appeared relative stable as per heavy
92 chain isotype, somatic hypermutations and clonal composition. Single IgG_E + memory
93 B cell and IgE+ pre-plasmablast transcriptomes encoded antibodies that were specific
94 for major grass pollen allergens and were able to elicit basophil activation at very low
95 allergen concentrations.
96 **Conclusion:** For the first time, we have shown that upon mucosal allergen exposure,
97 human IgE memory resides in allergen-specific IgG+ memory B cells. These rapidly
98 switch isotype and expand into short-lived IgE+ plasmablasts and serve as a potential
99 target for therapeutic intervention.

100 **INTRODUCTION**

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

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

133 **METHODS**

134 **Clinical trial samples**

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

143

144 **RNA extraction from PBMC, sorted cells and nasal biopsies**

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

150

151 **Immunoglobulin heavy chain sequencing and annotation**

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

on the first 17 nucleotides of the constant region, and annotation of V family, J family, CDR1, CDR2, and CDR3 was performed using migmap v0.9.8 (https://github.com/mikessh/migmap).[17] PCR cross-over events were removed by discarding sequences that contributed with <5% to a given CDR3-defined clonotype. VH sequences were clustered into clonal families using the DefineClones tool of the Change-O package v0.3.9.[18] Sequences were assigned to the same clonal family if they had the same V and J family annotation, if the CDR3 region had the same length and if the sequence identity between the CDR3 regions was >90% on nucleotide level. An IgE clonotype was defined as a clonal family that contains more than 50 IgE transcripts at a given time point. A more sensitive transcription cut-off was chosen to define IgG$_E$ clonotypes: A clonal family was required to contain at least 10 IgE and at least 10 IgG transcripts at any time point.

B cell FACS sorting

For B cell FACS sorting, PBMCs were stained with CD3 FITC, CD19 PE, IgD PerCP/Cy5.5, CD38 PE-Cy7, CD138 APC and CD27 Pacific Blue. Live/Dead Fixable Aqua Dead Cell Stain Kit (ThermoFisher, Waltham, MA, USA) was used to ensure sorting of viable cells. Naïve B cells (CD19+, CD27-, IgD+), Memory B cells (Double memory cells, CD19+CD27-IgD-; Classical memory, CD19+CD27+IgD-; and IgM memory; CD19+,CD27+,IgD+) and plasmablasts (CD19+/low, CD27+, CD38+, CD138-) populations were sorted into separate tubes. To prepare for single cell transcriptomics, single memory B cells from patient D04 were gated as (IgE+, CD19+, CD4-, CD8-) and sorted directly into 96-well PCR microtiter plates. Staining for surface IgE appeared unspecific, likely reflecting surface-bound IgE complexes on non-IgE memory B cells expressing the low affinity CD23 (FcεRII) receptor.

183

184 **Single cell transcriptomics**

185 The assay used to capture whole mRNA transcripts is adapted from the Smart-seq2
186 protocol.[19,20] Briefly, mRNA was captured using poly-dT oligos and directly reverse-
187 transcribed into full-length cDNA using the described template-switching LNA
188 oligo.[19,21] Whole transcriptome cDNA was amplified by PCR. Quality and quantity of
189 cDNA amplification were assessed by capillary electrophoresis using Fragment
190 Analyzer (Advance Analytical) and fluorescent dsDNA intercalating-dye based assay
191 (Picogreen, Invitrogen). Before sequencing, all libraries were purified using AMPure
192 XP beads (0.9:1). Samples were sequenced on the Illumina sequencing platform,
193 HiSeq2500 (Illumina). Libraries generating a total of 172 million uniquely mapped
194 reads (median of ~1.8 million total uniquely filtered mapped reads per cell).
195 Single-cell RNA-seq data were mapped against the human hg19 reference genome and
196 UCSC gene models using TopHat (v1.4.1., -library-type fr-unstranded). The single-cell
197 RNA-seq data was integrated with the single cell data from Croote et al.[22] using the R
198 library Seurat.[23]

199

200 **Antibody expression and characterization**

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

206

207 **Statistical Analysis**

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

210 **RESULTS**

211

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

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

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

235 IgE clonotypes (Fig E3, *B*), despite the daily high-dose administration of grass pollen
236 tablet for 4 weeks. This indicates that switching from IgG to IgE happens without
237 further affinity maturation.
238
239 **Stable composition of IgE repertoires during sublingual allergen immunotherapy**
240 The effect of grass SLIT-tablet on IgE repertoire development during one year of
241 treatment was investigated by analyzing longitudinal samples from three patients
242 selected for high levels of IgE transcripts at week 4 (D04, D07, D16). VH transcripts
243 for individual clonotypes tended to decline after 4 weeks of treatment indicating a
244 reduced number of peripheral IgE+ B cells (Fig 2, *A*). Nonetheless, IgE repertoires
245 remained diverse for over six months, and each time point had a substantial fraction of
246 private as well as shared IgE clonotypes (Fig 2, *B*). The IgE isotype was conserved for
247 most clonotypes throughout the six months of treatment. The IgE repertoire isolated at
248 week 4 (i.e. at the peak of the serological IgE response) yielded the highest number of
249 clonotypes which consistently constituted 51-52% of the repertoires at later time points
250 (Fig 2, B, top row). This consistent re-sampling rate indicates a relatively fixed grass-
251 tablet induced IgE repertoire. Sampling at later time points led to a progressively less
252 efficient sampling of the IgE repertoire likely explained by a lower number of IgE
253 producing B cells in the blood samples (i.e. contraction of the IgE repertoire) as evident
254 by the gradual drop in IgE transcripts over time.
255 The parallel trajectories of the total levels of IgE and IgG_E VH transcripts further
256 support simultaneous activation and co-evolution of two clonally related populations of
257 IgE+ and IgG_E+ B cells (Fig 2, *C*). For both repertoires, SHM levels remained constant
258 during treatment indicating no further affinity maturation (Fig 2, *D*). Thus, despite the
259 daily exposure to allergen in the course of one year of SLIT, the cellular IgE memory

