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ORIGINAL ARTICLE

RNA sequencing of single allergen-specific memory B cells after grass pollen immunotherapy: Two unique cell fates and CD29 as a biomarker for treatment effect

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Abstract

Background: Sublingual immunotherapy (SLIT) for grass pollen allergy can modify the natural history of allergic rhinitis and is associated with increased allergen-specific IgG_4. IgG_4 competitively inhibits functional IgE on the surface of effector cells, such as mast cells and basophils, from binding to allergens. To further understand the important role memory B-cell (Bmem) responses play in mediating the beneficial effects of SLIT, we assessed changes in allergen-specific Bmem subsets induced by SLIT for grass pollen allergy.

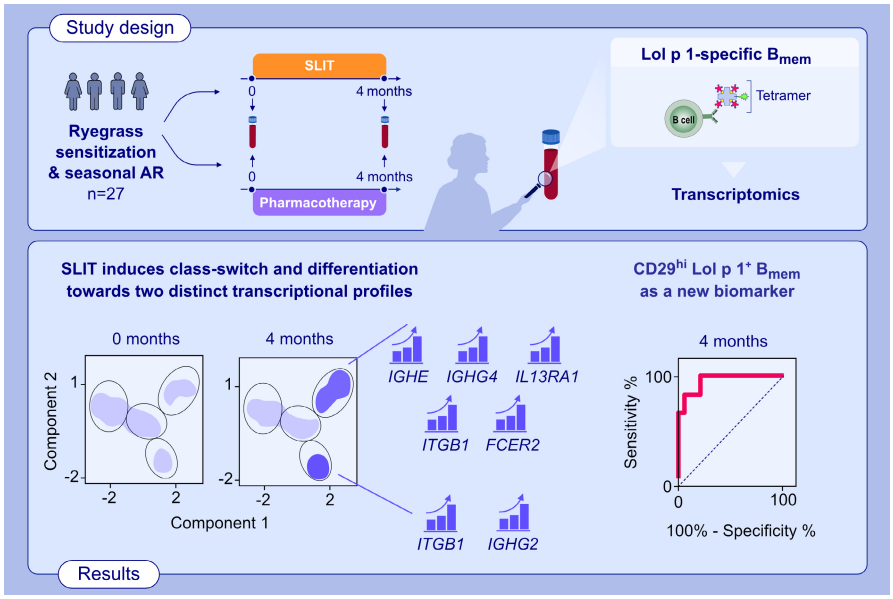
Methods: Blood samples were collected twice outside the pollen season from twenty-seven patients with sensitization to ryegrass pollen (RGP; *Lolium perenne*) and seasonal rhinoconjunctivitis. Thirteen received 4-month pre-seasonal SLIT for grass pollen allergy, and 14 received standard pharmacotherapy only. Single-cell RNA sequencing was performed on FACS-purified Lol p 1-specific Bmem before and after SLIT from four patients, and significant genes were validated by flow cytometry on the total cohort.

Results: Four months of SLIT increased RGP-specific IgE and IgG_4 in serum and induced two Lol p 1-specific Bmem subsets with unique transcriptional profiles. Both subsets had upregulated expression of beta 1 integrin *ITGB1* (CD29), whereas *IGHE* (IgE), *IGHG4* (IgG_4), *FCER2* (CD23), and *IL13RA1* were upregulated in one subset. There was an increase in the proportion of Lol p 1^+ Bmem expressing surface IgG_4, CD23, and CD29 after SLIT.

Conclusions: A clinically successful 4 months course of SLIT for grass pollen allergy induces two transcriptionally unique Bmem fates. Associated changes in surface-expressed proteins on these Bmem subsets can be used as early biomarkers for treatment effects.

KEYWORDS
allergen immunotherapy, biomarkers, grass pollen allergy, memory B cells, transcriptomics

Abbreviations: AIT, allergen-specific immunotherapy; AU, arbitrary units; BAT, basophil activation test; Bmem, memory B cells; DEG, differentially expressed gene; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cells; PCA, principal component analysis; RGP, ryegrass pollen; SLIT, sublingual immunotherapy; SPT, skin prick test.



GRAPHICAL ABSTRACT

Using recombinant Lol p 1 tetramers, allergen-specific B_{mem} from patients allergic to ryegrass pollen were examined before and after 4 months of SLIT. Single-cell RNA sequencing revealed that SLIT drives two unique transcriptional B_{mem} fates, typified by CD23, IgG4, IgE, CD29, and IL13Ra1 expression. Flow cytometry confirmation revealed that CD29 expression is the best positive predictor of SLIT effects. AR, allergic rhinitis; B_{mem}, memory B cells; FCER2, Fc epsilon receptor II; IGH, immunoglobulin heavy chain; IL13RA1, interleukin 13 receptor subunit alpha 1; ITGB1, integrin subunit beta 1; SLIT, sublingual immunotherapy.

1 | INTRODUCTION

Grass pollen allergy is the leading cause of seasonal allergic rhinitis (AR) worldwide, with 10%–30% of the global population exhibiting sensitization to one or more grass pollen allergens.[1] Allergies to grass pollens are a considerable burden on quality of life due to ongoing symptoms of rhinoconjunctivitis throughout the pollen season, anxiety in grass-covered areas such as public parks, the development of co-morbidities such as asthma and the financial costs of treatment and time off work or school.[2–5] Furthermore, grass pollen allergy is the underlying cause of thunderstorm asthma which can overwhelm healthcare institutions, exemplified by the November 2016 thunderstorm event in Melbourne, Australia, there were more than 3000 hospitalizations and 10 deaths.[6,7]

Allergen immunotherapy (AIT) can successfully modify the natural history of allergic disease and is associated with increased allergen-specific IgG_4 and increased circulating allergen-specific regulatory T(reg) and B(reg) cells.[8] Sublingual immunotherapy (SLIT) for grass pollen allergy can protect against thunderstorm asthma and prevent AR in the pollen season.[9] Such protection is associated with increased serum allergen-specific IgG_2 and IgG_4 and an increased IgG_4:IgE ratio.[9,10] IgG_4 antibodies generated by AIT have been demonstrated to competitively inhibit specific IgE from binding to the allergen, which can prevent the onset of allergic symptoms by inhibiting effector cell degranulation.[11–13] Furthermore, the protective effect of allergen-specific IgG can be further extended to other isotypes, particularly IgG_1 and possibly IgG_2, in preventing the binding of allergen-specific IgE on the surface of effector cells or the ligation of inhibitory Fcγ receptors.[14,15]

Despite the critical role of IgE in allergy pathogenesis and the role of AIT-induced IgG in protecting against allergic responses, our understanding of allergen-specific memory B cells (Bmem) and their role in AIT remains limited. IgE responses to allergens are driven by IL-13-expressing follicular helper T(fh) cells in the germinal center,[16] suggesting that this process leads to the formation of allergen-specific Bmem. AIT for HDM allergy has been demonstrated to induce relative expansion of circulating allergen-specific B cells expressing IgA or IgG_4, as well as IL-10$^+$IL-10Rα$^+$ Breg.[17] Single-cell transcriptomics of allergen-specific plasmablasts from peanut-allergic patients identified a unique transcriptional profile including expression of *IGHE* and *FCER2*,[18] but the transcriptional profile of allergen-specific Bmem remains largely unknown. We have previously reported that 4 months of pre-seasonal SLIT for grass pollen allergy can alleviate symptoms of AR and risk of thunderstorm asthma,[9] and that this is associated with increases in serum allergen-specific IgG_4 and proportions of total circulating IgG_4^+ B cells.[10]

Recent advances in the use of allergen tetramers to identify allergen-specific Bmem by flow cytometry have provided a novel opportunity to investigate the effect of AIT on this rare cell population.[19] We here applied fluorescent tetramers of the major RGP allergen Lol p 1 to purify circulating allergen-specific Bmem from RGP-allergic patients before and after 4 months of SLIT prior to the pollen season. The Bmem were subjected to single-cell RNA

sequencing to examine transcriptional changes induced by SLIT, subsequently evaluated at the protein level through multiparameter flow cytometry to identify early biomarkers for treatment success.

2 | METHODS

2.1 | Study design

Subjects with moderate to severe seasonal AR with or without asthma, and serum RGP-specific IgE of ≥0.35 kU_A/L (ImmunoCAP, Phadia) were recruited from the Allergy Clinics of The Alfred and Box Hill Hospitals (Alfred Ethics Committee project number 514/13). Exclusion criteria were immunodeficiency, AIT within the last 5 years, and treatment with continuous oral corticosteroids and/or β-blockers. The study was conducted according to the principles of the Declaration of Helsinki, and written informed consent from each participant was obtained prior to inclusion.

