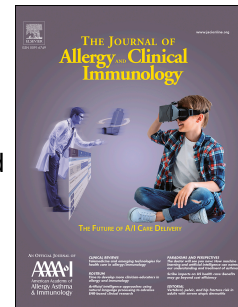


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Grass pollen immunotherapy alters chromatin landscape in circulating T follicular and regulatory cells

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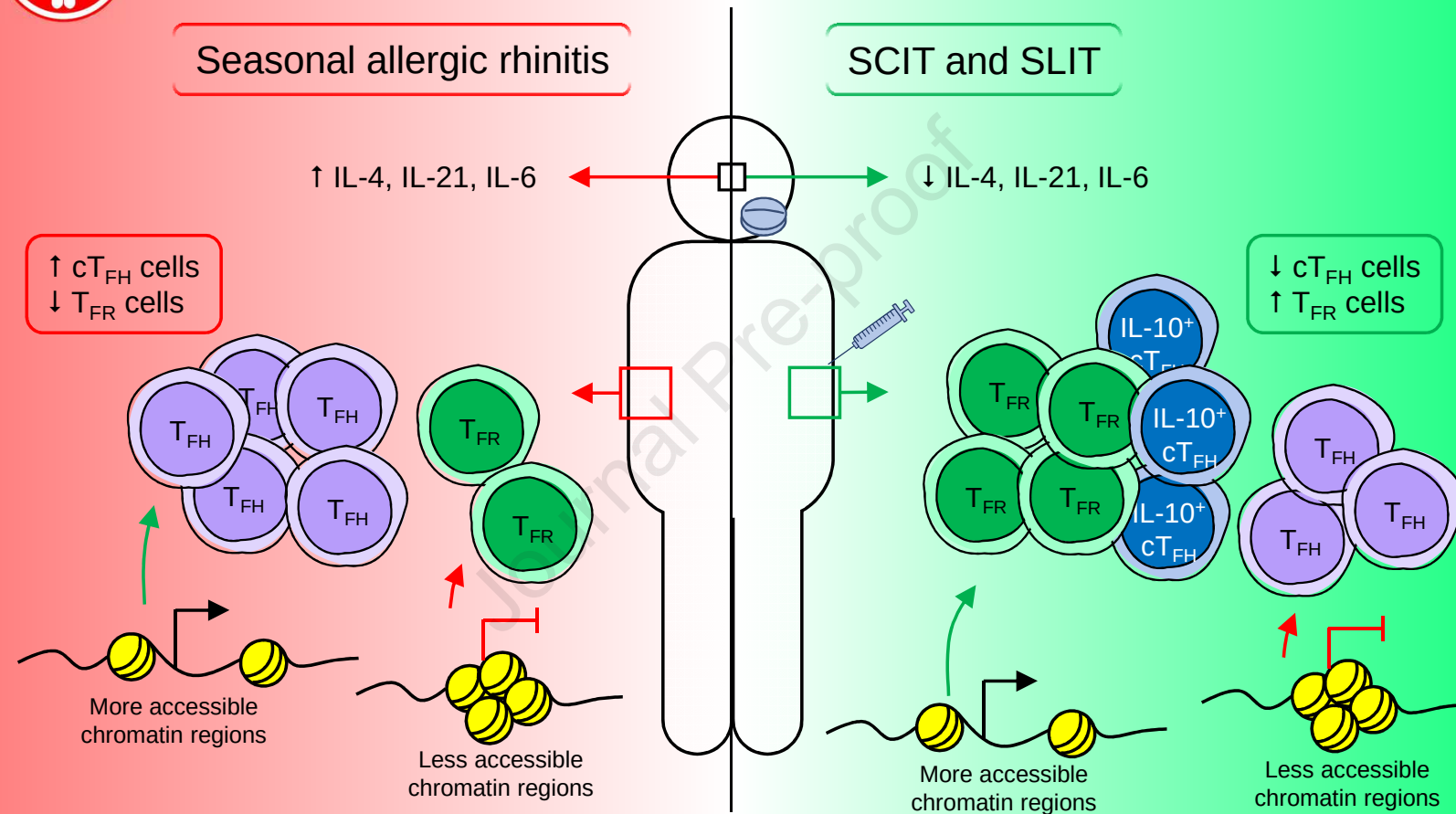
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Grass pollen allergen-specific SCIT and SLIT modulate cT_{FH} and T_{FR} cells and associated with altered chromatin landscape

Seasonal allergic rhinitis

SCIT and SLIT



Abbreviations: SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; cT_{FH} , circulating T follicular helper cells; T_{FR} , T follicular regulatory cells; $IL-10^+ cT_{FH}$, $IL-10$ -producing cT_{FH} cells; IL , interleukin.

Title: Grass pollen immunotherapy alters chromatin landscape in circulating T follicular and regulatory cells

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Key Messages

- Mathematical algorithms developed to manage high-dimensional data, i.e. viSNE, SPADE and FlowSOM reveal that cT_{FH} cells are a subset of T cells that are distinct from T_H2 and T_H2A cells in grass pollen allergics.
- IL-4⁺ and IL-21⁺ cT_{FH} cells show increased proliferative capacity following *in vitro* grass pollen allergen stimulation in grass pollen allergics (GPA) but not non-atopic healthy controls (NAC) and induces B cell class switching and plasma B cell differentiation.
- Chromatin landscape in cT_{FH} and T_{FR} cells are altered in grass pollen-induced seasonal allergic rhinitis and following SCIT and SLIT.

Capsule Summary

cT_{FH} cells are dysregulated in grass pollen allergics and restored following SCIT and SLIT treatment, and these changes are underscored by changes in the chromatin landscape.

Keywords

Allergy, seasonal allergic rhinitis, allergen-specific immunotherapy, T follicular helper cells, T follicular regulatory cells, chromatin accessibility, ATAC-seq.

Abbreviations

AIT, Allergen-specific immunotherapy; SCIT, Subcutaneous immunotherapy; SLIT, Sublingual immunotherapy; GPA, grass-pollen allergics; NAC, non-atopic healthy controls; T_{FH}, T follicular helper; T_{FR}, T follicular regulatory; Treg, T regulatory; Phlp, *Phleum pratense*; ATAC-seq, assay for transposase-accessible chromatin sequencing.

Abstract:

Background: Allergen-specific immunotherapy (AIT) is a disease-modifying treatment that induces long-term T cell tolerance.

Objective: To evaluate the role of circulating CXCR5⁺PD-1⁺T follicular helper (cT_{FH}) and T follicular regulatory (T_{FR}) cells following grass pollen subcutaneous (SCIT) and sublingual (SLIT) immunotherapy and the accompanying changes in their chromatin landscape.

Methods: Phenotype and function of cT_{FH} cells were initially evaluated in grass pollen-allergics (GPA, n= 28) and non-atopic controls (NAC, n=13) by mathematical algorithms developed to manage high-dimensional data and cell culture, respectively. cT_{FH} and T_{FR} cells were further enumerated in NAC (n=12), GPA (n=14), SCIT (n=10) and SLIT (n=8)-treated groups. Chromatin accessibility in cT_{FH} and T_{FR} cells was assessed by ATAC-seq to investigate epigenetic mechanisms underlying the differences between NAC, GPA, SCIT and SLIT.

Results: cT_{FH} cells were shown to be distinct from T_H2 and T_H2A cell subsets, capable of secreting IL-4 and IL-21. Both cytokines synergistically promoted B cell class switching to IgE and plasma cell differentiation. Grass pollen allergen induced cT_{FH} cell proliferation in GPA but not in NAC ($P<.05$). cT_{FH} cells were higher in GPA compared to NAC and were lower in SCIT and SLIT ($P<.01$). Time-dependent induction of IL-4, IL-21 and IL-6 were observed in nasal mucosa following intranasal allergen challenge in GPA but not in SCIT and SLIT groups. T_{FR} and IL-10⁺ cT_{FH} cells were induced in SCIT and SLIT (all, $P<.01$). ATAC-seq analyses revealed differentially accessible chromatin regions in all groups.

Conclusion: For the first time, we showed dysregulation of cT_{FH} cells in GPA compared to NAC, SCIT and SLIT and induction of T_{FR} and IL-10⁺ cT_{FH} cells following SCIT and SLIT. Changes in the chromatin landscape were observed following AIT in cT_{FH} and T_{FR} cells.

INTRODUCTION

Seasonal allergic rhinitis (SAR) is an immunoglobulin (Ig) E-mediated disease associated with inflammation of the nasal mucosa resulting upon exposure to prevalent common aeroallergens¹. Near 20% (1 in 5) do not respond to conventional treatments and in those patients, grass pollen immunotherapy administered subcutaneously (SCIT) or sublingually (SLIT) is effective. SCIT and SLIT are the only disease-modifying treatments that confer long-term clinical benefit and allergen tolerance²⁻⁵, as well as improvement in the quality of life⁶.

