

**EVALUATION OF THE ROLE OF T FOLLICULAR CELLS IN
GRASS POLLEN-INDUCED SEASONAL ALLERGIC RHINITIS AND
FOLLOWING ALLERGEN-SPECIFIC IMMUNOTHERAPY**

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Doctor of Philosophy

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DECLARATION OF ORIGINALITY

I hereby declare that the work presented in this thesis is my own, under the supervision of Dr Mohamed Shamji, Professor Stephen Durham and Dr David Fear, except where due references are made. The rational, hypotheses and experimental designs presented in this thesis are my own.

I performed all flow cytometry, cell culture, quantitative real-time polymerase chain reaction (qPCR) and assays for transposase-accessible chromatin (ATAC) techniques. Clinical data such as symptom scores, presented in Chapter 3, Chapter 4, Chapter 5 and Chapter 7 were obtained from Professor Stephen Durham, who was the clinical investigator for those studies at the Allergy Clinic in Royal Brompton Hospital. Symptom and rescue mediation scores from the multicentre, randomised, double-blind placebo-controlled study of *Lolium perenne* peptides presented in Chapter 6 was obtained from ASIT Biotech S.A. who conducted the phase III trial in Europe. Data presented in Chapter 6 was from a single clinical site at Ghent University, lead by Professor Claus Bachert, who was able to collect blood samples for the mechanistic analysis. The ATAC-seq analysis was performed in collaboration with Professor Kari Nadeau group at Stanford University, who performed the sequencing. I performed the bioinformatics analyses with her supervision.

Statistical analysis, evaluation and interpretation are my own. This thesis has been written independently, with advice and suggestions from my supervisors.

Hanisah Sharif

2020

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ABSTRACT

T follicular helper (T_{FH}) cells are a subset of $CD4^+$ T cells that produce IL-4 and IL-21 and drive B cell maturation and clonal expansion into antibody-secreting plasma cells, the key to IgE-mediated allergic responses. T_{FH} and B cell interactions are regulated by T follicular regulatory (T_{FR}) cells. Although effective in grass pollen allergics (GPA), allergen-specific immunotherapy (AIT), administered subcutaneously (SCIT) or sublingually (SLIT) is associated with adverse events. Thus, warranting the need for alternative approaches. This thesis aims to evaluate the role of and enumerate circulating $CXCR5^+PD-1^+$ T_{FH} cells and T_{FR} cells in GPA, non-atopic healthy controls (NAC) and AIT-treated patients. Isolated naïve and memory B cells were stimulated with IL-4 and IL-21 to investigate their effect on germinal centre (GC) reactions, B cell class-switching and differentiation.

T_{FH} cells were shown to be distinct from T_H2 and T_H2A cells and capable of secreting IL-4 and IL-21. IL-4 alone promotes GC responses and B cell class-switching; and drives plasma cell differentiation and IgE^+ cells in the presence of IL-21. Enumeration of T_{FH} cell subsets in a cross-sectional study demonstrated high T_{FH} cells in GPA compared to NAC, which was lower in SCIT and SLIT. Furthermore, T_{FR} cells were induced in SCIT and SLIT and differential chromatin accessibility was observed in T_{FH} and T_{FR} cells from all groups. In a separate RDBPC study, the administration of short-course peptide immunotherapy attenuated proportion of T_{FH} cell subsets and induced T_{FR} cells, which persisted until the end of the grass pollen season. Additionally, *in vitro* stimulation study of depigmented (DPG Phl p) and DPG-polymerised (DPG-POL Phl p) depigoids, demonstrated a lower proliferation of T_{FH} cell subsets in response to DPG-POL Phl p compared to the native protein and DPG Phl p.

Collectively, these findings demonstrated the role of T_{FH} cells in the pathomechanism of seasonal allergic rhinitis (SAR) and T_{FR} cells induction following SCIT and SLIT, underscored by changes in the chromatin landscape. Peptide immunotherapy and polymerised depigoids are associated with modulation of T_{FH} and T_{FR} cells, highlighting their potential use that is safe and effective.

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LIST OF ABBREVIATIONS

AICDA/AID	Activation-induced cytidine deaminase
AIT	Allergen-specific immunotherapy
APC	Antigen-presenting cell
AR	Allergic rhinitis
ATAC-seq	Assay for transposase-accessible chromatin with high-throughput sequencing
BACH2	BTB domain and CNC homolog 2
BCA	Bicinchoninic acid
BCL6	B cell lymphoma 6
BCR	B cell receptor
bnAbs	Broadly neutralising antibodies
C1Q	Complement component
CCR	C-C chemokine receptor
CD	Cluster of differentiation
COP	Contiguous overlapping peptides
CRC	CXCL12-expressing reticular cell
CSR	Class-switch recombination
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAO	Diamine oxidase
DC	Dendritic cell
DPG Phl p	Depigmented Phl p allergoids
DPG-POL Phl p	Depigmented and polymerised Phl p allergoids
EC ₅₀	Half maximal effective concentration
FDC	Follicular dendritic cell
FoxP3	Forkhead box P3
GC	Germinal centre
GO	Gene ontology
GPA	Grass pollen allergics
H3K27me3	Trimethylation of histone 3 lysine 27 (repressive mark)
H3K4me3	Trimethylation of histone 3 lysine 4 (permissive mark)
HDM	House dust mite
HIV	Human immunodeficiency virus

HLA	Human leukocyte antigen
ICAM	Intracellular adhesion molecules
ICOS	Inducible T cell costimulator
IFN	Interferon
Ig	Immunoglobulin
IgG ₄ -RD	IgG ₄ -related disease
IL	Interleukin
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
iT _R 35	IL-35-producing inducible T regulatory
iT _{reg}	Inducible T regulatory
JAK	Janus kinase
Lol p	<i>Lolium perenne</i> (ryegrass)
LPP	<i>Lolium perenne</i> (ryegrass) peptide
LTRA	Leukotriene receptor antagonist
MAPK	Mitogen-activated protein kinase
MG	Myasthenia gravis
MHC	Major histocompatibility complex
MST	Minimal spanning tree
MST	Multiple sclerosis
NAC	Non-atopic healthy controls
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B
nT _{reg}	Naturally occurring T regulatory
PD-1	Programmed cell death protein 1
Phl p	<i>Phleum pratense</i> (timothy grass)
PI3K	Phosphatidyl-inositol 3-kinase
PKC	Protein kinase C
pSS	Primary Sjögren syndrome
RA	Rheumatoid arthritis
RDBPC	Randomised double-blind placebo control
ROR _γ t	retinoid orphan receptor γ t
RUNX	Runt-related transcription factor
SAP	SLAM-associated protein
SAR	Seasonal allergic rhinitis
SATB1	Special AT-rich sequence binding protein 1
SCIT	Subcutaneous immunotherapy
SHM	Somatic hypermutation

SLAM	Signalling lymphocyte activation molecule
SLE	Systemic lupus erythematosus
SLIT	Sublingual immunotherapy
SLO	Secondary lymphoid organ
SOM	Self-organising map
SPADE	Spanning-tree progression analysis of density-normalised events
SPIRE	Synthetic peptide immuno-regulatory epitopes
SPT	Skin prick test
STAB	Stabilin
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokine
T _{conv}	T conventional
TCR	T cell receptor
T _{FH}	T follicular helper
T _{FR}	T follicular regulatory
TGF	Transforming growth factor
T _H	T helper
T _H 2A	Allergen-specific T helper 2
TNF	Tumour necrosis factor
T _R 1	Inducible T regulatory type I
T _{reg}	T regulatory
TSDR	T _{reg} cell-specific demethylated region
t-SNE	t-distributed stochastic neighbour embedding
UPR	Unfolded protein response
VAS	Visual analogue scale
VCAM	Vascular cell adhesion molecule
viSNE	t-SNE-based visualisation
XBP1	X-box binding protein 1

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PUBLICATIONS RELATED TO THESIS

Peer-reviewed publications

Sharif H, Singh I, Kouser L, Mösges R, Bonny MA, Karamani A, Parkin RV, Bovy N, Kishore U, Robb A, Katotomichelakis M, Holtappels G, Derycke L, Corazza F, von Frenckelf R, Wathélet N, Duchateau J, Legon T, Piroton S, Durham SR, Bachert C, Shamji MH. Immunologic mechanisms of a short-course of *Lolium perenne* peptide immunotherapy: A randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol*. 2019;144:738–49.

Sharif H, Acharya S, Dhondalay GKR, Varricchi G, Krasner-Macleod S, Laisuan W, Switzer A, Lenormand M, Kashe E, Parkin RV, Yi Y, Koc M, Fedina O, Vilà-Nadal G, Marone G, Eifan A, Scadding GW, Fear DJ, Nadeau KC, Durham SR, Shamji MH. Grass pollen immunotherapy alters chromatin landscape in circulating T follicular and regulatory cells (*in press*).

Sharif H, Layhadi JA, Singh I, Robb A, Parkin RV, Kouser L, Vilà-Nadal G, Penagos M, Rey-Garcia H, Eifan A, Durham SR, Shamji MH. Alternative approach for seasonal allergic rhinitis: *Lolium perenne* peptide hydrolysates are hypoallergenic compared to native protein whilst maintaining immunogenicity *in vitro* (*in preparation*).

T follicular cells in health and disease (*in preparation*).

Peer-reviewed abstracts

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Award-winning presentations

European Academy of Allergy and Clinical Immunology (EAACI) Congress, Lisbon, Portugal (2019) – Best oral abstract presentation

Sharif H, Acharya S, Dhondalay GK, Krasner-Macleod S, Parkin R, Scadding G, Durham S, Nadeau KC, Shamji M. Altered chromatin landscape in T follicular cells in seasonal allergic rhinitis and following allergen-specific immunotherapy.

American Academy of Allergy, Asthma and Immunology (AAAAI) and World Allergy Organization (WAO) Joint Congress, Orlando, Florida, USA (2018) – Featured poster presentation

Sharif H, Karamani A, Parkin R, Kouser L, Singh I, Robb A, Gabriele H, Derycke L, von Freneckell R, Wathelet N, Duchateau J, Legon T, Piroton S, Mösges R, Durham SR, Bachert C, Shamji MH. Short Course of *Lolium Perenne* Peptides (LPP) Immunotherapy Deletes Circulating IL-4⁺IL-21⁺ T follicular helper cells and Induces FoxP3⁺ T follicular regulatory cells: A Randomized Controlled Trial.

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Sharif H, Fear D, Laisuan W, Zhang J, Yi Y, Parkin R, Fedina O, Vila Nadal G, Scadding G, Durham SR, Shamji MH. IL-10⁺ T follicular helper cells (CXCR5⁺PD-1⁺CD4⁺ T_{FH}10) promote immune tolerance and regulate B cell function following allergen immunotherapy: A proof-of-concept cross-sectional study. *European Academy of Allergy and Clinical Immunology (EAACI) Digital Congress, (2020)*.

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CHAPTER 1 INTRODUCTION

1.1 Allergic rhinitis (AR): Clinical features and prevalence

Allergic rhinitis (AR) is an immunoglobulin (Ig) E-mediated inflammatory disease of the nasal mucosa elicited by aeroallergens, such as grass pollens, trees, moulds, house dust mites (HDM) and animal dander. AR is characterised by symptoms of rhinorrhoea, paroxysmal sneezing, nasal itching, nasal congestion, and watery eyes¹. AR can be classified according to the aetiology, into perennial and seasonal (SAR). SAR can easily be identified due to the onset of seasonal symptoms following exposure to seasonal allergens including pollens and trees, that peaked during spring and summer months².

AR affects 10 – 30% of the population worldwide. Its prevalence is still increasing, not only in the developed countries but also in developing countries³ of which around 67% consists of grass pollen and HDM allergy⁴. In western Europe, AR affects 25% of the population⁵. In the United Kingdom (UK), 15 – 25% of the population is suffering from SAR in both children and adults^{6,7}. SAR symptoms present as a major clinical problem that impaired quality of life of the patients due to sleep disturbance, poorer work performance and school grades and therefore, is considered as a socio-economic burden^{8,9}.

1.2 Pathophysiology of seasonal allergic rhinitis (SAR)

1.2.1 Sensitisation phase

Following initial exposure to allergens, antigen-presenting cells (APCs) such as dendritic cells (DCs) in the mucosal lining process the allergens and present the allergen peptides on the human leukocyte antigen (HLA) on the surface of APCs. The peptide-MHC II complex on APCs then binds to T cell receptor (TCR) on naïve T cells that drive in the activation of the

naïve T cells and differentiation into allergen-specific interleukin (IL)-4- and IL-13-producing T helper (T_H) 2 cells. The binding of CD40L on T_H2 to CD40 on B cells promotes isotype class-switching of B cells. IL-4 and IL-13 bind to their receptors on B cells and activates Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling and expression of STAT6, that results in the activation of the ϵ germline transcription¹⁰, resulting in the production of allergen-specific IgE antibodies. Allergen-specific IgE then binds to high-affinity receptor Fc ϵ RI on mast cells and basophils, the process referred to as allergen sensitisation. Subsequent exposures to the allergen result in an allergic reaction. The pathophysiology of AR consists of two phases following re-exposure to allergens, the early and late phase allergic responses (Figure 1.2.1).

1.2.2 Early phase allergic response

The early phase allergic responses occur almost immediately, within minutes of allergen exposure and last for about 2 to 3 hours in sensitised individuals. Following re-exposure of the allergens, allergens bind to allergen-specific IgE on the surfaces of mast cells and basophils. The cross-linking of allergens to IgE results in the degranulation of mast cells and basophils and the release of pro-inflammatory mediators including histamines, leukotrienes and prostaglandins¹¹ (Figure 1.2.1). The pro-inflammatory mediators promote arteriolar dilation and vascular permeability, increasing mucous secretion and sinusoidal filling, that leads to nasal blockage and mucosal oedema formation, and thus resulting in rhinorrhoea, a symptom of immediate type I hypersensitivity¹².

1.2.3 Late phase allergic response

The early phase response is then followed by the late phase allergic response that occurs within 4 to 12 hours following allergen exposure¹². The late phase allergic response is characterised by the recruitment of eosinophils, basophils, T cells, and monocytes into the

lining of the nasal mucosa. It is associated with prolonged tissue oedema and persistent symptoms, particularly nasal congestion. Tumour necrosis factor (TNF) produced by mast cells induces nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) signalling pathways¹³, and subsequently upregulates the endothelial expression of leukocytes adhesion molecules, such as E-selectin and intracellular adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)¹⁴. In addition to driving allergen-specific IgE production, IL-4 secreted by T_H2 cells promotes the expression of VCAM-1, but not ICAM-1 and E-selectin^{15,16}. The expression of endothelial adhesion molecules facilitates the infiltration of eosinophils into the lining of the nasal mucosa¹⁷ and promotes further production of mucous. Furthermore, IL-5 secreted by T_H2 cells induces proliferation of eosinophils in the bone marrow and IL-9 by T_H9 drives differentiation and recruitment of mast cells¹⁸. Moreover, epithelial cells secrete chemokines including RANTES (CCL5), eotaxin, and thymus and activation regulated chemokine (TARC/CCL17) that promote recruitment and infiltration of eosinophils, basophil and T cells^{19–21} (Figure 1.2.1). Collectively, this results in sustained inflammation which is the hallmark of the late phase allergic response.

