

Single-cell immunoprofiling after immunotherapy for allergic rhinitis reveals functional suppression of pathogenic T_H2 cells and clonal conversion



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Background: Allergic rhinitis is a growing problem worldwide. Currently the only treatment that can modify the disease is antigen-specific immunotherapy, but its mechanism of action is not fully understood.

Objective: We comprehensively investigated the role and changes of antigen-specific T cells before and after sublingual immunotherapy (SLIT) for Japanese cedar pollinosis.

Methods: We cultured peripheral blood mononuclear cells obtained both before and 1 year after initiating SLIT and used a combination of single-cell RNA sequencing and repertoire sequencing. To investigate biomarkers, we used cells from patients participating a phase 2/3 trial of SLIT tablets for Japanese cedar pollinosis and cells from outpatients with good and poor response. **Results:** Antigen-stimulated culturing after SLIT led to clonal expansion of T_H2 and regulatory T cells, and most of these

CD4⁺ T cells retained their CDR3 regions before and after treatment, indicating antigen-specific clonal responses and differentiation resulting from SLIT. However, SLIT reduced the number of clonal functional T_H2 cells but increased the trans-type T_H2 cell population that expresses muscadin (*MSC*), *TGF-β*, and *IL-2*. Trajectory analysis suggested that SLIT induced clonal differentiation of the trans-type T_H2 cells differentiated into regulatory T cells. Using real-time PCR, we found that the *MSC* levels increased in the active SLIT group and those with good response after 1 year of treatment.

Conclusion: The combination of single-cell RNA sequencing and repertoire analysis helped reveal part of the underlying mechanism: SLIT promotes the expression of *MSC* on pathogenic T_H2 cells and suppresses their function. *MSC* may be a potential biomarker of SLIT for allergic rhinitis. (*J Allergy Clin Immunol* 2022;150:850-60.)

Key words: Allergic rhinitis, sublingual immunotherapy, Japanese cedar pollinosis, T cell, single-cell RNA sequencing, repertoire sequencing, muscadin

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Allergic rhinitis (AR) is a common disease and has one of the highest prevalence rates in the world. According to the 2020 Japanese nasal allergy guidelines, its prevalence was 30% in children in 2019 and nearly 50% among individuals aged 10 to 50 years.¹ A pollinosis unique to Japan, called Japanese cedar pollinosis (JCP), which spreads as a result of a postwar reforestation policy in the 1950s, causes strong nasal symptoms during the pollen-dispersal period from February to April; unusually, its prevalence has been increasing by 10% every 10 years.² Although AR is not a life-threatening disease, its high prevalence and nasal symptoms can have a significant impact on work productivity. In fact, the European Union estimates yearly loss in productivity due to AR to be €30-50 billion.³ In Japan, the cost of a single season's prescription for JCP is 200-300 billion yen (\$2.1-3.2 billion). Thus, alternative treatments are needed.² However, individuals are often unaware of the decline in their productivity, and it is necessary to establish not only symptomatic but also curative treatments.

The advent of immunotherapy, which currently is the only treatment that can modify the disease for AR, has revolutionized the treatment of AR.⁴ In recent years, sublingual immunotherapy (SLIT), administered as tablets against mites, grass pollens, and, in Japan, Japanese cedar, has been effectively used for

Abbreviations used

AR:	Allergic rhinitis
JCP:	Japanese cedar pollinosis
MSC:	Musculin
PBMC:	Peripheral blood mononuclear cell
PCA:	Principal component analysis
scRNA-Seq:	Single-cell RNA sequencing
scVDJ-Seq:	Single-cell repertoire sequencing
SLIT:	Sublingual immunotherapy
TCR:	T-cell receptor
TNSS:	Total nasal symptom score
Tpath2:	Pathogenic T _H 2
TRA:	TCR α chain
Trans T _H 2:	Functionally impaired T _H 2 cell population
TRB:	TCR β chain
Treg:	Regulatory T
UMAP:	Uniform manifold approximation and projections

suppressing symptoms and is known to be better than symptomatic treatments such as antihistamines.⁵ However, SLIT is effective only if administered every day in the nonpollen season and for long time. Therefore, it is difficult to establish if the drug is being widely used, and its mechanism of action remains unclear. One report has indicated that SLIT increases the number of antigen-specific regulatory T (Treg) cells and that IgG₄ antibody blocks IgE activity.⁶ However, it is not known how Treg cells are induced during SLIT, and because certain studies have shown no Treg proliferation, depending on classification and function, this aspect remains unresolved.⁷⁻¹⁰ It is known that pathogenic T_H2 (Tpath2) cells are involved in the pathogenesis of allergic diseases,^{9,11,12} so we have been investigating antigen-specific T_H2 cell involvement in allergic inflammation; specifically, we wondered how Tpath2 cells are maintained locally and their involvement in inflammation and fibrosis.^{13,14} In humans, CRTH2-positive, CD161-positive T_H2 cells produce large amounts of T_H2 cytokines and express ST2, which further propagates allergic reactions.^{15,16} However, SLIT reduces Tpath2 cell numbers. We have previously reported that this reduction in functional T_H2 cells partially accounts for the effects of SLIT.¹⁷ However, the process or processes facilitating the reduction in Tpath2 cells and their relationship with Treg cells and other T-cell subsets remain unclear.

We therefore used a combination of single-cell RNA sequencing (scRNA-Seq) and single-cell repertoire sequencing (scVDJ-Seq) to comprehensively investigate the interrelationships between antigen-specific T cells by SLIT. Our data suggest that a mechanism of SLIT-induced immunodesensitization may include differentiation of T_H2 cells into Treg cells.

METHODS

Study design

The characteristics of the subjects are summarized in Table I. First, for scRNA-Seq and scVDJ-Seq, we used 14 peripheral blood mononuclear cell (PBMC) samples, which were obtained from 7 outpatients with JCP before and 1 year after initiating SLIT administration (2000JAU, CEDARTOLEN). The PBMCs were collected, frozen during the nondispersal period from 2014 to 2016 at the hospital's outpatient department and thawed for performance of scRNA-Seq and scVDJ-Seq in 2021 and 2022. Subjects 1 to 7 were patients whose total nasal symptom scores (TNSS) were severe during

the pollen season before SLIT initiation. These 7 patients were asked to describe their symptoms during 1 week of the peak pollen season and if the use of SLIT reduced their symptoms. To investigate biomarkers, we used 2 groups of PBMCs for real-time PCR. The first group comprised 22 patients whose specimens had been collected at Chiba University as part of the TO-206 study by Torii Pharmaceutical Co Ltd,¹⁸ which was a phase 2/3 trial of SLIT tablets for JCP (JapicCTI-142579). Placebo or SLIT tablet treatment was initiated in summer 2014; patients were instructed to record their symptoms during the following year's pollen season in the same diary. Symptoms during the peak pollen dispersal week were subsequently analyzed. As in previous trials,¹⁹ guidelines of the Japanese Society of Allergy and Immunology were followed for the TNSS, and the 3 symptoms of sneezing, runny nose, and nasal obstruction were scored on a scale of 0 to 4. The PBMCs in the second group for PCR were obtained from 36 patients who started SLIT in 2014 or 2015 using the same procedure for the scRNA-Seq experiments. We used samples from before and 1 year after the start of treatment. All samples were collected during the pollen's nondispersal period.

In this experiment, we classified disease as exhibiting good or poor response on the basis of whether SLIT was effective in alleviating symptoms; the 2 groups were compared thereafter. Patients who reported an improvement of >2 points in the TNSS after SLIT were considered to have disease with good response; all other patients were considered to have poor disease response.

All subjects provided written informed consent, and the study was approved by the ethics committee of Chiba University Graduate School of Medicine.

Cell culture

PBMCs were purified from whole blood using the Ficoll-Hypaque technique and cultured as previously described.¹⁶ RPMI 1640 (Sigma-Aldrich, St Louis, Mo), supplemented with fetal calf serum (10%), L-glutamine (2 μ mol), penicillin (100 U/mL), streptomycin (100 μ g/mL), HEPES (10 mmol), 2-mercaptoethanol (55 μ mol), nonessential amino acids (1%), and sodium pyruvate (1 mmol) (all from Gibco; Thermo Fisher Scientific, Waltham, Mass), was used as the culture medium. PBMCs obtained from patients were precultured (1×10^6 cells/mL) at 37°C overnight, and cells were stimulated with Sugi ("Japanese cedar" in Japanese) basic protein (2 μ g/mL; Hayashibara, Okayama, Japan) in the presence of recombinant human IL-2 (1 U/mL; Kyowa, Tokyo, Japan) for 5 days. Sugi basic protein is composed of major Japanese cedar pollen allergens Cry j 1 and Cry j 2. For real-time PCR, cultured PBMCs were immediately processed in TRIzol reagent (Invitrogen; Thermo Fisher Scientific). To compare patients with good and poor disease response, 25 ng/mL of phorbol-12-myristate-13-acetate and 1 μ g/mL of ionomycin (Sigma-Aldrich) were added at the final 4 hours of culture because the available number of cells was small.