SAR consists of a biphasic response that occurs within 15-20 minutes and 6-12 hours⁷. The former requires allergen-IgE induced cross-linking of FcεRI on mast cells and the latter involves recruitment of T_H2 cells into the local target organ. Allergen-specific T_H2A cells are a subset of T_H2 cells and share similar function as they secrete type 2 cytokines. They have recently been reported as a key driver of allergen-induced inflammation. T_H2A cells are characterized by the expression of CCR4, CD161 and CD49d and lack the expression of CD27. They have been shown to be elevated in allergic patients and associated with the clinical response following SCIT and SLIT^{8,9}. Moreover, other T cell subsets such as T follicular helper (T_{FH}) cells have been shown to have the capacity to promote pro-allergic responses¹⁰⁻¹³. T_{FH} cells are a distinct subset of T cells within the germinal center (GC) that provide signals to B cells to differentiate into antibody-producing plasma cells¹⁴. T_{FH} cells express high levels of the chemokine receptor, CXCR5, that aids their migration into the B cell follicle in response to a CXCL13 gradient¹⁵, and the transcription factor, Bcl-6¹⁶. Following the initial discovery, numerous studies have also identified T_{FH} cells in peripheral blood¹⁷. These circulating T_{FH} (cT_{FH}) cells are a surrogate of GC T_{FH} cells and have lower *BCL6* expression¹⁸. T_{FH} cells are responsible for providing B cell survival signals and plasma cell differentiation through the expression of cytokines IL-4 and IL-21¹⁴, involving a STAT3-dependent pathway¹⁹. The interaction between B cells and T_{FH} cells is tightly regulated by T follicular regulatory (T_{FR}) cells^{20,21} to prevent autoimmunity and/or allergy. T_{FR} cells share characteristics with cT_{FH} cells in the expression of follicular markers, CXCR5 and PD-1, suggesting a similar follicular origin²⁰. However, unlike cT_{FH} cells, T_{FR} cells express the

transcription factor of T regulatory (Treg) cells, FoxP3. Murine knockout studies of T_{FR} cells have shown characteristics of overactive GC response, highlighting their role in moderating GC reactions²⁰, although their role in SAR has not been extensively studied.

Immune mechanisms of tolerance induction following SCIT and SLIT are associated with induction of B regulatory cells, immune deviation from T_H2 to T_H1 responses and deletion of T_H2 and T_H2A cells. Moreover, FoxP3⁺ Treg cells and inducible IL-10⁺ and IL-35⁺ Treg cells have been reported to contribute towards tolerance induction following allergen-specific immunotherapy (AIT)²²⁻²⁴. However, the effect of grass pollen-SCIT and -SLIT on cT_{FH} and T_{FR} cells that are important in modulating IgE-mediated pro-allergic response remains to be elucidated.

Here, using flow cytometry-based unbiased machine learning algorithms, we demonstrate that cT_{FH} cells have a distinct phenotype compared to conventional T_H2 and T_H2A cells⁸. Moreover, IL-4- and IL-21-producing cT_{FH} cells are dysregulated in patients with SAR. We also demonstrate that IL-4 and IL-21 are involved in driving GC reactions, class switching of B cells to IgE and differentiation into antibody-producing plasma cells. Furthermore, in a cross-sectional study, we show that cT_{FH} and T_{FR} cells are modulated following grass pollen SCIT and SLIT, resulting in an altered chromatin accessibility landscape.

METHODS

Subjects

To conduct initial validation experiments to determine the phenotype and function of cT_{HH}, T_H2 and T_H2A cells, 28 untreated patients with moderate-severe SAR (GPA) and 12 non-atopic healthy controls (NAC) were recruited (Table I). In a cross-sectional, controlled, proof-of-concept study of SCIT and SLIT, 14 GPA, 13 NAC, 10 patients receiving SCIT (timothy grass, *Phleum pratense* [Phlp], Aquagen SQ, 100 000 SQ-U, ALK-Abello, Hørsholm, Denmark) and 8 patients receiving SLIT (Grazax tablet, 75 000 SQ-U, ALK-Abello) were recruited (Table II). Details on inclusion and exclusion criteria and rhinoconjunctivitis total symptom score (RTSS) questionnaire are described in the Online Repository. The study complied with Good Clinical Practice and was approved by the National Research Ethics Service (Camberwell St Giles office, London, UK), and by the Research Office of the Royal Brompton and Harefield NHS Foundation Trust. Informed, written consent was obtained from all patients.

Cell staining by flow cytometry and quantitative PCR

Methods for peripheral blood mononuclear cells (PBMCs) isolation, cell staining, and quantitative PCR are described in the Online Repository.

B cell stimulation and culture

Naïve and memory B cells were stimulated with CD40L (R&D Systems), in the absence and presence of IL-4 (R&D Systems), IL-21 (BioLegend) and/or IL-10 (R&D Systems) for up to 14 days.

Nasal cytokines measurements

Nasal allergen challenge was performed with Aquagen (ALK-Abelló) Phlp extract. Nasal fluid samples were collected and processed as described previously²⁵ and nasal IL-4, IL-21 and IL-6 were analyzed. Additional information is provided in the Online Repository.

ATAC-seq

ATAC-seq was performed on sorted cT_{FH} ($CD4^+CXCR5^+PD-1^+CD25^-CD127^+$) and T_{FR} ($CD4^+CXCR5^+PD-1^+CD25^+CD127^-$) cells as previously described²⁶. The analysis was performed as previously described²⁷ (Fig E1) using Partek® Flow® (Partek, MO, USA). Additional information is provided in the Online Repository.

Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 7.02 (GraphPad Software, La Jolla, USA). Non-parametric Mann-Whitney test was used for statistical comparisons between different groups of patients and Wilcoxon signed-rank test for comparisons within the same group. A *P* value of $< .05$ was considered statistically significant.

RESULTS

cT_{FH} cells are distinct from conventional T_H2 and T_H2A cells

Phenotypic composition of cT_{FH}, T_H2 and T_H2A cells in GPA was evaluated by multiparametric flow cytometry and further confirmed by visualization tools developed to manage high-dimensional data, i.e. viSNE, SPADE and FlowSOM. CXCR5⁺PD-1⁺ cT_{FH} cells (Fig 1, A) were interrogated by viSNE²⁸ which utilizes the Barnes-Hut implementation of tSNE algorithm²⁹, allowing a two-dimensional visualization whereby phenotypically similar cells form a cluster. CD4⁺ cells showed a cluster of CXCR5-expressing cells that co-expressed PD-1 indicating a cT_{FH} cell population (Fig 1, B, orange arrow). A proportion of CD4⁺ cells with high CRTH2 expression (Fig 1, B, green arrow) revealed low expression of CD27, indicating a T_H2 cell subset. Moreover, we confirmed that T_H2A cells⁸ resided within the TH2 subset and expressed CD49d and CD161 (Fig 1, B, red arrow).

SPADE (Spanning-tree Progression Analysis of Density-normalized Events) analysis connected hierarchical clusters of phenotypically similar cells in 'steps' using a minimal spanning tree (MST). We confirmed that clusters of CXCR5-expressing cells that co-express PD-1 (cT_{FH} cells) and T_H2 and T_H2A cell clusters that express low CD27 and high CRTH2 expression were separated into different branches, thus highlighting the distinct phenotype between these cell subsets (Fig 1, C). Furthermore, an unbiased approach of FlowSOM³⁰, that allows grouping of cell subsets into metaclusters (clusters of cell clusters), confirmed that cT_{FH}, T_H2 and T_H2A cell subsets form distinct metaclusters (Fig 1, D and E).

The expression of CXCR5, PD-1, CRTH2, CD27, CD49d and CD161 was also investigated in cT_{FH}, T_H2 and T_H2A cells. cT_{FH} cells demonstrated higher expression of CXCR5, PD-1 and CD27, but lower CRTH2 expression, compared to T_H2 and T_H2A cell subsets. cT_{FH} and T_H2 cells showed lower expression of CD49d and CD161 compared to T_H2A cells (Fig 1, F and G; Table E1). Moreover, T_H2 and T_H2A cells constituted a larger proportion of IL-4-producing cells compared to cT_{FH} cells whilst cT_{FH} cells constituted a higher proportion of IL-21-expressing cells compared to the T_H2 and T_H2A

cells (Fig 1, *H* and *I*). These findings, therefore, demonstrated the distinct phenotype of T_{FH} and T_{H2} and T_{H2A} cells.

Synergistic effect of IL-4 and IL-21 in driving IgE-producing plasma B cell differentiation

Having demonstrated that cT_{FH} cells express IL-4 and IL-21, their role in promoting clonal expansion and differentiation of B cells that drive allergic inflammation was investigated. The role of IL-21 in promoting plasma cell differentiation has previously been described¹⁶. However, the effect of IL-21 on class switch recombination (CSR), particularly to IgE, and the synergistic effect of IL-4 and IL-21 have yet to be fully elucidated and remain controversial. GC reactions, marked by the upregulation of *BCL6* expression, occur when T_{FH} cells interact with B cells during the cognate T_{FH} and B cell interaction in the GC and the production of IL-4 and IL-21. The latter lead to the induction of B cell proliferation and class switching, indicated by an increase in *AICDA* expression. A proportion of these B cells then undergo plasma cell differentiation, denoted by the upregulation of *PRDM1* and *XBPI*. IL-4 stimulation resulted in an upregulation of *BCL6* and *AICDA* in naïve B cells. Although stimulation with IL-21 upregulated *AICDA* expression, relative to CD40L alone, in the presence of IL-4, IL-21 downregulated *BCL6* and *AICDA* expression in naïve B cells compared to that induced by IL-4 alone (Fig 2, *A*). These findings suggest that IL-4 drives GC reaction and B cell class switching whilst IL-21 inhibits these processes in the presence of IL-4 (Tables E2 and E3).