260 response, composed of proliferating IgE+ and IgG_E+ B cells, appeared relatively stable
261 with no signs of isotype switching, clonal skewing or further mutagenesis.
262

263 **Nasal and blood IgE repertoires are clonally related**

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

269 IgE transcripts were present in all nasal biopsies except one, but at lower levels and
270 comparable to blood baseline samples (Fig 3, *B*). In accordance, IgE repertoire
271 diversity, i.e. the number of clonotypes per sample (Fig 3, *C*), and the transcript level
272 of individual IgE clonotypes (Fig 3, *D*) were low and comparable to blood baseline
273 values. There was a clear clonal relationship between blood and nasal IgE repertoires
274 (Fig 3, *E*) which increased at week 4 during SLIT. Similar to the blood repertoire, nasal
275 repertoires contained a significant and consistent fraction of IgG_E (Fig 3, *E* and
276 Repository Fig E4). Thus, the IgE and IgG_E memory responses in blood, induced by
277 oral allergen provocation, appeared closely associated to the quiescent nasal IgE
278 repertoire.
279

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

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

285 week 4 (Fig 4, *A-B*). Naïve B cells were evenly distributed in a multitude of small
286 clusters of IgM or IgD isotypes whereas plasmablasts were of relatively large sized
287 clusters with all isotypes represented (except IgD) in accordance with a repertoire
288 shaped by clonal expansion. Memory B cells contained a multitude of small clusters of
289 all isotypes but were dominated by a few large IgE clonotypes (Fig 4, *B*).
290

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

293 To address the observed transcriptional heterogeneity in the memory B cell
294 compartment, we performed indexed single cell FACS sorting followed by single-cell
295 transcriptomic profiling of single memory B cells of subject D04 week 4 sample
296 (experimental flow outlined in Fig 5, *A*).
297 Transcriptomic data were integrated with a reference dataset of 973 single cells
298 prepared from CD19+ B cells[22] and clustered by tSNE analysis of normalized gene
299 expression counts (Fig 5, *B*). Most of the sorted memory B cells (85/93) had the
300 expected naïve/memory phenotype. However, 8 cells clustered as plasmablasts
301 indicating phenotypic heterogeneity within the population of sorted memory B cells.
302 The expression of signature genes of the memory/naïve (*MS4A1* and *IRF8*) and
303 plasmablast (*PRDM1* and *IRF4*) populations was consistent with previous reports[22]
304 (Repository Fig E5). In accordance with the negative selection for CD38 expression in
305 the FACS sorting protocol, the 8 cells with a plasmablast phenotype differed from the
306 reference population in CD38 expression (Fig 5, *C*). Two transcriptomes contained
307 productive IgE transcripts, and they both clustered with the plasmablast-like subset of
308 cells. Thus, it appears that the CD38- CD27+ pre-plasmablast population is enriched in
309 IgE+ cells explaining the large number of IgE transcripts in sorted memory B cells.

310 For 64 single cell transcriptomes, it was possible to retrieve the full sequence of
311 cognate pairs of antibody heavy and light chain variable region genes. These cognate
312 pairs were aligned to the total antibody VH repertoires (Fig 5, *D*) which allowed for
313 selection of 11 antibody sequences based on clonal relationship to IgE and/or IgG
314 clonotypes. An additional 5 antibodies were selected based on the presence of sterile
315 germline transcripts of the IGHE locus (GLTε)[22] indicative of active involvement in
316 TH2 inflammatory response.[25] Fourteen of the 16 antibodies bound to grass extract and
317 were mostly specific for major allergens (Table 1).
318 Antibodies were of high affinity and able to trigger basophil activation at very low
319 concentration when combined (Fig 5, *E*). Eleven transcriptomes of the memory/naïve
320 phenotype encoded allergen-specific antibodies of the IgG1, IgG2 and IgG4 isotypes
321 which in 7 cases were co-expressed as IgE according to VH repertoire sequencing
322 thereby demonstrating a direct link between ongoing IgE memory response and
323 allergen-specific IgG memory B cells. Interestingly, most of the selected B cell
324 transcriptomes contained CD23 transcripts (15/16) and 10/12 of the GLTε+
325 transcriptomes encoded allergen-specific antibodies. Further, the co-existence of
326 clonally related IgG_E+ memory B cells and IgE+ plasmablasts agrees with the
327 difference in transcriptional levels (IgE>>IgG_E; Fig 1, *B*) and the slower kinetics in
328 synthesis of allergen specific IgG relative to IgE in the early phases of SLIT (Repository
329 Fig E6).
330

331 **DISCUSSION**

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

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

356 of allergen for one year, this implies that allergen-exposure, as such, is not the main
357 cause for diversification of the IgE repertoire.
358