Single-cell RNA sequencing and single-cell repertoire sequencing

Memory CD4⁺ cells (CD3⁺CD4⁺CD45RO⁺) were sorted on a FACS Aria III device (Becton Dickinson, San Diego, Calif) from PBMCs and then encapsulated in droplets. The scRNA-Seq libraries were prepared using Chromium Next GEM Single Cell 5' Reagent Kits (v1.1) according to the manufacturer's protocol (10x Genomics, Pleasanton, Calif). Full-length T-cell receptor (TCR) V(D)J segments (scVDJ-Seq) were enriched from amplified complementary DNA derived from 5' libraries via PCR amplification using a Chromium Single-Cell V(D)J Enrichment Kit for Human T Cells according to the manufacturer's protocol (10x Genomics). The generated scRNA-Seq and scVDJ-Seq libraries were sequenced using 150-cycle paired-end reads on a NovaSeq 6000 device (Illumina, San Diego, Calif). We used the following reagents for flow cytometry: fluorescein isothiocyanate-anti-CD3 (Becton Dickinson), phycoerythrin-anti-CD4 (BioLegend, San Diego, Calif), PECy7-anti-CD45RO (BioLegend), and 7AAD (Becton Dickinson).

scRNA-Seq and scVDJ-Seq analysis

Sequence reads from all samples were processed and scRNA-Seq data were aggregated using the Cell Ranger (v6.1.1) pipeline with GRCh38 (v5.0.0) as

TABLE I. Patient characteristics

Characteristic	scRNA-Seq		PCR 1		PCR 2	
	Good	Poor	Active	Placebo	Good	Poor
No. of patients	4	3	14	6	21	15
Age (years), median (IQR)	28 (23-28)	41 (33-51)	44.5 (39.5-47)	45.5 (41.8-48.5)	47 (40-50)	45 (31.5-57.5)
Sex (M/F), no.	3/1	1/2	3/11	3/3	9/12	6/9
Pre-SLIT blood cedar IgE level (UA/mL), median (IQR)	12.6 (3.2-27.0)	20.7 (20.2-27.5)	16.2 (9.1-32.2)	17.9 (9.5-22.3)	17.8 (7.2-49.0)	10.5 (7.9-32.0)
Pre-SLIT TNSS, median (IQR)	8.5 (7.8-8.5)	6 (5.5-6.5)	9.5 (7.3-10)	8.0 (8.0-8.8)	9.0 (8-11)	6.0 (4.5-9.0)
Post-SLIT TNSS, median (IQR)	2.9 (2.6-3.4)	6.8 (5.9-7.9)	4.1 (3.0-5.2)	7.6 (4.5-10.7)	4.6 (3.2-6.2)	6.0 (4.4-8.4)

Good, Good response; IQR, interquartile range; Poor, poor response.

reference. Next, scVDJ-Seq data were aggregated using the Cell Ranger (v6.1.1) 'vdj' pipeline with GRCh38 (v5.0.0) as reference. A TCR diversity metric containing clonotype frequency and barcode information was obtained. For TCR, only cells with at least 1 productive TCR α chain (TRA) and 1 productive TCR β chain (TRB) were used in further analysis. Each unique TRA-TRB pair was defined as a clonotype. The integration process of scRNA-Seq and scVDJ-Seq in the same cell was performed using the 'cellranger' multi-pipeline (v6.1.1). Aggregated data were further analyzed using "Seurat".²⁰ Specifically, we log normalized the expression matrix, regressed the data against the total number of unique molecule identifiers detected per cell, performed a principal component analysis (PCA), and used PCA dimensions 1 to 50 to find clusters via uniform manifold approximation and projections (UMAP). We visualized single-cell gene expression as UMAP overlays and a volcano plot. We also visualized T-cell clones as UMAP overlays. Single-cell gene expression was visualized as density plots by the Nebulosa R package (v1.0.1) algorithm.²¹ The percentage of cell clones was visualized as a pie chart and as a stacked bar graph; these were generated by 'ggplot2' (ggplot2.tidyverse.org/index.html). The Venn diagram was realized by 'gplot' (v3.1.1) (github.com/talgalili/gplots). To estimate the stage of differentiation, pseudotime trajectory analysis was performed using the Monocle3 package for R (v3.0) algorithm,²² based on gene expression patterns.

Data and materials availability

All data are available in the main text or in the Online Repository at www.jacionline.org. The raw and analyzed data we use in this report are available under accession number GSE200107 in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo).

Real-time PCR

Total RNA was isolated from PBMCs, and complementary DNA was synthesized as previously described.¹⁶ Semiquantitative real-time PCR was performed, and relative gene expression was calculated by the comparative C_t method. All procedures were performed according to the manufacturer's instructions. TaqMan Gene Expression Assay probes (Applied Biosystems; Thermo Fisher Scientific) were used for *MSC* and *IL2*.

Statistical analyses

Values are shown as means $\pm$ SEMs. The Mann-Whitney U test was used for intergroup comparisons and the Wilcoxon signed-rank test for intragroup comparisons. $P < .05$ was considered to indicate statistical significance. All statistical analyses were performed by GraphPad Prism (v6.03) software (GraphPad Software, La Jolla, Calif).

RESULTS

Functional subsets and clonal proportions of CD4⁺ memory T cells

PBMCs that were obtained before and 1 year after SLIT from those with good ($n = 4$) and poor ($n = 3$) response were used.

After culturing and stimulation with Japanese cedar antigen, CD3⁺CD4⁺CD45RO⁺ T cells were isolated and analyzed simultaneously by scVDJ-Seq for TCR and scRNA-Seq. From the obtained sequencing data, 102,037 cells were detected using the single-cell analysis pipeline. Of these cells, 20,537 were from patient 1; 9,579 from patient 2; 23,041 from patient 3; 17,491 from patient 4; 14,259 from patient 5; 25,317 from patient 6; and 11,039 from patient 7. V(D)J data for TCR were combined for all cells of high quality, corrected for read depth and mitochondrial read counts, and subjected to PCA. On the basis of the expression levels of known transcription factors and marker genes, 7 T-cell clusters (naive, Treg, T_H2, T_H17, cytotoxic CD4, natural killer T, and interferon response cells) among CD4⁺ T memory cells in PBMCs were identified and are shown in UMAP (Fig 1, A), which shows each subject (Fig 1, B) as well as a representative gene expression that determined subsets (Fig 1, C). Among these cells, a unique TCR configuration was seen in 67.2% to 78.5% (Fig 1, D), whereas more than 1 clonal memory CD4⁺ T-cell type accounted for 32.8% in those with poor response and 21.5% in those with good response, and clones with >5 cells constituted 10.3% in those with poor response and 4.7% in those with good response of all cells. Next, to compare clonal T-cell clusters (unique, 2-5 cells, and >5 cells), relative proportions for each of these clusters were calculated on the basis of scVDJ-Seq data and depicted on the UMAP (Fig 1, E). As the number of clones increased, the clones accumulated in effector cells. We show that T cells with multiple clones were predominantly T_H2, T_H17, Treg, or cytotoxic CD4, and that, notably, clonal population was highest in the T_H2 cell cluster (Fig 1, F).

Alterations in T-cell subsets and clonality before and after SLIT

To investigate T-cell responses, we compared T-cell subset profiles before and after SLIT against Japanese cedar antigen. Although a large individual difference was present, no consistent changes in overall T-cell subsets were seen before and after SLIT in both those with poor and good response if the subsets were not classified by clone count (Fig 2, A and B). Therefore, we reanalyzed the cells by categorizing them as unique groups of cells that form a single clone and as clonal populations that form multiple clones. It was observed that unique and 2-5 cell populations did not show significant changes before and after SLIT, whereas >5 clonal cell populations revealed that T_H2 and Treg cells only expanded in those with good response (Fig 2, C). Results are shown in Fig E1 in this article's Online Repository at www.jacionline.org for each T-cell subset. Next, to evaluate the variation in antigen specificity due to changes in TCR repertoire before

and after SLIT, we compared amino acid sequences of TRA and TRB in the CDR3 region in all patients. T-cell clones with high clonal frequency in the top 7 CDR regions showed overlap of 60% to 80% before and after SLIT (Fig 3, A). Moreover, if the number of clones was limited to >5, there was an 80% overlap in the CDR regions of both TRA and TRB in pre- and post-SLIT specimens (Fig 3, B).