An upregulation of *PRDM1* was observed in response to stimulation with either IL-21, dual IL-4/IL-21 and to a lesser extent with IL-4 in naïve B cells when compared to unstimulated samples (Fig 2, *B*; Table E4). Additionally, a significant upregulation of *PRDM1* expression was observed in response to dual IL-4/IL-21 stimulation compared to IL-4 and IL-21 alone (Fig 2, *B*; Table E4). *XBPI* expression was upregulated in naïve B cells in response to both IL-4 and IL-21 as well as dual IL-4/IL-21 (Fig 2, *C*; Table E5). These data suggest the differential roles of IL-4 and IL-21 in the different stages of plasma cell differentiation and clonal expansion. While IL-4 is crucial for late-phase plasma cell differentiation and secretory function, IL-21 plays a role in both the initial and late phase plasma cell differentiation. A higher proportion of CD38⁺CD27^{hi} plasmablasts was observed following

stimulation with dual IL-4/IL-21 in memory and naïve B cells (Fig 2, *D*, left, and E2, *B*). Increased proportions of CD38-expressing cells were observed following stimulation with IL-4, IL-21 and dual IL-4/IL-21 compared to CD40L alone (Fig 2, *D*, right, and E3). Furthermore, FlowSOM analysis showed that clusters of cells highly expressing CD38 and CD27 were more abundant when stimulated with IL-4 and IL-21 (Fig 2, *E*). When compared to CD40L alone, IL-21 and dual IL-4/IL-21 stimulation resulted in higher proportions of CD38⁺CD27^{hi} plasmablasts from naïve B cells. Additionally, higher plasmablasts were demonstrated following stimulation of memory B cells with IL-4 (Fig 2, *F*; Table E6). These plasmablasts co-expressed IgE (Fig 2, *G*). The proportion of IgE⁺ plasmablasts were higher in response to dual IL-4/IL-21 stimulation in naïve B cells and in response to IL-4, IL-21 and dual IL-4/IL-21 in memory B cells, compared to CD40L alone (Fig 2, *H*, and E2, *B*; Table E7). The expression of ϵ germline transcript (ϵ GLT) was upregulated in response to IL-4 compared to CD40L alone in naïve B cells (Fig 2, *I*; Table E8). However, IgE⁺ cells were higher in response to stimulation with IL-4, IL-21 and dual IL-4/IL-21 in memory, but not naïve B cells (Fig 2, *J* and E2, *C*; Table E9). Moreover, there were higher IgE levels observed in response to IL-4 and dual IL-4/IL-21 stimulation in naïve B cells, and to a lesser extent when stimulated with IL-21 alone (Fig 2, *K*). A similar trend was observed in memory B cells (Fig 2, *K*; Table E10). Higher IgG⁺ cells were observed in response to IL-21 and dual IL-4/IL-21 stimulation in naïve and memory B cells. IL-4 stimulation also resulted in higher IgG⁺ cells in memory B cells (Fig 2, *L*, and E2, *D*; Table E11).

Higher allergen-driven proliferation of cT_{FH} cells in GPA compared to healthy controls

The effect of *in vitro* allergen stimulation on the proportion of cT_{FH}, T_H2 and T_H2A cells in GPA and NAC were then investigated. PBMCs were stimulated with Phlp and proliferation of T cell subsets was assessed by CellTrace Violet (Fig 3, *A*). A dose-dependent increase in the proliferation of IL-4⁺ and IL-21⁺ cT_{FH}, T_H2 and T_H2A cells were observed in GPA, but not in NAC (Fig 3, *B – E*). The proliferation of IL-4⁺ cT_{FH} cells, IL-21⁺ cT_{FH} and T_H2, and T_H2A cells were higher in GPA compared to NAC (Fig 3, *B – E*; Table E12). This highlights the role of the allergen-driven proliferation of T cell subsets in GPA, which was not observed in NAC.

Dysregulation of IL-4⁺, IL-21⁺ and dual IL-4⁺IL-21⁺ cT_{FH} cells in GPA and restoration following allergen-specific SCIT and SLIT

In a controlled, cross-sectional study of SCIT and SLIT, cT_{FH} cells were enumerated in NAC, GPA, SCIT and SLIT. RTSS in GPA was higher compared to NAC and was lower following SCIT or SLIT compared to GPA (Fig 4, A). Previous studies have identified a proportion of cT_{FH} cells that express ICOS as an activated cT_{FH} cell subset^{31,32}. In this study, blood samples were collected during the grass pollen season and natural exposure to allergens leads to the activation of cT_{FH} cells. Here, the proportion of activated ICOS⁺ cT_{FH} cells were higher in GPA compared to NAC and were lower in SCIT and SLIT compared to GPA (Fig 4, B). Similarly, IL-4⁺ cT_{FH} cells, IL-21⁺ cT_{FH} and dual IL-4⁺IL-21⁺ cT_{FH} cells were all found to be higher in GPA compared to NAC and were lower in SCIT and SLIT compared to GPA (Fig 4, C – E). No differences were observed between SCIT and SLIT (Table E13), suggesting the potential role of IL-4 and IL-21-producing cT_{FH} cells in the pathophysiology of SAR and modulation following SCIT and SLIT.

Time-course induction of IL-4, IL-21 and IL-6 in GPA following nasal allergen challenge

In the same cohort of patients in the cross-sectional study, a time-course induction of IL-4, IL-21 and IL-6 levels was observed in the nasal fluid of GPA. This was inhibited in SCIT and SLIT. No changes were observed in NAC (Fig 5, A – C, Tables E14 – E16). This further highlights the importance of these cytokines in driving local allergic inflammation.

Altered epigenetic landscape of cT_{FH} cells in GPA compared to NAC, SCIT and SLIT

We then investigated the chromatin landscape by assay for transposase-accessible chromatin sequencing (ATAC-seq) in NAC, GPA, SCIT and SLIT. Highly accessible chromatin regions allow the interactions between enhancers and promoters and binding of transcription factors, thus regulating gene expression. There were 567 regions that were found to be more accessible in GPA compared to NAC, 715 regions compared to SCIT and 604 regions compared to SLIT (Fig 6, A left). In total, we observed 680 differentially accessible regions between all patient groups (Fig 6, B). The nearest genes

to the differentially accessible regions were identified and gene ontology (GO) analysis was performed. Regions that were more accessible in GPA compared to NAC were associated with genes that belong to the GO terms associated with various metabolic processes (Fig 6, C). Regions that were more accessible in GPA compared to SCIT and SLIT were associated with metabolic processes and regulation of cytokine secretion that includes endoplasmic reticulum (Fig 6, D and E). Accessible regions in SLIT compared to SCIT were mainly associated with the regulation of protein transport and secretion (Fig 6, F). Pathway enrichment analysis identified the more accessible regions in GPA compared to NAC to be associated with various signaling pathways (Fig 6, G), whilst those compared to SCIT and SLIT were associated with signaling and metabolic pathways as well as tight junction (Fig 6, H and I). Pathways that were associated with more accessible regions in SLIT compared to SCIT includes signaling pathways as well as epigenetics processes (Fig 6, J), highlighting the differential chromatin accessibility landscape in cT_{FH} cells of GPA, NAC, SCIT and SLIT.

Induction of T_{FR} cells and IL-10⁺ cT_{FH} following allergen-specific SCIT and SLIT

T_{FR} cells regulate T_{FH} and B cell interaction and exert their suppressive function through the expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)³³. In the same cohort of patients, suppressive CTLA-4⁺ T_{FR} cells were lower in GPA compared to NAC and were higher in SCIT and SLIT compared to GPA (Fig 7, A). In parallel to this, IL-10⁺ cT_{FH} cells were lower in GPA compared to NAC and were higher in SCIT and SLIT compared to GPA (Fig 7, B; Table E17). Moreover, IL-10 stimulation resulted in the upregulation of *PRDM1* and *XBPI* expression in the naïve B cells stimulated with dual IL-4/IL-21 (Fig 7, C and D). Although a similar trend was observed in memory B cells, no significant differences were observed (Fig 7, C and D; Tables E4 and E5). The proportion of plasmablasts were higher in the presence of IL-10 in memory, but not naïve B cells stimulated with dual IL-4/IL-21 (Fig 7, E; Table E6). IL-10 stimulation resulted in a higher proportion of IgE⁺ cells in naïve B cells in the presence of dual IL-4/IL-21. In contrast, a reduction in IgE⁺ cells was observed in memory B cells, although significance was lost at day 14 (Fig 7, F; Table E9). IL-10 stimulation also resulted in a higher proportion of IgG⁺ cells in the presence of IL-4 in memory B

cells (Fig 7, G; Table E11). These findings, therefore, suggest the role of T_{FR}, Treg cells and IL-10 in the immune tolerance induction following SCIT and SLIT.

Epigenetic landscape of T_{FR} cells in GPA, NAC, SCIT and SLIT

ATAC-seq was carried out to identify differentially accessible regions in T_{FR} cells in GPA and following SCIT and SLIT. There were 141 regions found to be more accessible in GPA compared to NAC, 184 regions compared to SCIT and 139 regions compared to SLIT (Fig 7, H). Moreover, a total of 221 differentially accessible regions in T_{FR} cells were observed between NAC, GPA, SCIT and SLIT (Fig 7, I). GO analysis of the nearest gene revealed that more accessible regions in GPA compared to NAC were associated with regulation of cytokine and protein secretion (Fig 7, J). Various catabolic processes were associated with accessible regions in SCIT (Fig 7, K) and various cellular and biological processes were associated with accessible regions in SLIT (Fig 7, L) compared to GPA. Regions that were more accessible in SCIT compared to SLIT were mainly associated with various metabolic processes as well as enzymatic activity (Fig 7, M). Pathway analysis on regions that were more accessible in SCIT compared to GPA was associated with metabolic processes and pathways (Fig 7, N). Furthermore, regions that were more accessible in SCIT compared to SLIT were associated with various signaling and metabolic pathways (Fig 7, O), suggesting that changes in chromatin accessibility may potentially contribute to tolerance induction following SCIT and SLIT.