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

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

381 later increase in allergen-specific serum IgG, and hence not associated with a
382 concurrent IgG_E+ plasmablast response. iv) IgE transcript levels were consistently
383 higher than levels for IgG_E supporting that it is preferentially isotype-switched IgE+ B
384 cells that leave the memory state and differentiate into plasmablasts. v) The similar and
385 constant levels of SHM in IgE and IgG_E repertoires, even within clonotypes, indicate
386 isotype switching outside germinal centers and hence absence of affinity maturation.
387 Thus, allergen-specific IgG+ memory B cells, capable of rapid extra-follicular isotype
388 switching to IgE, are likely the progenitors of the IgE-secreting plasmablasts forming
389 the serological IgE memory response at the site of inflammation, as recently proposed
390 by Gould and colleagues.[1] Moreover, the observation of high ratios of allergen-specific
391 to total IgE and low levels of IgG in the nasal mucosa of rhinitis patients corroborates
392 these findings.[37]
393
394 One important question remains: Do long lived IgE+ memory B cells exist? The low
395 levels of IgE transcripts at baseline in blood and nasal samples could represent a rare
396 population of long-lived IgE+ memory B cells giving rise to the subsequent IgE
397 plasmablast response whereas long-lived plasma-cells are not likely to be found in
398 blood.[38] Considering the simultaneous presence of IgG_E transcripts in these baseline
399 samples, such IgE transcripts could also be the result of homeostatic self-renewal of
400 IgG_E+ memory B cells turning into IgE+ plasmablasts by microbial products or
401 bystander T cell help.[39] Further, such allergen-specific plasma cells in allergic subjects
402 have previously been identified,[8,22] and B cell cultures from allergic, but not from
403 healthy donors, expressed IgE by T cell bystander activation suggesting differences in
404 the state of activation of memory B cells in these donors.[40] In further support, mouse

405 studies suggest that lifelong food allergy is the consequence of recurrent activation of
406 memory B cells leading to relatively short-lived plasma cells.[3]
407 IgE class switching can occur directly from IgM to IgE or from sequential
408 rearrangements via IgG1, IgG2 or IgG4.[7,10,41] The intermittent IgG phase allows for
409 affinity maturation and was proposed as the mechanism involved in the production of
410 affinity-matured IgE antibodies in memory responses.[7,42] Prior studies using deep
411 sequencing of human IgE repertoires show that IgE VH genes are most closely related
412 to clonal lineages of IgG, particularly IgG1, and share extensive patterns of
413 hypermutation with this isotype.[10,33] In agreement, peanut allergen-specific antibodies
414 isolated from antigen-specific B cells were in most cases derived from class-switched
415 cells expressing IgG.[35] We found direct evidence of indirect switching by identifying
416 single IgG1+, IgG2+ and IgG4+ memory B cells expressing allergen-specific
417 antibodies which were simultaneously expressed as clonal variants in the IgE repertoire.
418 Since IgE clonal families often were of the same lineage as IgG, showed no clonal
419 relationship to IgM and contained SHM at levels comparable to IgG, we conclude that
420 direct switching from IgM to IgE has an insignificant role in allergen-specific IgE
421 memory responses. Our previous work showed indirect evidence, switching from all
422 IgG subclasses and less from IgM,[43] and we now prove the inferred antibody
423 production. Similar to others,[10,33,35] a limitation of the current study is that it cannot
424 formally exclude the existence of IgE+ memory B cells given the limited sampling
425 depth of single-cell transcriptomics. Second, the daily exposure of high allergen doses
426 might lead to different cellular dynamics than the daily low-level exposure during a
427 pollen season. Thus, IgE memory responses induced by sublingual application of SLIT
428 tablets might not fully represent a memory response to natural allergen exposure
429 considering that the end-result of SLIT is clinical tolerance.

430

431 Our findings have several clinical implications. The relatively fixed composition of the
432 IgE repertoire during the first year of SLIT demonstrates why long-term
433 immunotherapy is not associated with any signs of disease progression, such as *de novo*
434 sensitizations. Moreover, we demonstrate that antigen exposure *per se* is not a driving
435 factor for IgE repertoire diversification. Finally, the existence of a distinct population
436 of allergen-specific IgG+ memory B cells, prone to isotype switching and IgE secretion,
437 can explain the life-long persistence of allergy and is an obvious new target for
438 therapeutic intervention.
439

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584

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

611 minimum of 3 time points (upper panel) and, in the case of IgG_E, of all clonotypes
612 belonging to the IgG_E repertoires at each time point (lower panel). Each dot represents
613 the average nonsynonymous mutation rate among transcripts contained in any
614 particular clonotype.
615
616 **Figure 3. The IgE repertoire of the nasal mucosa from 7 grass patients at baseline**
617 **and after 12 months of grass SLIT-tablet treatment. (A)** Isotype distribution profiles
618 of pooled nasal antibody repertoires from the 7 donors. The corresponding data from
619 pooled blood PBMC repertoires of these seven donors at baseline is shown for
620 comparison (left column). **(B)** Transcript levels of total IgE of individual donors in
621 nasal biopsies and blood. **(C)** Number of IgE clonotypes per nasal sample in
622 comparison to matched blood baseline from the same donors. **(D)** Transcript levels of
623 individual IgE clonotypes in nasal biopsies and blood (at baseline) as indicated. **(E)**
624 Pooled analysis of the overlap of IgE repertoire from the seven selected donors. Overlap
625 in IgE clonotype usage (dark colors) of the nasal repertoires and the blood at baseline
626 (upper row) or at the blood IgE peak point of 4 weeks into SLIT (lower row). The total
627 number of identified IgE clonotypes at each time point is indicated in brackets.
628
629 **Figure 4. Cellular phenotypes of IgE memory B cell responses. (A)** FACS sorting
630 of PBMCs of patient D04 collected at week 4. Cells were bulk sorted using phenotypic
631 markers for naïve B cells, plasmablasts and memory B cells as indicated. **(B)** VH
632 repertoire sequencing of the FACS sorted populations. Waterfall plot of the
633 transcriptional levels and isotype distribution for individual clonotypes. Only the 100
634 most frequent clonotypes are shown for each sample of sorted cells.