Functional changes in T_H2 and Treg cells after SLIT

Because it has been suggested that SLIT induces clonal expansion of T_H2 and Treg cells with the same antigen specificity, we immunoprofiled T_H2 and Treg cells that showed clonal expansion after SLIT and performed PCA to increase the resolution and reevaluate the immune profiles (Fig 4, A). Consequently, on the basis of the expression of *IL-5*, *IL-13*, *PTGDR2* (CRTH2), and *KLRB1* (CD161), T_H2 cells could be divided into 2 clusters: a highly functional T_{path}2 cell population and a functionally impaired T_H2 cell population (Trans T_H2) (Fig 4, B). Next, we performed trajectory analysis to identify the differentiation pathway in these cell populations and evaluated pseudo-time based on similarity in gene expression profiles. Thus, a differentiation trajectory from T_{path}2 cells to Trans T_H2 cells was observed among T_H2 cells (Fig 4, C), along with a continuum of intermediate cells between Trans T_H2 cells and the Treg cell populations, indicating transdifferentiation of Trans T_H2 cells to Treg cells. Moreover, a comparison of TCR clones in these cell populations before and after SLIT showed that the clonal expansion of Treg cells (Fig 2, C) and Trans T_H2 cells as well as a decrease in T_{path}2 cells were observed in those with good response with multiple clones after treatment, especially in those with >5 clones (Fig 4, D and E). The results for each subject are presented in Fig E2 in the article's Online Repository at www.jacionline.org. These results therefore indicate the possibility of clonal transdifferentiation of Trans T_H2 cells into Treg cells.

Genetic shift from T_H2 cells to Treg cells

We next comprehensively compared gene expression profiles of each cell population to search for functional molecules that facilitate Trans T_H2 cell-mediated differentiation to Treg cells and immune tolerance after SLIT. We found that myosin (MSC), a transcription factor that represses the transcriptional program of T_H2 cells, and *LPAR6*, a T-cell migration inhibitory receptor, were highly upregulated in Trans T_H2 cells compared to T_{path}2 cells (Fig 5, A). MSC was also highly and specifically expressed at the end of differentiation trajectories in Trans T_H2 cells (Fig 5, B). Additionally, while the expression of *IL-5* was lower in Trans T_H2 cells (Fig 4, B), that of *IL-2* and *TGF-β*—that is, molecules that induce Treg cell differentiation—was higher. A comparison of Trans T_H2 cells and Treg cells revealed greater expression of *FOXP3*, inhibitory cytokines such as *TGF-β* and *IL-10RA*, and *CTLA4*, the inhibitory immune checkpoint factor, in Treg cells. Regarding the changes after SLIT within each T-cell subset, MSC and *IL-2* expression was upregulated after SLIT in Trans T_H2, whereas *TGF-β* expression was upregulated after SLIT in both Trans T_H2 and Treg cells (Fig 5, C).

Potential as a biomarker

Given these changes in the expression of MSC and *IL-2* and their strong expression in Trans T_H2 cells, we investigated if these molecules can be used as biomarkers for SLIT from the perspective of suppression of T_H2 cell function in specimens obtained from a previous clinical trial of SLIT tablets for JCP.¹⁸ The study scheme is depicted in Fig 6, A. We were able to use only specimens shared by Chiba University Hospital. Specifically, in addition to 6 patients in the placebo group, there were 16 patients in the active drug group. However, 2 patients whose symptoms worsened after SLIT were excluded, resulting in 14 patients in the active drug group who showed symptom suppression. To evaluate their utility as biomarkers, we analyzed the expression of MSC and *IL-2* in whole PBMCs by PCR after antigen stimulation. In contrast to samples from the placebo group that showed no particular increase in MSC after SLIT, the active drug group showed a significant increase in MSC at 1 year after therapy initiation (Fig 6, B). We also observed an increasing trend in *IL-2* expression after 1 year of therapy (Fig 6, C). Additionally, we analyzed 36 outpatients to confirm whether MSC can serve as biomarkers of SLIT efficacy. PBMCs before and 1 year after treatment were cultured and PCR performed. A significant increase in MSC expression was observed in those with good response after SLIT (Fig 7, A). However, no significant changes were observed in *IL-2* expression (Fig 7, B). These results demonstrate the usefulness of evaluating the efficacy of MSC in PBMCs as an assessment of curative response to SLIT.

DISCUSSION

In our study, using blood samples derived from patients whose disease was or was not responsive to SLIT, we investigated its mechanistic aspects by subjecting T cells to scRNA-Seq and scVDJ-Seq. We found that in T cells with multiple clones after antigen-stimulated culture, T_H2 and Treg cell numbers increased after SLIT, suggesting that differentiation occurred mostly within the same clone. However, this clonal expansion occurred in Trans T_H2 cells whose function was suppressed. Thus, our results point toward a mechanism wherein SLIT suppresses antigen-specific T_H2 cell function and induces their differentiation into a Treg cell population. Further, MSC, which is known to suppress the function of T_H2 cells, may be involved as the transcription factor, and T_H2 cells with suppressed functions, such as lower CRTH2 and *IL-5* secretion, could enhance their expression of *IL-2* and *TGF-β* to become Treg cells. Finally, we investigated whether MSC and *IL-2*, which are expressed on Trans T_H2 cells just before their differentiation into Treg cells, are useful as biomarkers for SLIT. We found that drug administration led to an increase in MSC expression in patients with disease responsive to SLIT.

Antigen-stimulated CD4⁺ T cells differentiate into various subsets, such as T_H2 and T_H17, depending on their environment; such plasticity persists even after differentiation from the naive state, and despite changes in cytokines secreted and surface antigens expressed.²³ In the present study, 1 year of SLIT resulted in an increase in Trans T_H2 cells that expressed the transcription factor MSC but whose function was suppressed. MSC, a member of the helix-loop-helix family of transcription factors, was first described in mouse skeletal muscle progenitor cells and later identified as a transcriptional repressor in B-cell lymphoma.^{24,25} Wu et al²⁶ reported that MSC suppresses T_H2 cell function by inhibiting GATA3 but that it promotes their differentiation into Treg