DISCUSSION

We show for the first time, using unbiased machine learning algorithms and visualization tools, that cT_{FH} cells represent a subset of T cells distinct from T_{H2} and T_{H2A} cells, capable of secreting IL-4 and IL-21. IL-4 stimulation of B cells resulted in the upregulation of class switching, which was inhibited in the presence of IL-21. Moreover, IL-4, IL-21 and dual IL-4/IL-21 stimulation upregulated plasma cell differentiation markers. Grass pollen-driven proliferation of cT_{FH} cells was higher in GPA compared to NAC. More importantly, *ex vivo* enumeration of cT_{FH} cell subsets following SCIT or SLIT showed a lower frequency of cT_{FH} cell subsets compared to untreated GPA. We also report for the first time that T_{FR} cells and IL-10⁺ T_{FH} cells were induced in SCIT and SLIT. These findings highlight the potential role of IL-4- and IL-21-secreting cT_{FH} cells in SAR and the role of T_{FR} cells following grass pollen-specific SCIT and SLIT.

T_{H2} cells are known as primary cells that drive allergic inflammation. However, in the GC, T_{FH} cells interact with B cells, promoting allergic inflammation. We phenotyped T_{FH}, T_{H2} and T_{H2A} cells according to their surface marker expression using viSNE, SPADE and FlowSOM clustering. These analyses identified T_{FH} and T_{H2} and T_{H2A} cells as distinct subsets of cells and therefore, likely to play a distinct role in driving allergic inflammation. We also showed that in addition to the T_{H2} cytokine, IL-4, a proportion of cT_{FH} cells also express IL-21, in line with the previous studies¹⁴.

The functions of IL-4 in driving antibody production by B cells have been established¹⁶. However, the synergistic effect of IL-4 and IL-21 in human have not been fully elucidated, particularly with respect to IgE production. Here, we showed that IL-4 stimulation resulted in the upregulation of *BCL6*, a marker of the GC response, and *AICDA*, a critical component of the class switching and somatic hypermutation pathways in B cells. However, in the presence of both IL-4 and IL-21, the expression of *BCL6* and *AICDA* was downregulated compared to IL-4 alone, highlighting the potential inhibitory role of IL-21 on GC reactions and CSR. Indeed, previous studies have demonstrated a timely secretion of IL-4 and IL-21 by T_{FH} cells following infection. IL-21 are predominantly expressed during the early course of infections prior to migration of T_{FH} cells to the B cell follicle, while IL-4

expression is heightened following the migration of T_{FH} cells into the B cell follicle^{34,35}. Furthermore, IL-21⁺ and IL-4⁺ T_{FH} cells display distinct localization within the GC, which consists of the dark and light zone. IL-4⁺ T_{FH} cells are predominantly localized distal to the dark zone and express high CD40L, while IL-21⁺ T_{FH} cells express high CXCR4 and localize closer to the dark zone³⁶. This suggests a role for IL-21⁺ T_{FH} cells in promoting somatic hypermutation and affinity maturation, while IL-4⁺ T_{FH} cells are crucial in the class switching of GC B cells. While the effect of IL-4 stimulation on IgE class switching has been explored, the role of IL-21 on CSR to IgE has not been fully elucidated. Despite the inhibitory effect of IL-21 observed on CSR in this study, we reported a higher proportion of IgE⁺ cells in the presence of dual IL-4/IL-21 stimulation. This suggests the potential role of IL-21 in driving differentiation to IgE⁺ cell occurs post-CSR. Previous studies investigating the effect of IL-21 on IgE production yield contradicting results. Studies performed on IL-21R-deficient mice reported aberrant IgE production, and that rIL-21 administration suppressed IgE production^{37,38}. On the other hand, studies performed on sorted B cells showed elevated IgE production by IL-21^{39,40}, which is consistent with our observations. These discrepancies are potentially due to the different experimental models used in these studies. Furthermore, whilst stimulation with IL-21 and dual IL-4/IL-21 can upregulate the expression of *PRDM1* and *XBPI*, IL-4 stimulation only resulted in the upregulation of *XBPI* expression. *PRDM1* expression is known to be essential for the initiation of plasma cell differentiation and *XBPI* is expressed further downstream and is crucial for the unfolded protein response (UPR) that is important for the secretory function of plasma cells¹⁶. This, therefore, indicates that while IL-4 is crucial for the UPR, IL-21 plays a key role in the initiation stage of plasma cell differentiation and antibody-secretory function of plasma cells. Collectively, our findings confirmed that IL-4 promoted GC reactions and CSR, whilst IL-21 inhibited these processes. Despite this, IL-21 showed synergistic effect with IL-4 in driving the differentiation of plasmablasts, IgE⁺ cells and the secretion of tIgE, of which the latter promote IgE-mediated pro-allergic responses.

T_{FH} cell differentiation and interaction with B cells occurs in the GC and therefore can act on local target organs such as the nasal mucosa. There was a local induction of IL-4 and IL-21 in GPA following nasal allergen challenge which was inhibited in SCIT or SLIT. This observation was

consistent with the proportion of IL-4⁺ and IL-21⁺ cT_{FH} cells demonstrated in the same patient cohort, which was elevated in GPA, but was lower in SCIT- and SLIT-treated groups. Hence, suggesting the potential contribution of cT_{FH} cells on the elevation of nasal IL-4 and IL-21 levels. Furthermore, this suggests that the increase in cT_{FH} cells observed in GPA is associated with the production of IgE, subsequently leading to allergic inflammation. Previous studies have also reported increased proportions of T_{FH} cells in patients with house dust mite (HDM) allergy and mice exposed to HDM^{41,42}. Serum IgE in patients with HDM allergy has also been shown to correlate with the proportion of cT_{FH} cells⁴¹. Studies on autoimmune diseases and IgG₄-related disease (IgG₄-RD) have shown that dysregulation of cT_{FH} cells leads to impaired antibody production in autoimmune diseases, and increased Ig levels in patients with IgG₄-RD⁴³⁻⁴⁵. These findings highlighted the important role of cT_{FH} cells in maintaining immune homeostasis. Moreover, IL-6 was induced as early as one hour in GPA, which was attenuated in SCIT and SLIT. IL-6 has previously been shown to be crucial in the initial phase of T_{FH} cell differentiation in mice⁴⁶ by upregulating *BCL6* expression and IL-21 secretion^{47,48}. The secreted IL-21 has an autocrine role where it creates a positive feedback loop on T_{FH} cells to support the induction of *BCL6* expression, which is indispensable for T_{FH} cell differentiation^{49,50}. IL-6 and IL-21 signaling induce STAT1 and STAT3 expression and result in the expression of T_{FH} cell markers CXCR5, PD-1 and ICOS^{45,51}. Early induction of IL-6 in GPA lead to the differentiation of T_{FH} cells, subsequently secreting IL-4 and IL-21 cytokines in the local target organs. The autocrine role of IL-21⁵⁰ potentially results in the persistent induction of T_{FH} cell cytokines IL-4 and IL-21, which was observed for up to 8 hours in GPA. This is a proof-of-concept, cross-sectional study to assess the effect of SCIT and SLIT on the proportion of cT_{FH} and T_{FR} cells, and will need to be further validated in a large, prospective RDBPC study that investigate the time-course effect of SCIT and SLIT.

T_{FR} cells have previously been reported to suppress T_{FH} and B cell interaction through the expression of CTLA-4 by cell-to-cell contact manner³³. However, due to the limited sample availability, it was not feasible to perform suppression assays in this study. We observed induction of CTLA-4⁺ T_{FR} cells and IL-10-producing cT_{FH} cells in SCIT- and SLIT-treated patients, which corroborates with previous

studies demonstrating the induction of T_{FR} cells following AIT in HDM allergic patients⁴¹ and immunotherapy using allergen peptides in GPA⁵². Previously studied mechanism of AIT have been associated with the induction of IL-10⁵³ from allergen-specific T cells.

In the presence of IL-4 and IL-21, IL-10 stimulation drives plasmablast differentiation in memory B cells and the production of IgE⁺ cells from naïve B cells. Furthermore, IgG⁺ B cells were higher in the presence of IL-10 and IL-4 in memory B cells. However, these were not generated to a similar extent from naïve B cells. These data highlight the potential differential role of IL-10 on naïve (allergen-inexperienced) and allergen-experienced memory B cells, where it may play a protective role when interacting with the latter. Previous studies have shown conflicting findings. In a PBMC culture, IL-10 has been shown to negatively regulate IgE production by IL-4 when added to antigen-inexperienced B cells and induced IgE production when added to IgE-switched B cells⁵⁴. Another study reported an increase in IgE production following IL-10 stimulation on PBMCs and purified pan B cells⁵⁵. In the same study, IL-10 promoted proliferation of B cells and generation of CD38⁺ cells, which is consistent with our observations. Moreover, an upregulation of IgG₄ but not IgE was shown following IL-10 stimulation in the presence of IL-4 in CD27⁺ memory B cell culture⁵⁶. These findings are in agreement with our observations in this study. These discrepancies may be due to the different experimental model used in these studies. There are very limited studies that have investigated the effect of IL-10 on B cells in the presence of IL-4 and IL-21. Further studies are warranted to better understand the role of IL-10 in B cell proliferation and class switching.