635

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

651

Table 1

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

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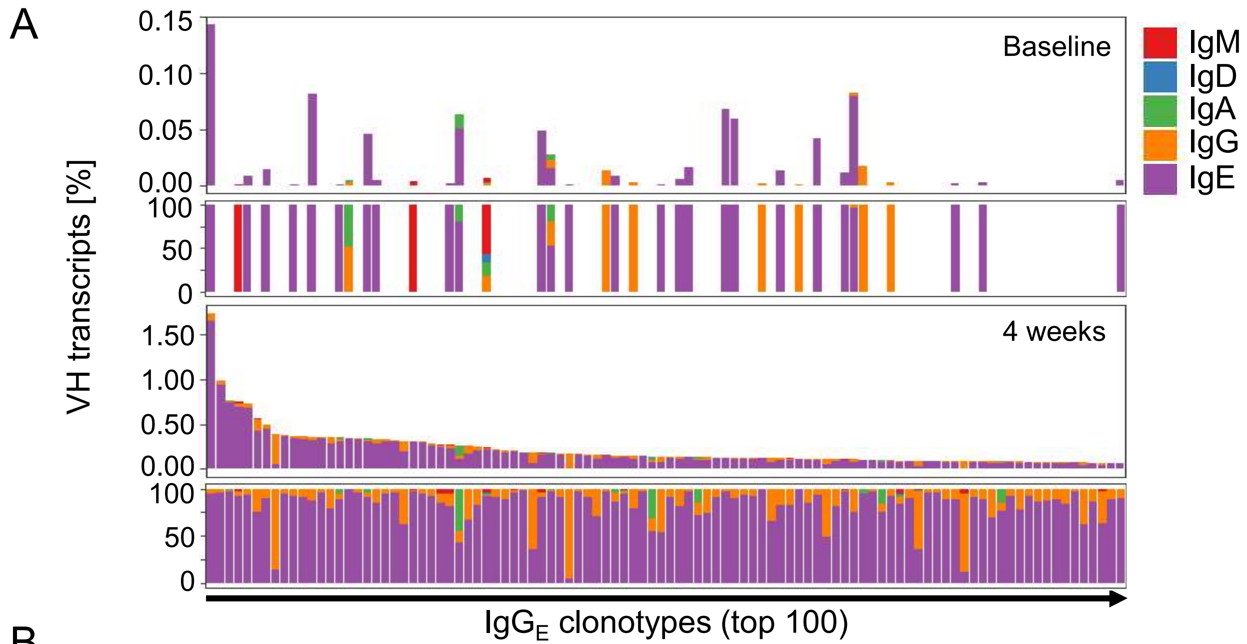