All patients were permitted standard pharmacotherapy with anti-histamines and topical intranasal corticosteroids and/or anti-histamines for local symptom relief. Subjects that received SLIT as per standard protocol were treated with a commercial 5-grass pollen SLIT tablet (Oralair®; Stallergenes) using a 4 months (May/June through to September/October) regimen completed prior to the Australian pollen season in 2019. Treatment with Oralair® involved dissolution under the tongue (at least 2 min) followed by swallowing the residue. The treatment regimen comprised: day 1–1 tablet 100 IR (index of reactivity); day 2–2 tablets 100 IR; day 3 to day 120–1 daily tablet 300 IR. Two years after recruitment, patients were contacted by phone and asked to report whether or not they had noticed a change in symptoms of allergic rhinitis in the subsequent spring after receiving 4 months of SLIT.

2.2 | Blood sampling and ELISA

Heparinized blood samples were processed within 24 h of collection for PBMC isolation and storage. Serum was isolated concurrently. Serum RGP-specific-IgE levels were measured by ImmunoCAP and Lol p 1-specific IgE was measured by a semi-quantitative in-house ELISA, as described previously.[10,20,21] Briefly, ELISA plate wells were coated with recombinant monomeric, non-biotinylated Lol p 1 (MyBiosource), blocked with 5% skim milk powder in PBS, and incubated with serial dilutions of serum samples. For Lol p 1-specific IgG_2 and IgG_4 ELISA, standard curves were generated with chimeric Lol p 1-specific IgG_2 or IgG_4 monoclonal antibodies generated from a previously described mouse hybridoma producing Lol p 1-specific antibody (see next section).[22] For Lol p 1-specific IgE ELISA, separate wells were incubated with a range of concentrations of purified recombinant human IgE (clone AbD18705; Bio-Rad) to generate a standard curve for relative quantification of IgE in serum. Antibody bound to Lol p 1 was detected using polyclonal rabbit anti-hIgE (Agilent), biotinylated anti-$hIgG_2$ (clone HP6002, Thermo Fisher Scientific), or biotinylated anti-$hIgG_4$ (clone HP6025, Sigma Aldrich) followed by polyclonal goat anti-rabbit HRP (Promega). ELISA were developed using TMB (Thermo Fisher Scientific) before the reaction was stopped with 1 M HCl and absorbance measured at OD 450 nm on a Multiskan Microplate Spectrophotometer (Thermo Fisher Scientific). Wells without allergens were used to determine background values. Arbitrary units (AU) were calculated by subtracting background values from allergen-specific Ig absorbance values.

2.3 | Production of recombinant Lol p 1 and anti-Lol p 1 IgG_2 and IgG_4 antibodies

Production of and validation of recombinant Lol p 1 in Spodoptera frugiperda 21 (Sf21) insect cells was described previously.[23] The Lol p 1.0101 isoform[24,25] was generated with the N-terminal leader sequence of insect allergen Api m 1 for secretion, a 6-His tag for purification and a BirA tag for targeted biotinylation. To prevent unwanted effects of catalytic activity, a mutation was introduced (H104V), as published previously.[26] Purified, recombinant Lol p 1 was tetramerized with fluorochrome-conjugated streptavidin (PE and APC or BV711 and BUV395 conjugates; BD Biosciences) at a 4:1 molar ratio of Lol p 1:streptavidin.

Chimeric IgG_2 and IgG_4 monoclonal antibodies specific to Lol p 1 were generated based on the *Igh* and *Igκ* variable regions from a previously-described mouse hybridoma producing a Lol p 1-specific antibody.[22] The PCR-amplified (Table S1)[27,28] *Igh* variable region was cloned into the pWin98 expression vector containing human IgG_2 and into the pBAR981 expression vector containing human IgG_4 constant regions. The *Igκ* variable region was cloned into pWin187. Resultant plasmids were transfected into Expi293F cells (Thermo Fisher Scientific, Scoresby, Australia). Anti-Lol p 1 IgG_2 and IgG_4 were purified using a Protein A column (GE Healthcare Amersham Biosciences) and Lol p 1-specificity confirmed by ELISA.[23]

2.4 | Flow cytometry and cell sorting

Isolation of Lol p 1-specific Bmem for single-cell transcriptomics was performed using fluorescence-activated cell sorting. Briefly, ten million PBMC were incubated with a 10-color cocktail of 2 Lol p 1 allergen tetramers (APC and PE) and 7 antibodies against B-cell markers (CD19, CD38, CD27, IgM, IgD, IgG, IgA) for 15 minutes at room temperature in 300 µl total volume (Table S2). Lol p 1-specific Bmem were defined (Lol p $1^+CD19^+CD27^+CD38^{dim}$, Lol p $1^+CD19^+CD27^-CD38^{dim}IgM^-IgD^-IgG^+$ and Lol p $1^+CD19^+CD27^-CD38^{dim}IgM^-IgD^-IgA^+$), and sorted from four patients before and after SLIT on a 6-laser FACSAria™ Fusion (BD Biosciences).

Detailed immunophenotyping of Lol p 1 specific Bmem was performed on 27 paired samples from RGP-allergic patients. B-cell

subsets were defined as described previously.[29–31] Briefly, within the CD19$^+$ B-cell population, the proportions of IgM$^+$ Bmem (CD38^{dim}CD27$^+$IgM$^+$), IgG$^+$ Bmem (CD38^{dim}IgD$^-$IgM$^-$IgG$^+$) and IgA$^+$ Bmem (CD38^{dim}IgD$^-$IgM$^-$IgA$^+$) were determined.

Data were acquired on a 5-laser BD LSRFortessa X-20 with instrument set-up and calibration performed using standardized EuroFlow SOPs, as described in detail previously.[32,33] All flow cytometry data were analyzed with FACS DIVA v8.0.1 (BD Biosciences) and FlowJo v10 software packages (FlowJo LLC).

2.5 | Single-cell transcriptome profiling

FACS purified Lol p 1$^+$ Bmem from four patients were labeled with sample tags according to the manufacturer's instructions (Human Single-Cell Multiplexing Kit, BD Biosciences). To ensure our transcriptomic analysis of Lol p 1-specific Bmem was representative of the wider cohort, we used PBMC from four subjects with an allergen-specific immunoglobulin profile representative of all patients recruited for SLIT (Figure 1). Single-cell capture, cDNA synthesis, and library preparation were performed using the BD Rhapsody Single-Cell Analysis System (BD Biosciences) for whole transcriptome analysis, according to manufacturer's instructions. Libraries were sequenced on a single run using an Illumina MiSeq platform (Genomics Hub, The Walter and Elisa Hall Institute of Medical Research). Raw sequences were processed using the SevenBridges platform (SevenBridges) to identify sample tags, cell barcodes, and the number of transcripts per cell. Cells without identifiable sample tags were excluded from further analysis. From the four patients, 512 Lol p 1-specific Bmem were identified for single-cell transcriptomic analysis. Patient 1 contributed 130 cells (27 pre-SLIT, 103 post-SLIT), patient 2 contributed 193 cells (96 pre-SLIT, 97 post-SLIT), patient 3 contributed 105 cells (45 pre-SLIT, 60 post-SLIT), and patient 4 contributed 84 cells (21 pre-SLIT, 63 post-SLIT). Single-cell transcriptomics data were analyzed with SeqGeq v1.6 software package (FlowJo LLC). Read counts were normalized to a total library size of 10,000 reads per cell. Dimensionality reduction by principal component analysis (PCA) determined 25 principal components derived from the top 200 genes with most dispersed gene expression. T-distributed stochastic neighbor embedding (tSNE) analysis was performed with 25 principal components from PCA. Pseudotime analysis to construct single-cell trajectories was determined with Monocle v.2 plugin. Data dimensionality was reduced by reversed graph embedding with a gene expression filter of one and a cell expression filter of ten.