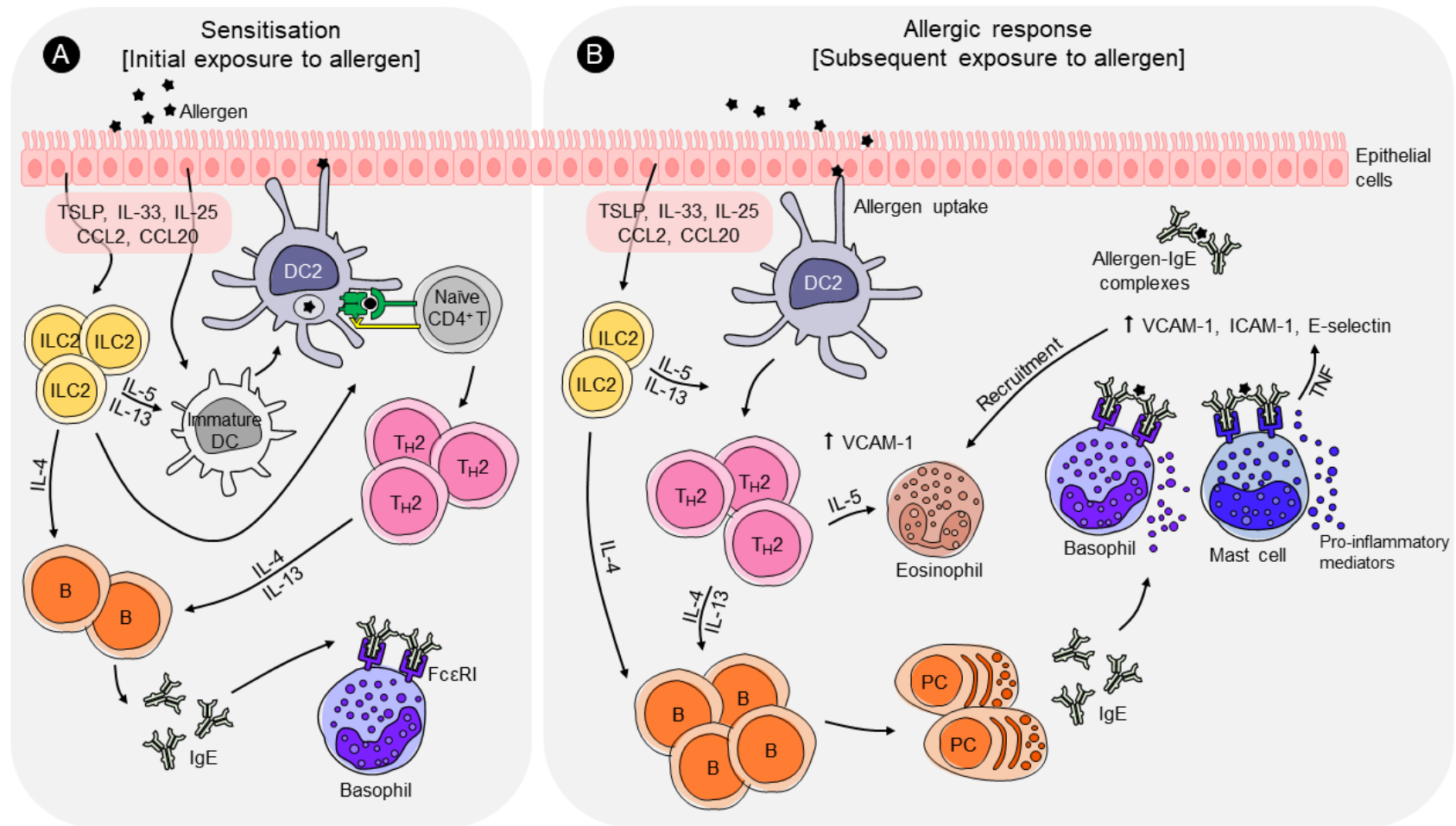


Figure 1.2.1 Pathomechanism of allergic rhinitis. A Sensitisation phase. Initial exposure to allergen promotes differentiation of IL-4- and IL-13-producing T_H2 cells. This promotes class-switching of B cells and the production of allergen-specific IgE. Allergen-specific IgE binds to high-affinity receptor FcεRI on mast cells and basophils. **B** Following subsequent re-exposure to the allergen, the allergens bind to the IgE on the surfaces of mast cells and basophils and results in the degranulation and release of pro-inflammatory mediators. This early phase is followed by the late phase allergic response, characterised by the recruitment of T cells, eosinophils and basophils. IL-5 secreted by T_H2 cells promote the recruitment of eosinophils and induce the production of IgE antibodies by B cells. DC, dendritic cell; T_H2, T helper 2 cell; PC, plasma cell.

1.3 Management of SAR

1.3.1 Allergen avoidance

Although allergen avoidance is the most effective way to improve clinical symptoms in patients with SAR, this is not always feasible²². Exposure to seasonal allergens could be limited by keeping windows closed, using an air conditioner and limiting outside activities during spring and summer seasons, where seasonal allergens are at their peak²³. As this is not always possible, patients are encouraged to use multiple measures for optimal management of symptoms.

1.3.2 Antihistamines

The non-sedating second-generation oral antihistamines such as loratadine are the first-line pharmacological treatments for SAR. They are effective in relieving sneezing, itching and rhinorrhoea when taken prior to exposure to the allergen or when symptoms are at its peak²³. The sedating first-generation oral antihistamines such as chlorpheniramine are equally effective, however, they have been reported to impair cognition performance and functioning and potentially have anticholinergic effects, and therefore are not regularly prescribed for treatment of SAR²⁴.

1.3.3 Intranasal corticosteroids

Intranasal corticosteroids such as fluticasone propionate are the first-line treatment for patients with mild persistent or moderate to severe symptoms. They are effective in reducing the inflammation of the nasal mucosa and have been demonstrated to have higher efficacy in reducing nasal congestion and rhinorrhoea compared to antihistamines and leukotriene receptor antagonists (LTRAs)²⁵. Furthermore, intranasal corticosteroids have been reported to be effective in reducing ocular symptoms and improving the quality of life of patients with

SAR²⁶. A proper administration of the nasal spray is essential of an optimal response and is most effective when administered prior to exposure to the allergens²³.

1.3.4 Leukotriene receptor antagonists (LTRAs)

LTRAs are equally effective as antihistamines in relieving SAR symptoms. However, LTRAs have been reported to be more efficient in improving night-time symptoms (sleep disturbance) due to night-time awakening and nasal congestion²⁷. Although LTRAs are not as effective as intranasal corticosteroids in relieving SAR symptoms and improving quality of life of patients²⁸, they are prescribed in occasions where antihistamines or intranasal corticosteroids are not well tolerated²³.

1.4 Disease-modifying treatments of SAR: Allergen-specific immunotherapy (AIT)

Although pharmacotherapy interventions are effective in reducing symptoms and improving the quality of life of most patients with SAR, a small proportion of patients do not respond to even high-dose pharmacotherapy treatments. Allergen-specific immunotherapy (AIT) is beneficial for the treatment of SAR in these patients that typically exhibit moderate to severe symptoms⁸. AIT was first reported in 1911 by Leonard Noon and John Freeman, where prophylactic inoculation with grass pollen extracts in patients with SAR before the pollen season results in the desensitisation and improved ocular symptoms following allergen exposure^{29,30}. AIT involves the repeated administration of high-dose allergens, either subcutaneously (subcutaneous immunotherapy [SCIT]) or sublingually (sublingual immunotherapy [SLIT])⁸, for at least three years to confer clinical benefits³¹. SCIT has been shown to be highly effective in the treatment of SAR^{32,33} and is considered as a gold standard and SLIT emerged as a safer alternative while maintaining efficacy^{34,35}. Both SCIT and SLIT have been reported to confer long-term clinical benefit and tolerance in patients^{34–37} and

prevention of disease progression^{38,39} following cessation of treatment. AIT is currently the only disease-modifying therapy for IgE-mediated allergies^{37,40}.

1.4.1 Alternatives to SCIT and SLIT

Although SCIT and SLIT using whole allergen extracts are clinically effective and induce long-term immune tolerance, they are associated with adverse events including anaphylaxis⁴¹. Furthermore, the long administration period of at least three years to confer clinical benefits³¹ leads to poor patient compliance. Alternative approaches are actively being researched to reduce the allergenicity of current immunotherapy approaches, whilst maintaining efficacy.

1.4.1.1 Peptide immunotherapy

Short linear peptides with immunomodulatory properties have been proposed to be an alternative and safer approach to conventional immunotherapy which uses whole allergen extracts. Linear allergen peptides are short and have a non-conformational structure. Consequently, they lack the ability to cross-link IgE on the surface of mast cells and basophils, thus inducing allergen-specific non-responsiveness of T_H2 cells (anergy)⁴². Two types of T cell epitope-based peptide immunotherapy have been explored in allergy: synthetic peptide immuno-regulatory epitopes (SPIRE)⁴³ and contiguous overlapping peptides (COPs)⁴⁴.

1.4.1.2 Synthetic Peptide Immuno-Regulatory Epitopes (SPIRE)

SPIRE comprises of short T cell epitope peptide mixtures of 13 – 17 amino acids long^{43,45}. SPIRE identifies and uses a mixture of synthetic immunodominant T cell epitopes within major allergens. A phase IIb study carried out in cat allergic patients treated with major cat allergen peptide, Fel d 1, referred to as Cat-PAD, showed persistent tolerance towards cat allergen for up to two years after the treatment⁴³. However, the phase III study resulted in a strong placebo

effect and it was not significant when compared to the treated group. It is important to note that participants from the phase III study were exposed to cat and this may have resulted in the induction of IgG antibodies that may have been protective even in the placebo-treated group. However, the clinical and immunologic findings of this study are yet to be published. Allergen peptide treatment using SPIRE for grass allergic patients using epitopes from major grass pollen allergens also showed reduced symptoms in treated patients compared to placebo for 6 months following the end of treatment⁴⁶. Despite its efficacy, due to its limited number of epitopes, there is a limited number of candidates that could recognise the specific epitopes. In addition, it requires an adjuvant to improve the stability of the epitope-T cell complex and the expression of the epitopes on APCs⁴⁷.

1.4.1.3 Contiguous Overlapping Peptides (COPs)

COPs are made up of long synthetic peptides of up to 80 amino acids. It comprises of all potential T cell epitopes with defective IgE conformations⁴⁸. Phase I/IIa and IIb trial studies on patients with birch pollen allergy reported improved clinical symptoms and rescue medication scores in COP-treated groups compared to placebo^{44,49}. The study involves five subcutaneous administration of Bet v 1 COPs over two months. Repeated administration of COPs induces Bet v 1-specific IgG₄⁴⁴, suggesting a similar mechanism of action of blocking antibodies in conventional AIT. However, at higher concentrations, COPs induce late asthmatic responses and skin reactions^{44,48}. Furthermore, patients that received Bet v 1 COPs continued to show improved symptoms and rescue medication scores in the following season compared to placebo, without any additional treatment or booster injections. Although a lower level of Bet v 1-specific IgG₄ was observed in the active groups, IgG₄ levels were 2 to 3-fold higher compared to the placebo group⁵⁰. Although the results are currently very promising, further studies are needed to confirm these findings and to elucidate their mechanism.

1.4.1.4 B cell epitope-based vaccines

B cell epitope-based peptide vaccines are synthetic peptides developed based on IgE binding epitope-mapping data, to induce production of 'protective' blocking IgG antibodies⁵¹. BM32 is a well-studied recombinant B cell epitope-based grass pollen allergy vaccine made from derivatives of four major allergens from timothy grass: Phl p 1, Phl p 2, Phl p 5 and Phl p 6⁵². Peptides from IgE binding sites of these allergen derivatives were fused to HBV (hepatitis B virus)-derived PreS to form a recombinant fusion protein that was adsorbed to an adjuvant (aluminium hydroxide)⁵². A phase II study that involves the administration of 3 monthly subcutaneous injections of BM32 reported reduced SPT and ocular symptoms compared to placebo. Furthermore, basophil activation was inhibited which was accompanied by induction of IgG, without eliciting IgE production⁵². Phase IIb study performed in patients with grass pollen allergy showed a reduction in symptoms and medication scores, however, no statistical significance was observed between the active and placebo groups⁵³. Despite that, IgG was induced and blunting of IgE during pollen season was observed in BM32-treated group⁵³. Collectively, these findings demonstrated the potential of BM32 in the treatment of allergy, although larger studies will need to be performed.

1.4.1.5 Allergoids

The nature of allergens is such that they possess hydrolytic enzymatic activities that may contribute to their allergenicity. One approach to reduce allergenicity and risk of anaphylaxis is to chemically modify natural (native) allergen extracts to impede with their IgE binding capacity⁵⁴. Various methods are used to deactivate enzymatic activity of an allergen extract, including polymerisation using glutaraldehyde and depigmentation (mild acid treatment), and the chemically modified allergens are termed allergoids. The allergoids undergo polymerisation and depigmentation (depigoids), resulting in the deactivation of the enzymatic activity and removal of pigments. The resulting allergoids have a high molecular weight of

1 – 3 MDa while conserving the major allergen sequences^{55,56}. Furthermore, the reduced IgE binding results in increased solubility and thus allows the administration of higher allergen dose during immunotherapy^{57,58}. Randomised controlled studies in patients with birch pollen allergy reported a reduction in symptoms and the use of rescue medication in the treated group compared to placebo and were very well tolerated^{59,60}. Despite the different study design of either 4 injections over 3 weeks⁵⁹ or 4 up-dosing injections with maintenance injections over 18 months⁶⁰, both studies demonstrated induced IgG₄ and blunting of IgE following depigoid treatment^{59,60}. The reduction observed in the actively treated group and IgG₄ was comparable to and consistent with conventional immunotherapy with whole allergen extracts^{59,61,62}. An *in vivo* study has shown that IgG induced following depigoid treatment is capable of blocking IgE binding sites for native allergen protein, despite recognising different epitopes from native allergen extracts⁶³. This, therefore, suggests a similar immune tolerance mechanism of depigoids and conventional SCIT and SLIT. However, the responder analysis revealed that about 30% of the actively treated group did not respond to the treatment⁵⁹. Clinical trial studies performed in patients with HDM allergy demonstrated similar clinical efficacy^{64,65}. A retrospective study performed on children that received at least one year of treatment with depigoids reported a similar reduction in clinical symptoms and the use of rescue medication⁶⁶. Collectively, these findings highlight the potential of depigoids as a safer alternative to conventional SCIT and SLIT.

1.5 Mechanism of immune tolerance following SCIT and SLIT

Immune tolerance induction following AIT occurs in multiple phases. Initial AIT dose in both SCIT and SLIT results in an early reduction in the degranulation of mast cells and basophils, and histamine release, although the mechanism has not yet been elucidated^{67,68}. Moreover, pro-inflammatory cytokines including IL-4, IL-5, IL-13 and IL-9 is reduced. This is accompanied by a reduction of eosinophil proliferation and infiltration into inflammatory sites due to attenuated eotaxin expression. Exposure to high dose allergen administration in AIT induces

expression of regulatory DC markers, complement component 1 (C1Q) and stabilin-1 (STAB1)⁶⁹. Moreover, the DCs secrete IL-12, IL-27 and IL-10 and the expression of CD86 is attenuated⁷⁰. In allergic murine models, CD86 blockade, but not CD80 diminished airway eosinophilia and hyperresponsiveness⁷¹, suggesting that CD86 may be more relevant in allergy pathomechanism. IL-10 have previously been shown to downregulate CD86^{72,73} and induce T cell anergy⁷⁴. Furthermore, IL-10 in the presence of transforming growth factor (TGF)- β drives proliferation of naïve T cells into allergen-specific T regulatory (T_{reg}) cells and induction of inducible type 1 T_{reg} (T_{R1}) cells, observed during early phases of AIT^{75,76}. Additionally, the CpG sites within the FoxP3 gene locus of memory T_{reg} cells demonstrated reduced methylation following administration of SLIT⁷⁷, resulting in upregulation of FoxP3 expression and suppressive function of T_{reg} cells. The T_{reg} cell subsets dampen the T_H2 cells driven responses through the release of regulatory cytokines including IL-10 and TGF- β and the expression of CTLA-4 and cell-bound TGF- β ⁷⁸. TGF- β signalling upregulates FoxP3 expression and suppresses GATA3 expression⁷⁹, further promoting differentiation of naturally occurring T_{reg} (nT_{reg}) cells. In addition, TGF- β stimulates IgA class-switching, which has been observed following AIT⁸⁰.