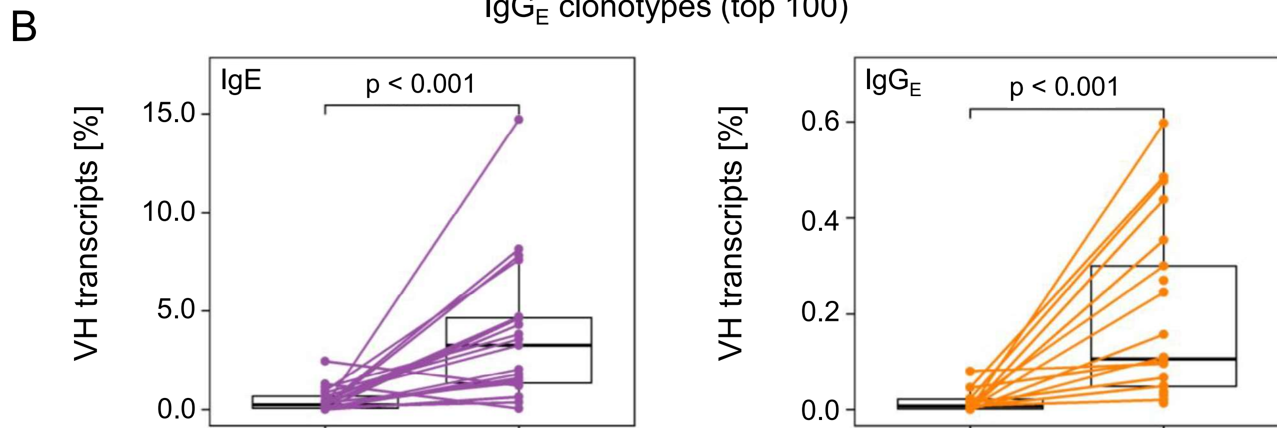

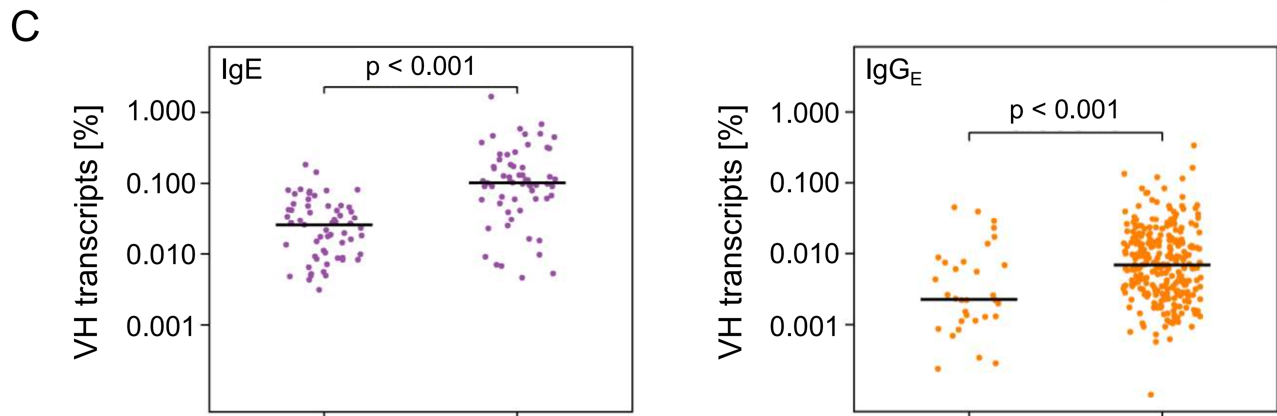

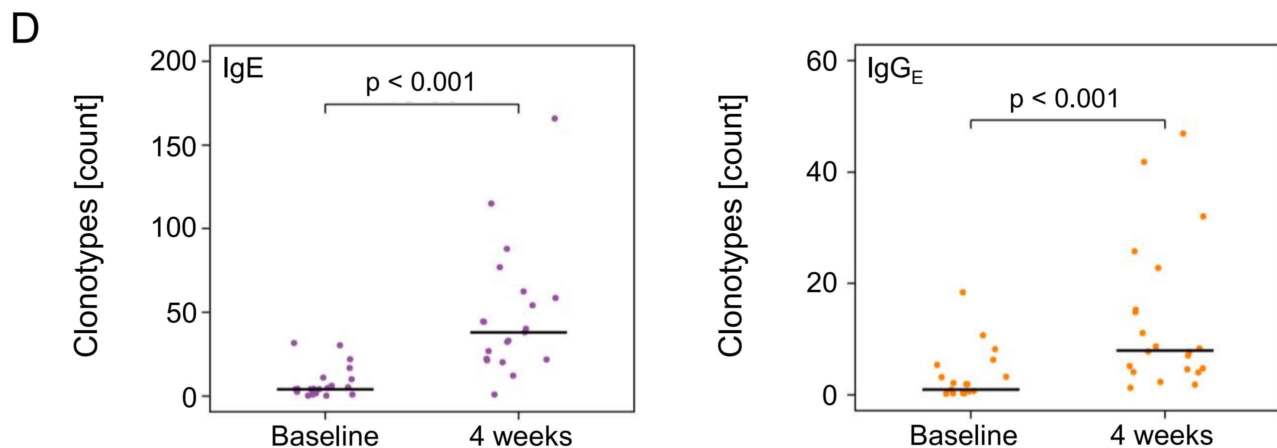

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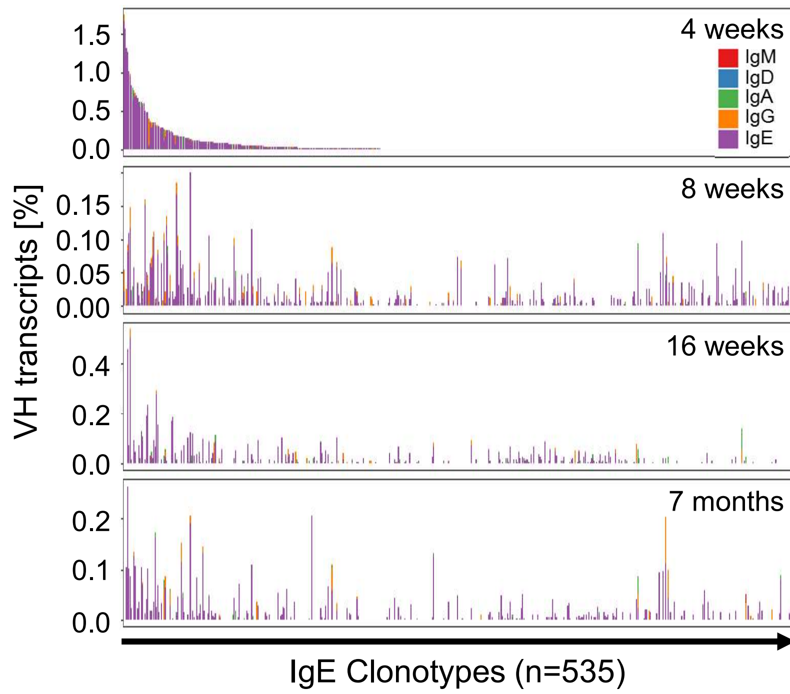