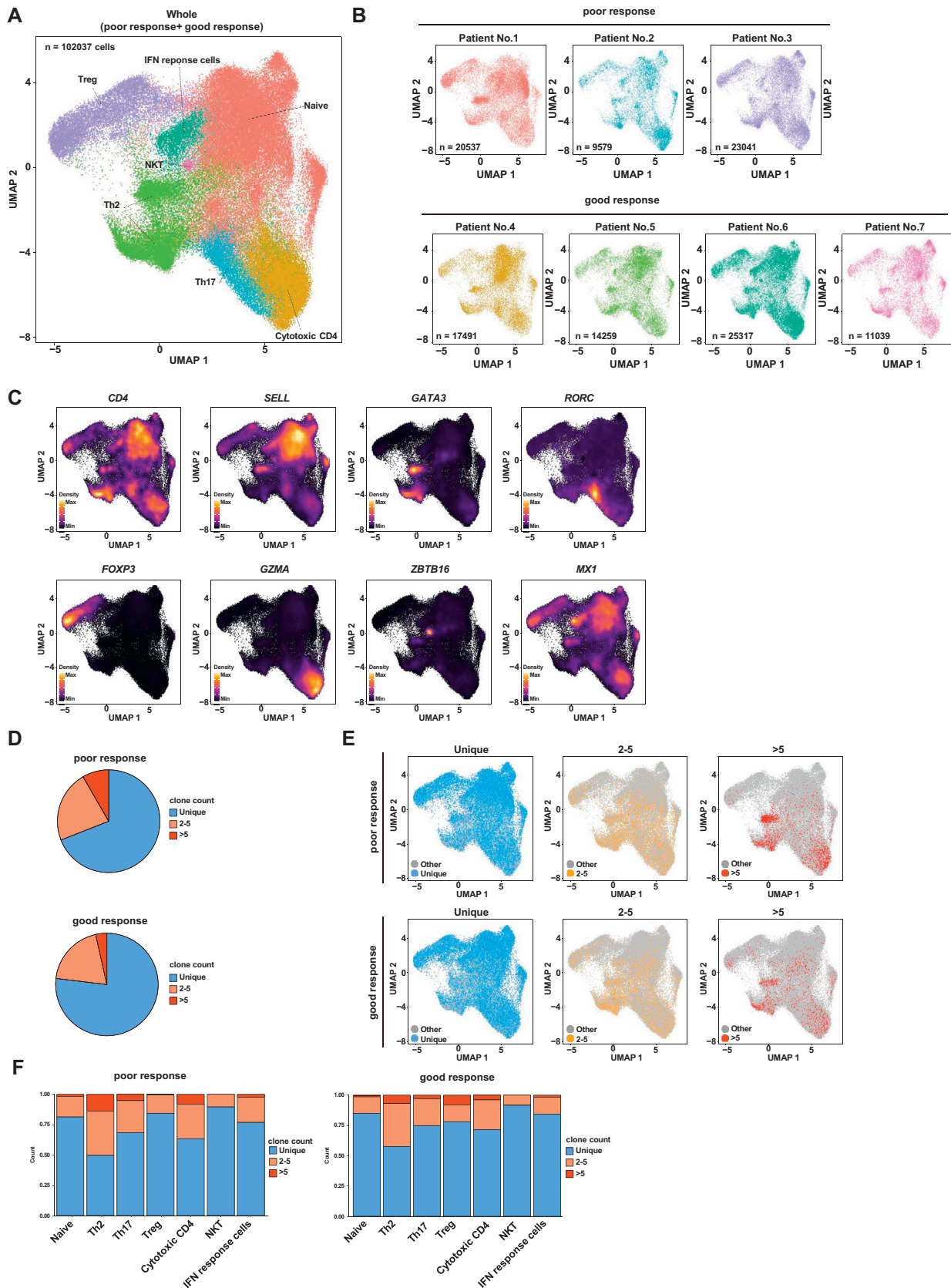
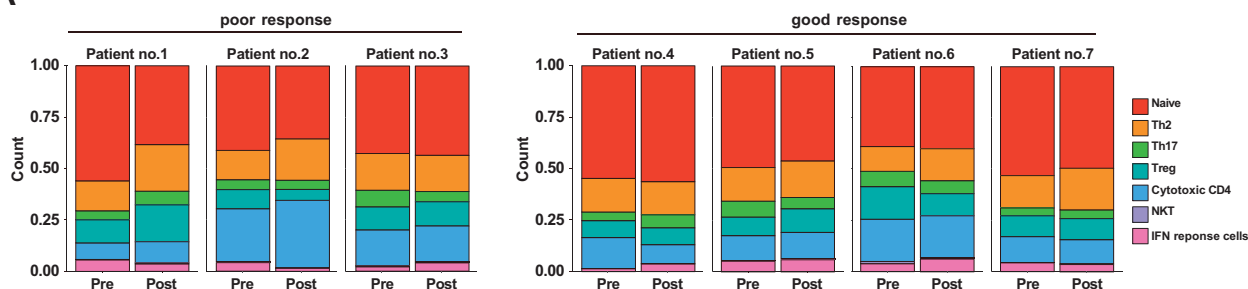
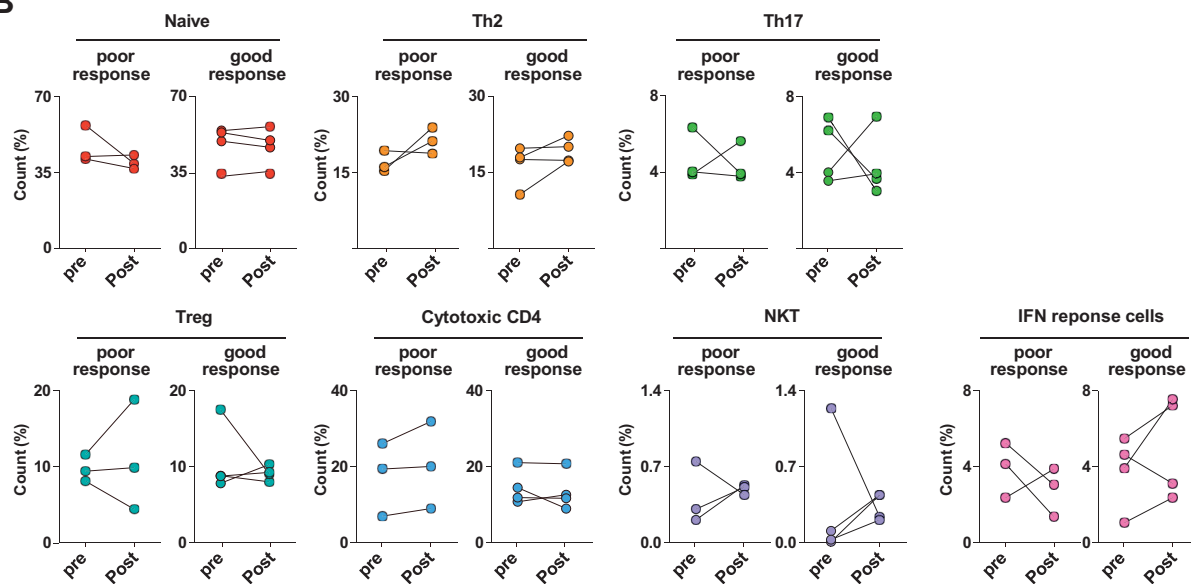


FIG 1. Changes in T-cell subsets seen with scRNA-Seq and repertoire analysis. **(A)** UMAP generated by single-cell analysis of memory CD4⁺ T cells isolated after culturing PBMCs with cedar antigen before and after SLIT. The number of cells analyzed is indicated. **(B)** Cell distribution on UMAP for each subject. **(C)** Gene expression intensities that characterize each subset are shown on the UMAP. Black indicates low expression; yellow, high expression. Logarithmic scale used. **(D)** Proportion of TCR amino acid sequence duplication across 14 samples. Amino acid sequences that were of a single clone type (unique; blue), sequences with multiple clones (2-5; orange), and groups with more than 5 clones (red) are shown in those whose disease had good and poor response. **(E)** The distribution of T cells with unique, multiple clones of 2-5, or multiple clones of >5 shown

A



B



C

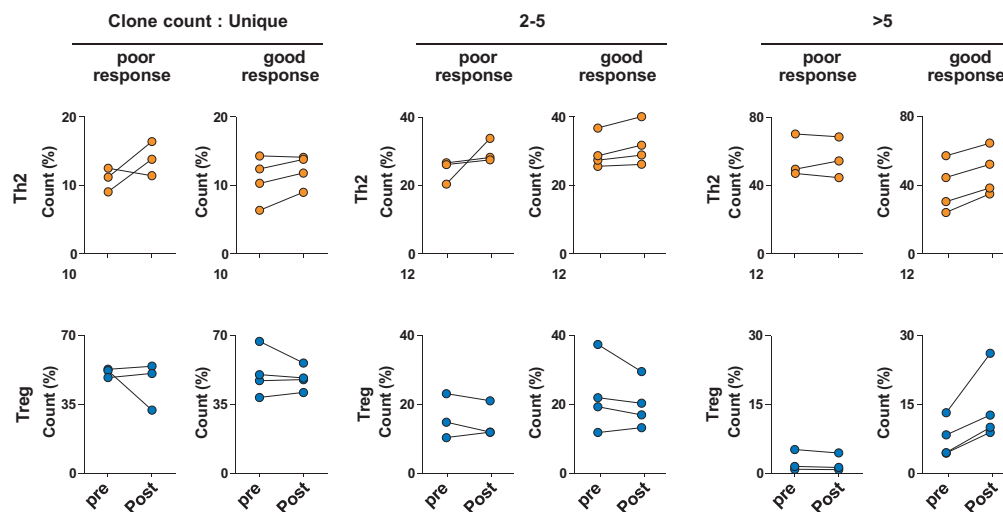
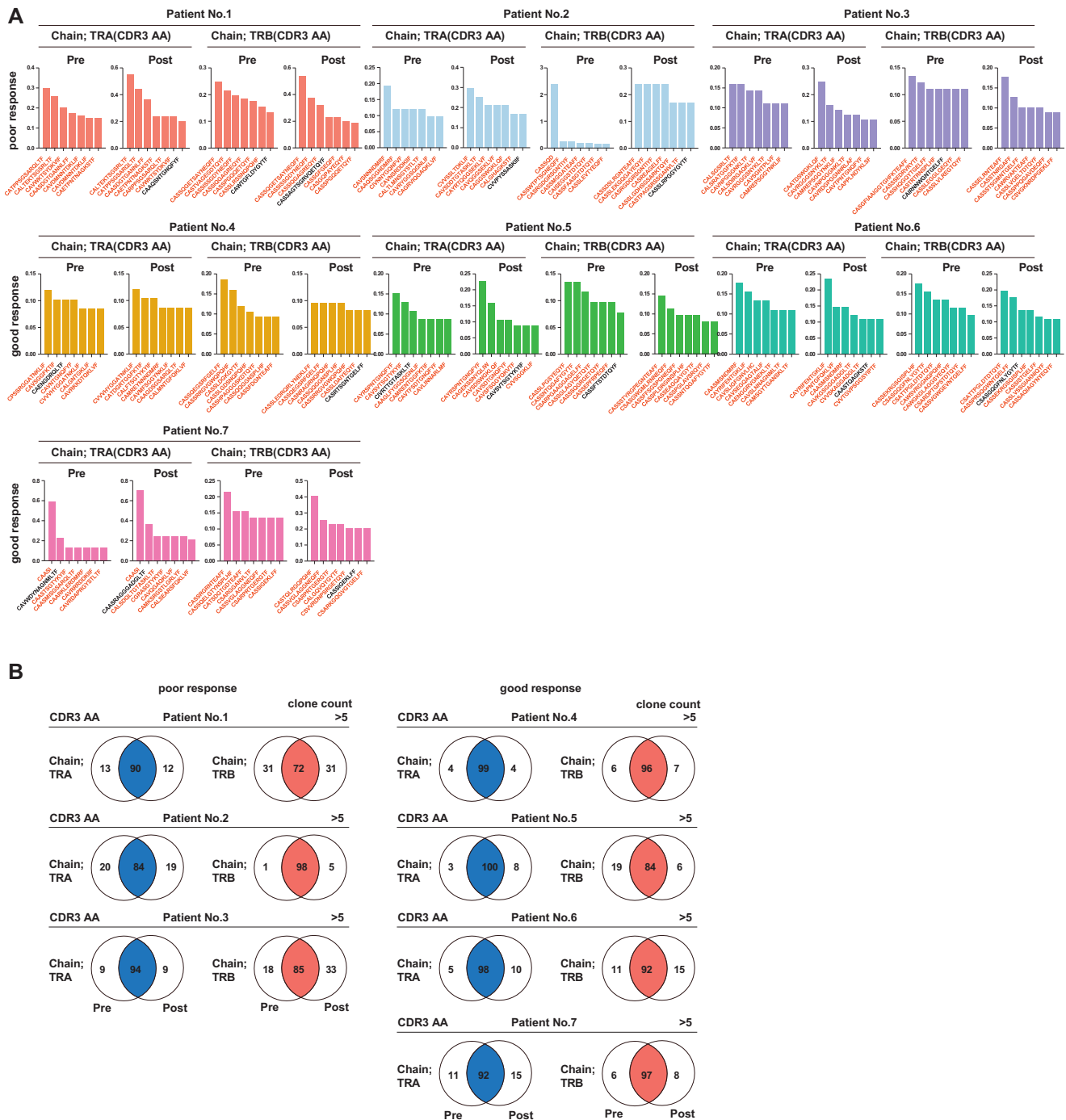