We further speculated that these changes in the peripheral blood are underscored by modifications in the chromatin landscape. NAC, GPA, SCIT and SLIT showed differential chromatin accessibility which was associated with various GO terms and pathways. We observed differentially accessible chromatin regions between GPA and NAC, SCIT and SLIT. Regions that were more accessible in cT_{FR} of GPA compared to NAC, SCIT and SLIT were associated with the breakdown of macromolecules and the biosynthesis of lipid molecules. The catabolism of proteins and other macromolecules provides the energy that is crucial for cell survival^{57,58} and leads to the upregulation

of lipid synthesis, of which the associated chromatin regions were observed to be more accessible in GPA. This consequently contributes to T_{FH} cell growth and proliferation. These findings are in agreement with other studies which have shown that the inhibition of lipid biosynthesis pathways can result in the attenuation of T cell survival and proliferation^{59,60}. Additionally, the accessible regions were associated with ribosome biogenesis which has been shown to play a role in supporting T cell cytokine production. The activation of genes that are involved in ribosome synthesis was shown to be dependent on ERK-MAPK signaling⁶¹, which was consistently more accessible in GPA compared to NAC, SCIT and SLIT in our cohort. We also demonstrated higher chromatin accessibility in regions associated with PI3K-Akt, MAPK and AMPK signaling in GPA. These pathways are known to play a role in glucose homeostasis⁶¹, a process known to be important for cell differentiation and growth. Regions associated with toll-like receptor (TLR) signaling pathway was also more accessible in GPA compared to NAC. To complement this, we also observed higher cT_{FH} cells and their cytokine-producing subsets in GPA compared to NAC, SCIT and SLIT. These findings not only validate the previous study highlighting the role of TLR signaling in promoting T cell responses and enhancing cytokine production⁶², but also suggest that the higher proportion of cT_{FH} cells observed in GPA could be due to the predisposition of cT_{FH} cell differentiation and cytokine production. This, however, needs to be further confirmed in a larger study.

Regions that were more accessible in SLIT compared to SCIT were associated with protein secretion and transport. Metabolite transport plays a role in activated T cells and promotes cell growth and proliferation⁶³, suggesting that cT_{FH} cells in SLIT were more active compared to SCIT. This is in conjunction with the modest differences in IL-4⁺IL-21⁺ cT_{FH} cells between SLIT and SCIT observed in our cohort. Interestingly, chromatin regions associated with ribosome biogenesis and MAPK signaling, which are known to be crucial for cell differentiation and growth⁶¹ were found to have differential chromatin accessibility between the different patient groups. Chromatin regions associated with these pathways were more accessible in SLIT compared to SCIT, highlighting the potential differences in the mechanism underlying the two modes of AIT. However, these regions were more accessible in GPA compared to SLIT (Fig E4).

On the other hand, chromatin regions that were more accessible in T_{FR} cells in GPA were associated mainly with negative regulation of gluconeogenesis, cytokine and protein secretion. As discussed above, gluconeogenesis, which refers to the generation of glucose, that is important for the proliferation of T cells^{57,58,64} were attenuated in GPA. These findings coincide with our observation where the proportion of T_{FR} cells were lower in GPA compared to NAC. Chromatin regions that were more accessible in SCIT compared to GPA were associated with various catabolic processes of macromolecules, which is important for cell survival and growth^{57,58,60}. Furthermore, SLIT showed highly accessible chromatin regions associated with negative regulation of cell differentiation and proliferation and various molecular functions. These differences were reflected when chromatin accessibility between SCIT and SLIT were investigated. SCIT showed higher accessibility in regions associated with metabolic processes of macromolecules such as lipids, proteins and nucleic acids compared to SLIT, and MAPK activity (Fig E4). Furthermore, regions associated with ribosome biosynthesis were also more accessible in SCIT compared to SLIT, which is crucial in the cytokine production of T cells. Despite these differences observed at the chromatin levels, there was no difference in the proportion of T_{FR} cells at cellular levels. These findings suggest that the mechanism of SCIT involves the induction of T_{FR} cell differentiation and survival, whilst SLIT resulted in changes in the molecular function of T_{FR} cells, which is important in cell organization and structure. The differences observed in the chromatin landscape of cT_{FH} and T_{FR} cells provide further insights on the importance of metabolic and molecular pathways in the mechanism of tolerance induction following SCIT and SLIT. However, this needs to be investigated further. The allergen-specificity of the T cell subsets that were investigated in this study was not determined due to the limitation of blood samples collected, in addition to the small proportion of cT_{FH} and T_{FR} cell subsets present within the peripheral blood. However, this can be achieved by performing peptide-MHC class II tetramer staining with intracellular CD154 assays^{8,65}, which will need to be validated in future studies.

In summary, for the first time, we show that cT_{FH} cells play a role in the pathophysiology of SAR, in addition to T_{H2} and T_{H2A} cells. SCIT and SLIT treatment resulted in the modulation of cT_{FH} cells and

477 induction of T_{FR} and $IL-10^+$ cT_{FH} cells, with underlying changes in the chromatin landscape,
478 suggestive of epigenetics modifications.

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TABLE

Table I Patients' demographics for initial validation experiments of the phenotypic characteristics and function of cT_{FH} cells

Characteristics	NAC	GPA
Sex, n, Male:Female	5:7	15:13
Age (y), mean $\pm$ SEM	48 $\pm$ 4	38 $\pm$ 2
Disease duration (y), mean $\pm$ SEM	n.d.	16.96 $\pm$ 1.63
Phlp IgE (kU _A /L), mean $\pm$ SEM	n.d.	40.09 $\pm$ 8.15
Total IgE (kU/L), mean $\pm$ SEM	13.87 $\pm$ 4.25	339.50 $\pm$ 108.70***
Cosensitization (SPT $\geq$ 3 mm), n (%)		
Tree	0 (0.0)	12 (26.7)
House dust mite	0 (0.0)	13 (28.9)
Cat epithelia	0 (0.0)	12 (26.7)
Dog epithelia	0 (0.0)	7 (15.6)

*** $P < .001$ compared to GPA, Mann-Whitney test.

655 Table II Patients' demographics for the cross-sectional, proof-of-concept study of SCIT and SLIT

Characteristics	NAC	GPA	SCIT	SLIT
Sex, n,				
Male:Female	2:11	9:5	9:1	2:6
Age (y), mean $\pm$ SEM	38 $\pm$ 4	33 $\pm$ 3	43 $\pm$ 4	40 $\pm$ 5
Disease duration (y), mean $\pm$ SEM	n.d.	17.36 $\pm$ 3.03	18.80 $\pm$ 2.99	33.50 $\pm$ 5.53
Phlp IgE (kU _A /L), mean $\pm$ SEM	n.d.	32.50 $\pm$ 10.68	30.96 $\pm$ 12.00	23.50 $\pm$ 7.81
Total IgE (kU/L), mean $\pm$ SEM	n.d.	179.60 $\pm$ 41.04	405.90 $\pm$ 213.20	151.60 $\pm$ 65.59
Cosensitization, (SPT $\geq$ 3 mm), n (%),				
Tree	0 (0.0)	6 (13.3)	7 (53.9)	3 (27.3)
House dust mite	0 (0.0)	8 (17.8)	5 (38.5)	2 (18.2)
Cat epithelia	0 (0.0)	7 (15.6)	6 (46.2)	3 (27.3)
Dog epithelia	0 (0.0)	7 (15.6)	2 (15.4)	4 (36.4)

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FIGURE LEGENDS

Fig 1. cT_{FH} cells are a distinct population than T_H2 and T_H2A cells. **A** Gating strategy to identify cT_{FH} (CD4⁺CXCR5⁺PD-1⁺) cells in peripheral blood. **B** Unbiased viSNE plots showing a proportion of CD4⁺ cells express CXCR5, PD-1, CRTH2, CD27, CD49d and CD161. Colors indicate the median expression level of labeled cell markers. **C** SPADE trees of CD4⁺ cells revealed that cT_{FH} and T_H2 (CD4⁺CRTH2⁺CD27⁻)/T_H2A (CRTH2⁺CD27⁺CD161⁺CD49d⁺) cells formed distinct nodes. Color of nodes represents the intensities of labeled cell markers. **D** FlowSOM analysis identified cT_{FH} and T_H2 cells as two separate metaclusters. **E** cT_{FH} cells and T_H2 and T_H2A cells formed different islands in overlaid viSNE plots. T_H2A cells are within the T_H2 cell population. Red, orange and green events indicate cT_{FH}, T_H2 and T_H2A cells, respectively. **F** MFI of CXCR5, PD-1, CRTH2, CD27, CD49d and CD161 in cT_{FH}, T_H2 and T_H2A cells. **G** Heatmap of CXCR5, PD-1, CRTH2, CD27, CD49d and CD161 MFI in cT_{FH}, T_H2 and T_H2A cells of individual patients (n = 13). Red indicates high expression and green indicates low expression of the markers. **H** Proportions of cT_{FH}, T_H2 and T_H2A cells within IL-4⁺ and IL-21⁺ CD4⁺ cells. **I** Differences in the expression of IL-4 and IL-21 in cT_{FH}, T_H2 and T_H2A cells. **P* < .05, ***P* < .01, ****P* < .001, Mann-Whitney test.

Fig 2. Dual IL-4 and IL-21 drives differentiation of naïve and memory B cells into plasmablasts.

A Relative *BCL6*, *AICDA*; **C** *PRDM1*; and **D** *XBPI* expressions in naïve B cells. **D** Differentiation of memory B cells into CD38⁺CD27^{hi} plasmablasts in response to stimulation with IL-4 and IL-21 as observed by conventional FACS analysis and unbiased viSNE analysis. Colors in viSNE plots indicate the intensity of CD38 within B cells. **E** Unbiased FlowSOM analyses showing clusters of CD38⁻ and CD27-expressing B cells (left panel). Colors in the individual clusters indicate the intensity of CD27 (yellow), CD38 (red), CD19 (aqua) and CD138 (blue). Heatmap of the population abundance of individual clusters (right panel). **F** Proportions of CD38⁺CD27⁺ plasmablasts following stimulations of naïve and memory B cells. **G** viSNE plots showing a proportion of CD38-expressing cells also co-express IgE. **H** The proportion of IgE⁺ plasmablasts following stimulations of naïve and

memory B cell. **I** ε GLT expression in naïve B cells. **J** The proportion of IgE⁺ cells; **K** tIgE levels in culture supernatants; and **L** the proportion of IgG⁺ cells, following stimulation of naïve and memory B cells. All qPCR data were normalized to 18S. Error bars represent SEM. * $P < .05$, ** $P < .01$, *** $P < .001$, Wilcoxon paired signed-rank and Mann-Whitney tests.