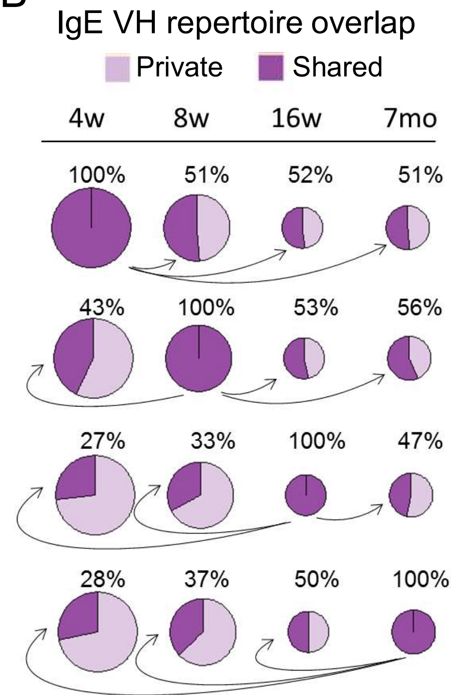

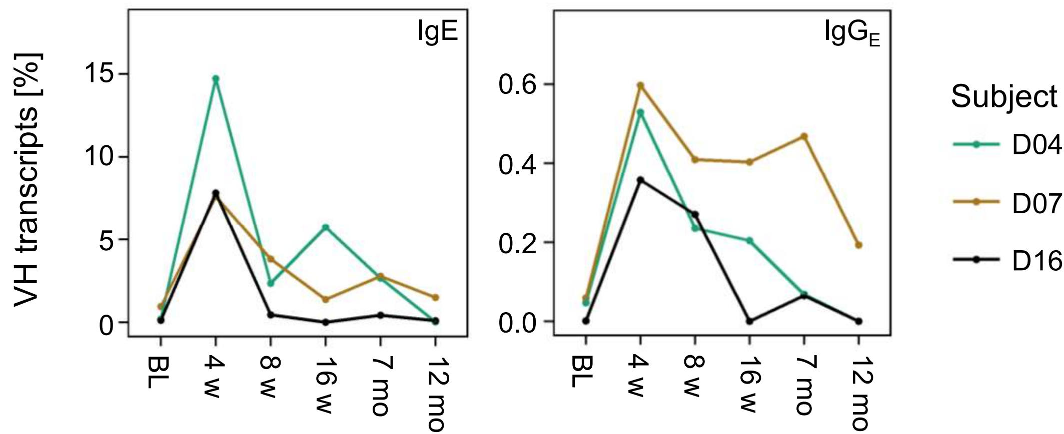

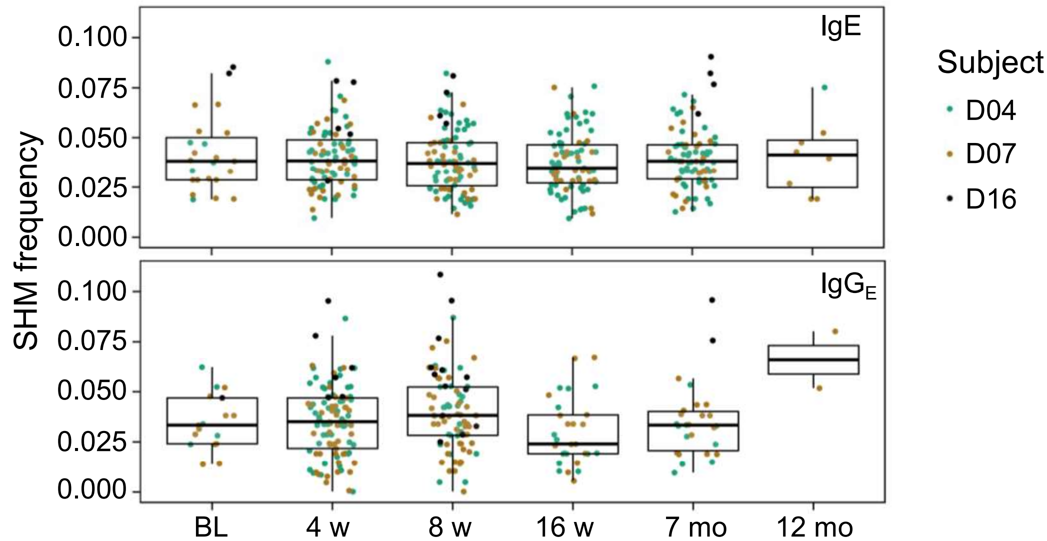

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