FIG 2. Changes in T-cell subsets before and after SLIT. **(A)** Proportion of T-cell subsets in each subject before and after SLIT. *Pre* indicates before sublingual immunization; *post*, 1 year after. **(B)** Proportional shifts in each T-cell subset before and after SLIT. **(C)** Proportional change in T_H2 , T_H17 , and Treg cells in those with poor and good response before and after SLIT, categorized as clones of TCRs that are unique, 2-5, or >5.

on the UMAP. *Left to right*, Distribution of unique cells (blue), distribution of cells with multiple clones of 2-5 (orange), and distribution of cells with multiple clones of >5 (red). **(F)** The proportion of each T-cell subset by number of clones (unique, 2-5, and >5) are presented in 3 different colors.



cells by enhancing Foxp3 expression. They have also demonstrated that Foxp3 alone cannot induce Treg differentiation without repressing other lineage-specific transcriptional programs, and that MSC forms a complex with GATA3 to act as a repressor and thereby suppress T_H2 function. Although that report

was based on *in vitro* and mouse models, we show that this phenomenon can actually occur in humans after SLIT. Additionally, comparison of gene expression profiles between Tpath2 cells and Trans T_H2 cells showed that SLIT enhanced the expression of TGF- β and IL-2 in Trans T_H2 cells, and because Treg cells can

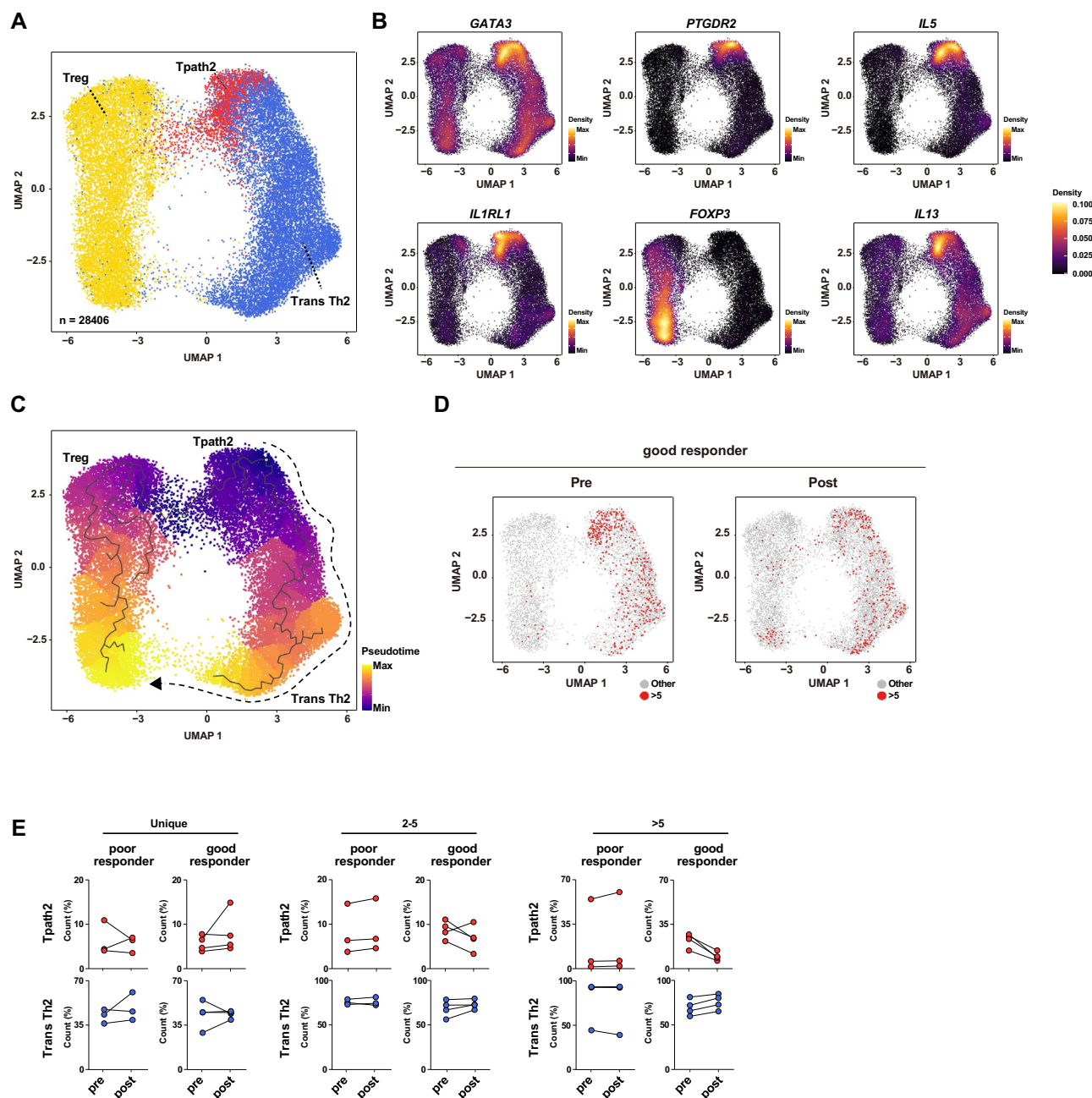


FIG 4. Functional modification of T_H2 and Treg cells after SLIT. **(A)** UMAP focusing on T_H2 and Treg cells, generated from a total of 14 samples. From PCA, T_H2 cells were categorized as Tpath2 (red) or Trans T_H2 (blue); Treg cells are presented in yellow. **(B)** Gene expression intensity shown in the UMAP: GATA3 (GATA3), *PTGDR2* (CRTH2), *IL1RL1* (ST2), *IL5* (IL-5), *FOXP3* (Foxp3), and *IL13* (IL-13). Black indicates low expression; yellow, high expression. **(C)** Pseudo-time trajectory analysis of T_H2 and Treg cell differentiation induced by SLIT. **(D)** Cell variation in unique and multiple-clone groups before and after SLIT. Gray dots indicate unique cells, generated from a total of 14 samples; red dots indicate multiple-clone cells, generated from good-response samples before and after treatment. Pre indicates before SLIT; post, 1 year after SLIT. **(E)** Proportional shifts in T_H2 subsets compared between those with poor and good response. The unique group and the multiple-clone group are shown before and after SLIT.

differentiate in the presence of TGF- β and IL-2 under *in vitro* assay conditions,²⁷ it is possible that Trans T_H2 cells create their own environment for Treg differentiation. Once the T_H2 cell becomes a Treg and expresses Foxp3, Foxp3⁺ Treg cells cannot produce IL-2 by themselves because Foxp3 represses the transcription of IL-2 gene.²⁸ Thus, IL-2 signaling from other

T cells would be essential for Treg homeostasis,²⁹ and it may be produced by Trans T_H2 cells themselves.