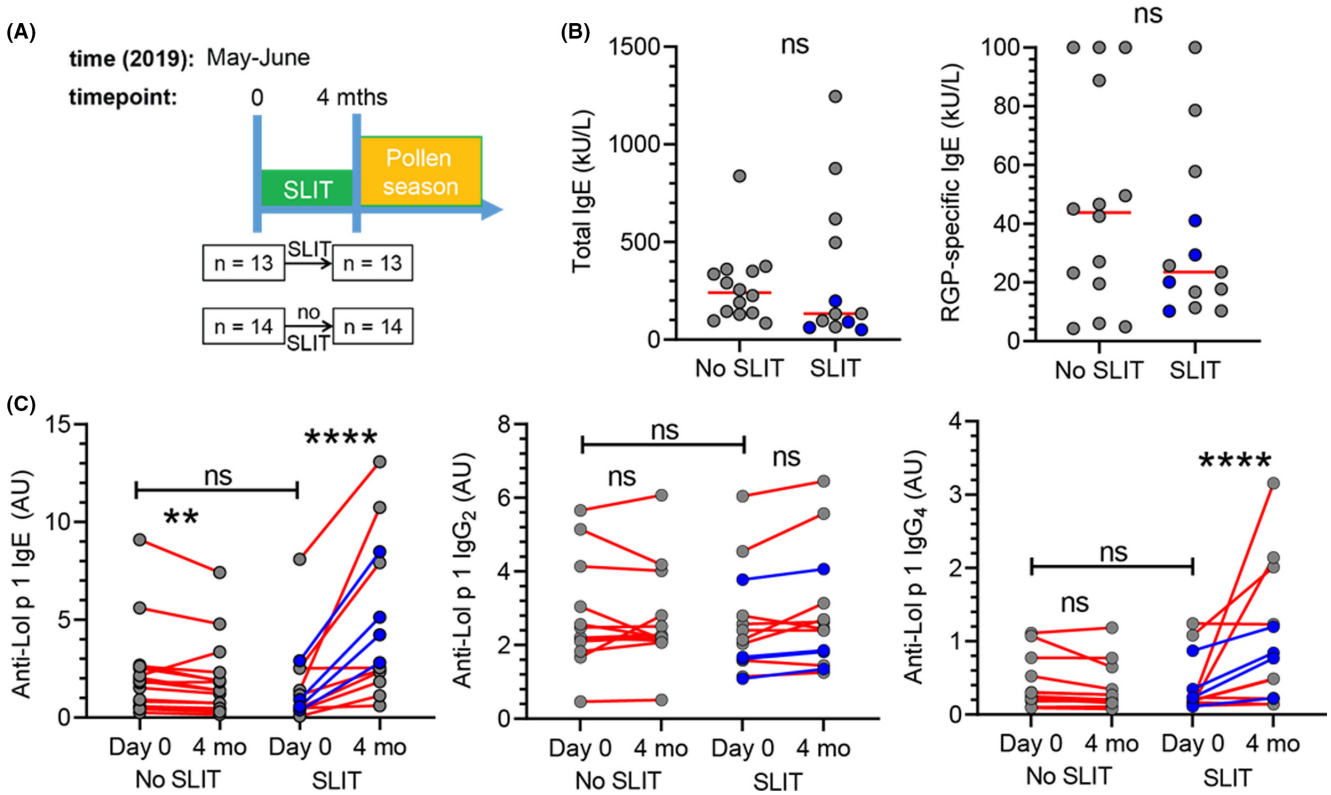


FIGURE 1 Four months of SLIT increases Lol p 1-specific IgE and IgG$_4$ in serum. A, Patient recruitment in May and June 2019 and timeline of 4 months pre-seasonal SLIT for grass pollen allergy. B, Total and RGP-specific IgE in serum at timepoint 0. Red bars, median values. Statistics, Mann–Whitney U-test; ns, not significant. C, Lol p 1-specific IgE, IgG$_2$ and IgG$_4$ in serum at $t = 0$ and 4 months. B, C, Data from the four subjects that were included for transcriptomic analysis of Lol p 1-specific Bmem are highlighted in blue. Statistics, Wilcoxon signed rank test; $^{**}p < 0.01$; $^{****}p < 0.0001$

2.6 | Statistics

Differential gene expression from the combined transcripts of all cells before vs after SLIT was illustrated by volcano plots and significance determined by Bonferroni corrected Mann–Whitney *t*-test. Genes with a *Q*-value <0.05 and absolute fold change ≥1.5 were considered significantly and relevantly different between the analyzed subsets. Differences in proportions of *IGHC* transcripts were statistically analyzed with the chi-square test.

Statistical analyses of flow cytometric data were performed using Graphpad Prism (v8.4.1): Paired data were analyzed with the non-parametric Wilcoxon signed rank test and unpaired data with the non-parametric Mann–Whitney *U*-test. For all tests, $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Participant characteristics

In an investigator-initiated study, thirteen RGP-allergic patients were recruited after their decision to commence SLIT (median 46 years, 31% female), and fourteen patients who received standard pharmacotherapy only (median 32 years, 64% female) (Figure 1A). All 27 patients suffered from moderate to severe seasonal AR with or without asthma, and had serum RGP-specific IgE of ≥0.35 kU_A/L (ImmunoCAP).

3.2 | Four-month SLIT increases serum allergen-specific IgE and IgG_4 levels

Meta-analyses and clinical trials have confirmed the clinical efficacy of SLIT with a 5-grass mix.[9,10,34] Our prior study demonstrated that one 4 months course of pre-seasonal SLIT reduced self-reported symptoms of allergic rhinitis, and that this effect increased with successive courses over the following 2 years.[23] Of the 13 patients in our active study arm accessible 2 years later, 12 (92%) reported clinical benefit, thereby validating the efficacy of SLIT in this cohort.[23] There were no significant differences in total, RGP-specific or Lol p 1-specific IgE at the time of recruitment between patients recruited to receive SLIT and those who did not receive SLIT (Figure 1B,C). Four months of SLIT increased Lol p 1-specific IgE and IgG_4 (Figure 1C). In patients who did not receive SLIT, the intervening 4 months saw a slight but significant decrease in Lol p 1-specific IgE, whereas Lol p 1-specific IgG_2 and IgG_4 levels were unchanged (Figure 1C).

3.3 | Increased expression of *IGHE* and *IGHG4* transcripts in Lol p 1-specific Bmem after SLIT

To investigate the immunological effects of SLIT, we conducted single-cell transcriptomics on Lol p 1-specific Bmem from four patients before and after 4 months of SLIT. Following electronic gating of Bmem (details in Methods), Lol p 1-specific Bmem were identified by double-positivity for two fluorescent Lol p 1 tetramer conjugates using APC and PE fluorochromes (Figure 2A). Outliers for total reads per gene (<150 or >15,000) and cells per gene (<30) were excluded, followed by dimensionality reduction with PCA based on the 200 most dispersed genes (Figure 2B–D). This resulted in 745 cells that formed 2 main clusters. One enriched for the myeloid gene *CD14* (233 cells; Figure 2E) was excluded from further analysis as this was deemed to comprise monocytes. The main cluster of 512 cells expressed the canonical B-cell gene *MS4A1* (CD20).

To investigate the Ig gene usage of these Bmem, we assessed the expression of the *IGH* constant regions (Figure 3A), as well as expression of the *IGK* and *IGL* light chain regions. Each cell expressed one predominant *IGH* isotype and a single *IGK* or *IGL* constant region (Figure 3B). The proportions of *IGHE* and *IGHG4* among total *IGH* transcripts were significantly increased in Lol p 1-specific Bmem after SLIT (Figure 3C). There were no differences in the proportion of *IGK* or *IGL* expression in Lol p 1-specific Bmem before and after SLIT (data not shown). Thus, after 4 months SLIT, both allergen-specific IgE and IgG4 serum levels as well as Bmem expressing *IGHE* and *IGHG4* were increased (Figure 1C).

3.4 | Expansion of two transcriptionally distinct Bmem subsets after SLIT

The transcription profiles of the 512 Lol p 1-specific Bmem included those for $n = 189$ pre- and 323 post-SLIT. Direct comparison of the gene transcripts between these pre- and post-SLIT samples showed that only 12 transcripts were >1.5-fold upregulated and 3 were >1.5 fold downregulated (Figure 4A). To further investigate the transcriptional changes following SLIT on Bmem, we identified differentially expressed genes (DEG) between Lol p 1-specific Bmem before ($n = 189$) and after SLIT ($n = 323$). Most notably, SLIT increased expression of genes with known immunological function including *IGHE* (IgE), *ITGB1* (β1 integrin; CD29), *PPP1R18* (phostensin), and oncogene *PARM1* (Figure 4A). In contrast, expression of *JUN* (c-jun) and *CD69* was reduced after SLIT.