T_{reg} cells suppression of T_H2 cells results in a delayed shift from a T_H2 to T_H1 cell response. This results in a reduction of *IL5:IFNG* mRNA ratio, and higher interferon (IFN)- γ production following exposure to naturally-occurring allergens⁸¹. Furthermore, IL-12 secreted by DCs activates STAT4 that drives IFN- γ gene expression and secretion, that promotes T_H1 cell differentiation and suppress *IL4* gene locus, further inhibiting differentiation of T_H2 cells. Additionally, T_H2 cells have increased susceptibility to apoptosis following AIT⁸², further enhancing the shift to T_H1 cell responses. Moreover, SCIT upregulates local *IL12* expression in macrophages, that contributes to T_H1 cell responses and inhibition of local late-phase reaction⁸³. Although the role of IL-27 in AIT has yet to be determined, patients with SAR demonstrated lower IL-27 levels following stimulation with lipopolysaccharide (LPS) compared

to healthy controls. Moreover, IL-27 has the capacity to suppress GATA3 expression, IL-4 production and T_H2 cell responses. In contrast, IL-27 upregulates *IL-10* and *IFNG* expression, further suppressing T_H2 cell responses and promoting T_H1 cell responses⁸⁴.

A transient increase in allergen-specific IgE is detected following SLIT, but not SCIT³¹, which gradually plateaued and decreased over time^{31,85}. Induction of blocking IgG, IgG₄ and IgA antibodies following AIT have been well documented^{86–89}. Studies have reported the induction of IgG₁ and IgG₄ antibodies following SCIT^{86,89–92}, and IgG₁, IgG₄ and IgA following SLIT^{34,93,94}. These blocking antibodies compete with IgE antibodies for the binding of allergen, thereby, inhibiting the formation of allergen-IgE complexes. This impedes cross-linking of FcεRI on mast cell and basophil, thus inhibiting degranulation and histamine release. Moreover, the binding of allergen-IgE complexes to low-affinity FcεRII (CD23) on B cells is inhibited, preventing IgE-facilitated antigen presentation to T cells, thus impeding IgE-driven T_H2 cell responses⁹⁵. The blocking activity of IgG and IgA antibodies can be evaluated using IgE-facilitated allergen binding (IgE-FAB) assay that measures the binding of allergen-IgE complexes to CD23 (low-affinity FcεRII) on B cells⁹⁶. Both SCIT and SLIT have been reported to maintain the inhibitory function of the blocking antibodies following cessation of treatment^{36,89}, potentially contributing to the long-term immune tolerance. Collectively, both SCIT and SLIT induce persistent immune tolerance which is associated with the induction of T_{reg} cells, dampening of T_H2 cell and a shift to a T_H1 cell response, accompanied by the induction of 'protective', blocking IgG and IgA antibodies (Figure 1.5.1).

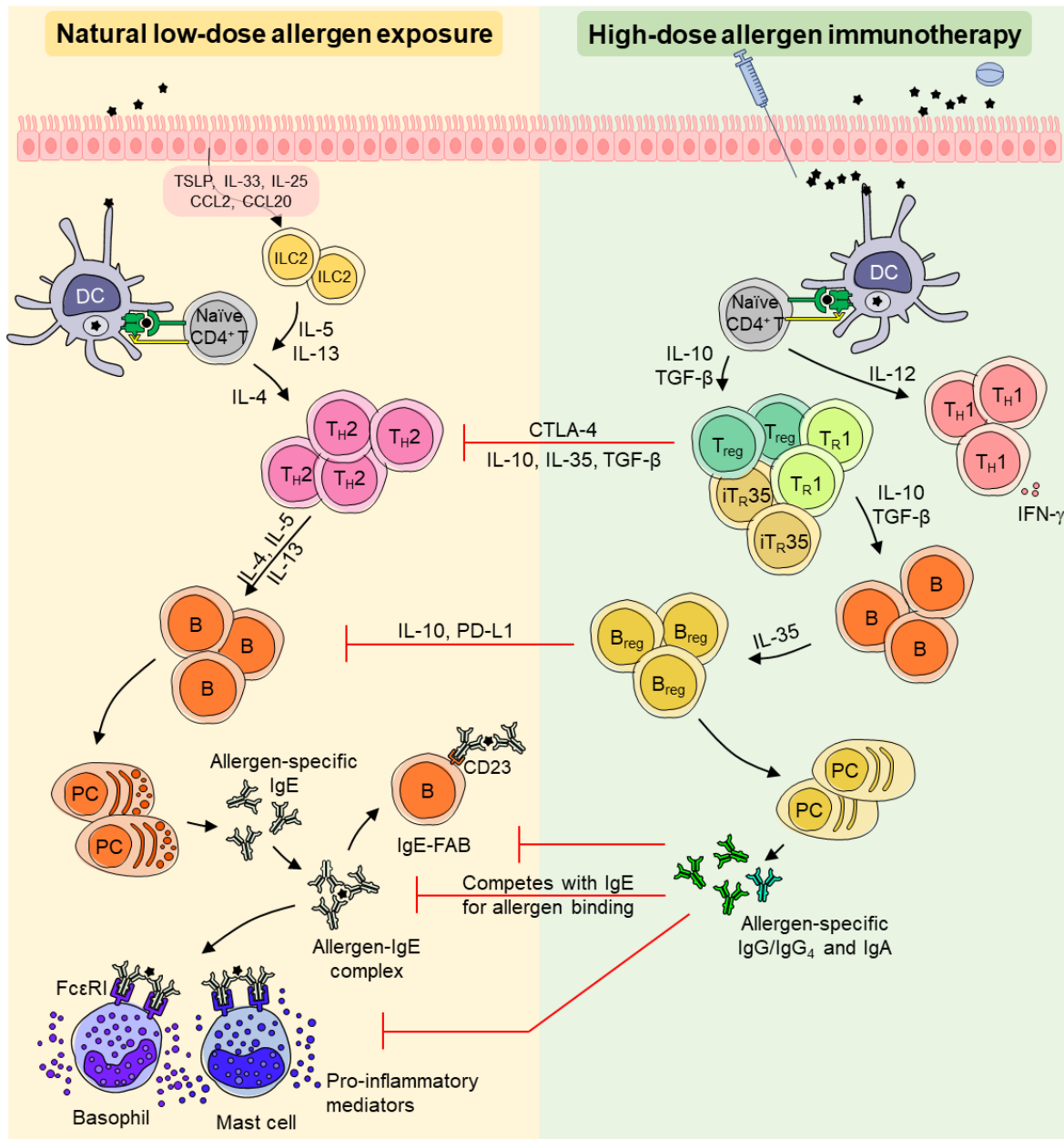


Figure 1.5.1 Mechanism of immune tolerance induction following AIT. Exposure to low dose allergen drives a T_H2 cell response that promotes IgE production by B cells. IgE binds to an allergen and the allergen-IgE complexes cross-link $Fc\epsilon R1$ on mast cells and basophils and low-affinity CD23 ($Fc\epsilon R2$) on B cells. High-dose SCIT and SLIT stimulate IL-10 and IL-12 secretion by DCs and induced proliferation of T_{reg} cell subsets in the presence of TGF- β . T_{reg} cells subsets dampen T_H2 cell responses, inducing a delayed shift towards a T_H1 response. Furthermore, IL-10 secreted by T_{reg} cell subsets induced the formation of B_{reg} cells and production of blocking IgG, IgG₄ and IgA antibodies. These blocking antibodies compete with IgE for allergen binding, thus inhibiting the formation of allergen-IgE complexes. This impedes the cross-linking of allergen-IgE complexes on $Fc\epsilon R1$ on mast cells and basophils and to CD23 on B cells and hence, IgE-facilitated allergen presentation to T cells. DC, dendritic cell; T_H , T helper cell; T_{reg} , T regulatory cell; T_{R1} , type 1 T_{reg} cell; iT_{R35} , IL-35-producing T_{reg} cell; B_{reg} , B regulatory cell; PC, plasma cell.

1.5.1 T cell subsets in immune tolerance induction following SCIT and SLIT

Immune tolerance induction following SCIT and SLIT is associated with the deletion of T_H2 and T_H2A cells, immune deviation from a T_H2 to a T_H1 cell response, and induction of T_{reg} cells.

1.5.1.1 Dampening of T_H2 cell responses

T_H2 cells express the C-C chemokine receptor (CCR) 3, CCR4 and CCR8 that aids their migration into sites of inflammation and infection⁹⁷. Although initial studies suggested that CCR3 as the main chemokine expressed by T_H2 cells, polarisation of naïve T cells into T_H2 cells results in the upregulation of CCR4 expression, suggesting that CCR4 are predominantly expressed chemokine of T_H2 cells⁹⁷. The ligands for the chemokine receptors expressed by T_H2 cells are highly expressed at sites of helminthic infections or allergic reactions, particularly in mucosal tissues, thus resulting in increased migration of T_H2 cells to these tissues. T_H2 cells developed in response to helminthic infection and allergen, which induce persistent and repeated chronic T cell stimulation.

Differentiation of T_H2 cells from naïve CD4⁺ T cells is mediated by the cytokine IL-4^{98,99}. Upon activation of T cells through TCR signalling, TCF-1 interacts with β -catenin, upregulating Wnt signalling in an IL-4-independent manner, providing the initial production of IL-4, to initiate T_H2 cell differentiation¹⁰⁰. Following the persistent and repeated exposure to the antigen, the level of IL-4 is elevated, thus promoting polarisation of naïve T cells into T_H2 cells. This results in IL-4 secretion, creating a positive amplification loop of T_H2 cell differentiation and response. IL-4 activates STAT6, which stimulates the expression of transcription factor GATA3, a member of the GATA family of transcription factors^{101,102}. GATA3 is the master regulator of T_H2 cell differentiation, that causes DNA demethylation and hyperacetylation of the T_H2 cell effector cytokine *IL5* gene locus, of which is in the same genetic locus as IL-4, and IL-13

cytokines¹⁰³. GATA3 expression induces chromatin remodelling resulting in increased accessibility of the locus for the binding of other transcription factors, which subsequently resulted in a positive loop that drives differentiation towards a T_H2 cell phenotype and inhibit polarisation of naïve CD4⁺ T cells into other T_H cell subsets¹⁰⁴. GATA3 expression is regulated by the expression of special AT-rich sequence binding protein 1 (SATB1) through the Wnt/ β -catenin signalling pathway¹⁰⁵. Activation of STAT6 by IL-4 upregulates SATB1, however, following Wnt signalling, SATB1 undergoes deacetylation and recruits β -catenin, which reverse GATA3 suppression, thus driving commitment towards T_H2 cell lineage¹⁰⁵. The binding of IL-4 and IL-13 to IL-13 receptor (IL-13R) present of cells such as B cells, DCs and basophils, results in the signalling through the JAK-STAT pathway^{106,107}, that upregulates STAT6 expression, promoting T_H2 and B cell differentiation.

IL-4 produced by T_H2 cells function to stimulate B cell Ig class-switching to IgE, key in eosinophil-mediated responses against helminthic infection and hypersensitivity reaction and development of allergies^{107–110}. IL-4 have also been shown to induce class-switching to IgG₄ in humans and inhibit class-switching to IgG₂ isotypes, which are stimulated by IFN- γ . IL-13 shares similar homology with IL-4, and play similar roles against infections and hypersensitivity reactions, although IL-13 is not involved in the development of T_H2 cells. IL-5 function to activate and stimulate eosinophil differentiation, which serves to combat against helminthic infections. Eosinophils express Fc receptors specific for IgE and IgG antibodies and thus, are able to bind microbes opsonised (marked to increase susceptibility to phagocytosis) by these antibodies¹⁰⁷.

T_H2 cell responses are inhibited following AIT. Previous studies have reported a reduction in *IL4* mRNA and T_H2 cytokines in the nasal mucosa and following a nasal allergen challenge^{31,111}. A short-course SCIT treatment in ragweed-allergic patients was associated

with lower IL-4⁺ cells in the nasal biopsies compared to placebo, following allergen challenge after the pollen season, but not before the pollen season, suggesting that the priming effect of natural allergen exposure following the SCIT treatment may be required to achieve allergen desensitisation¹¹¹. Another study reported similar findings, whereby timothy grass pollen SCIT- and SLIT-treated patients demonstrated lower nasal fluid T_H2 cell cytokines, including IL-4, IL-5 and IL-13, compared to untreated allergics, following nasal allergen challenge¹¹². More recently, allergen-specific T_H2A cells have been described that was identified using tetramer analysis^{113–115}. These allergen-specific T_H2A cells are characterised by the expression of CCR2, CD161 and CD49d and lack the expression of CD27¹¹³. They have been shown to be elevated in pollen allergic patients during the pollen season compared to non-atopic controls^{113,116}. The phenotype of these T_H2A cells was also consistent across different allergic diseases, including timothy grass, alder pollen, HDM and peanut¹¹³. In the Gauging Responses in Allergic Rhinitis to SCIT versus SLIT (GRASS) trial, SCIT and SLIT were associated with clinical improvement and lower frequency of these tetramer-positive CCR2⁺CCR4⁺CD27⁻CD161⁺ T_H2A cells following two years of treatment^{31,116}. Moreover, in the same study the nasal IL-4, IL-5 and IL-13 were also lower following nasal allergen challenge in SCIT- and SLIT-treated groups compared to placebo, which was lost at year 3 (one-year post-treatment). These findings suggest that SCIT and SLIT need to be administered for at least three years for long-term clinical benefit, which is associated with the reduction in the T_H2 and T_H2A cell responses.

1.5.1.2 Immune deviation towards T_H1 cell response

Unlike T_H2 cells, T_H1 cells are identified by the expression of C-X-C chemokine receptor (CXCR) 3 and CCR5 that aids their recruitment and migration to sites of infections⁹⁷. CXCR3 and CCR5 bind to their ligands CXCL9, CXCL10 and CXCL11 for CXCR3, and CCL3, CCL4 and CCL5 for CCR5⁹⁷, mediating the infiltrations of T_H1 cells into infection and inflammatory sites¹¹⁷. The ligands for CXCR3 and CCR5 are found to be abundant in tissues during innate

immune responses. Thus, high T_H1 cell responses are observed where the infection elicit strong innate immune responses, such as in response to viruses and bacteria.

Infection by viruses and bacteria, as well as protein antigens administered with strong adjuvants, stimulate the differentiation of naïve T cells into T_H1 cells. These infections and immunisations lead to the activation of DCs, macrophages and natural killer cells, and production of cytokines, such as IL-12, IL-18, and IFN- γ ^{118–120}. Once differentiated, T_H1 cells produce IFN- γ , further promoting T_H1 cell development⁹⁹. Activated T cells may help to increase the cytokine production by DCs, through the binding of CD40L on activated T cells and CD40 on the DCs, which resulted in the secretion of IL-12. IL-12 activates the STAT4, that enhances IFN- γ production, thus amplifies the cycles of T_H1 cell differentiation¹²¹. Furthermore, IFN- γ activates the STAT1, that stimulates the expression of transcription factor T-bet, which belongs to a T-box family of transcription factors¹²². T-bet expression, in conjunction with runt-related transcription factor (RUNX) 3 induces chromatin accessibility in the *IFNG* gene locus, further promoting the production of IFN- γ , which creates a positive amplification loop of polarisation of naïve T cells towards a T_H1 cell response^{123,124}. This also results in the suppression of *IL4* gene locus, and RUNX3 and RUNX1 attenuate GATA3 and retinoid orphan receptor γ t (ROR γ t), respectively, in the presence of T-bet, consequently, inhibiting the polarisation of naïve $CD4^+$ T cells into T_H2 and T_H17 cells^{123,124}. In addition to combatting intracellular microbes, T_H1 cells are also involved in the delayed-type hypersensitivity, which is a component of inflammatory diseases and granulomatous inflammation. This is typically observed in tuberculosis and other inflammatory disorders.