In the present study, about 70% of the cells were unique (that is, with no overlap in TCR amino acid sequence), and more than 1 clone was found in approximately 20% to 30% of the cells, which is relatively high. Similarly, a study that analyzed T-cell

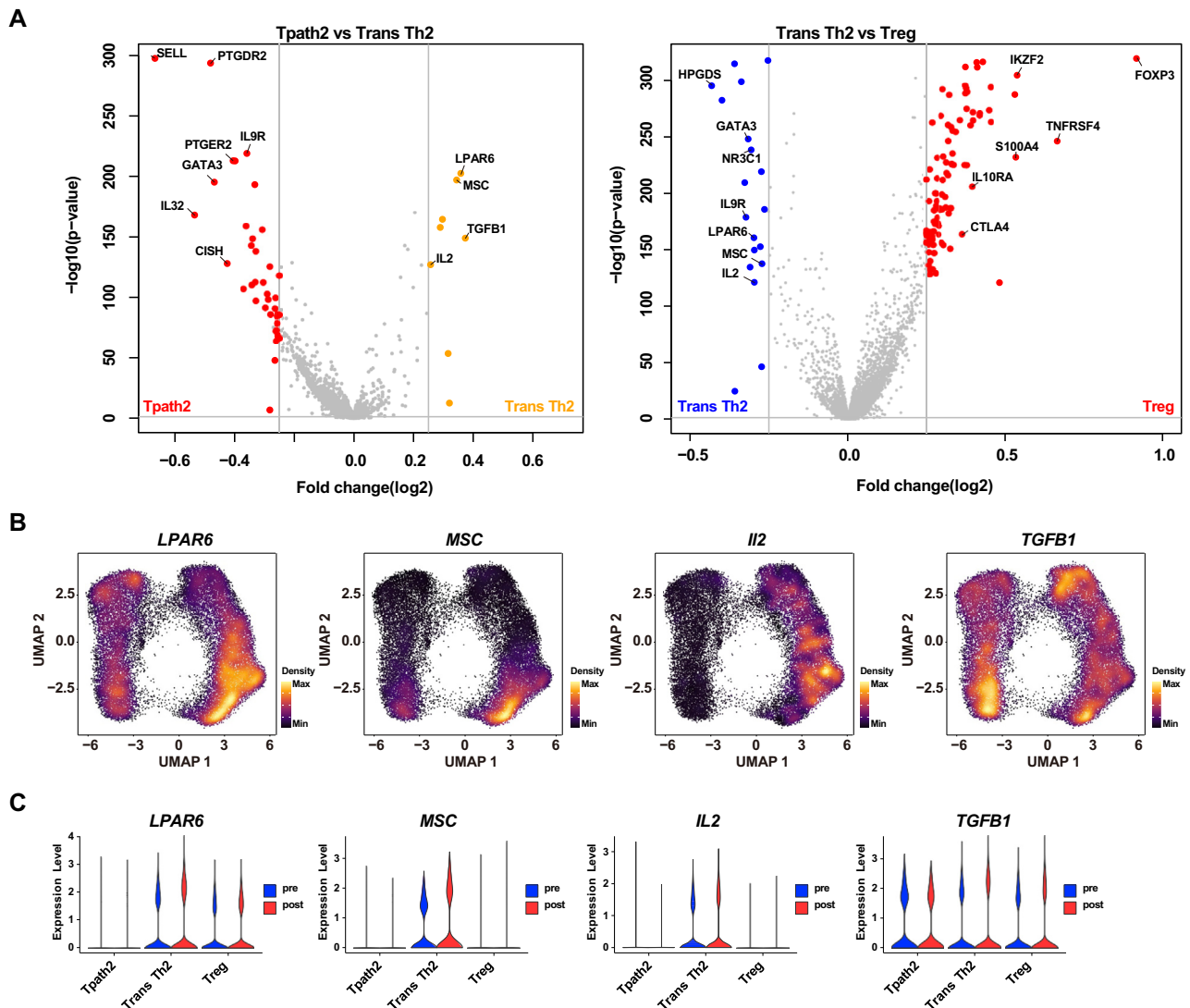


FIG 5. Comparison of gene expression between functional T_H2 cells, T_H2 cells with suppressed function, and Treg cells. **(A)** Differences in gene expression between Tpath2 and Trans T_H2 cells (left), and between Trans T_H2 and Treg cells (right) are shown as a volcano plot. The names of the characteristic genes that showed differences are shown. **(B and C)** Gene expression intensities of *LPAR6* (lysophosphatidic acid receptor 6), *MSC* (musculin), *IL2* (IL-2), and *TGFB1* (TGF- β) are shown on the UMAP (B) and comparisons in each subset before and after by violin plots (C).

repertoires in PBMCs in about 19 humans, including Covid-19 patients, showed that about 90% of the cells were unique.³⁰ Given the result that T_H2 was the most abundant cell type in more than 1 clone, we hypothesized that we could categorize dividing cells on the basis of antigen specificity after stimulation with Japanese cedar antigen. We have previously described methods used to capture dividing cells after antigen stimulation, such as flow cytometry or using tetramers. However, although it is difficult to determine antigen specificity,¹¹ because this study used scRNA-Seq combined with antigen-stimulated culture and repertoire analysis using scVDJ-Seq to compare changes before and after SLIT, we were able to demonstrate that about 80% of the most frequent amino acid sequences remained identical at 1 year after SLIT, attesting to the usefulness of combining specific antigen stimulation with scVDJ-Seq and scRNA-Seq.

Previous studies have reported the involvement of higher Treg cell numbers and IgG₄ in symptom reduction after SLIT;⁴ however, the results described here, as well as results reported by us and others previously,^{11,17} imply a role for suppression of T_H2 function. Further, although it is possible that MSC expression-induced inhibition of T_H2 cells may be due to repeated sublingual antigen stimulation, the role of plasmacytoid dendritic cells or the effects of induced Breg or Treg cells on Tpath2 cells could not be ascertained. Thus, a system that permits efficient expression of MSC will enable a better understanding of the underlying mechanism or mechanisms. In general, women are more susceptible to collagen or allergic diseases, partly because the expression of Foxp3 and its protein complex on the X chromosome is disrupted by hormonal changes and inflammatory cytokines.³¹⁻³³ In the present study, scRNA-Seq was performed in a larger number of male

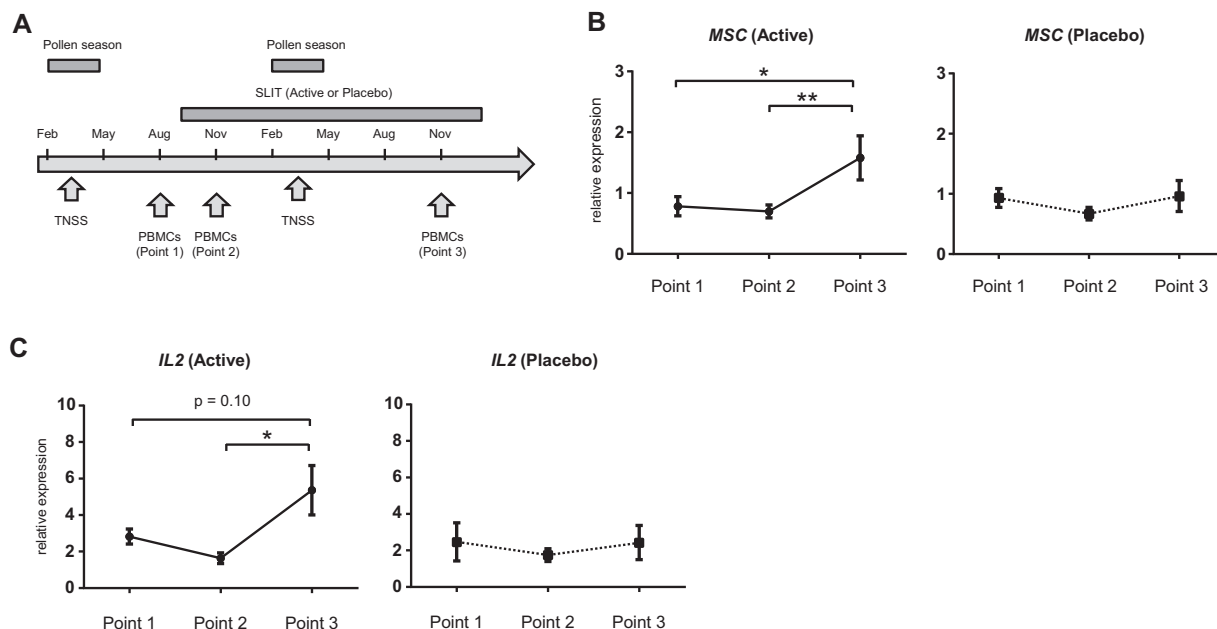


FIG 6. Analysis of *MSC* and *IL-2* in specimens from the phase 2/3 clinical trial. **(A)** Outline of the medication and follow-up procedures in clinical trial for SLIT tablets. **(B and C)** The expression of *MSC* **(B)** and *IL-2* **(C)** in PBMCs cultured with Japanese cedar antigen and *IL-2* by real-time PCR in patients provided active SLIT (n = 14) or placebo (n = 6). **P* < .05, ***P* < .01.