A large number of genes were significantly different ($Q < 0.05$) with a <1.5-fold change after SLIT (Table S3), potentially due to heterogeneity in the populations before and/or after SLIT. To assess this at the single-cell level, all $n = 268$ genes with $Q < 0.05$ were used to generate PCA and tSNE plots (Figure S2B; Table S4). Both analyses did not yield distinct clusters of Bmem before and after SLIT. To identify clusters of transcriptional profiles associated with changes induced by SLIT, we conducted a pseudotime analysis on the same 268 genes. The B-cell cluster expressing *IGHM* and *IGHD* was designated pseudotime zero due to not being Ig class-switched, followed by 3 more clusters. Cluster 2 had a higher pseudotime value, followed by clusters 3 or cluster 4, which contained predominantly Ig class-switched B cells (Figure 4B,C). After SLIT, the proportion of Bmem in cluster 1 (pseudotime 0–2)

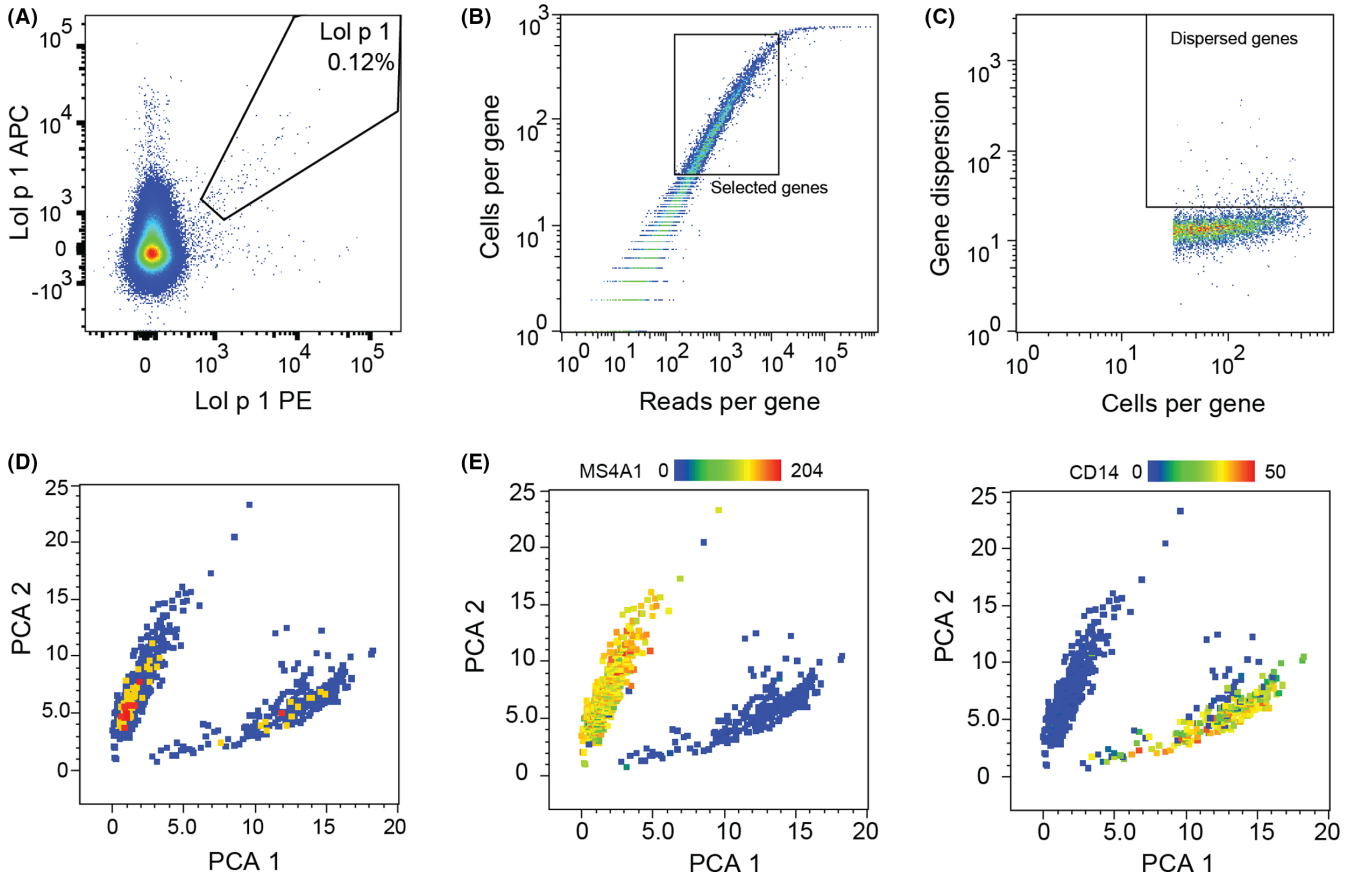


FIGURE 2 Identification of Lol p 1-specific B cells for single-cell transcriptomic analysis. Single-cell RNA sequencing of PBMC sorted for Lol p 1-specific Bmem by FACS from four patients before and after SLIT was conducted using the BD Rhapsody Whole Transcriptome Analysis pathway. A, Exemplary plot of Lol p 1-specific Bmem sorted for single-cell transcriptomics. Number indicates proportion of Bmem. B, Selection of genes used for the identification of B cells (square gate), excluding those with low expression (insufficient sequencing) or high and ubiquitous expression (housekeeping genes). C, The top 200 genes with highest dispersion in expression among all cells were used to generate D, PCA plot with two distinct clusters. E, Heatmaps of expression patterns for canonical B-cell gene *MS4A1* and myeloid gene *CD14* identified separate clusters. Monocytes (lower cluster; 233 cells) were excluded from further analyses

decreased, with relatively more Bmem in the most differentiated states: cluster 3 (pseudotime 5–6) and cluster 4 (pseudotime 6–8) (Figure 4C). Heat maps of *IGH* gene expression demonstrated that increasing pseudotime is associated with a shift from *IGHM* to expression of class-switched *IGH* isotypes, particularly *IGHE* in cluster 4 (Figure 4D,F). Indeed, Bmem in cluster 4 were enriched for *IGHE*, *IGHG1*, and *IGHG4* expression whereas cluster 3 predominantly expressed *IGHM*, *IGHG3*, and *IGHG1* (Figure 4G; Figure S3A,B). Given that SLIT increased the proportion of B cells in clusters 3 and 4, this suggests that SLIT induces class-switching and differentiation toward two distinct transcriptional profiles.

3.5 | Gene expression associated with transcriptional profiles induced by SLIT

DEG analysis of distinct pseudotime clusters confirmed increases in *IGHE*, *IGHG4*, and *IGHG1* in cluster 4 compared to cluster 1, whereas cluster 1 was enriched for *IGHM* and *IGHD* (Figure 5A,B; Table S5). Compared to cluster 1, cluster 4 also had increased expression of immune-related genes such as *PARM1*, *IL13RA1*, *ITGB1*, *PPP1R18*, *SELL*, and *FCER2* and decreased expression of *CXCR4* and *CD69* (Figure 5B). Similarly, cluster 3 also had increased expression of *ITGB1* and *PPP1R18* alongside decreased *CXCR4* expression compared to cluster 1 (Figure 5B; Table S6). Furthermore, cluster 3 exhibited increased expression of *FGR*, *SIGLEC10*, *CD99*, and *TLE3* and decreased expression of *TXNIP*, *BTG1*, and *FCER2* compared to cluster 1. Comparing clusters 3 and 4, cluster 4 had increased expression of *SELL*, *FCER2*, *IGHE*, *IL13RA1*, *IL4R*, *PARM1*, and *ITGB1* whereas cluster 3 had increased expression of *IGHM*, *IGHD*, *CD1C*, *PLAC8*, *SYK*, *FGR*, *FCGR2B*, *FCRLA*, *FCRL2*, *FCRL3*, *FCRL5*, and *ITGAX* (Figure 5B; Table S7). Due to unique expression patterns of *ITGB1*, *FCER2* and *IL13RA1* in pseudotime cluster 4 compared to clusters 1–3, as well as the association of this cluster with IgE and IgG_4 which we have observed previously to be increased by SLIT, we wished to visualize the expression of these genes relative to their space in pseudotime to further characterize these cells (Figure 5C). Expression of these genes changed as pseudotime progressed reflecting the changes in Bmem transcriptional profiles along a SLIT-induced differentiation pathway.