In AIT, T_H2 cell responses were inhibited and resulting in an immune deviation and induction of T_H1 cells. Previous studies have reported higher levels of nasal IFN- γ and a reduction in the *IL5:IFNG* mRNA ratio upon exposure to naturally-occurring allergens following grass pollen

SCIT, which negatively correlated with the clinical response^{81,91}. Another study also demonstrated the time-course induction of T_H1 cell cytokine, IFN- γ , from a T_H2 cell (IL-4) phenotype, thus, confirming the immune deviation following AIT¹²⁵. This finding was supported by another study that reported an increase in IL-12 mRNA⁺ cells in skin biopsies from SCIT-treated patients, but not in untreated allergics⁸³. In this study, the frequency of IL-12 mRNA⁺ cells positively correlated with the frequency of IFN- γ mRNA⁺ cells, and negatively correlated with IL-4 mRNA⁺ cells⁸³. A study performed on birch allergic patients showed an attenuated circulating Bet v 1-specific T_H2 to IFN- γ ⁺ T_H1 cell ratio following one year of SCIT treatment¹²⁶. Several studies have suggested that the immune deviation could be due to the apoptosis of T_H2 cells. Cells obtained from AIT-treated grass pollen allergic patients were shown to have an increased proportion of IL-4⁺ cells undergoing apoptosis compared to untreated allergics⁸². Similarly, T_H1 cells were shown to have increased expression of the anti-apoptotic protein, Bcl-2 following one year of SLIT treatment compared to baseline, thus suggesting a potentially persistent T_H1 cell population that was induced following SLIT¹²⁷.

1.5.1.3 Induction of natural T regulatory (nT_{reg}) and inducible T_{reg} (iT_{reg}) cells

The functions of the aforementioned T effector cell subsets require regulation to prevent autoimmune diseases or allergy, which leads to the identification of T_{reg} cells¹²⁸. T_{reg} cells have been recognised by the expression of their master transcription regulator, FoxP3^{129,130}. Treg cells have also been described to have low expression of IL-7R (CD127) and high CD25 expression. In the thymus, naïve CD4⁺ T cells differentiate into precursors to T_{reg} cells that express CD25, GITR and high expression of CD24, that do not express FoxP3. These cells then further differentiate into FoxP3-expressing cells that have suppressive capabilities and loses the expression of CD24¹³¹. The differentiation of naïve T cells to functional T_{reg} cells depends on the TCR and CD28 signalling and requires TGF- β and IL-2. In addition to TCR stimulation, IL-2 signalling activates STAT5, upregulating the expression of FoxP3 and other

T_{reg} cell-associated markers¹³². Although a lot of active enhancers and hypomethylated regions of T_{reg} cells overlaps with T effector cells, T_{reg} cell-specific regions were found to encode for the genes *FOXP3*, *IL2RA* and *CTLA4*. The *FOXP3* gene locus consists of three conserved non-coding DNA sequence (CNS) that act as transcription enhancers, CNS1, CNS2 and CNS3¹³³. CNS1 contains binding sites for NFAT and SMAD transcription factors, that mediates TGF- β signalling for the expression of FoxP3 in the development of T_{reg} cells^{133,134}. CNS2 is activated by TCR signalling and IL-2 and contains the binding sites for CREB, STAT5 and RUNX^{131,133}. TGF- β also induces the expression of RUNX1 and RUNX3 that binds to CNS2, thus upregulating the expression of FoxP3¹³⁵. CNS3 contains the binding site for the transcription factor c-Rel, which is activated following signalling through TCR and CD28. Binding of c-Rel to CNS3 also results in the expression of FoxP3¹³³. The stability of FoxP3 expression depends on the demethylation and expression of T_{reg} cell-specific demethylated region (TSDR) in the *FOXP3* gene locus¹³⁶.

The binding of SATB1 to T_{reg} cell-specific super-enhancers is required to increase chromatin accessibility and activate the super-enhancers¹³⁷. SATB1 also binds to the *FOXP3* gene locus and its downregulation is crucial for the expression of FoxP3. FoxP3 suppresses SATB1 by binding to the *SATB1* gene locus. The loss of SATB1 suppression by FoxP3 results in the loss of T_{reg} cell suppressive function, thus resulting in an intensification of effector T cell responses and production of effector T cell cytokines¹³⁸. The expression of FoxP3 amplifies the expression of T_{reg} cell-specific markers such as CD25 and CTLA-4, both of which contributes to the suppressive activity of T_{reg} cells.

Following the initial reports of naturally occurring FoxP3⁺ T_{reg} (also known as nT_{reg}) cells, other T_{reg} cell subsets have been identified such as T_R1 and iT_R35. These are inducible T_{reg} (iT_{reg}) cell subsets and secrete, regulatory (anti-inflammatory) cytokines, such as TGF- β , IL-10 and

IL-35¹³⁹. iT_{reg} cells share similar differentiation cues as nT_{reg} cells. However, they have distinct phenotypes. T_R1 cells are characterised by the expression of CD49b and lymphocyte-activation gene 3 (LAG3)¹⁴⁰. They express the transcription factors BLIMP-1 and IRF4 and mainly secrete the cytokines IL-10, TGF- β and granzyme B¹⁴¹. Unlike nT_{reg} cells, FoxP3 expression in T_R1 cells is transient. iT_R35 are characterised by the expression of CCR7 and their ability to secrete IL-35, and the expression of FoxP3 is indispensable for the development of iT_R35¹⁴¹. However, studies on iT_R35 cell development and phenotype are currently very limited, and thus need to be further studied.

T_{reg} cell subsets are crucial in for self-tolerance, prevention of the development of autoimmune disease and maintaining immune homeostasis^{142,143}. Moreover, the induction of nT_{reg} and iT_{reg} cell subsets has been associated with immune tolerance following immunotherapy treatment in patients with allergic diseases¹⁴⁴. Previous studies have demonstrated higher proportions of FoxP3⁺, and CD25⁺FoxP3⁺ cells in the nasal mucosa in SCIT-treated groups compared to untreated grass pollen allergics. Moreover, the same study reported a seasonal increased in FoxP3⁺ and CD25⁺FoxP3⁺ cells in SCIT-treated group but not placebo¹⁴⁵. Another study demonstrated higher FoxP3-expressing cells in the sublingual mucosal biopsies following SLIT compared to placebo⁸⁷. Epigenetic changes in the T_{reg} cell population was previously observed in patients with grass and HDM allergy and following SLIT treatment. The study showed induction of CD25^{hi}CD127^{lo}FoxP3⁺ T_{reg} cells with suppressive capacity following SLIT, but not placebo⁷⁷. Furthermore, a reduction in the CpG methylation within the *FOXP3* regions was observed following SLIT treatment, which was accompanied by an increase in the FoxP3 transcript⁷⁷. *In situ* hybridisation study of nasal mucosal biopsies following two years of AIT demonstrated an increase in the frequency of IL-10 mRNA⁺ cells, which correlated with the frequency of IL-10⁺ cells¹⁴⁶. A two-year SCIT treatment was shown to have higher TGF- β mRNA⁺ cells in nasal biopsies compared to placebo during the pollen season, which positively correlates to the levels of serum IgA₂⁸⁰. Similarly, HDM SLIT study reported an induction of

FoxP3⁺ Treg cells that have the capacity to suppress the proliferation of T effector cells and cytokine production⁷⁶. A time-course SCIT study reported the early induction of IL-10 production that paralleled suppression of late-phase responses in the skin that was observed at two to four weeks. Following this, induction of serum blocking IgG₄ and IgA antibodies were observed at six to twelve weeks and associated with a reduction in basophil histamine release and IgE-facilitated allergen binding to B cells⁹².

1.5.2 Immunoglobulin (Ig) responses following SCIT and SLIT

As described in section 1.5, a successful AIT involves the modulation in the cellular responses as well as humoral responses. Although an initial rise in IgE levels is often observed following SCIT and SLIT, patients remain asymptomatic upon exposure to sensitising allergens¹⁴⁷. Furthermore, studies have reported that the level of IgE was reduced following long-term SCIT treatment^{31,85}. This is accompanied by an induction of allergen-specific IgG, IgG₄ and IgA with blocking activity and competes with IgE for allergen binding^{86–89}. The formation of allergen-IgE complexes was reduced and thus, reducing the amount of IgE-mediated allergen presentation to T cells¹⁴⁸. In 1935, Robert Cooke *et al.* first reported that the passive transfer of serum from SCIT treated patients were able to suppress ragweed sensitivity in another¹⁴⁹. Then onwards, numerous studies have been performed and shown induction of IgG₄ following SCIT and SLIT^{90,91,150,151}. Two years of grass pollen IT results in the blunting of IgE, which was associated with an induction of blocking IgG and IgG₄ which correlates with clinical improvement¹⁴⁶. Similarly, a study on HDM SLIT reported an increased Der p 2-specific IgG₄ following SLIT but not placebo⁷⁶. These IgG₄ antibodies have been shown to be induced according to the duration and dose of the immunotherapy⁸⁶. IgG₄ antibodies consist of a Fab-arm exchange that contributes to its bispecific nature with two distinct antigen-binding sites¹⁵². In AIT, the induction of IgG, IgG₄ and IgA with blocking activity have been well described^{86–89}. These blocking antibodies compete with IgE for allergen binding, thus inhibiting allergen-IgE complexes formation. This subsequently hinders the cross-linking of

FcεRI on mast cells and basophils, and therefore degranulation and histamine release. Moreover, the binding of allergen-IgE complexes to low-affinity FcεRII on B cells is inhibited, preventing IgE-facilitated antigen presentation to T cells, thus impeding IgE-driven T_H2 cell responses^{95,153,154}, which can be evaluated using the IgE-FAB assay⁹⁶. The IgE-FAB assay demonstrated that IgG₁ and IgG₄ blocking activity following SCIT was time- and dose-dependent, that peaked between 3 – 6 months following administration of SCIT^{86,87}. The blocking activity of IgG antibodies persists for at least two years following cessation of SCIT treatment, despite a reduction in the IgG levels⁸⁹. Similarly, SLIT induced IgG₄ antibodies with blocking activity as assessed by IgE-FAB, that persisted for at least one year following discontinuation of a 3-year treatment period³⁶. This, therefore, suggests the induction of blocking antibodies-secreting memory B cells, that leads to the long-term immune tolerance following discontinuation of SCIT and SLIT.

1.6 Discovery of T follicular helper (T_{FH}) cells

More recently, a distinct subset of T cells located in the germinal centre (GC) within secondary lymphoid organs (SLOs) and tissues, T follicular helper (T_{FH}) cells were identified. T_{FH} cells were first reported in 2000 in human as CD4⁺ T cells that express high levels of CXCR5 in the tonsils^{155–157}. The existence of T_{FH} cells was controversial and it was not until B cell lymphoma 6 (BCL6) was identified in 2009 to be the master regulator of the T_{FH} lineage that it was accepted as a distinct subset of T cells^{158–160}. The expression of CXCR5 aids their migration into the B cell follicle where they play a crucial role in providing help to B cells and induces humoral responses^{161–164}. In the same study by Breitfeld *et al* in 2000, T_{FH} cells were shown to produce IL-21¹⁵⁷ and were identified as the main producers of IL-4 cytokine in secondary lymphoid tissues. Unlike T_H2 cells where the expression of IL-4 is regulated by GATA3, IL-4 expression is regulated by SLAM-associated protein (SAP) and protein kinase Cθ (PKCθ) in T_{FH} cells. Gene profiling of human and murine T_{FH} cells reported a distinct profile of T_{FH} cells from other T cell subsets. Unlike other T_H cell subsets, T_{FH} cells do not express

TBX21 (encodes for T-bet), *GATA3*, and they have upregulated *BCL6*, *ASCL2*, *IL21*, *PDCD1* (encodes for PD-1) and *ICOS*¹⁶⁵. Thereafter, numerous studies have identified the roles of these markers in T_{FH} cell development, differentiation, migration, homeostasis and function. In addition to CXCR5, programmed cell death protein 1 (PD-1) has been identified as a marker for T_{FH} cells and potentially regulates the interaction between T_{FH} and B cells¹⁶⁶. These markers in addition to BCL6, ICOS and SAP are upregulated in pre-T_{FH} cells following migration into the GC, whilst the expression of CD127, PSGL1 and EB12 is downregulated.

The expression of BCL6 is crucial for the initial cognate interaction of T_{FH} and B cells in the T:B cell border¹⁶⁷. Following their discovery in the SLOs, a circulating counterpart of T_{FH} cells have been identified in human peripheral blood and have been studied in various diseases^{168,169}. These circulating T_{FH} cells also express CXCR5 and PD-1, however, they have lost the expression of BCL6¹⁶⁸. Circulating T_{FH} cells have been proposed to represent the memory counterpart of GC T_{FH} cells. A study using tetramers showed that tetanus toxoid-specific T_{FH} cells were present in the peripheral blood following the administration of a booster injection¹⁷⁰. A heterogeneous population of circulating T_{FH} cells have been reported based on the expression of the chemokine receptors and activation markers. T_{FH} cells that express ICOS have been shown to express Ki-67, a marker for an active proliferative state. ICOS⁺ T_{FH} cells, therefore, represent an activated population of T_{FH} cells, whilst ICOS⁻ T_{FH} cells are regarded as a quiescent subset. Moreover, they can be further identified into the T_{H1}-, T_{H2}- and T_{H17}-like T_{FH} cells by the expression of chemokine receptors CXCR3 and CCR6, which is predominantly expressed by T_{H1} and T_{H17} cells, respectively. Similar to T_{H1} cells, CXCR3⁺CCR6⁻ T_{FH} cells also express T-bet and secretes IFN- γ , and thus, referred to as T_{FH1} cells. CXCR3⁻CCR6⁻ T_{H2}-like T_{FH} cells express GATA3 and produces IL-4, IL-5 and IL-3, and are therefore termed T_{FH2} cells. CXCR3⁻CCR6⁺ T_{H17}-like T_{FH} cells exhibit T_{H17}-like phenotype through the expression of ROR γ t and IL-17, and therefore, referred to as T_{FH17} cells¹⁷¹. Multiple studies have reported the differential functions of these subsets of T_{FH} cells. T_{FH2} and

T_{FH}17 have the capacity to provide B cells help and drive antibody production by B cells, which was lacking in T_{FH}1 cells^{170,171}, highlighting that B cell help by T_{FH} cells is mainly driven by T_{FH}2 and T_{FH}17 cells. T_{FH}1 cells, in contrast, have been shown to contribute to the induction of protective antibodies following the administration of an influenza vaccine, although this is more prominent during an activated state¹⁷². Although some studies have investigated the importance of T_{FH} cells in human, it is noteworthy that most of these studies were performed in mice, and the roles of T_{FH} cells in human diseases are currently under active investigations.

1.7 T_{FH} cell differentiation and GC reaction

T_{FH} cell differentiation occurs in several phases, all of which involves sequential antigenic stimulations from DCs and GC B cells. T_{FH} cell differentiation can be broadly divided into three phases according to the site of the interactions (Figure 1.7.1)¹⁷³. The differentiation and maintenance of T_{FH} cells depend on continuous antigenic stimulation by APCs^{174,175}. Antigen presentation during the first phase of T_{FH} cell differentiation is mainly mediated by DCs (and by GC B cells during high-dose antigen exposure). GC B cells are responsible for the completion of the subsequent phases and contribute to the stabilisation of BCL6 expression^{176–178}.