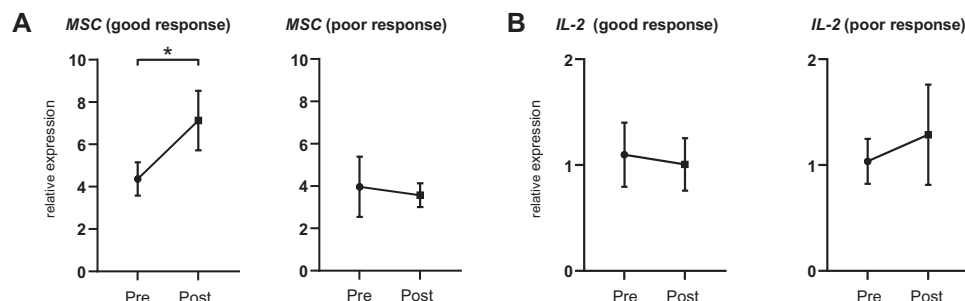


FIG 7. Analysis of *MSC* and *IL-2* expression in specimens collected from patients with good and poor response. The expression of *MSC* **(A)** and *IL-2* **(B)** in PBMCs cultured with Japanese cedar antigen was analyzed by real-time PCR in those with good (n = 21) and poor (n = 15) response. **P* < .05.

subjects with good response; therefore, the possibility of sex affecting Foxp3 cannot be denied. However, the effect of SLIT has not been reported to vary by sex,^{34,35} and no differences due to sex were observed in the variation of *MSC* (see Fig E3 in the Online Repository at www.jacionline.org). Further, for experimental systems, additional studies are warranted. T cells have various subpopulations, including T_H1, T_H2, T_H17, Treg, and Tfh, and each has a different maturation period. We thus could not determine how the duration of this experiment's culture period affected the increase of the other subpopulations.

Currently there are no biomarkers for evaluating the efficacy of SLIT,³⁶ and any increase in Treg numbers should be reflected in a greater expression of Foxp3; however, this has also not been clearly established. Thus, presumably the effect of SLIT resides in actual functionality of the cells, which is difficult to assess according to an increase or decrease in transcription factor levels. IL-5 is a

pathogenic factor in allergic diseases; we have previously reported that T cells expressing CRTH2 and CD161 produce large amounts of IL-5 but that SLIT reduces its levels; however, we did not find any difference in Foxp3 expression in our study.¹⁷ Therefore, we checked for higher *MSC* expression and found a significant increase within the active drug group and those with good response to SLIT. This observation not only confirms a role for this factor in the mechanism of action of SLIT but also indicates its usefulness as a potential biomarker of long-term effects.

To summarize, we described the mechanism of symptom reduction due to SLIT by combining single-cell analysis and repertoire analysis. Specifically, promoting the expression of *MSC* in T_{path}2 cells may contribute to SLIT-induced reduction in symptoms by suppressing T_H2 cell function and allowing them to differentiate into Treg cells. Various methods of allergen-specific immunotherapy are currently being devised.^{37,38} Because *MSC*

expression may be used as a marker of its efficacy, developing methods to effectively produce MSC can enhance the effectiveness of this therapy.

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Clinical implications: MSC expression was higher in the group of active patients whose disease was responsive to SLIT, indicating that these molecules may be potential biomarkers of SLIT efficacy.

REFERENCES

- Okubo K, Kuroto Y, Ichimura K, Enomoto T, Okamoto Y, Kawauchi H, et al. Japanese guidelines for allergic rhinitis, 2020. *Allergol Int* 2020;69:331-45.
- Yamada T, Saito H, Fujieda S. Present state of Japanese cedar pollinosis: the national affliction. *J Allergy Clin Immunol* 2014;133:632-9.e5.
- Bousquet J, Anto JM, Bachert C, Baiardini I, Bosnic-Anticevich S, Walter Canonica G, et al. Allergic rhinitis. *Nat Rev Dis Primers* 2020;6:95.
- Nolte H, Maloney J. The global development and clinical efficacy of sublingual tablet immunotherapy for allergic diseases. *Allergol Int* 2018;67:301-8.
- Durham SR, Creticos PS, Nelson HS, Li Z, Kaur A, Meltzer EO, et al. Treatment effect of sublingual immunotherapy tablets and pharmacotherapies for seasonal and perennial allergic rhinitis: pooled analyses. *J Allergy Clin Immunol* 2016;138:1081-8.e4.
- Kucuksezer UC, Ozdemir C, Cevhertas L, Ogulur I, Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy and allergen tolerance. *Allergol Int* 2020;69:549-60.
- Francis JN, Till SJ, Durham SR. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;111:1255-61.
- Kerstan A, Beyersdorf N, Stoevesandt J, Trautmann A. Wasp venom immunotherapy expands a subpopulation of CD4⁺CD25⁺ forkhead box protein 3-positive regulatory T cells expressing the T-cell receptor Vβ2 and Vβ5.1 chains. *J Allergy Clin Immunol* 2012;130:994-6.e3.
- Bonvalet M, Moussu H, Wambre E, Ricarte C, Horiot S, Rimaniol AC, et al. Allergen-specific CD4⁺ T cell responses in peripheral blood do not predict the early onset of clinical efficacy during grass pollen sublingual immunotherapy. *Clin Exp Allergy* 2012;42:1745-55.
- Bacher P, Scheffold A. The effect of regulatory T cells on tolerance to airborne allergens and allergen immunotherapy. *J Allergy Clin Immunol* 2018;142:1697-709.
- Wambre E, DeLong JH, James EA, Torres-Chinn N, Pfützner W, Möbs C, et al. Specific immunotherapy modifies allergen-specific CD4⁺ T-cell responses in an epitope-dependent manner. *J Allergy Clin Immunol* 2014;133:872-9.e7.
- Nakayama T, Hirahara K, Onodera A, Endo Y, Hosokawa H, Shinoda K, et al. Th2 cells in health and disease. *Annu Rev Immunol* 2017;35:53-84.
- Morimoto Y, Hirahara K, Kiuchi M, Wada T, Ichikawa T, Kanno T, et al. Amphiregulin-producing pathogenic memory T helper 2 cells instruct eosinophils to secrete osteopontin and facilitate airway fibrosis. *Immunity* 2018;49:134-50.e6.
- Shinoda K, Hirahara K, Iinuma T, Ichikawa T, Suzuki AS, Sugaya K, et al. Thy1⁺IL-7⁺ lymphatic endothelial cells in iBALT provide a survival niche for memory T-helper cells in allergic airway inflammation. *Proc Natl Acad Sci U S A* 2016;113:E2842-51.
- Hirahara K, Aoki A, Kiuchi M, Nakayama T. Memory-type pathogenic Th2 cells and ILC2s in type 2 allergic inflammation. *J Allergy Clin Immunol* 2021;147:2063-6.
- Iinuma T, Okamoto Y, Morimoto Y, Arai T, Sakurai T, Yonekura S, et al. Pathogenicity of memory Th2 cells is linked to stage of allergic rhinitis. *Allergy* 2018;73:479-89.
- Ihara F, Sakurai D, Yonekura S, Iinuma T, Yagi R, Sakurai T, et al. Identification of specifically reduced Th2 cell subsets in allergic rhinitis patients after sublingual immunotherapy. *Allergy* 2018;73:1823-32.
- Gotoh M, Yonekura S, Imai T, Kaneko S, Horikawa E, Konno A, et al. Long-term efficacy and dose-finding trial of Japanese cedar pollen sublingual immunotherapy tablet. *J Allergy Clin Immunol Pract* 2019;7:1287-97.e8.
- Yonekura S, Okamoto Y, Sakurai D, Iinuma T, Sakurai T, Yoneda R, et al. Efficacy of desloratadine and levocetirizine in patients with cedar pollen-induced allergic rhinitis: a randomized, double-blind study. *Int Arch Allergy Immunol* 2019;180:274-83.
- Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell* 2015;161:1202-14.
- Alquicira-Hernandez J, Powell JE. Nebulosa recovers single cell gene expression signals by kernel density estimation. *Bioinformatics* 2021 Jan 18;btab003. doi: 10.1093/bioinformatics/btab003. Epub ahead of print.
- Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, et al. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol* 2014;32:381-6.
- Nakayama S, Takahashi H, Kanno Y, O'Shea JJ. Helper T cell diversity and plasticity. *Curr Opin Immunol* 2012;24:297-302.
- Robb L, Hartley L, Wang CC, Harvey RP, Begley CG. Musculin: a murine basic helix-loop-helix transcription factor gene expressed in embryonic skeletal muscle. *Mech Dev* 1998;76:197-201.
- Robb L, Brodnicki T, Copeland NG, Gilbert DJ, Jenkins NA, Harvey RP. Assignment of the human helix-loop-helix transcription factor gene musculin/activated B-cell factor-1 (MSC) to chromosome 8q21 and its mouse homologue (Msc) to the proximal region of chromosome 1. *Genomics* 1999;57:318-9.
- Wu C, Chen Z, Dardalhon V, Xiao S, Thalhammer T, Liao M, et al. The transcription factor musculin promotes the unidirectional development of peripheral Treg cells by suppressing the Th2 transcriptional program. *Nat Immunol* 2017;18:344-53.
- Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol* 2010;28:445-89.
- Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 2006;126:375-87.
- Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, et al. An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol* 2016;17:1322-33.
- Wang P, Jin X, Zhou W, Luo M, Xu Z, Xu C, et al. Comprehensive analysis of TCR repertoire in Covid-19 using single cell sequencing. *Genomics* 2021;113:456-62.
- Polanczyk MJ, Hopke C, Huan J, Vandenbark AA, Offner H. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. *J Neuroimmunol* 2005;170:85-92.
- Ishikawa A, Wada T, Nishimura S, Ito T, Okekawa A, Onogi Y, et al. Estrogen regulates sex-specific localization of regulatory T cells in adipose tissue of obese female mice. *PLoS One* 2020;15:e0230885.
- Nie J, Li YY, Zheng SG, Tsun A, Li B. FOXP3⁺ Treg cells and gender bias in autoimmune diseases. *Front Immunol* 2015;6:493.
- Bernstein DI, Kleine-Tebbe J, Nelson HS, Bardelas JA Jr, Sussman GL, Lu S, et al. SQ house dust mite sublingual immunotherapy tablet subgroup efficacy and local application site reaction duration. *Ann Allergy Asthma Immunol* 2018;121:105-10.
- Yonekura S, Okamoto Y, Sakurai D, Okubo K, Gotoh M, Kaneko S, et al. An analysis of factors related to the effect of sublingual immunotherapy on Japanese cedar pollen induced allergic rhinitis. *Allergol Int* 2018;67:201-8.
- Pfaar O, Agache I, de Blay F, Bonini S, Chaker AM, Durham SR, et al. Perspectives in allergen immunotherapy: 2019 and beyond. *Allergy* 2019;74(suppl 108):3-25.
- Wraith DC, Krishna MT. Peptide allergen-specific immunotherapy for allergic airway diseases—state of the art. *Clin Exp Allergy* 2021;51:751-69.
- Bousquet J, Pfaar O, Togias A, Schünemann HJ, Ansotegui I, Papadopoulos NG, et al. 2019 ARIA Care pathways for allergen immunotherapy. *Allergy* 2019;74:2087-102.