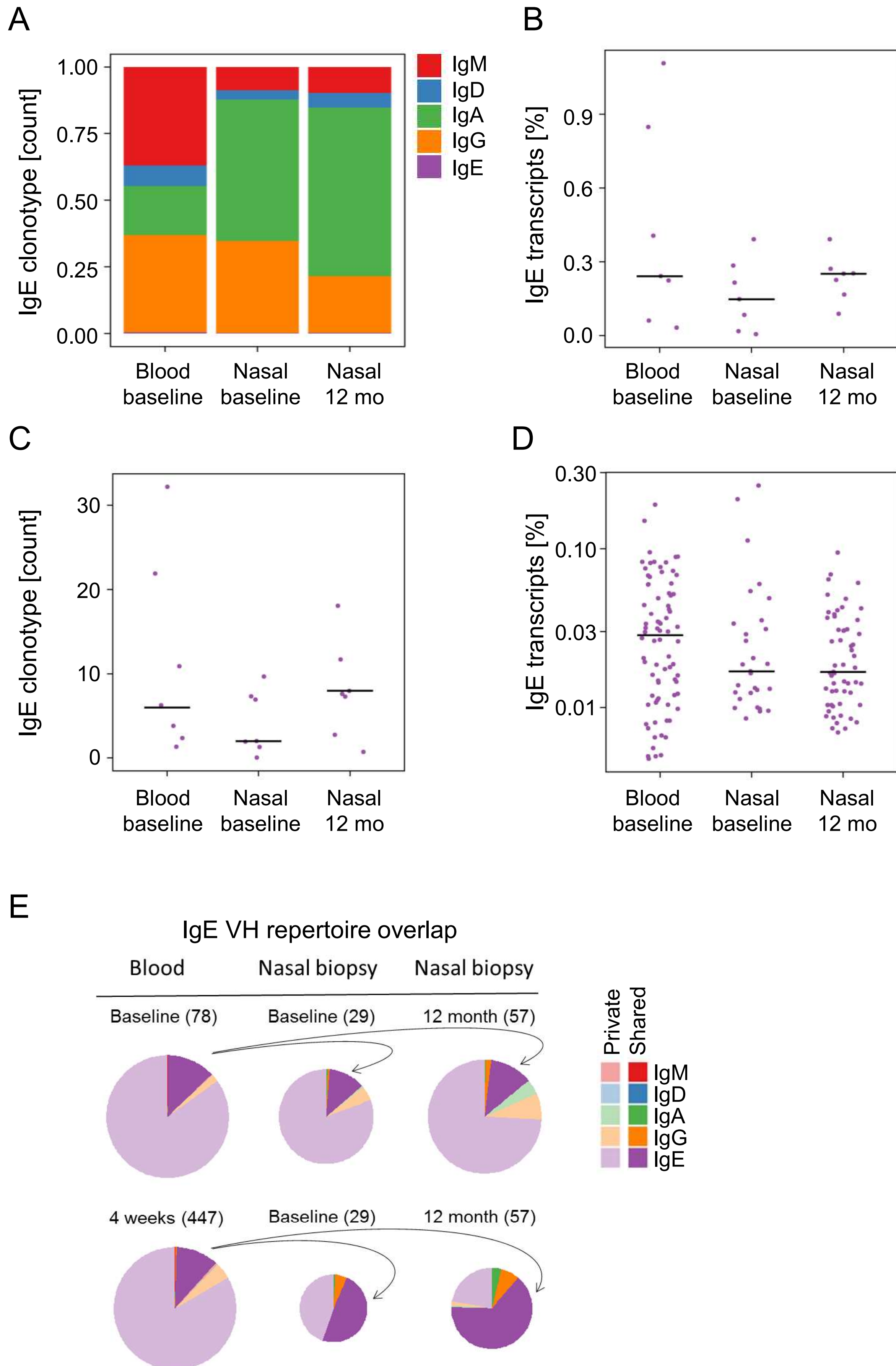

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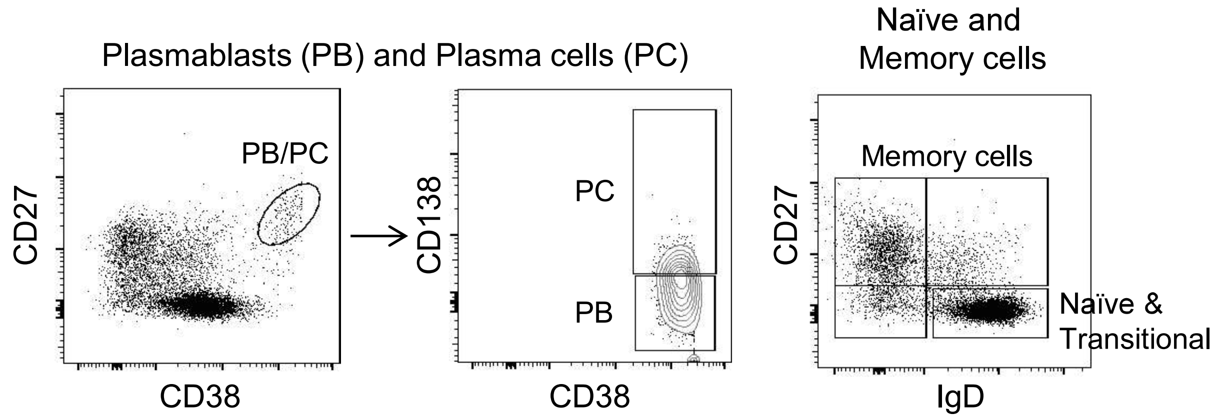