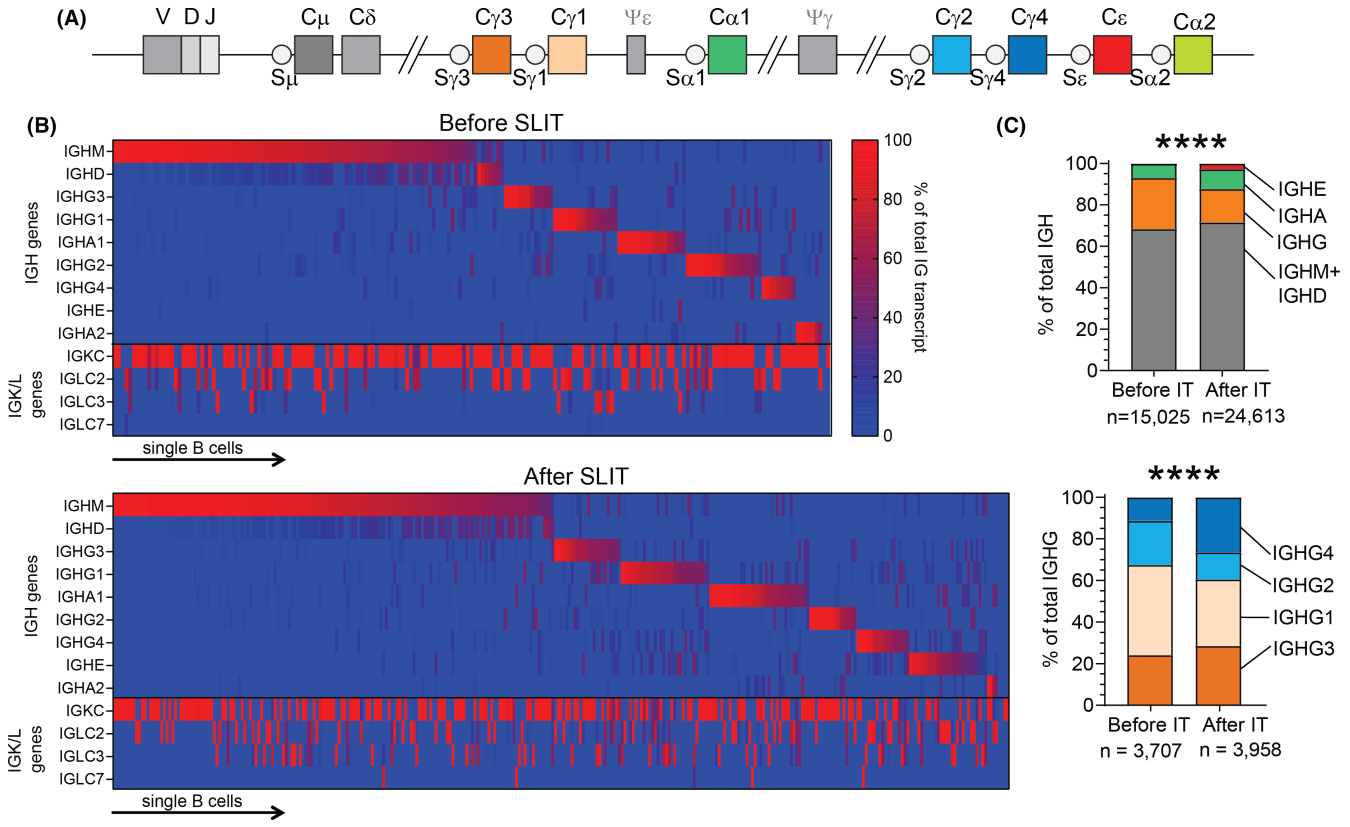


FIGURE 3 SLIT increases proportions of *IGHE* and *IGHG4* transcripts in Lol p 1-specific Bmem. A, Schematic of the constant genes in the human *IGH* locus depicting their position relative to the variable domain of the VDJ exon. B, Proportion of transcripts for *IGH* or *IGK* and *IGL* genes in single Lol p 1-specific Bmem before ($n = 189$) and after SLIT ($n = 323$) isolated from four allergic patients used for single-cell transcriptomic analysis. Cells arranged left-to-right based on maximal expression of *IGH* transcripts in the order of *IGH* genes in the human *IGH* locus (*IGHM* to *IGHA2*). C, Proportion of total *IGH* or *IGHG* gene transcripts in these Lol p 1-specific Bmem before and after SLIT. Statistics for the proportional distribution of *IGH* and *IGHG* in (C), χ^2 test; ****$p < 0.0001$

3.6 | Confirmation of changes in IgG4, CD29, and CD23 by flowcytometric immunophenotyping of Lol p 1-specific Bmem

To validate SLIT-induced changes in gene expression identified by transcriptomics, we used flow cytometry to assess the expression of IgM, IgG, IgG_4, and IgA isotypes and CD29 (*ITGB1*), CD23 (*FCER2*), and CD213A1 (*IL13RA1*) on Lol p 1-specific Bmem at timepoints 0 and 4 months from patients who received or did not receive SLIT. Following electronic gating of $CD19^+$ B cells and removal of $CD3^+$ T cells, Lol p 1-specific B cells were identified by double-positivity for two fluorescent Lol p 1 tetramer conjugates using small molecule fluorophores BUV395 and BV711 (Figure 2A, Figure S1A–E). Lol p 1 tetramers did not bind to $CD3^+$ T cells (Figure S1C), nor did B cells bind directly to the streptavidin-fluorochrome conjugates used to generate these tetramers (Figure S1D). We did not observe $CD14^+$ monocyte contamination in our identification of Lol p 1-specific B cells (Figure S4A,B). In conjunction with additional gating on $IgG2^+$, $IgG4^+$ or IgA^+, B cells that were double-positive for both tetramers constitute a bona fide population of Lol p 1-specific B cells. In patients who received SLIT, the number of Lol p 1-specific Bmem was increased (Figure 6A). Both proportion and number of IgG^+ (all IgG isotypes) and more specifically IgG_4^+ Lol p 1-specific Bmem increased after SLIT, whereas the proportion of IgM^+ Lol p 1-specific Bmem decreased (Figure 6B, Figure S5A). Subjects who did not receive SLIT had no observable change in proportion or number of Lol p 1-specific Bmem over the 4-month time course (Figure 6A,B). SLIT also increased the proportion and number of $CD29^{hi}$ Lol p 1-specific Bmem and was further associated with increases in CD29 and CD23 staining on Lol p 1-specific Bmem (Figure 6C–F, Figure S5B). These flow cytometry data correspond with our transcriptomic data that identified increased proportions of Lol p 1-specific Bmem expressing *FCER2* (CD23) and *ITGB1* (CD29) after SLIT (Figure 5). The one subject who did not report a benefit of SLIT (indicated by red dots and black lines in Figure 6) displayed decreases in total, IgG^+ and $CD29^{hi}$ Lol p 1-specific Bmem. In contrast, we did not observe any change in CD213A1 staining on Lol p 1-specific Bmem from patients who received SLIT (Figure 6G). Staining of CD29, CD23, and CD213A1 on Lol p 1-specific Bmem from patients who did not receive SLIT was unchanged (Figure 6D–G).