Fig 3. Allergen-driven proliferation of T cell subsets in grass pollen allergics (GPA) but not in non-atopic healthy control (NAC). **A** Representative plots illustrating proliferated IL-4⁺ and IL-21⁺ cT_{FH}, T_H2 and T_H2A cells in response to stimulation with timothy grass (Phlp) in GPA as assessed by CellTrace Violet. Proportions of proliferated **B** IL-4⁺ cT_{FH} (of CD4⁺ cells); **C** IL-21⁺ cT_{FH} (of CD4⁺ cells); **D** T_H2 (of CRTH2⁺CD27⁻ T_H2 cells); and **E** T_H2A (of CD4⁺ cells) cells, in response to stimulation with 0.1, 1, 10 and 25 µg/mL timothy grass (Phlp) in GPA (n = 16) and NAC (n = 6). Graphs are represented as the change from baseline (unstimulated). Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic healthy controls. * $P < .05$, Mann-Whitney test.

Fig 4. Dysregulation of cT_{FH} cells in patients with GPA compared to NAC and AIT-treated patients (SCIT and SLIT). **A** Rhinoconjunctivitis total symptom score (RTSS) of NAC (n = 13), GPA (n = 14), SCIT (n = 10) and SLIT (n = 8). **B** Proportions of activated ICOS⁺ cT_{FH}; **C** IL-4⁺ cT_{FH}; **D** IL-21⁺ cT_{FH}; and **E** dual IL-4⁺IL-21⁺ cT_{FH} cells, in NAC, GPA, SCIT and SLIT. NAC, non-atopic healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients; SLIT, sublingual immunotherapy-treated patients. * $P < .05$, ** $P < .01$, *** $P < .001$, Mann-Whitney.

Fig 5. Time-course induction of IL-4, IL-21 and IL-6 in GPA following nasal allergen challenge. **A** Time-course measurement of nasal fluid IL-4; **B** IL-21; and **C** IL-6, in NAC (blue), GPA (red), SCIT (green, closed circle) and SLIT (green, open circle) following nasal allergen challenge. Error bars represent SEM. Statistical significance in are represented as * for NAC, # for SCIT and ^ω for

SLIT, compared to GPA and ⁺ for individual timepoints in GPA compared to baseline (0 hours). NAC, non-atopic healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients; SLIT, sublingual immunotherapy-treated patients. *,[#],^ω,⁺*P* < .05, **,^{ωω},⁺⁺*P* < .01, ***,⁺⁺⁺*P* < .001, Wilcoxon paired signed-rank tests.

Fig 6. Differentially accessible chromatin regions in the cT_{FH} cells of GPA, NAC and AIT-treated patients (SCIT and SLIT). **A** Number of accessible regions in cT_{FH} cells of GPA compared to NAC, SCIT and SLIT. **B** Heatmap of the accessible chromatin landscape in NAC, GPA, SCIT and SLIT. Color represents the intensity of chromatin accessibility. Each row represents individual regions. Gene ontology (GO) enrichment analysis of biological processes for significantly accessible regions in **C** GPA compared to NAC; **D** GPA compared to SCIT; **E** GPA compared to SLIT; **F** SLIT compared to SCIT. Pathway enrichment analysis for significantly accessible regions in **G** GPA compared to NAC; **H** GPA compared to SCIT; **I** GPA compared to SLIT; **J** SLIT compared to SCIT. NAC, non-atopic healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients; SLIT, sublingual immunotherapy-treated patients; GO, gene ontology.

Fig 7. Induction of T_{FR} and IL-10⁺ cT_{FH} cells following SCIT and SLIT. **A** Proportions of CTLA-4⁺FoxP3⁺ T_{FR}; and **B** IL-10⁺ cT_{FH} cells, in NAC, GPA, SCIT and SLIT. Effect of IL-10 on **C** the relative *PRDM1*; and **D** *XBP1* expressions in naïve and memory B cells stimulated with CD40L, IL-4 and IL-21. **E** Proportions of CD38⁺CD27^{hi} plasmablasts; **F** IgE⁺; and **G** IgG⁺ cells following stimulations of naïve and memory B cell. **H** Number of accessible regions in T_{FR} cells of GPA compared to NAC, SCIT and SLIT. **I** Heatmap of the accessible chromatin landscape in NAC, GPA, SCIT and SLIT. Color represents the intensity of chromatin accessibility. Each row represents individual regions. Gene ontology (GO) enrichment analysis of significantly accessible regions in **J** GPA compared to NAC; and **K** SCIT compared to GPA; **L** SLIT compared to GPA; and **M** SCIT compared to SLIT. Pathway enrichment analysis for significantly accessible regions in **N** SCIT

734 compared to GPA; and **O** SCIT compared to SLIT. Error bars represent SEM. NAC, non-atopic
735 healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients;
736 SLIT, sublingual immunotherapy-treated patients; GO, gene ontology. $*P < .05$, $**P < .01$, $***P <$
737 $.001$, Mann-Whitney and Wilcoxon paired signed-rank tests.