1.7.1 Pre-T_{FH} cell differentiation in the T cell zone

The first phase involves the differentiation of T_{FH} cells from naïve CD4⁺ T cells initiated following interactions with DCs in the T cell zone of SLOs. Upon exposure to an antigen, naïve T cells are primed by activated DCs through the binding of the TCR to MHC II on DCs. CD28 on T cells is upregulated and ligates to its ligands CD80/CD86 on DCs, resulting in the activation of PKC θ signalling. Moreover, activated DCs secretes IL-6 and IL-12, which upregulates BCL6 expression and IL-21 secretion^{160,179}. Secreted IL-21 has an autocrine role¹⁸⁰ where it creates a positive feedback loop on GC T_{FH} cells to support the induction of

BCL6 expression, which is indispensable for T_{FH} cell differentiation^{158,160,179,181,182}. IL-6 and IL-21 signalling induce STAT1 and STAT3, whilst IL-12 activates STAT4 expression^{179,183,184}. This results in the expression of T_{FH} cell markers CXCR5, PD-1, ICOS and CXCR4^{159,160,166,183}. In addition to IL-6 and IL-12, IL-27 secreted by DCs further induces IL-21 secretion and ICOS expression by GC T_{FH} cells through activation of STAT3¹⁸⁵. BCL6 also binds to the promoter regions of T-bet, GATA3 and ROR γ t and thus, inhibiting their expression and differentiation of T_H1, T_H2 and T_H17 cells, respectively^{159,160,186}. BCL6 expression is accompanied by the simultaneous downregulation of its antagonist, BLIMP-1¹⁵⁸. Moreover, BCL6 downregulates the expressions of CCR7 and EBI2, and the expressions of CXCR5, CXCR4 and sphingosine-1-phosphate receptor 2 (S1PR2) are upregulated^{166,187,188}. The T cell zone expresses high CCL19 and CCL21, ligands for the CCR7, whilst CXCL13, a CXCR5 ligand, is predominantly expressed in the B cell follicle. The CXCR5 expression and CCR7 repression, therefore, allow the migration of pre-T_{FH} cells from the T cell zone to the interface between T cell zone and the B cell follicle (T:B border) within the SLOs¹⁶⁶, where the second phase of T_{FH} cell differentiation and maturation occurs.

1.7.2 Pre-T_{FH} and GC B cell interactions at the T:B border

At the T:B border, pre-T_{FH} cells interact with activated GC B cells and provide signals and support for the formation and initiation of a GC response in the B cell follicle. The cognate interactions between pre-T_{FH} and GC B cells are bidirectional and contribute to the maturation of both pre-T_{FH} and GC B cells¹⁸⁹. Activated B cells provide costimulatory signals during the cognate interaction with pre-T_{FH} cells through the expression of MHC II, CD40, CD80/CD86, ICOSL, OX40L, PD-L1 and PD-L2 on B cells. Co-stimulation through CD80/CD86-CD28 induces the expression of ICOS, PD-1, OX40 and CXCR5 on pre-T_{FH} cells¹⁸⁹. The MHC II-TCR and ICOSL-ICOS signalling elicited calcium signalling, induces the production IL-4 and IL-21 by pre-T_{FH} cells¹⁹⁰. The calcium spike following ICOS-ICOSL signalling also activates the transcription factors, nuclear factor of activated T cells (NFAT), in a PI3K-dependent manner,

that drives T_{FH} cell differentiation¹⁹¹. In addition, PI3K signalling induces the expression of c-Maf, which upregulates IL-21 and IL-4¹⁹². ICOSL-ICOS signalling increased CD40 expression on B cells, upregulates ICOSL expression on GC B cells, thus creating a positive feedback loop that enhances T_{FH} and B cell interactions¹⁹³. Similar to ICOS, OX40 activates PI3K and Akt signalling pathways and enhances NFAT production that contributes to the survival of T_{FH} cells^{194,195}. Simultaneous signalling through OX40-OX40L and ICOS-ICOSL and the expression of SAP is crucial for stable cognate interaction between pre-T_{FH} and GC B cells^{196,197}. The expression of BCL6 and CXCR5 are maintained during this phase for the maintenance of T_{FH} cell differentiation and subsequently promotes the migration of T_{FH} cells into the GC within the B cell follicle.

1.7.3 GC T_{FH} and B cell interactions within the GC

The third, functional and maintenance phase of T_{FH} cell differentiation occurs within the GC. The GC is formed following antigen exposure and pre-T_{FH} and B cell interactions at the T:B border. Fully developed GC is polarised into dark and light zones, sites for proliferating GC B cells (centroblasts) and non-proliferating GC B cells (centrocytes), respectively¹⁹⁸. GC B cells (centroblasts and centrocytes) cycles between the dark and light zones during B cell maturation process¹⁹⁸. Also present in the GC are the CXCL13-producing follicular DCs (FDCs) and CXCL12-expressing reticular cells (CRCs)^{199,200}. The chemokines produced by these cells aids localisation of GC B and T_{FH} cells during the GC response. Fully differentiated T_{FH} cells localised in the light zone of the GC, referred to as GC T_{FH} cells, express high CXCR5, PD-1 and BCL6¹⁶⁶. GC T_{FH} cells provide signals to centrocytes through the expression of CD40L and production of IL-4 and IL-21, that mediates GC B cell proliferation and survival.

Following GC T_{FH} and B cell interaction, centroblasts undergo proliferation and somatic hypermutation (SMH) in the dark zone. Engagement of CD40L on GC T_{FH} cells to CD40 on

GC B cells induces the enzyme activation-induced cytidine deaminase (AID), initiating SHM, although it mainly functions in isotype class-switching of centrocytes. SHM refers to the point mutations of the variable (V) genes of the Ig heavy (H) and light (L) chains of the antibody expressed by GC B cells, introduced into the DNA during the repair of DNA strand breaks^{201,202}. SHM results in affinity maturation of antibodies and occurs at an extremely high rate of about 1 in 10^3 V gene base pairs per cell division²⁰³. GC B cells express BCL6, which is crucial for their survival during the high proliferation rate during SHM and class-switch recombination (CSR). Furthermore, BCL6 binds to the p53 promoter region, thus suppressing p53 and PIAS2 expression, inhibiting the expression of cell cycle arrest genes *p53* and *CDKN1A* (encodes for p21)^{204,205}. This, therefore, inhibits apoptosis and allows the GC B cells to withstand the stress during the high proliferation of GC B cells while sustaining the DNA breaks during SHM and CSR. The centroblasts progress to become non-proliferating centrocytes following signalling through B cell receptor (BCR) and phosphorylation of BCL6 by mitogen-activated protein kinases (MAPKs)²⁰⁶. Furthermore, the binding of NF- κ B-mediated IRF4 onto the BCL6 promoter region suppresses *BCL6* gene expression²⁰⁷, thus resulting in the transition of centroblasts to centrocytes. Centroblasts loses the expression of CXCR4 and migration into the light zone down the CXCL12 gradient.

Engagement of CD40L on GC T_{FH} cells to CD40 on centrocytes in the light zone activates the enzyme AID, inducing isotype class-switching (Ig class-switching) and differentiation into memory B cells^{208,209}. An Ig is composed of two L and two H chains, consisting of variable (V) regions that recognise antigen, and constant (C) regions that mediate the effector functions. Isotypes refers to the classes of antibodies, consisting of five major isotypes namely IgA, IgD, IgE, IgG and IgM, classified based on the differences in the C_H regions (α , δ , ϵ , γ , and μ , respectively). IgG isotype is further divided into four subclasses: IgG₁, IgG₂, IgG₃ and IgG₄; whilst IgA is divided into two subclasses: IgA₁ and IgA₂. A V gene, D (diversity) segment and J (joining) genes are rearranged, a process called V(D)J recombination, to form a single V(D)J

exon that encodes for the V region of an Ig²¹⁰. CSR is a mechanism whereby the DNA encoding for the target C_H is excised, resulting in the recombination of the V(D)J region to a downstream targeted C_H locus. CSR involves germline transcripts, that are found at the upstream μ locus and the downstream targeted C_H locus, mediating isotype switching from IgM to either IgG, IgE or IgA. Germline transcripts are not translated into proteins but are crucial for isotype class-switching²⁰⁸. In addition to CD40-CD40L signalling, ICOSL-ICOS co-stimulation is indispensable for isotype class-switching²¹¹. Suppression of BCL6 expression in centrocytes is further mediated by IRF4, that results in the termination of the centroblast-centrocytes growth cycle. Suppression of BCL6 upregulates BLIMP1 expression^{212,213}, and drives the differentiation of centrocytes into plasma cells. Additionally, BLIMP1 regulates the expression of X-box binding protein 1 (XBP1), which is a component of unfolded protein response (UPR) and is essential for the development of antibody secretory function of plasma cells^{214,215}. Moreover, IL-4⁺ T_{FH} cells induce *PRDM1* (encodes for BLIMP1) expression and thus, contributes to promoting plasma cell differentiation in the light zone²¹⁶. IL-4⁺ GC T_{FH} cells are predominantly located distal to the dark zone and express high CD40L. On the other hand, IL-21⁺ GC T_{FH} cells have high CXCR4 expression and are localised closer to the dark zone, suggesting their role in promoting SHM and affinity maturation of centroblasts²¹⁶. Continuous antigenic stimulation is crucial for the maintenance of GC T_{FH} cells which then persist as long-lived memory T_{FH} cells.

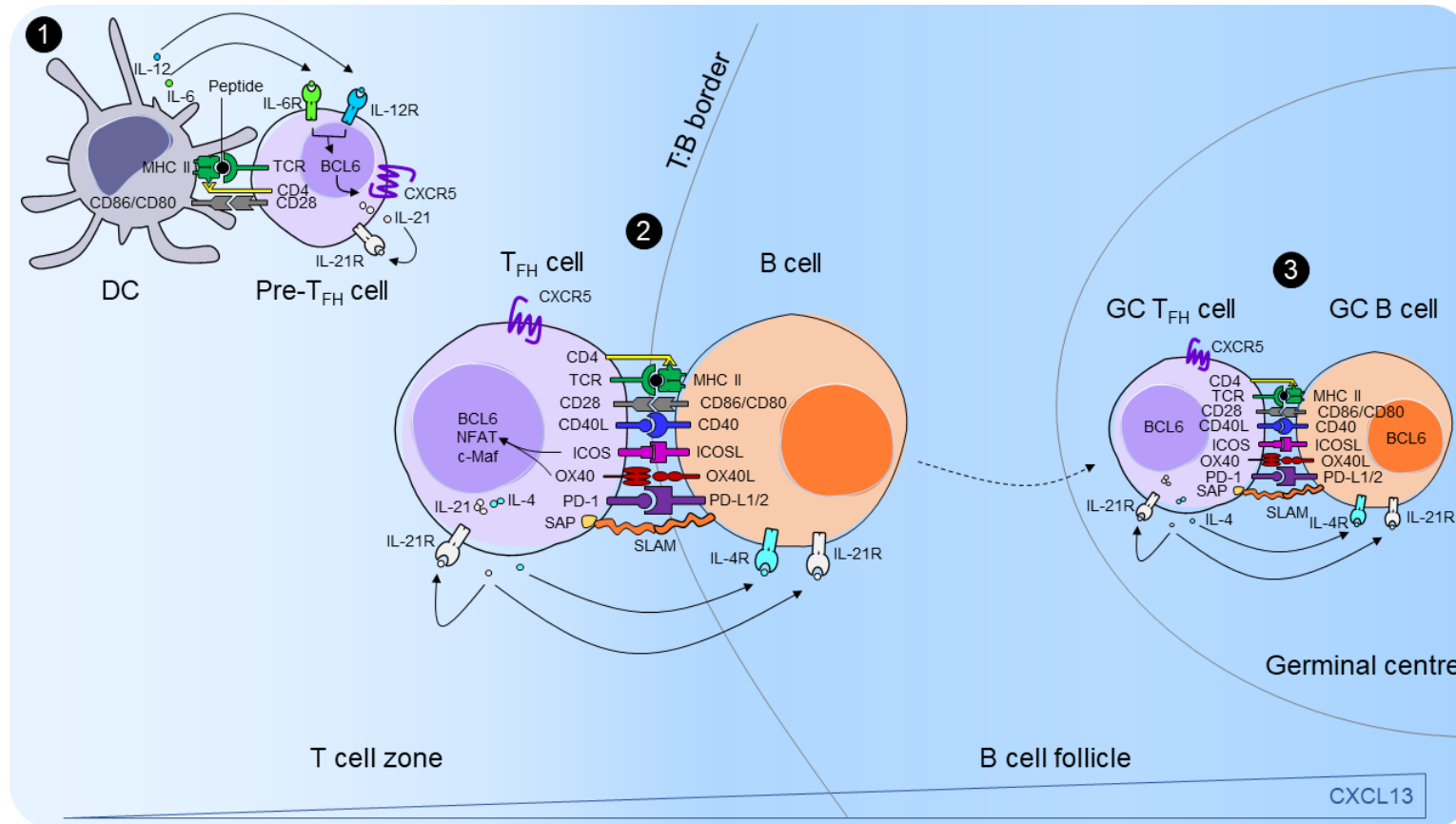


Figure 1.7.1 T_{FH} cell differentiation. T_{FH} cell differentiation occurs as a multistep process. The first phase occurs in the T cell zone where naïve CD4⁺ T cells differentiate into pre-T_{FH} cells following interactions with DCs in the secondary lymphoid organs (SLOs). IL-6 and IL-12 from DCs upregulate BCL6 and induce IL-21 secretion. Secreted IL-21 creates a positive feedback loop in supporting BCL6 expression. Pre-T_{FH} cells downregulate CCR7 and upregulate CXCR5 and migrate to the T:B border in response to the CXCL13 gradient. The second stage of T_{FH} cell differentiation occurs at the T:B border and involves the cognate interactions between T_{FH} and B cells through the expression of CD40L, CD28, ICOS, OX40, PD-1 and SAP. ICOS-ICOSL and OX40-OX40L signalling induces expression of NFAT and c-Maf and upregulates IL-4 and IL-21 secretion. Expression of BCL6 and CXCR5 are maintained to promote migration of T_{FH} cells into the GC. The third stage of T_{FH} cell differentiation is the effector stage that occurs within the GC, where GC T_{FH} cells promote affinity maturation, class-switching and proliferation of GC B cells into memory B and antibody-producing plasma cells. T_{FH}, T follicular helper cell; GC, germinal centre.

1.8 Discovery of T follicular regulatory (T_{FR}) cells

Whilst the crosstalk between GC T_{FH} and B cells is critical for antibody production and protection against foreign pathogens, aberrant GC reaction leads to the development of diseases. The stochastic nature of SHM has the capacity to generate high-affinity autoantibodies and thus will lead to the development of autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS). Furthermore, other diseases including B cell lymphoma, allergic responses, and chronic inflammation resulted from abnormal GC responses and antibody production. Tight regulation of GC responses is therefore critical to maintain immune tolerance. It has been established and well accepted that T_{reg} cells play a role in regulating and maintaining immune homeostasis and the humoral response. Studies using scurfy mice (FoxP3⁺ T_{reg} cells deficient mice) showed elevated serum IgG, IgM, IgA and IgE²¹⁷ and increased production of autoantibodies²¹⁸. In human, FoxP3 mutations lead to the development of immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome with high IgE levels and eosinophil counts²¹⁹, further highlighting the role of T_{reg} cells in regulating humoral immunity.