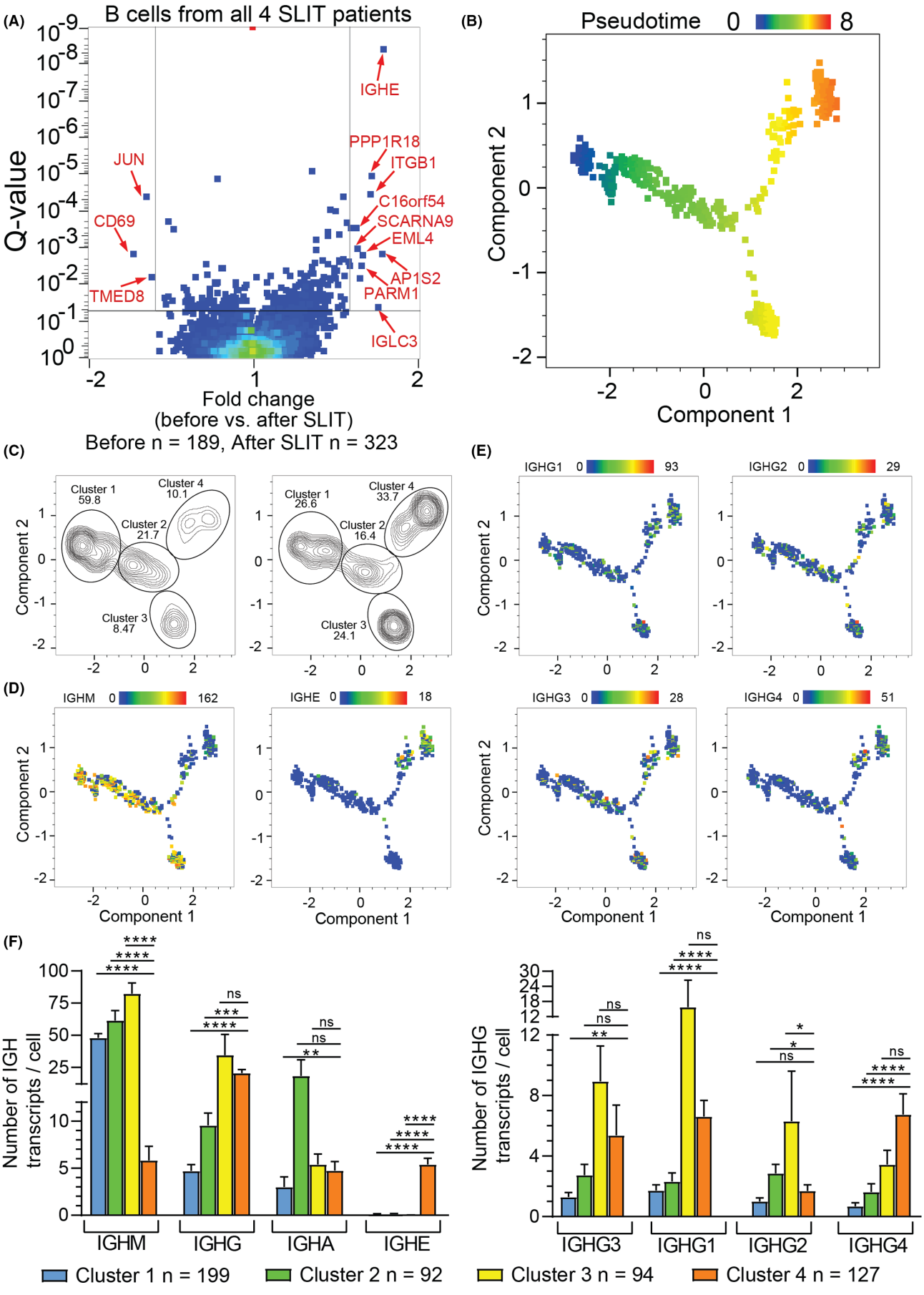


FIGURE 4 SLIT increases the proportion of *IGHE*, *IGHG1* and *IGHG4*-expressing Lol p 1-specific Bmem and promotes differentiation toward two transcriptionally distinct clusters A, DEG in Lol p 1-specific Bmem before and after SLIT. Genes with >1.5-fold change and $q < 0.05$ identified by red arrows. Genes with >1.5-fold change and $q < 0.05$ identified by red arrows. B, Pseudotime clustering based on the 268 genes ($Q < 0.05$) identified in 4A. C, B-cell clusters identified by pseudotime analysis before or after SLIT. D, Heat map of *IGHM*, *IGHE* and E, *IGHG1-4* gene expression in B cells clustered by pseudotime analysis. F, *IGH* gene expression in Bmem from the four distinct pseudotime clusters. Statistics: Mann–Whitney *U*-test;*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant

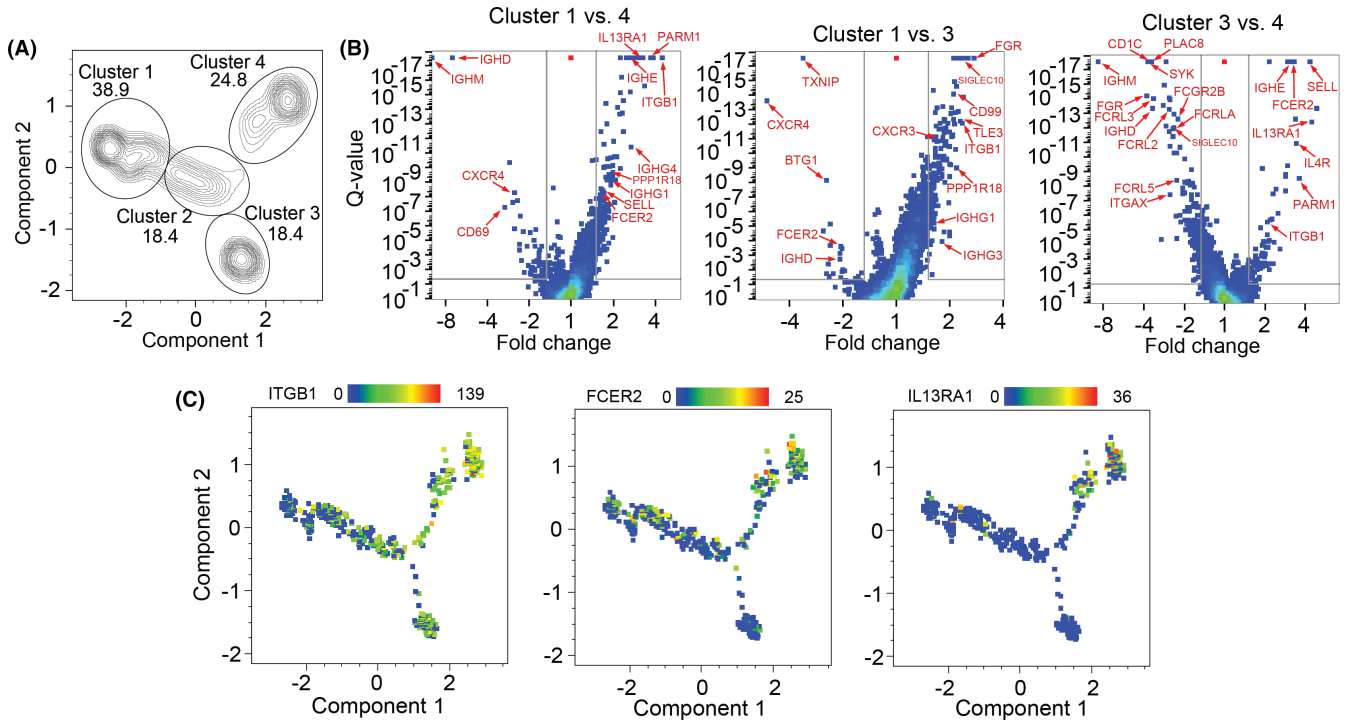


FIGURE 5 Lol p 1-specific Bmem generated by SLIT show increased expression of *ITGB1* (CD29), *FCER2* (CD23), and *IL13RA1* (CD213A1). A, Pseudotime clustering of all Lol p 1-specific Bmem from four patients before and after SLIT using genes identified in Figure 3A. Numbers indicate proportion of clusters. B, Volcano plots of DEG comparing pseudotime clusters 1 to 4, 1 to 3, and 3 to 4. Significant genes ($p > 0.05$) with largest fold change or known immunological interest indicated by red arrows. C, Heatmap of gene expression for *ITGB1*, *FCER2*, and *IL13RA1* on pseudotime plot from A

3.7 | CD29^{hi}-expressing Lol p 1^{+} Bmem as biomarker

To evaluate the potential of each marker as a predictor of treatment outcome, receiver-operator curves (ROC) were generated for treated vs untreated patients. To evaluate the effects of 4 months SLIT, the first evaluation was the ratio of markers after 4 months divided by the pre-treatment values (Figure 7A) for total Lol p 1^{+} Bmem, CD29^{hi} Lol p 1^{+} Bmem, and for expression levels of CD29, CD23, and CD213A1 on Lol p 1^{+} Bmem. None of the areas under the curve (AUC) were very high with CD29^{hi} Lol p 1^{+} Bmem being the most predictive with an AUC of 0.72 (Figure 7A). Subsequently, for a comparison of SLIT vs no SLIT, we only evaluated the markers at 4 months. These yielded higher AUC for Lol p 1^{+} Bmem (0.79) and CD29^{hi} Lol p 1^{+} Bmem (0.95), while the AUC for expression levels of CD29, CD23, and CD213A1 did not improve (Figure 7B). Thus, at 4 months of treatment, CD29^{+} Lol p 1^{+} Bmem numbers are a very strong indicator of treatment effect and more specific than total Lol p 1+ Bmem.