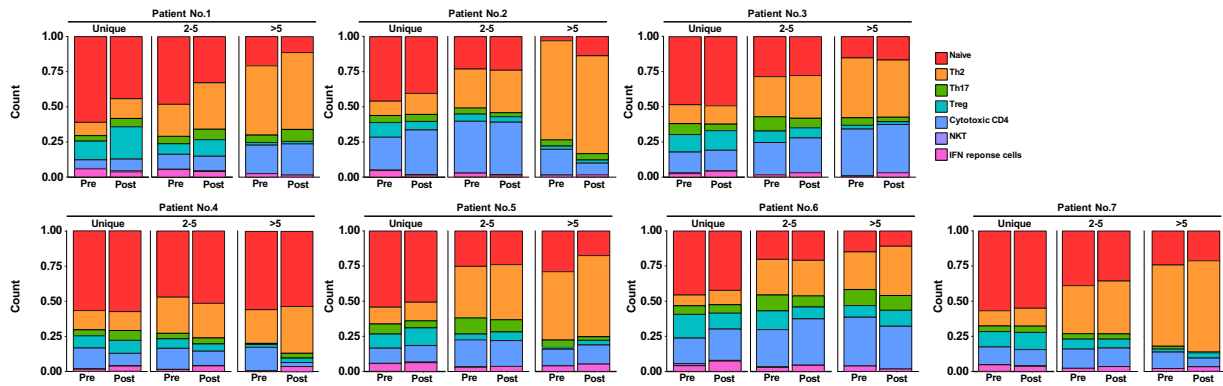


FIG E1. Proportional change in T-cell subsets in each subject before and after SLIT, categorized as clones of TCRs that are unique, 2-5, or >5.

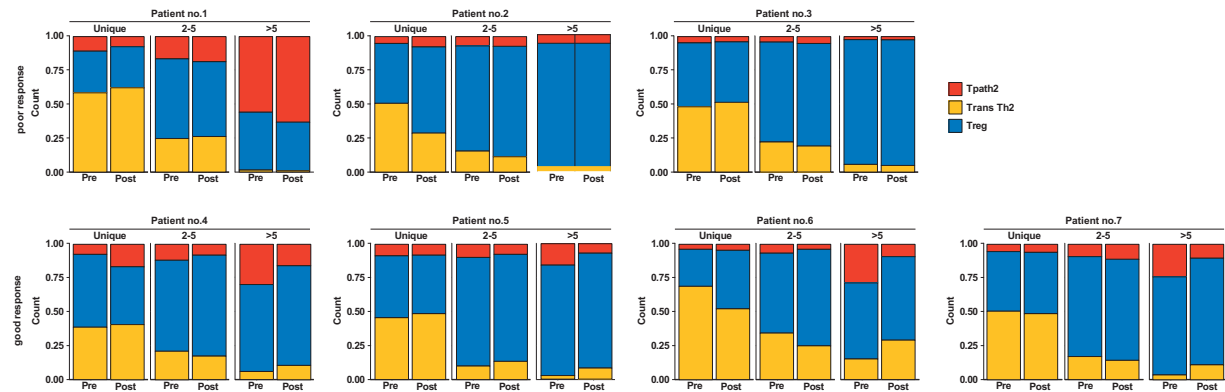


FIG E2. Proportional change in Tpath2, Trans T_H2, and Treg cells in each subject before and after SLIT, categorized as clones of TCRs that are unique, 2-5, or >5.

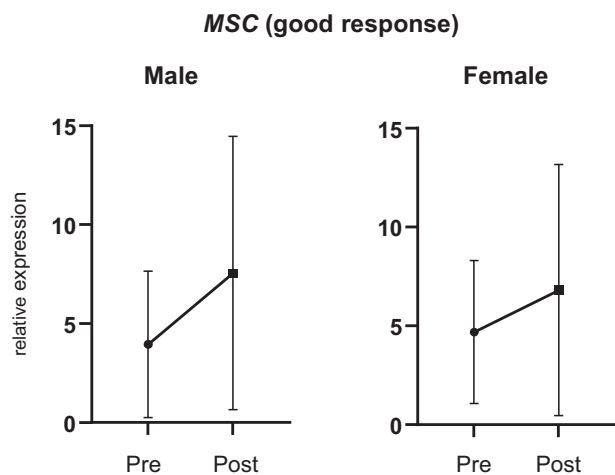


FIG E3. Comparison by sex in good-response analysis of MSC in specimens from outpatients. Data are SDs and means. The expression of MSC in PBMCs cultured with Japanese cedar antigen and IL-2 by real-time PCR are those with good response shown in [Fig 7](#). Nine patients were male and 12 female. No significant difference was found between groups.

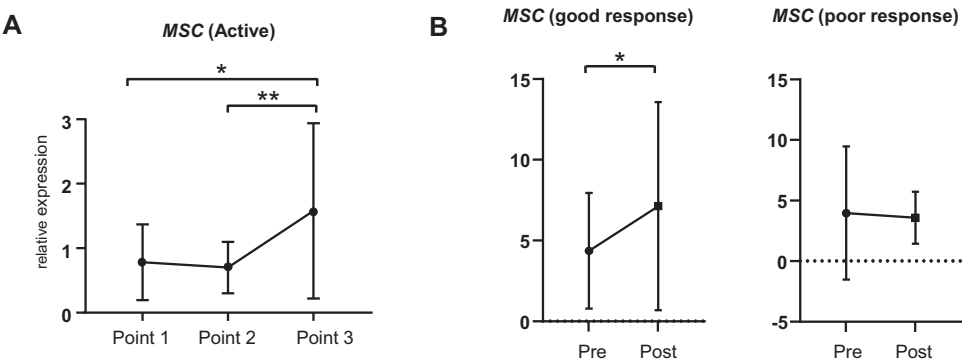


FIG E4. Variation of MSC. (**A** and **B**) Recreated figures regarding MSC for Fig 6, B (**A**), and Fig 7, B (**B**), with data shown as SDs and means.

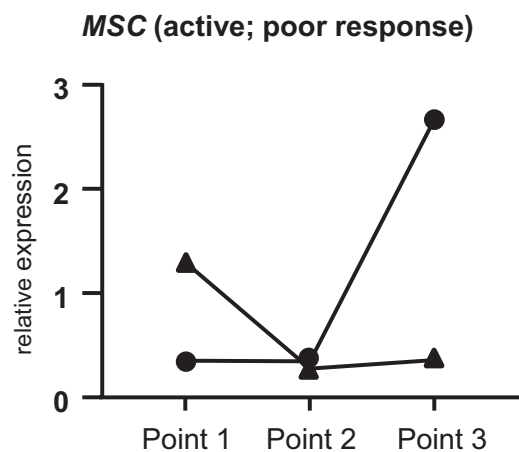


FIG E5. Analysis of MSC in poor-response specimens from the phase 2/3 clinical trial. The 2 lines show the values for each of the 2 excluded individuals in [Fig 6](#).