In 2004, CD4⁺CD25⁺CD69⁻ T_{reg} cells were found to have the ability to migrate into GCs and directly suppress GC reactions²²⁰. Upon activation, these cells express CXCR5 that facilitates their migration into the CXCL13-rich GC. Regulation of GC responses and Ig production by these CXCR5-expressing T_{reg} cells is mediated by suppressing T_{FH} cell-induced AID expression in GC B cells and CXCL13 production, thus interfering the crosstalk between GC T_{FH} and B cells^{220,221}. However, it was not until 2011 that this population of cells were recognised as a distinct subset of CD4⁺ T cells. Three independent studies investigated this newly discovered subset of CD4⁺ T_{reg} cell, that shared characteristics with, and yet distinct from both T_{FH} and T_{reg} cells, where the term T follicular regulatory (T_{FR}) cells were then coined^{222–224}. They constitute about 5 – 25% of the follicular T cell population in mice²²². Similar to T_{FH} cells, T_{FR} cells express CXCR5, transcription factor BCL6, PD-1 and ICOS that accounts

for their similar follicular origin^{222–224}. Deletion of *BCL6* or *SH2D1A* (encodes for SAP) and B cell knockout studies selectively impaired T_{FR} cell development, but not T_{reg} cells. Furthermore, the transcriptional analysis revealed distinct gene signatures from T_{FH}, T_{reg} and T effector cell subsets, highlighting the distinct profile of T_{FR} cells^{222–224}.

1.9 Differentiation of T_{FR} cells

T_{FR} cells differentiate from T_{reg} cell precursors and occur as a multistage process. Upon exposure to an antigen, T_{reg} cells upregulate CCR7 and CD62L, that aid trafficking into SLOs²²⁵. Similar to T_{FH} cells, T_{FR} cells require continuous antigenic stimulation for their differentiation and maintenance. The initiation phase of T_{FR} cell differentiation involves signalling from DCs through CD80/CD86-CD28 and ICOSL-ICOS signalling in the T cell zone. This results in the CXCR5 expression in T_{FR} cells which is regulated by NFAT2²²⁶. IRF4 expression upregulates CTLA-4 in T_{FR} cells^{227,228}. CTLA-4 expression, in turn, regulates T_{FR} cell differentiation and ICOS expression²²⁸. ICOS expression is maintained by the upregulation of transcription factor TRAF3 and is essential for the migration of T_{FR} cells into the GC and GC maintenance²²⁹. As with T_{FH} cells, BCL6 expression is indispensable for the development of T_{FR} cells. mTORC1 signalling induces STAT3 expression, which binds to *TCF7* (encodes for TCF1) and results in the expression of BCL6^{230,231}, driving T_{reg} cell differentiation into T_{FR} cells. IL-2 signalling induces STAT5 expression and leads to the expression of BLIMP1, which is a BCL6 repressor. T_{FR} cells express CD25 during the initial differentiation phase, however, they eventually lose the CD25 expression to maintain the expression of BCL6. At this stage, BLIMP1 expression is downregulated to allow the expansion of T_{FR} cells²³². Whilst the downregulation of BLIMP1 is crucial for the expansion of T_{FR} cells, complete abolishment of BLIMP1 has been associated with the loss of suppressive function of T_{FR} cells, thus highlighting a delicate balance between BCL6 and BLIMP1 expression to achieve efficient differentiation while maintaining the suppressive phenotype. A proportion of T_{FR} cells exit the lymph node and become circulating memory-like T_{FR} cells, while the rest migrate to the T:B

border in response to the CXCL13 gradient as effector T_{FR} cells with suppressive functions²³³. Similar to T_{FH} cells, T_{FR} cells undergo developmental and maintenance phase during interaction of T_{FR} and GC B cells at the T:B border. The maintenance of the balance between T_{FH} and T_{FR} cell ratio is crucial for a normal GC response. Upon antigen exposure, both T_{FH} and T_{FR} cells undergo differentiation, with T_{FH} cell differentiation occurring at a higher speed. Expansion of T_{FH} cells allows the formation of the GC, which return to baseline following GC resolution²³⁴. Currently, there have been very limited studies that investigate the T_{FR} cell differentiation and maintenance due to the dependency of T_{FR} cells on T_{FH} and B cells for their development. Furthermore, T_{FR} cells share a substantial amount of stimulatory signals and markers with T_{FH} and T_{reg} cells, making it a very challenging area to explore without affecting the development of the other T cell subsets. In mice, T_{FR} cells are predominantly localised in the GCs, while in human, they are primarily found at the T:B border and B cell follicle.

1.10 Mechanism of immune suppression by T_{FR} cells

In vivo murine studies have shown the exclusive suppressive function of T_{FR} cells on B cell responses and antibody production, which was not observed with T_{reg} cells^{227,235}. Henceforth, *in vitro* studies were performed to unravel the mechanism and function of T_{FR} cell suppression on GC T_{FH} and B cell interactions (Figure 1.10.1). Depletion of T_{FR} cells prior to GC formation results in an exacerbated GC response and production of autoantibodies. However, depletion of T_{FR} cell post GC formation results in an increased plasma cell differentiation²³⁶, suggesting that the regulation of GC responses by T_{FR} cells mainly occurs during the early GC reaction. In addition to suppression of antibody production, T_{FR} cells suppress T_{FH} cells BCL6 expression, cell growth and the production of IL-4 and IL-21^{233,237}. Although suppression of T_{FR} cells on T_{FH} cells affects T_{FH} cell effector function, no changes were observed in the T_{FH} cell developmental programme. In B cells, T_{FR} cells downregulate the expressions of *POU2AF1* (encodes for Oct activator from B cells [OCAB]), *XBP1* (encodes for XBP1) and *AICDA* (encodes for AID)^{220,221,237}. Moreover, the expression of GL7 and CD80 (B7-1),

normally expressed in activated B cells, and antibody class-switching is attenuated in the presence of T_{FR} cells^{233,237}. The suppression of these markers will, therefore, lead to a defective B cell development, CSR and secretory function of B cells. In addition, T_{FR} cells upregulate the expressions of *PAX5*, *BACH2* and *IRF4* in B cells, thus interfering with B cell differentiation into plasma cells²³⁷. The observed effector function of T_{FR} cells is not achieved with T_{reg} cells, further highlighting the specialised function of T_{FR} cells in regulating GC response. It has also been observed that T_{FR} cell suppression leads to changes in the metabolic profile of T_{FH} and GC B cells. Genes associated with mTOR pathways, Myc and glycolysis are downregulated following the suppression of GC B and T_{FH} cells by T_{FR} cells²³⁷. These pathways have previously been implicated in the GC response and metabolism^{238,239}. In addition, T_{FR} cells suppress glycolysis, which is critical for antibody production, thus resulting in defective GC response, and suppression of IL-4 and IL-21 production by T_{FH} cells²³⁷.

The exact mechanism of T_{FR} cells has yet to be fully elucidated, although cell-to-cell contact appears to be crucial for the execution of T_{FR} cell suppressive function²³⁷. One relatively well-studied T_{FR} cell suppressive mechanism is through the expression of CTLA-4^{228,240}. Despite the T_{FR} cell expansion following the deletion of CTLA-4, the T_{FR} cell population demonstrate a defective suppression of the GC response, thus resulting in exacerbated T_{FH} differentiation and increased antibody production²²⁸. The suppression of CTLA-4 is potentially mediated by trans-endocytosis of both CD80 (B7-1) and CD86 (B7-2)²⁴¹, thus impeding the cognate GC T_{FH} and B cell interactions. Furthermore, the binding of CTLA-4 to CD80/CD86 may potentially facilitate an effective suppression of GC response by the production of inhibitory mediators. The effect of IL-10 from T_{FR} cells on GC responses yielded varying findings. Although well known as a regulatory cytokine, IL-10 levels were elevated in T_{FH} and B cell co-culture experiments²³³. Furthermore, IL-10 from T_{FR} cells has been shown to promote GC response by inducing FOXO1 expression and driving B cell division in the GC²⁴². In breast cancer, IL-10

levels were elevated from T_{FR} cells, with a concurrent increase in IL-10 production by GC T_{FH} and B cells, ²⁴³. However, selective deletion of IL-10 receptor results in higher T_{FH} cell differentiation, highlighting the contradictory role of IL-10 from T_{FR} cells on GC responses. T_{FR} cells express T_{reg} cell markers that are responsible for T_{reg} cell suppressive function and therefore, potentially share a similar mechanism of effector functions. However, studies on inhibitory mediators secreted by T_{FR} cells are currently lacking, and thus further studies are needed to fully elucidate T_{FR} cell suppressive mechanisms.

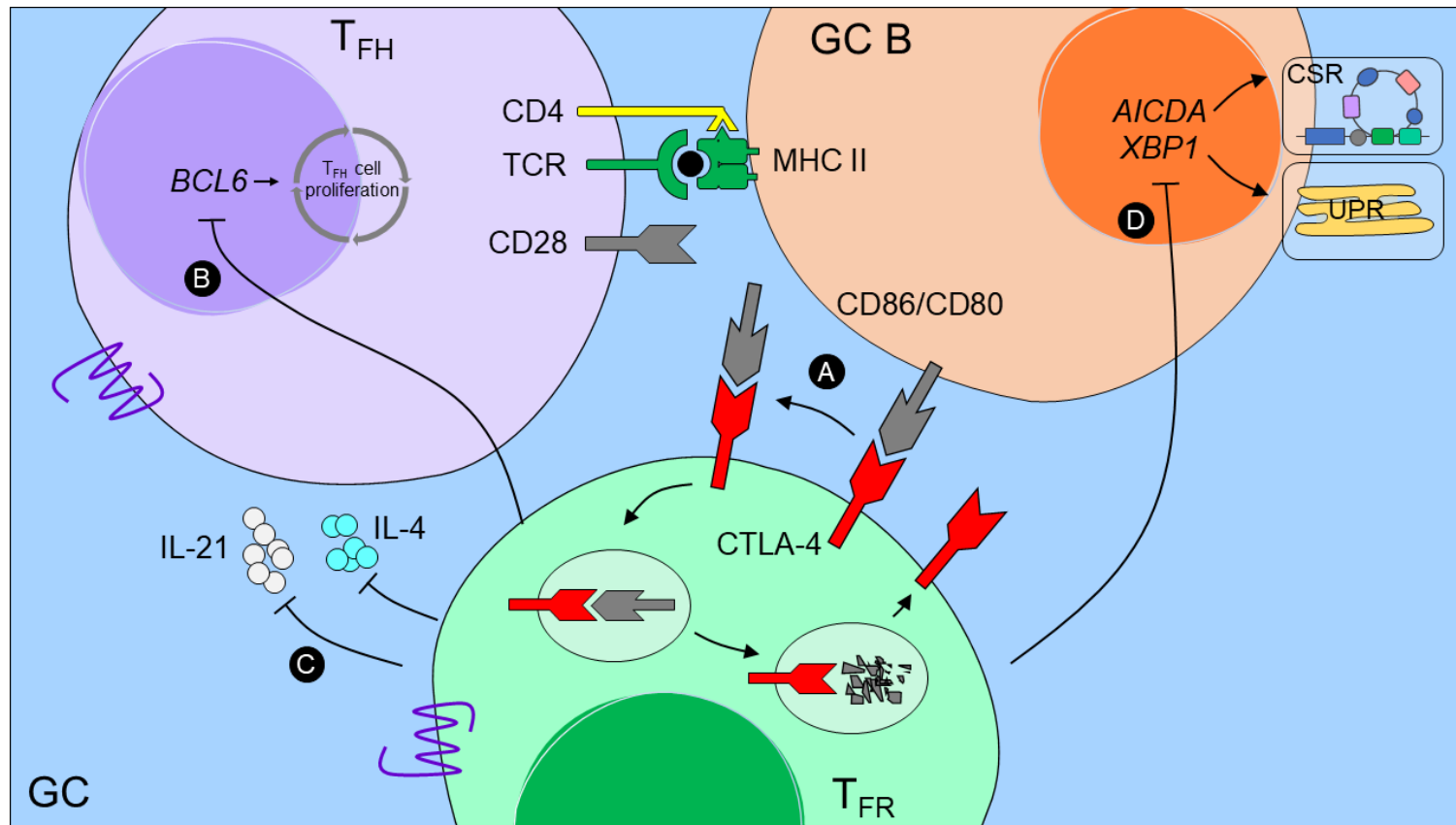


Figure 1.10.1 Proposed mechanism of T_{FR} cell suppression on T_{FH} and B cell interaction and development. **A** CTLA-4-mediated trans-endocytosis of CD80 (B7-1) and CD86 (B7-2) on B cells, interfering with the co-stimulation during cognate T_{FH} and B cell interaction; **B** Attenuation of BCL6 expression which is indispensable for T_{FH} cell development; **C** Suppression of IL-4 and IL-21 production by T_{FH} cells; and **D** Downregulation of AICDA that is crucial for class-switch recombination (CSR) and XBP1 expression, a component of the unfolded protein response (UPR) and is essential in B cell development and secretory function of plasma cells. T_{FH}, T follicular helper cell; T_{FR}, T follicular regulatory cell; GC, germinal centre; CSR, class-switch recombination; UPR, unfolded protein response.

1.11 T_{FH} and T_{FR} cells in human diseases

1.11.1 HIV and chronic infections

During human immunodeficiency virus (HIV) infection, T_{FH} cells are highly permissive to HIV-1, thus results in heightened HIV-1 replication within the GC in the SLOs²⁴⁴. HIV-infected individuals display higher T_{FH} cells compared to healthy controls²⁴⁵. Interestingly, T_{FR} cells are equally as high during HIV infection and are even more permissive to R5-tropic strain of HIV-1 compared to T_{FH} cells²⁴⁶. T_{FR} cells in HIV-infected individuals display higher CCR5 and Ki67 that potentially contribute to their permissiveness to HIV-1 and persistent HIV-1 replication *in vivo*²⁴⁶. The production of neutralising antibodies are the key to a successful antiviral vaccine²⁴⁷. A proportion of HIV-infected individuals generate HIV broadly neutralising antibodies (bnAbs)²⁴⁸ that are capable of neutralising a huge range of circulating HIV strains²⁴⁹. The proportion of HIV bnAbs positively correlates with circulating CXCR5⁺PD-1⁺CXCR3⁻ T_{FH} cells and is speculated to be crucial for the generation of HIV bnAbs^{170,250}.

In patients with hepatitis B and C viruses, T_{FR} cells correlate with blood viral load. Higher T_{FR} cells were associated with deteriorating clinical symptoms. During schistosomiasis, a helminthic disease caused by *Schistosoma japonicum* infection, the proportion of circulating T_{FH} and T_{FR} cells were elevated, with an increase in IgG antibody²⁵¹. During an influenza viral infection, the induction of high IL-2 cytokines inhibits T_{FR} differentiation by upregulating BLIMP1 expression. However, upon reaching the resolution phase, IL-2 levels dropped, T_{reg} cells downregulate CD25 and upregulate BCL6 expression, thus, driving differentiation of T_{reg} cells into T_{FR} cells. The T_{FR} cells then migrate into the GC where they regulate the formation of autoreactive B cells²⁵². As with T_{FR} cells, high IL-2 levels following viral infection activates STAT5 expression and inhibit the proliferation of T_{FH} cells. Expansion of T_{reg} cells during viral infection may, in this case, be to utilise the IL-2, which drives T_{reg} cell differentiation, thus reducing available IL-2 and promoting T_{FH} cell differentiation and GC formation²⁵³. In patients

with lymphocytic choriomeningitis virus (LCMV), T_{FR} cells were shown to promote GC response and plasma cell differentiation through the production of IL-10. Although widely reported to have regulatory functions, IL-10 secreted by T_{FR} cells have been previously discussed to promote B cell differentiation by upregulating FOXO1 expression²⁴².

1.11.2 Autoimmune diseases

As previously discussed, uncontrolled GC responses lead to the generation of self-reactive antibodies and the development of autoimmune diseases. Indeed, dysregulation of T_{FH} and T_{FR} cells have been demonstrated in various autoimmune diseases. Patients with multiple sclerosis (MS) have lower T_{FR} cells with elevated $T_{FH}:T_{FR}$ cell ratio compared to healthy controls¹⁶⁸. These circulating T_{FR} cells from MS patients demonstrated defective suppressive capabilities as assessed by the proliferation of conventional T cells¹⁶⁸, although no measurement of antibodies was performed in the study. Similarly, patients with myasthenia gravis (MG) have higher T_{FH} cells and a lower proportion of T_{FR} cells compared to healthy controls. This dysregulation was more prominent in patients with generalised MG compared to ocular MG, highlighting the heterogeneity in the pathogenesis of MG. Furthermore, $T_{FR}:T_{FH}$ cell ratio inversely correlates with clinical severity of MG patients, which was rescued following treatment with glucocorticoid²⁵⁴.