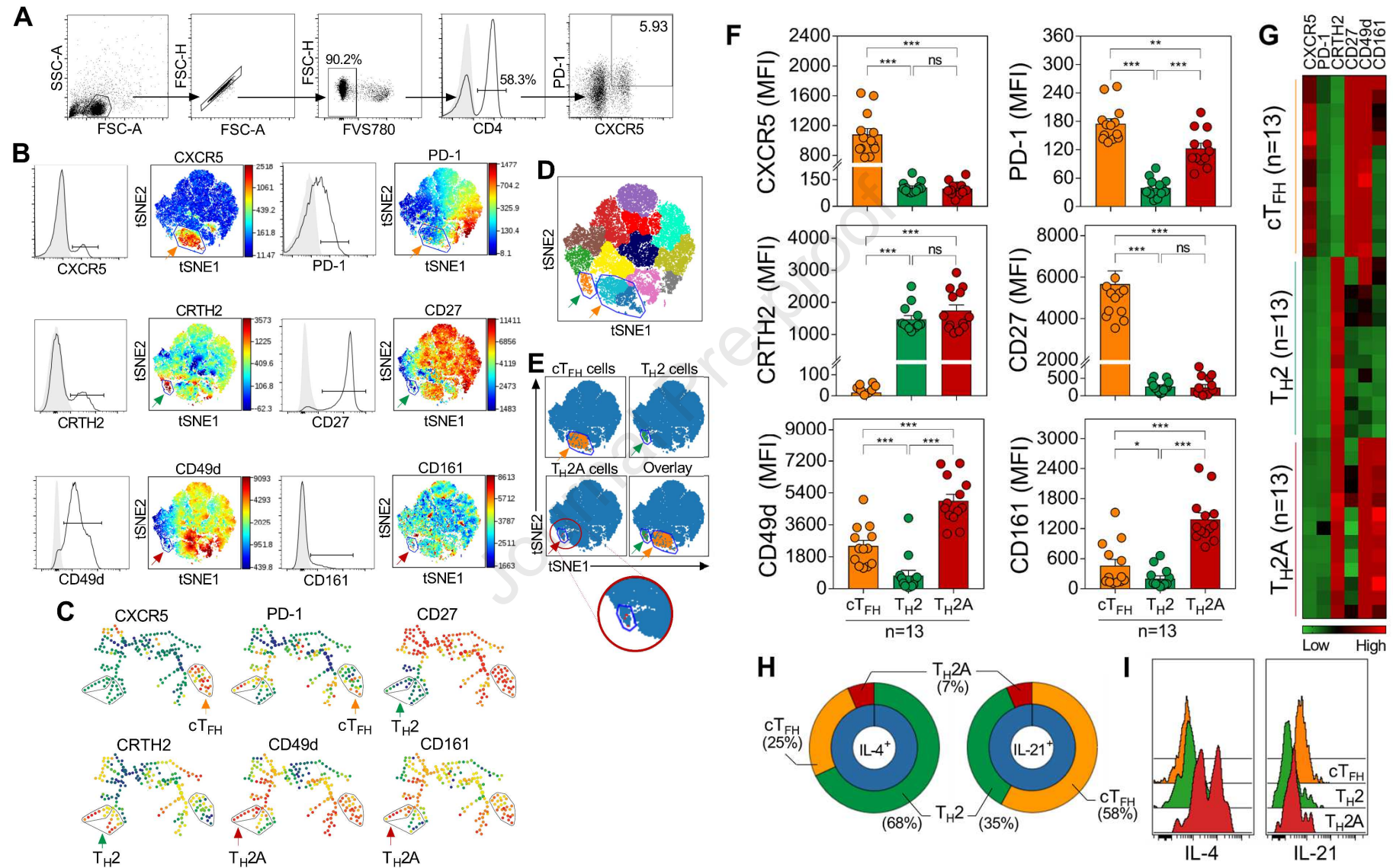


Figure 1

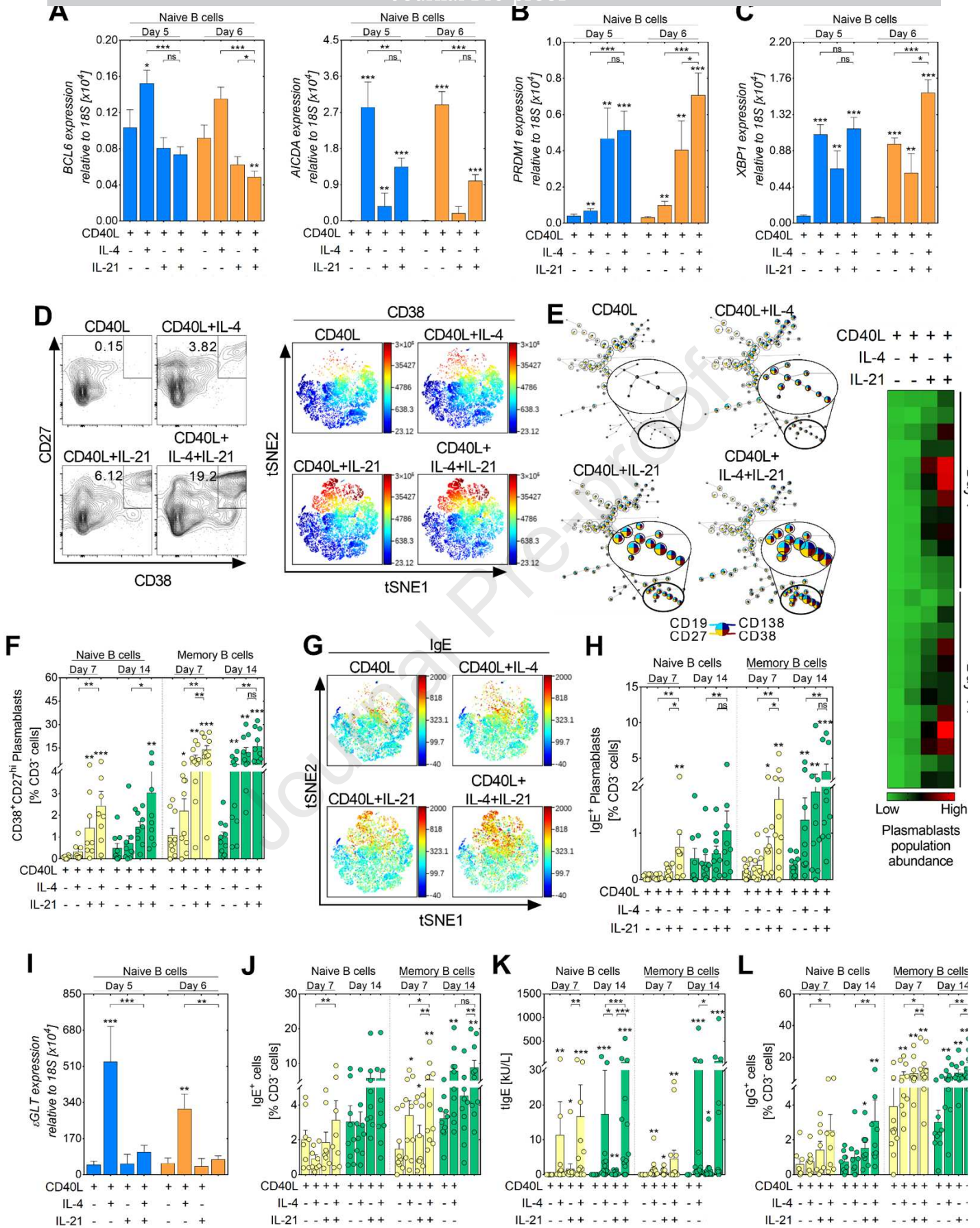


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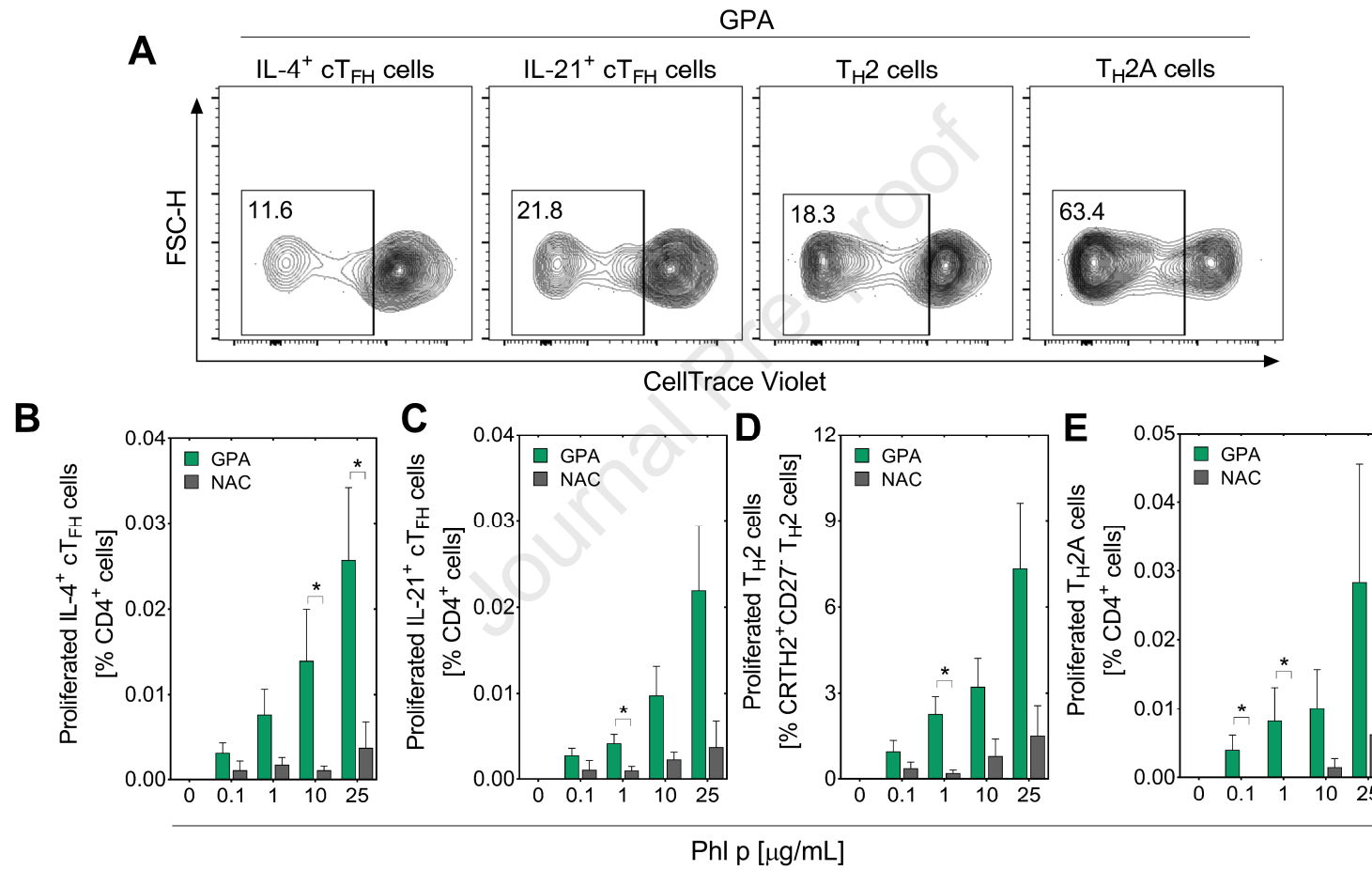


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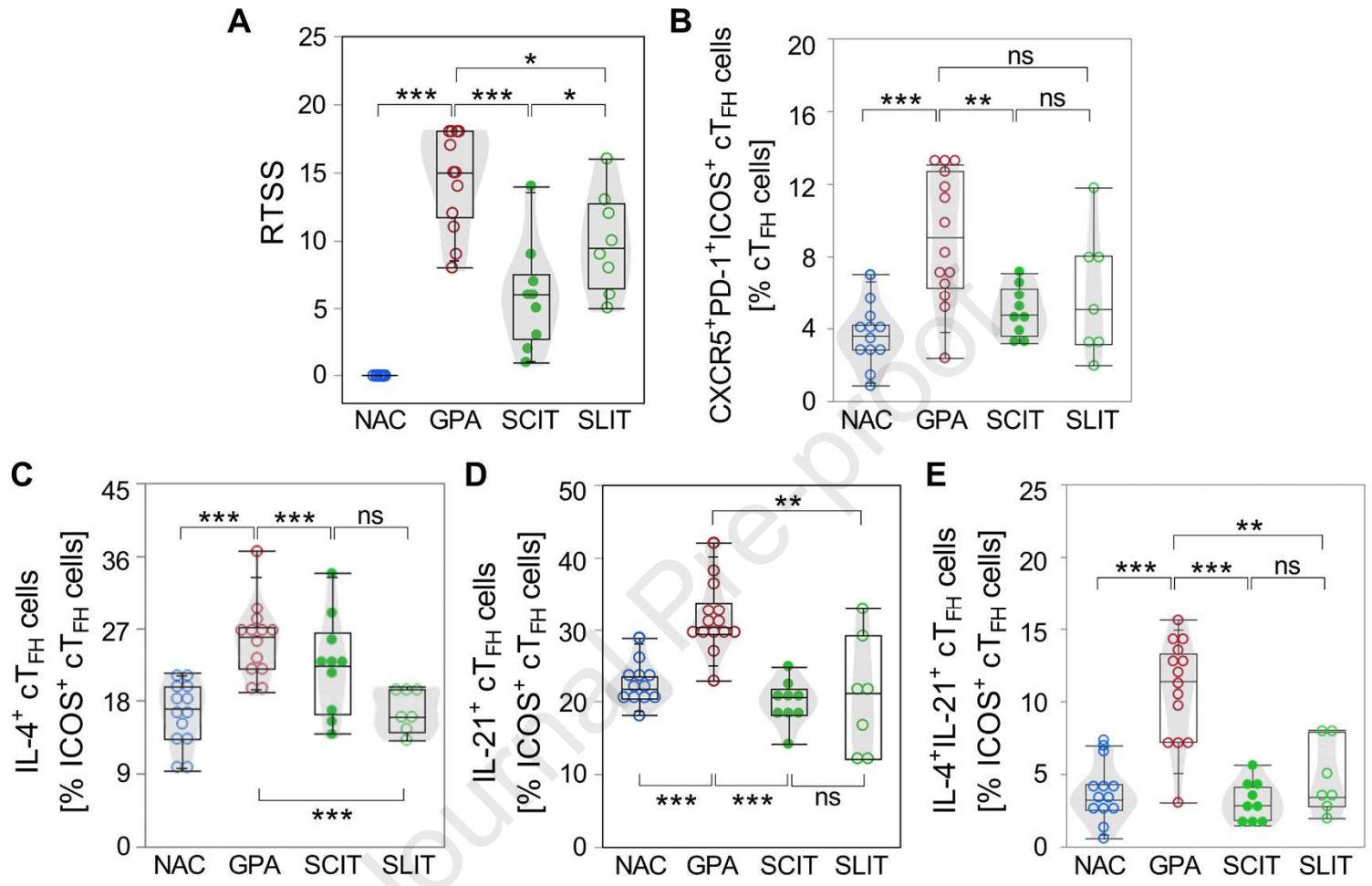