Taken together, we demonstrate SLIT induces class-switching from IgM to IgG, including IgG_4, in circulating allergen-specific Bmem and that these Bmem uniquely and highly express both CD29 and CD23. These changes at the transcript and protein level demonstrate that already after 4 months of SLIT for grass pollen allergy, allergen-specific Bmem display a change in their immunophenotype.

4 | DISCUSSION

We here describe for the first time the effect of SLIT for grass pollen allergy on the gene expression profile of allergen-specific Bmem. We have identified distinct Bmem transcriptional profiles and demonstrated, using pseudotime analysis, that SLIT induces differentiation toward two branches of distinct transcriptional clusters.

In this study, one course of 4 months SLIT resulted in an increase in allergen-specific IgE and IgG_4 in serum, consistent with our prior study on a separate cohort.[10] This is in accordance with a number of studies demonstrating that AIT induces transient increases in

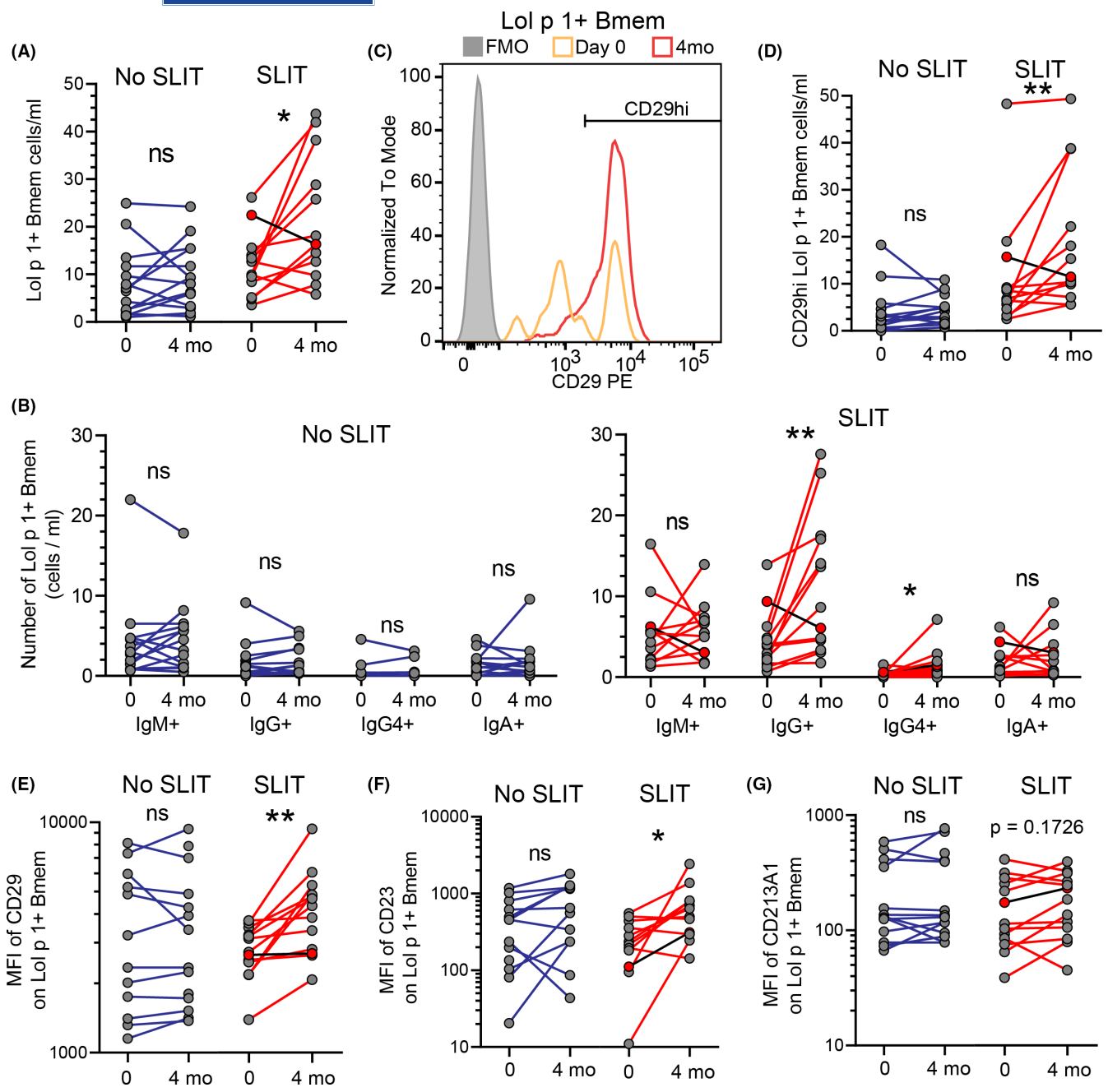


FIGURE 6 Increased numbers of circulating IgG_4^+ Lol p 1-specific Bmem and surface expression of CD29 and CD23 after SLIT. A, Number of circulating Lol p 1-specific Bmem and B, number of IgM^+, IgG^+, IgG_4^+, and IgA^+ Lol p 1-specific Bmem from PBMC of RGP-allergic patients before and after SLIT (red lines; SLIT $n = 13$) or from RGP-allergic patients that did not receive SLIT (blue lines; no SLIT $n = 14$). The only patient to report no clinical benefit of SLIT is indicated by red dots and black lines. C, Representative histogram of CD29 expression on Lol p 1-specific Bmem from one RGP-allergic patient before (orange) and after (red) SLIT. Fluorescence minus one (FMO) control from after SLIT in gray. D, Number of CD29hi Lol p 1-specific Bmem and MFI of E, CD29, F, CD23 and G, CD213A1 on Lol p 1-specific Bmem. Statistics, Wilcoxon signed rank test; $^*p < 0.05$; $^{**}p < 0.01$; ns, not significant

allergen-specific IgE.[35] This transient rise is suggested to result from IgE-expressing B cells, which are prone to apoptosis and differentiation to short-lived plasmablasts.[36–38] In contrast, IgG_4 appears to be maintained at higher levels for at least three consecutive years of SLIT,[10] suggesting that IgG_4^+ B-cell responses induced by SLIT may give rise to long-lived plasma cells that maintain allergen-specific IgG_4 levels long term. We did not observe a significant increase in Lol p 1-specific IgG2. Previously, we did observe that RGP-specific IgG2 increased after 4 months of SLIT. Potentially, the effect magnitude of a single component in this study (Lol p 1) was not sufficient to observe this effect.

SLIT induced differentiation toward a transcriptional profile associated with *IGHE*- and *IGHG4*-expressing Lol p 1-specific Bmem (pseudotime cluster 4). This was confirmed by flow cytometry that