Patients with systemic lupus erythematosus (SLE) display higher circulating T_{FH} cells that correlates with autoreactive antibody titers²⁵⁵. SLE patients have increased expression of ICOS²⁵⁶ that is crucial in the crosstalk between GC T_{FH} and B cells, resulting in enhanced production of antibodies. T_{FH} cells in SLE patients express IL-21, accompanied by an elevated serum IL-21 in SLE patients compared to healthy controls, which was associated with diseases severity²⁵⁷. Treatment with anti-SLE therapies improves the proportion of T_{FH} cells and serum IL-21 levels²⁵⁷, highlighting the role of T_{FH} cells in the pathogenesis of SLE.

Primary Sjögren syndrome (pSS) is a systemic autoimmune disease characterised by severe inflammation of the exocrine glands and the development of sicca symptoms. A higher proportion of circulating CXCR5⁺CCR6⁺ T_{FH} (T_{FH}17) cells was observed in patients with pSS which positively correlate to autoantibodies titers²⁵⁸. T_{FH}17 cells in pSS patients express PD-1, CD40L and IL-21, all of which contributes to the crosstalk between GC T_{FH} and B cells, thus promoting B cell proliferation and autoantibody production²⁵⁸. Interestingly, the proportion of circulating CXCR5⁺FoxP3⁺ T_{FR} cells in pSS patients is also higher compared to healthy controls, although the T_{FR} cell absolute number was decreased. Furthermore, the T_{FR}:T_{FH} cell ratio was increased, thus resulting in the aberrant production of autoantibodies²⁵⁹.

Rheumatoid arthritis (RA) is an autoimmune disease that primarily affects the joints. Patients with RA have higher circulating CXCR5⁺PD-1⁺ T_{FH} cells compared to healthy controls, although the difference was not as great as compared to patients with SLE. Patients with RA have increased phosphorylation of STAT3, that results in the induction of IL-6 and IL-21, thus driving T_{FH} cell differentiation. Moreover, the phosphorylation of STAT3 positively correlated with T_{FH} cells and plasma IL-6 levels in patients with RA²⁶⁰. On the other hand, the proportion of T_{FR} cells in patients with RA reported contradictory results and therefore requires further studies to elucidate the roles of T_{FR} cells in RA.

1.11.3 Chronic rhinosinusitis (CRS)

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a T_H2 cell-mediated inflammatory response associated with eosinophilic infiltration and local IgE production. T_{FH} cells have been implicated in driving GC B cell proliferation, survival, maturation and antibody production, resulting in the hyperglobulinaemia observed in patients with CRSwNP. The proportion of IL-21-producing cells were reported to be higher in patients with CRSwNP compared to

healthy controls^{261,262}. *IL21* expression was also upregulated in CRSwNP compared to healthy controls. Moreover, *BCL6* and *PRDM1* expressions were upregulated in patients with CRSwNP compared to CRSsNP (CRS without nasal polyps), which promote GC T_{FH} and B cell interactions, driving B cell differentiation into antibody-secreting plasma cells²⁶¹. Another study reported higher proportions of total CXCR5⁺ T_{FH} cells, IL-21⁺, IFN- γ ⁺ and IL-17⁺ T_{FH} (indicative of T_{FH2}, T_{FH1} and T_{FH17}, respectively) cell subsets in both eosinophilic and non-eosinophilic nasal polyp tissues. However, IL-4⁺ T_{FH} cells were only increased in eosinophilic polyp tissues, which correlated with IgE levels *in vivo*²⁶³. IL-4 and IL-21 also drive IgE production by B cells in eosinophilic polyp tissues. T_{FH} cells in nasal polyp tissues also correlated with GC B cells and plasma cells, thus highlighting the role of T_{FH} cells in driving GC B cell differentiation and antibody production in nasal polyps.

1.11.4 Allergic asthma

The indispensable role of T_{FH} cells in B cell maturation, survival and antibody production have been implicated in the development of asthma. A higher proportion of circulating CXCR5⁺ T_{FH} cells have been observed in patients with asthma, both in adults and children, compared to healthy controls, accompanied by a marked increase in total IgE and IL-21 levels in the plasma^{264,265}. Blocking IL-21 and IL-4 results in defective IgE production in patients with allergic asthma²⁶⁵. Moreover, the expression of CXCR5, BCL6 and ICOS was higher in patients with acute asthma compared to healthy controls²⁶⁴. MicroRNA (miRNA) studies reported the potential role of miR-192 on T_{FH} cell differentiation in patients with asthma. miRNA function to regulate gene expression at a post-transcriptional level and have been shown to control cell differentiation and apoptosis. Patients with asthma showed lower miR-192 expression compared to healthy controls^{264,266}, and further reduced following an allergen inhalation challenge²⁶⁶. CD4⁺ T cells transfected with miR-192 plasmid results in a lower expression of CXCR5, BCL6 and ICOS, and proportion of CXCR5⁺ T_{FH} cells compared to control group, suggested the role of miR-192 in the inhibition of T_{FH} cell differentiation^{264,266}.

1.11.5 Allergic rhinitis (AR)

The production of IgE is key in the pathophysiology of allergic diseases. Although IgE production has been associated with T_H2 cells through the production of IL-4, T_{FH} cells have now emerged as another IL-4-producing cell subset. Interestingly, deletion of CNS2 in mice abrogated IL-4 production, and subsequently antigen-specific IgE antibodies by T_{FH} cells, but not T_H2 cells^{267,268}, highlighting the critical role of T_{FH} cells in IgE production. Several murine studies reported that exposure to house dust mite (HDM) allergen or ovalbumin (OVA) promotes the development of T_{FH} cells and IgE production^{269,270}. In humans, an impaired T_{FH}:T_{FR} cell ratio was observed in patients with allergic rhinitis (AR)^{271,272}. Furthermore, T_{FH}2 cells have been reported to be higher in patients with AR with and without asthma²⁷³, and in patients who developed food allergy following liver transplantation²⁷⁴. Patients allergic to HDM demonstrated higher levels of T_{FH} cells and attenuated T_{FR} cell proportion compared to healthy controls^{271,275}. The T_{FH} cells from HDM allergic patients have enhanced capacity to induce IgE production compared to healthy controls²⁷⁵, whilst the T_{FR} cells have diminished suppressive capacity²⁷¹, further highlighting the role of T_{FH} and T_{FR} cells in the development of allergic diseases. To date, the majority of the studies performed to elucidate the functions of T_{FH} and T_{FR} cells were performed in murine models. There are very limited studies on the role of T_{FH} and T_{FR} cells on the pathophysiology of allergic diseases in human, and even fewer in SAR and following grass pollen SCIT and SLIT (Table 1.11.5.1).

Table 1.11.5.1 Summary of studies on T_{FH} and T_{FR} cells in AR in humans

Studies	Kim <i>et al.</i> , 2016 ²⁷⁶	Schulten <i>et al.</i> , 2018 ²⁷²	Yao <i>et al.</i> , 2019 ²⁷¹
Allergen	Cat, dog, cockroach, HDM, grasses, trees, weeds, moulds	Timothy grass	HDM
SCIT/SLIT	No	SCIT	SCIT
T _{FH} /T _{FR} cells	T _{FH} cells	T _{FH} cells	T _{FR} cells
Outcomes	↓ T _{FH} cells in AR versus controls	↓ T _{FH} cells in SCIT versus AR	↓ T _{FR} cells in AR versus healthy controls ↑ T _{FR} cells in SCIT versus untreated AR

HDM, house dust mite; ↓, lower; ↑, higher.

1.12 Hypotheses

This study hypothesised that T_{FH} and T_{FR} cells are dysregulated in patients with untreated SAR and are modulated following allergen-specific immunotherapy and novel immunotherapy approaches. The study was performed in several sections, with individual hypotheses outlined as below:

1. Circulating $CD4^+CXCR5^+PD-1^+$ T_{FH} cells are distinct subsets of T cells from $CD4^+CRTH2^+CD27^-$ T_{H2} and $CD4^+CRTH2^+CD27^-CD161^+CD49d^+$ T_{H2A} cells that strongly promote IgE-mediated allergic inflammation.
2. The proportion of pro-allergic circulating $IL-4^+$, $IL-21^+$, and dual $IL-4^+IL-21^+$ T_{FH} cells are higher in patients with grass pollen-induced allergy (GPA) compared to non-atopic healthy controls (NAC). Immune tolerance induction following AIT, administered either by SCIT or SLIT routes reduce the proportions of circulating dual $IL-4^+IL-21^+$ T_{FH} cells, similar to NAC.
3. Circulating $CD4^+CXCR5^+PD-1^+FoxP3^+$ T_{FR} cells that regulate T_{FH} cell effector function are lower in GPA compared to NAC and are restored following SCIT and SLIT. Moreover, there is an altered chromatin landscape in T_{FH} and T_{FR} cells from GPA, compared to NAC, SCIT- and SLIT-treated patients.
4. Peptide hydrolysates (1 to 10 kDa) from *Lolium perenne* (LPP) are hypoallergenic, have reduced capacity to cross-link $Fc\epsilon RI$ and elicit basophil histamine release compared to unmodified grass pollen protein. Moreover, they do not promote pro-allergic T_{FH} cell subsets responses *in vitro*.

5. In a proof-of-concept randomised, double-blind placebo-controlled (RDBPC) study, a short-course of subcutaneous administration of LPP during four visits over 3-week (170 µg cumulative dose of LPP) is associated with a reduction in grass pollen-induced basophil hyperresponsiveness and pro-allergic T_{FH} cell responses. In addition, FoxP3⁺ T_{FR} and T_{reg} cell subsets are also induced following administration of LPP immunotherapy, but not placebo.

6. Novel approaches of AIT using depigmented and polymerised allergen extract are hypoallergenic, have reduced capacity to cross-link FcεRI and elicit basophil histamine release compared to unmodified grass pollen protein. Moreover, they do not promote pro-allergic T_{FH} and T_{H2} cell subsets responses *in vitro*.

1.13 Aims and objectives in relation to hypotheses

Hypothesis 1

1. To phenotype circulating T_{FH}, T_{H2} and allergen-specific T_{H2A} cells by conventional flow cytometry.
2. To phenotype and characterise circulating T_{FH}, T_{H2} and T_{H2A} cells using the state-of-the-art unbiased approach of viSNE, SPADE and FlowSOM analyses.
3. To determine the function of IL-4, IL-21 and dual IL-4 and IL-21 on GC reaction, B cell class-switching and plasma cell differentiation by investigating the expression of *BCL6*, *AICDA*, *PRDM1* and *XBPI* genes, and ε germline transcript (GLT) by qPCR.

4. To enumerate B cell subsets following stimulation with IL-4, IL-21 and dual IL-4 and IL-21 by flow cytometry, and immunoglobulin secretions in culture supernatants by ImmunoCAP system.

Hypothesis 2

1. To enumerate pro-allergic circulating T_{FH} , IL-4⁺, IL-21⁺ and dual IL-4⁺IL-21⁺ T_{FH} cell subsets in GPA and NAC.
2. To evaluate the proportions of circulating T_{FH} cell subsets in patients following immunotherapy, administered by SCIT and SLIT, in a cross-sectional study of allergen-specific immunotherapy.
3. To evaluate the levels of IL-4 and IL-21 in nasal fluid collected following intranasal grass pollen allergen challenge in GPA, NAC, SCIT and SLIT.

Hypothesis 3

1. To enumerate circulating FoxP3⁺ T_{FR} (CXCR5⁺PD-1⁺FoxP3⁺) and T_{reg} (CD25⁺CD127⁻FoxP3⁺) cell subsets in GPA, NAC and following SCIT and SLIT.
2. To evaluate the differences in the chromatin landscape of GPA and NAC.
3. To identify the top 100 transcription binding sites in GPA compared to NAC.
4. To identify the gene ontology of the top accessible genes in GPA and NAC.
5. To identify the relevant pathways that are associated with the top accessible genes in GPA and NAC.
6. To investigate these changes in the chromatin landscape in SCIT- and SLIT-treated patients compared to GPA.

Hypothesis 4

1. To assess the allergenic properties of LPP in GPA and NAC, through *ex vivo* basophil activation test.
2. To investigate the immunogenicity properties of LPP in GPA and NAC, through *in vitro* stimulation of T cells with LPP candidates and enumeration of pro-allergic IL-4⁺, IL-21⁺ T_{FH} and T_{H2} cell subsets.

Hypothesis 5

1. To investigate basophil responsiveness in a subset of patients who participated in a multicentre, RDBPC study of LPP immunotherapy.
2. To enumerate pro-allergic circulating IL-4⁺, IL-21⁺ and IL-4⁺IL-21⁺ T_{FH} cells in patients treated with LPP immunotherapy and placebo.
3. To evaluate the proportions of FoxP3⁺ T_{FR}, FoxP3⁺ T_{reg}, and inducible IL-10 and IL-35 T_{reg} cells in patients treated with LPP immunotherapy and placebo.

Hypothesis 6

1. To assess the allergenic properties of modified (depigmented and polymerised) allergen extracts in GPA and NAC, through *ex vivo* basophil activation test.
2. To assess the immunogenicity properties of modified allergen extracts in GPA and NAC, through the *in vitro* stimulation of T cells and enumeration of pro-allergic IL-4⁺, IL-21⁺ T_{FH}, T_{H2} and T_{H2A} cell subsets.

CHAPTER 2 MATERIALS AND METHODS

2.1 Ethical statement

Four clinical studies are described in this thesis. All studies were performed according to Good Clinical Practice and informed, written consent was obtained from all patients prior to sample collection. The studies described in Chapter 3, Chapter 4, Chapter 5 and Chapter 7 were approved by the National Research Ethics Service and by the Research Office of the Royal Brompton and Harefield NHS Foundation Trust. The clinical study in Chapter 6 was approved by local regulatory authorities and independent ethics committees in Belgium. I was fully blinded throughout the entire study, and the data was locked internally by the data manager before the unblinding of the results.

2.2 Patients recruitment

Unless stated otherwise, patients were recruited from the Allergy clinic, Royal Brompton Hospital, London, UK, during the grass pollen season. Peripheral whole blood was collected from grass pollen allergics (GPA), non-atopic healthy controls (NAC) and those receiving AIT, either SCIT or SLIT. GPA patients were selected based on a medical history of moderate to severe seasonal grass pollen allergic rhinitis for at least two years, a positive skin prick test (wheal diameter ≥ 3 mm) to grass pollen mixture (ALK-Abelló, Hørsholm, Denmark) and elevated specific IgE (≥ 0.70 kU_A/L) to timothy grass (*Phleum pratense* [Phl p]) extract without any history of treatment with AIT. NAC individuals had no history of allergic diseases, a negative skin prick tests (wheal diameter < 3 mm) to timothy grass and other common aeroallergens and negative specific IgE (< 0.35 kU_A/L). AIT-treated patients received subcutaneous (monthly injections of 100 000 SQ-U *Aquagen* SQ, ALK-Abelló) or sublingual (daily *Grazax* tablet, 75 000 SQ-U, ALK-Abelló) timothy grass pollen allergen immunotherapy.

Exclusion criteria included a previous history of AIT within the last five years, perennial rhinitis, chronic or recurrent sinusitis, perennial asthma, current smoker or have a history of smoking > 5 packs per year, a clinical history of other allergies (including symptoms during the tree pollen season), or other significant medical illnesses. Patients had not used nasal corticosteroids, antihistamines or other anti-allergy medication for at least two weeks before the assessment. Women of childbearing potential who were not taking contraceptive precaution, pregnant or lactating were also excluded.