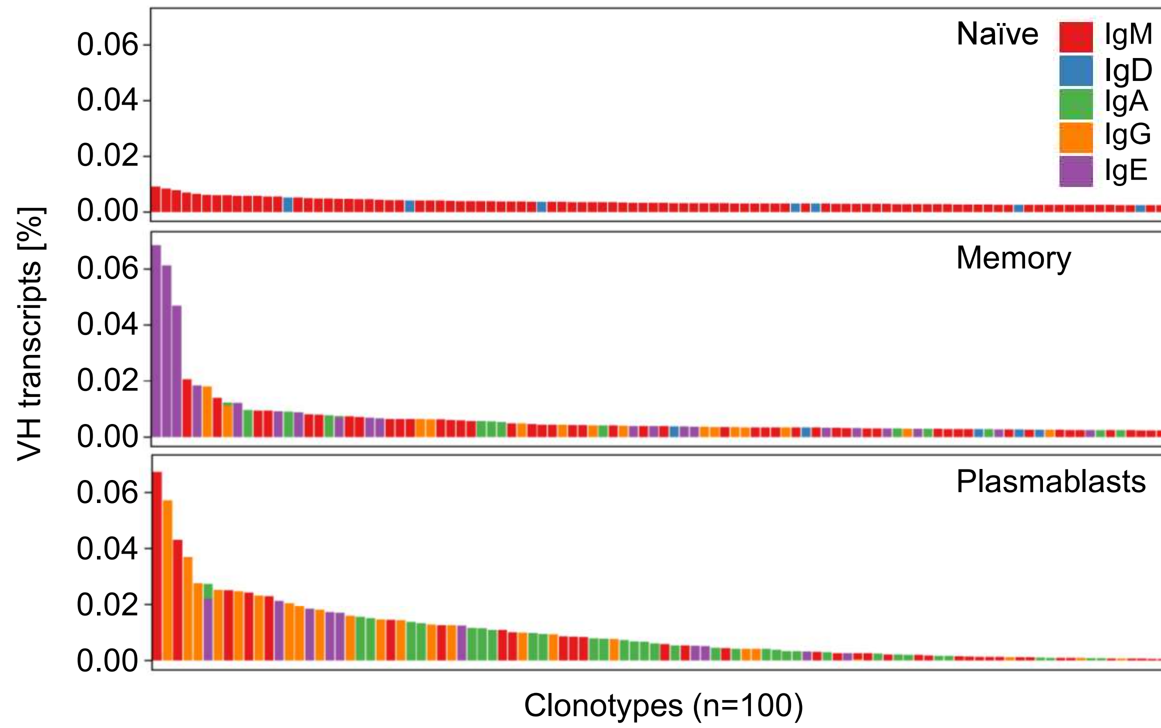

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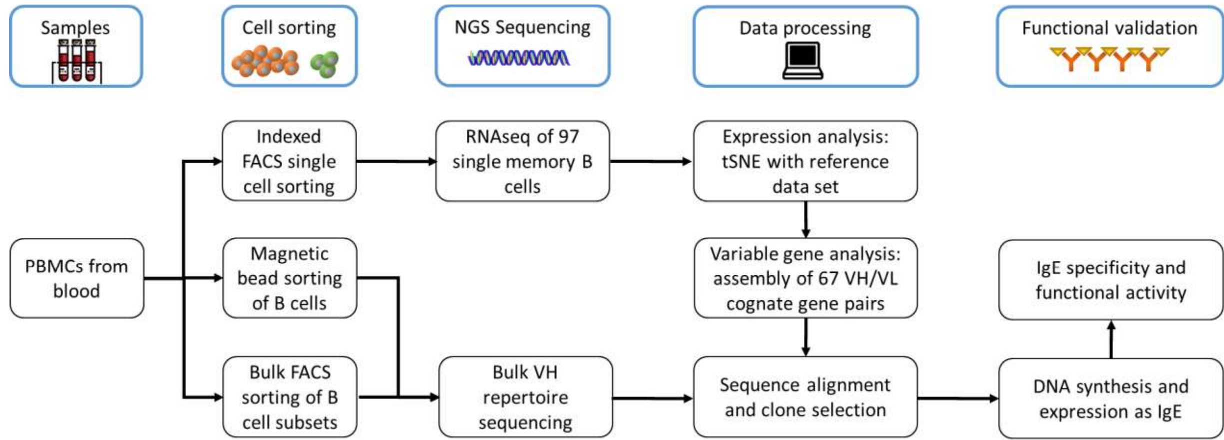

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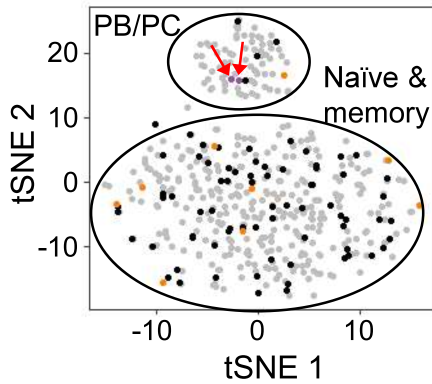

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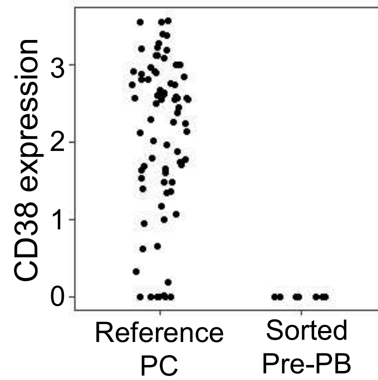

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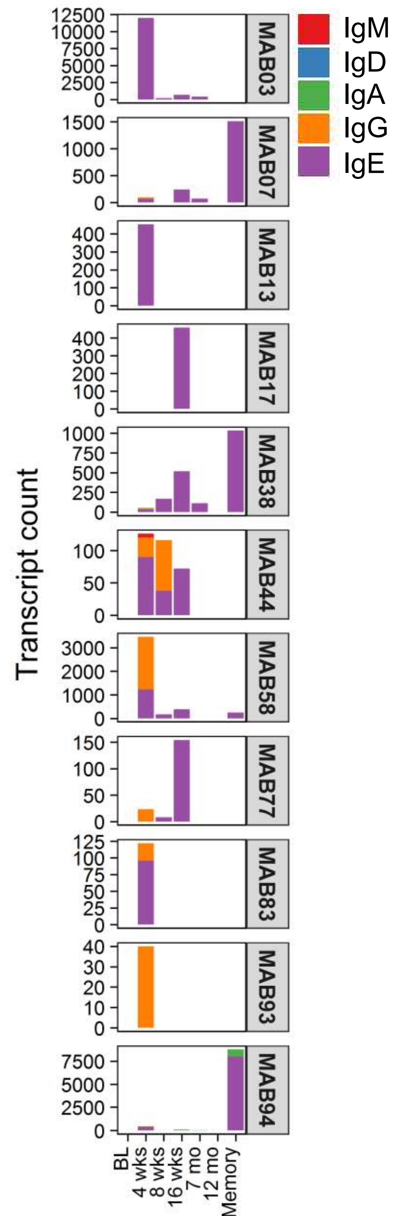

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