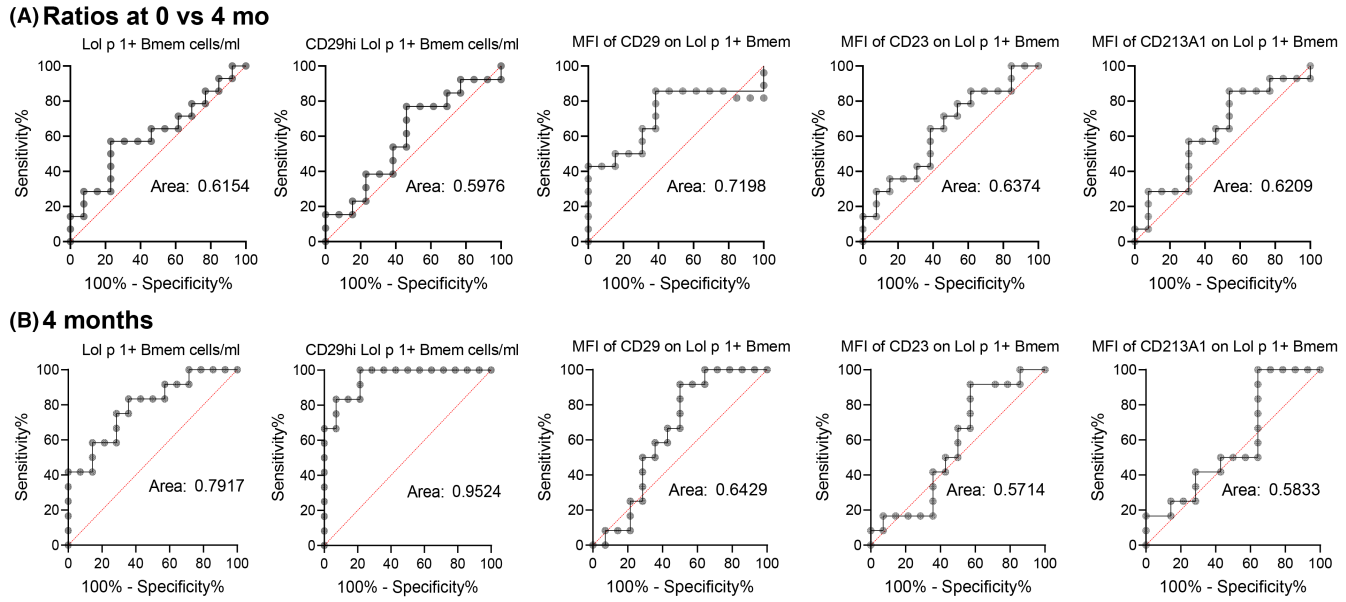


FIGURE 7 Positive predictive values of biomarkers for allergen immunotherapy. A, Receiver-operator curves of ratios before/after 4 months for patients treated with SLIT vs untreated RGP allergic patients. Shown are Lol p 1^+ Bmem numbers, $CD29^{hi}$ Lol p 1^+ Bmem numbers and MFI of CD29, CD23, and CD213A1 on Lol p 1^+ Bmem. B, Receiver-operator curves of data obtained at the 4-month timepoint for patients treated with SLIT vs untreated RGP-allergic patients. Shown are Lol p 1^+ Bmem numbers, $CD29^{hi}$ Lol p 1^+ Bmem numbers and MFI of CD29, CD23, and CD213A1 on Lol p 1^+ Bmem

identified an increased proportion and number of circulating IgG_4^+ Lol p 1-specific Bmem and an increased staining intensity of anti-IgE on Lol p 1-specific Bmem after SLIT, which may reflect the normally weak expression of IgE on the surface of B cells. This profile of Ig expression reflects what we observed for serum allergen-specific Ig titres, whereby SLIT increased Lol p 1-specific IgE and IgG_4. This suggests that transcriptional and flow cytometric profiling of antigen-specific memory B cells reflects the isotypic nature of antibody responses to SLIT. The profiling of allergen-specific Bmem can therefore provide valuable insight into the responses to various AIT and yield valuable biomarkers for disease progression or successful AIT for allergies due to the immunophenotype of allergen-specific Bmem reflecting the response to SLIT.

IL13RA1, *IL4R*, *IGHE*, *and IGHG4* transcript levels in Lol p 1-specific Bmem were increased in pseudotime cluster 4 compared to clusters 1 and 3. This is consistent with the role of the Th2 cytokines IL-13 and IL-4 in driving class-switching to IgE and IgG4, which was enhanced after SLIT. Cluster 4 also showed increased expression of *FCER2* (encoding CD23), a well-documented target of IL-4.[39,40]

Similar to our observation that *IGHE* expression and *FCER2* expression were concomitant in Lol p 1-specific Bmem, *FCER2* expression has previously been observed in *IGHE*-expressing plasmablasts in peanut-allergic patients.[18] The functional relevance of CD23 expression on class-switched B cells and plasma cells is largely unknown. Murine transitional B cells expressing CD23 are protected from BCR-induced apoptosis in contrast to those lacking CD23, exhibiting increased survival from an apoptotic pathway hypothesized to limit potentially autoreactive immature B cells.[41] Although the mechanism by which CD23-expressing B cells are protected from apoptosis is unclear, protection was reliant on anti-CD40 and IL-4, both signals of T cell help. Concomitant expression of Th2 cytokine receptors *IL13RA1* and *IL4R* in *IGHE*-expressing Bmem may suggest a requirement of T cells not only for class-switching, but also for promoting survival of IgE-expressing B cells, which otherwise are prone to apoptosis.[36–38]

SLIT was associated with an increased number of Lol p 1-specific Bmem highly expressing CD29. CD29 (β1 integrin) is commonly paired with CD49d (α4 integrin) to form the VLA-4 (α4β1) integrin that binds VCAM-1 and fibronectin to maintain leukocyte adhesion to the endothelium.[42] In vitro studies have demonstrated that CD29 expression on eosinophils enhances binding to nasal polyp endothelium,[43] and that histamine can induce expression of the CD29 ligand VCAM-1 on nasal polyp endothelium.[44] VCAM-1 is highly expressed on nasal polyps compared to the inferior turbinates from patients with diffuse sino-nasal polyps, and its expression is correlated with recurring development of nasal polyps in these patients.[45] Chronic allergic inflammation characterized by AR may therefore induce and maintain the expression of VCAM-1 in the upper airway to attract CD29-expressing allergen-specific Bmem to the site of allergen exposure and thereby draw circulating Bmem to their cognate antigen and promote allergen-specific immune responses. This is in accordance with the detection of *IGHE* germline transcripts in the bronchial mucosa of asthmatic patients,[46] which may indicate that *IGHE*- and *IGHG4*-expressing B cells that arise from the immune response to aeroallergens home to the site of allergen exposure. Upon repeated allergen exposure from immunotherapy, class-switched allergen-specific Bmem highly expressing CD29 may preferentially home to the

site of allergic inflammation, whereby natural allergen exposure may subsequently promote differentiation into IgG-producing plasmablasts that protect the host from allergic responses by producing IgG antibodies that competitively inhibit IgE on effector cells from binding to allergens. It remains unclear if CD29 expression increases on all circulating human Bmem upon exposure to their cognate antigen, or if persistent or repeated exposure is required. Repeated exposure leads to enhanced Ig class switching, and CD29 expression is typically higher on class-switched B cells than un-switched Bmem and naive B cells.[47]

The expansion of allergen-specific Bmem that highly express CD29 is strongly associated with efficacy of SLIT and may prove to be a potential new biomarker for successful immunotherapy. Furthermore, the one patient who did not report a clinical benefit from SLIT did not show a change in CD29 expression. Importantly, this marker can be assessed within a few months of treatment without the need of a pre-treatment assessment.

The Database of Allergy and Asthma Biomarkers (DAAB; version 2) includes two studies in humans that associate the expression of *ITGB1* in leukocytes with allergic disease.[48] One study identified >twofold lower *ITGB1* expression in $CD3^+$ T cells of patients with atopic dermatitis as compared to healthy controls,[49] while the other demonstrated a > 2 fold reduction in the expression of *ITGB1* in cells of the bronchoalveolar lavage from corticosteroid-resistant asthmatics compared to corticosteroid-sensitive asthmatics.[50] These studies did not address allergen-specific cells or evaluation after immunotherapy and so the finding of reduced CD29 expression does not contradict our result. The fact that the presence of disease might induce lower CD29 expression could actually help to improve the specificity of measurements of increased expression after SLIT.

One limitation of our study is that our cell sorting of Lol p 1-specific Bmem for single-cell transcriptomics was contaminated by monocytes. This is likely due to the rarity of these events, making it challenging to isolate these to more than 70% purity. It is unlikely that our tetramers bound to monocytes and that this affected in-depth immunophenotyping as all B cells were defined by expression of CD19 and at least one surface Ig isotype (IgM, IgD, IgG, IgA, IgE).

Our study demonstrates that AIT induces a unique transcriptional profile in circulating allergen-specific Bmem whereby B cells are class-switched to IgE and IgG_4, likely due to Th2 cytokines IL-4 and IL-13, and that this class-switching is associated with the expression of CD29 which may promote homing to inflamed tissue. Detection of changes in surface marker expression on allergen-specific Bmem by flow cytometry may provide a novel avenue for immune monitoring of successful therapy.

AUTHOR CONTRIBUTIONS

MCvZ and REO'H conceived the idea for the present study. REO'H, FT, and MH recruited patients and facilitated sample collection. CIM, NV, PMA, SR, BDW, and PMH conducted experiments. CIM analyzed the data. MCvZ and CIM wrote the manuscript with input from JMR, PMH, and REO'H. All authors revised and commented on the final version of the manuscript.

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CONFLICT OF INTEREST

MCvZ, REO'H, and CIM are inventors on a patent application related to this work. All the other authors declare that they have no relevant conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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