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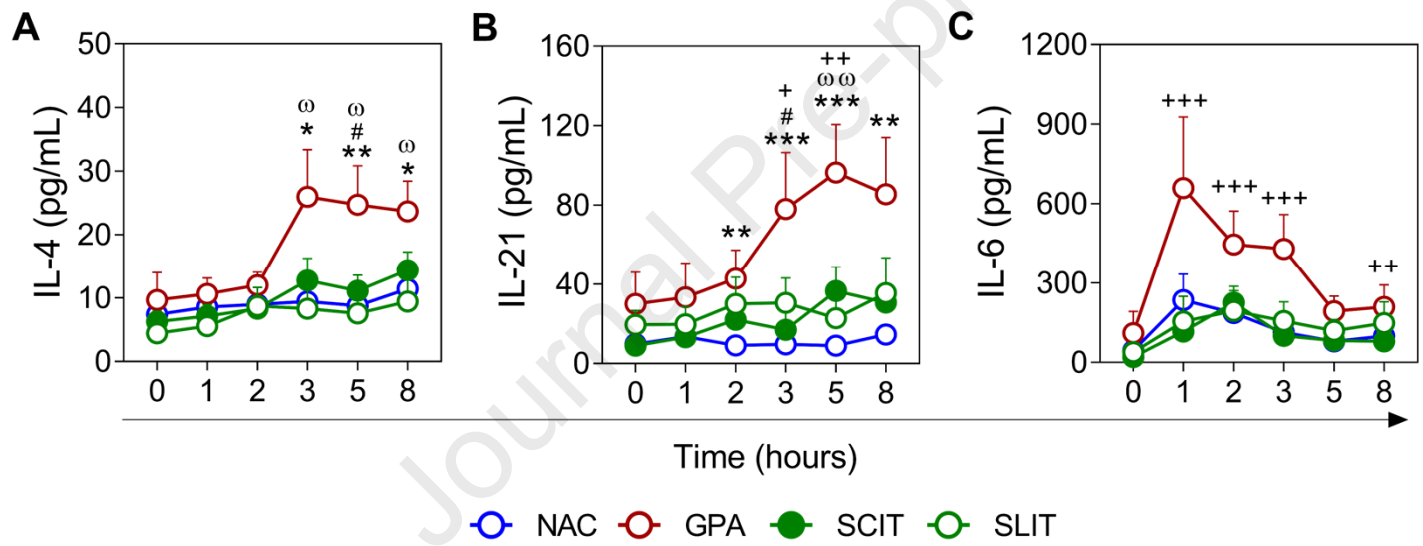


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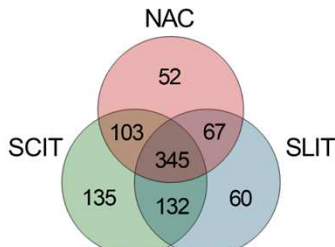
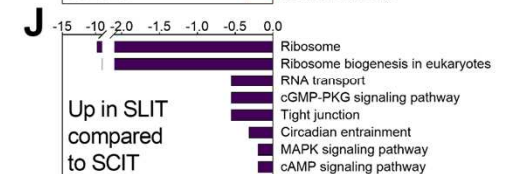
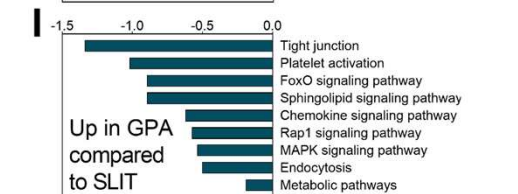
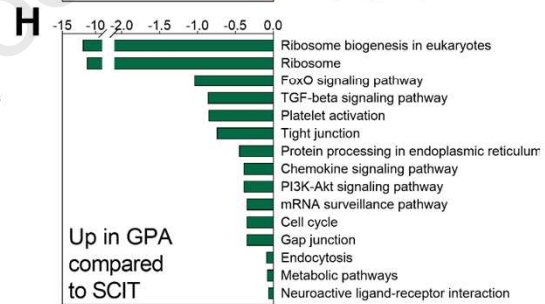
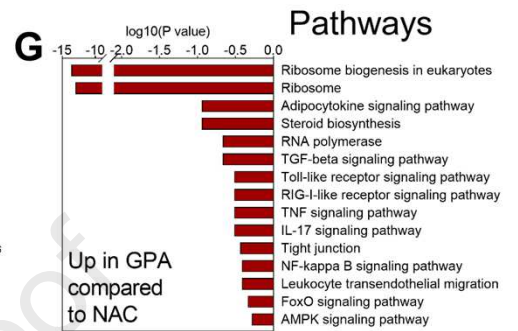
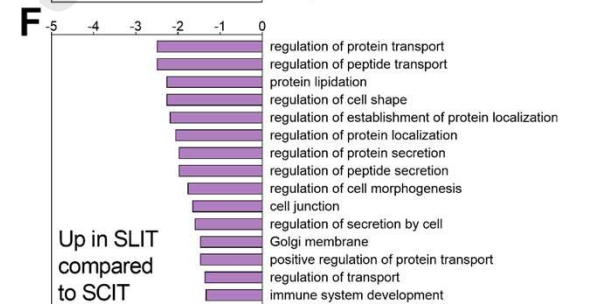
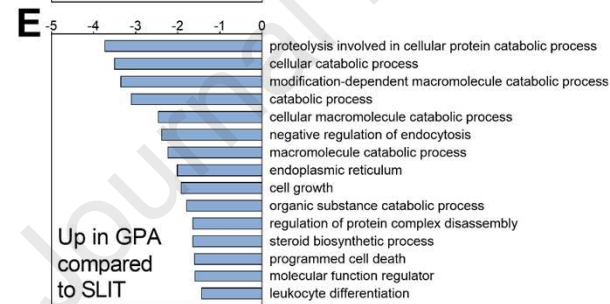
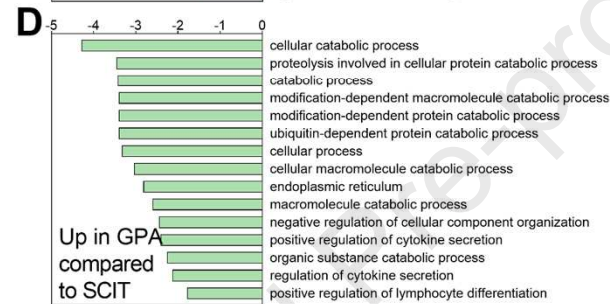
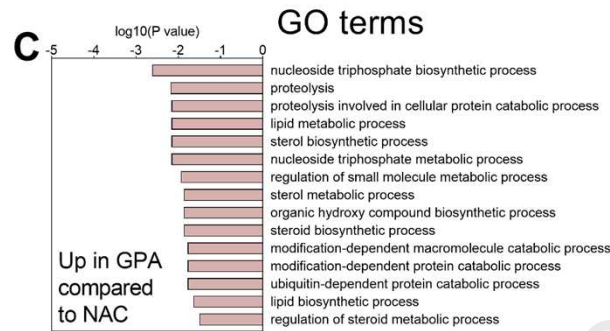
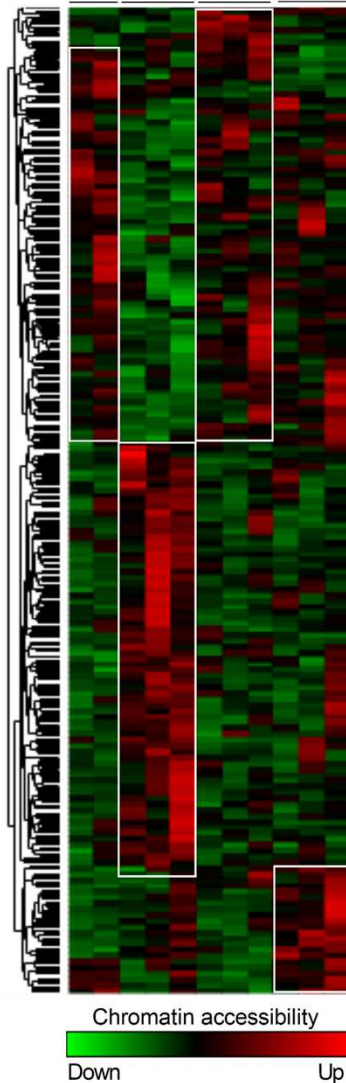
A More accessible in GPA vs**B** NAC GPA SCIT SLIT

Figure 6