2.3 Clinical studies

2.3.1 Proof-of-concept, cross-sectional study of SCIT and SLIT

For the initial validation experiments to determine the phenotypic characteristics and function of T_{FH} cells, 28 untreated moderate-to-severe GPA and 12 NAC were recruited during the grass pollen season (Table 2.3.1.1). A cross-sectional, controlled, proof-of-concept study of grass pollen SCIT and SLIT was conducted. Fourteen untreated moderate-to-severe GPA, ten patients treated with SCIT who were receiving monthly injections of 100 000 SQ-U *Aquagen* SQ (ALK-Abelló), who had reached a maintenance dose following a standard up-dosing protocol and eight patients treated with SLIT receiving daily 75 000 SQ-U *Grazax* tablet (ALK-Abelló) for at least six months were recruited for this study. All study participants completed the symptom questionnaires. Thirteen NAC were recruited as controls (Table 2.3.1.2). Blood samples were collected during the grass pollen season. Nasal fluid samples were collected from the same study participants following intranasal grass pollen allergen challenge outside of the grass pollen season.

Nasal allergen challenge was performed with *Aquagen* SQ (ALK-Abelló) Phl p extract as described previously¹¹². Briefly, 100 000 SQ-U/mL of *Aquagen* SQ was reconstituted to 33 333 SQ-U/mL in normal saline and added to disposable Bi-dose nasal applicator devices (Aptar Pharma, Louveciennes Cedex, France). Each participant received one 100 µL allergen spray per nostril by a trained examiner. All participants were asked to refrain from sniffing strongly or blow their nose in the 5 minutes following challenge. Nasal fluid samples were collected using synthetic polyurethane sponge (RG27 Grau, Gummi-Welz GmbH & Co., Neu-Ulm, Germany) inserted into each nostril. The sponges were left for 2 minutes before removal and transferred into a 2 mL centrifuge tubes and kept on ice. Elution buffer was added and the tubes were centrifuged at 4500 rcf, 4°C for 10 minutes. The nasal fluid was then transferred into a new tube and stored at -80°C prior to analysis.

Table 2.3.1.1 Patient demographics for the initial validation experiments of the phenotypic characteristics and function of T_{FH} cells

Characteristics	GPA (n = 28)	NAC (n = 12)
Sex, n (%)		
Male	15 (53.6)	5 (41.7)
Female	13 (46.4)	7 (58.3)
Age (y), mean ± SD	38 ± 12	48 ± 13
Disease duration (y), mean ± SD	16.96 ± 8.32	n.a.
Visual analogue scale (VAS), mean ± SD	70.61 ± 16.78	n.a.
Phl p IgE (kU _A /L), mean ± SD	37.03 ± 36.34	n.d.
Total IgE (kU/L), mean ± SD	409.40 ± 578.30	13.83 ± 14.66
Cosensitisation (SPT ≥ 3 mm), n (%)		
None (other than grass)	8 (28.6)	0 (0.0)
Silver birch	13 (46.4)	0 (0.0)
House dust mite (<i>D. pteronyssinus</i>)	12 (42.9)	0 (0.0)
Cat epithelia	12 (42.9)	0 (0.0)
Dog epithelia	7 (25.0)	0 (0.0)

n.a., not applicable; n.d., not detected; GPA, grass pollen allergics; NAC, non-atopic controls; SPT, skin prick test.

Table 2.3.1.2 Patient demographics for proof-of-concept, cross-sectional study of SCIT and SLIT

Characteristics	NAC (n = 13)	GPA (n = 14)	SCIT (n = 10)	SLIT (n = 8)
Sex, n (%)				
Male	2 (15.4)	9 (64.3)	9 (90.0)	2 (25.0)
Female	11 (84.6)	5 (35.7)	1 (10.0)	6 (75.0)
Age (y), mean $\pm$ SD	38 $\pm$ 13	33 $\pm$ 10	43 $\pm$ 13	40 $\pm$ 14
Disease duration (y), mean $\pm$ SD	n.a.	17.36 $\pm$ 11.32	18.80 $\pm$ 9.45	33.50 $\pm$ 15.63
Phl p sIgE (kU _A /L), mean $\pm$ SD	n.d.	32.50 $\pm$ 39.97	30.96 $\pm$ 37.94	23.50 $\pm$ 22.08
Total IgE (kU/L), mean $\pm$ SD	n.a	179.60 $\pm$ 153.60	405.90 $\pm$ 674.20	151.60 $\pm$ 185.50
Cosensitisation (SPT $\geq$ 3 mm), n (%)				
None (other than grass)	0 (0.0)	4 (28.6)	3 (23.1)	3 (27.3)
Silver birch	0 (0.0)	7 (50.0)	7 (53.8)	3 (27.3)
House dust mite	0 (0.0)	8 (57.1)	5 (38.5)	2 (18.2)
Cat epithelia	0 (0.0)	7 (50.0)	6 (46.2)	3 (27.3)
Dog epithelia	0 (0.0)	7 (50.0)	2 (15.4)	4 (36.4)

n.a., not applicable; n.d., not detected; NAC, non-atopic controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; SPT, skin prick test.

2.3.2 Pre-clinical evaluation of alternative immunotherapy approach using peptide hydrolysates

Four peptide candidates (peptide 1, 2, 3 and 4) derived from purified ryegrass (*Lolium perenne*, Lol p) allergen extracts (Lol p peptides [LPP]) were generated by ASIT Biotech S.A. (Brussels, Belgium) as an alternative approach to conventional SCIT and SLIT using whole allergen extracts. Previous studies have demonstrated extensive cross-reactivity between the members of the grass subfamily Pooideae²⁷⁷ that includes both Phl p and Lol p. Studies performing sequence analysis on Phl p 1 and Lol p reported that both allergens shared an extensive homology of about 30 – 90 % homolog sequence, that contributes to their cross-reactivity. Furthermore, Phl p 1 fusion protein was capable of blocking reactivity of other grass species²⁷⁸, further highlighting the extensive cross-reactivity between grass pollen species. Thus, this justifies the use of Lol p as an approach to treating other grass pollen-induced SAR.

Crude Lol p allergen extracts were initially extracted from a natural allergen source and then purified to remove the non-protein components, resulting in a purified Lol p extract. The purified Lol p protein extract was then denatured and underwent enzymatic hydrolysis to generate peptidic fragments with a molecular weight ranging between 1 to 10 kDa. The enzyme was then removed to ensure that the ratio of enzyme to peptide was less than 0.01%²⁷⁹. The protein and peptides were quantified using the bicinchoninic acid (BCA) assay validated by ASIT Biotech S.A. (Belgium). Grass pollen native and unmodified protein was used as a positive control in the study. For this study, sixteen GPA and six NAC were recruited (Table 2.3.2.1).

Table 2.3.2.1 Patient demographics for the pre-clinical peptide hydrolysates study

Characteristics	GPA (n = 16)	NAC (n = 6)
Sex, n (%)		
Male	8 (50.0)	3 (50.0)
Female	8 (50.0)	3 (50.0)
Age (y), mean $\pm$ SD	37 $\pm$ 13	46 $\pm$ 14
Disease duration (y), mean $\pm$ SD	16.69 $\pm$ 8.13	n.a.
Visual analogue scale (VAS), mean $\pm$ SD	73.06 $\pm$ 9.18	n.a.
Phl p IgE (kU _A /L), mean $\pm$ SD	41.64 $\pm$ 39.50	n.d.
Total IgE (kU/L), mean $\pm$ SD	291.00 $\pm$ 300.20	15.38 $\pm$ 14.42
Cosensitisation (SPT $\geq$ 3 mm), n (%)		
None (other than grass)	7 (43.8)	0 (0.0)
Silver birch	6 (37.5)	0 (0.0)
House dust mite (<i>D. pteronyssinus</i>)	7 (43.8)	0 (0.0)
Cat epithelia	4 (25.0)	0 (0.0)
Dog epithelia	2 (12.5)	0 (0.0)

n.a., not applicable; n.d., not detected; GPA, grass pollen allergics; NAC, non-atopic controls; SPT, skin prick test.

2.3.3 Phase III RDBPC trial study of *Lolium perenne* peptide (LPP) immunotherapy

The phase III study of LPP treatment was performed in 57 different sites across Europe. A total of 554 patients with a medical history of moderate-to-severe SAR were randomised into receiving LPP (372 patients) and placebo (PL; 182 patients). LPP treatment was administered prior to the grass pollen season between January 2016 and April 2016 (visit [V] 2). At each visit, patients received a first subcutaneous injection in one arm, followed by a second injection in the opposite arm 30 minutes later. The incremental doses over the four visits were: 2 x 5 µg at V2, 2 x 10 µg at V3, 2 x 20 µg at V4 and 2 x 50 µg at V5. Patients were then followed-up prior to the grass pollen season at V6 (between March and April 2016) and following the grass pollen season at V8 (August 2016; Figure 2.3.3.1). This thesis aims to investigate the mechanistic endpoints of LPP treatment on a sub-study site in Belgium, whereby 32 patients were randomised into receiving LPP (21 patients) and PL (11 patients; Table 2.3.3.1).

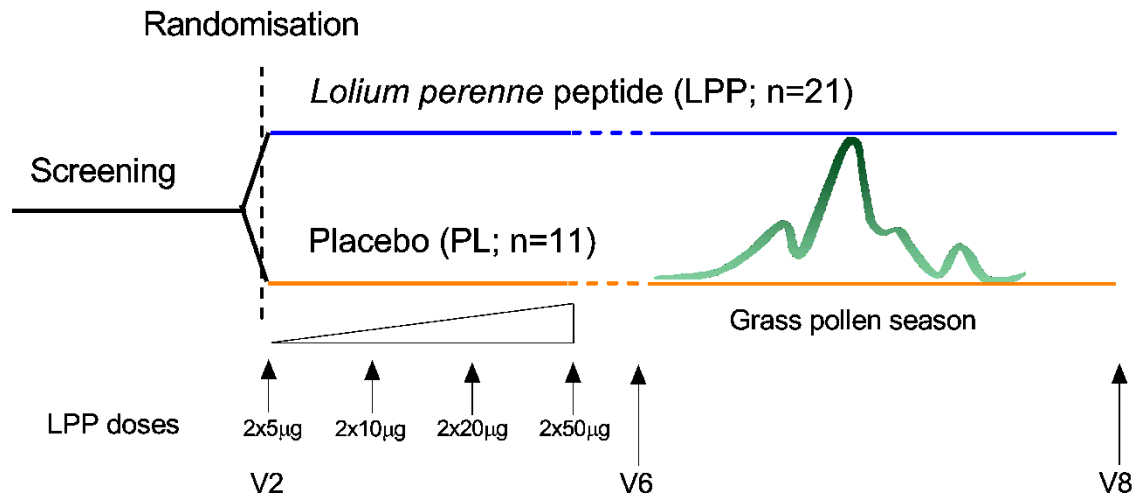


Figure 2.3.3.1 Study design for RDBPC phase III study. 32 patients were randomised into receiving LPP (21 patients) and placebo (PL; 11 patients) for a sub-study site in Belgium. LPP treatment was administered prior to the grass pollen season between January 2016 and April 2016 (V2). At each visit, patients received a first subcutaneous injection in one arm, followed by a second injection in the opposite arm 30 minutes later. A total of 170 µg of LPP was administered at the end of the treatment period. Patients were followed-up prior to the grass pollen season at V6 (between March and April 2016) and following the grass pollen season at V8 (August 2016). Blood samples were collected at V2, V6 and V8. LPP, *Lolium perenne* peptide; PL, placebo; V, visit.

Table 2.3.3.1 Patient demographics for a sub-study site in Belgium

Characteristics	LPP (n = 21)	PL (n = 11)
Sex, n (%)		
Male	8 (38.1)	5 (45.5)
Female	13 (61.9)	6 (54.6)
Age (y), mean $\pm$ SD	33 $\pm$ 11	33 $\pm$ 8
Disease duration (y), mean $\pm$ SD	18.19 $\pm$ 10.33	15.73 $\pm$ 9.95
Phl p IgE (kU _A /L), mean $\pm$ SD	27.65 $\pm$ 31.89	20.76 $\pm$ 25.58
Total IgE (kU/L), mean $\pm$ SD	219.83 $\pm$ 173.08	156.44 $\pm$ 211.28
Cosensitisation (SPT $\geq$ 3 mm), n (%)		
None (other than grass)	0 (0.0)	0 (0.0)
Silver birch	8 (38.1)	2 (18.2)
House dust mite (<i>D. farinae</i>)	3 (14.3)	1 (9.1)
House dust mite (<i>D. pteronyssinus</i>)	7 (33.3)	2 (18.2)
Cat epithelia	2 (9.5)	4 (36.4)
Dog epithelia	3 (14.3)	1 (9.1)

LPP, *Lolium perenne* peptide; PL, placebo; *D. farinae*, *Dermatophagoides farinae*; *D. pteronyssinus*, *Dermatophagoides pteronyssinus*; SPT, skin prick test.

2.3.4 Novel approaches to AIT using depigmented and polymerised allergen extract

Allergen extracts subjected to chemical modifications, termed allergoids, have been shown to have a lower capacity to form IgE-allergen complexes and cross-link with FcεRI on the surface of basophils and mast cells. Thus, they are thought to have a superior safety profile compared to conventional SCIT and SLIT that involves unmodified whole allergens. Laboratorios LETI S.L.U. (Madrid, Spain) have generated depigmented and polymerised allergoids of grass pollen (Phl p) allergen extracts as a potential alternative treatment for SAR. Briefly, the native grass pollen extract was prepared by extracting Phl p in 0.01 mol/L of PBS, dialysed against bi-distilled water with a cut-off membrane of 3.5 kDa and lyophilised. Depigmented allergoids (DPG Phl p) were generated by reducing the pH of reconstituted native extract in bi-distilled water using a mild acid treatment to remove low molecular weight substances. The extract was then further dialysed overnight against bi-distilled water with a cut-off membrane of 3.5 kDa (Millipore, Bedford, USA). Sodium hydroxide was added to neutralise the solution, which was then sterile-filtered and lyophilised as DPG Phl p extract. Polymerised allergoids (DPG-POL Phl p) were generated from the reconstituted DPG Phl p extract. DPG Phl p were polymerised with glutaraldehyde at room temperature for 8 hours before glycine was added to stop the reaction. The solution was then dialysed in 100 kDa dialysis membranes (Millipore) and lyophilised as DPG-POL Phl p extract. The presence of Phl p in the native protein extract, DPG Phl p and DPG-POL Phl p was confirmed by mass spectrometry performed by Laboratorios LETI S.L.U. (Spain) and were quantified using the BCA assay. Laboratorios LETI S.L.U. (Spain) have shown that unlike native protein and DPG Phl p, DPG-POL Phl p did not bind to IgE in the serum of GPA. Grass pollen native and unmodified protein was used as a positive control. For this study, sixteen GPA and eight NAC were recruited (Table 2.3.4.1).

Table 2.3.4.1 Patient demographics for the allergoids study

Characteristics	GPA (n = 16)	NAC (n = 8)
Sex, n (%)		
Male	10 (62.5)	3 (37.5)
Female	6 (37.5)	5 (62.5)
Age (y), mean $\pm$ SD	39 $\pm$ 11	41 $\pm$ 11
Disease duration (y), mean $\pm$ SD	20.19 $\pm$ 8.02	n.a.
Visual analogue scale (VAS), mean $\pm$ SD	72.13 $\pm$ 12.44	n.a.
Phl p IgE (kU _A /L), mean $\pm$ SD	36.04 $\pm$ 37.17	n.d.
Total IgE (kU/L), mean $\pm$ SD	478.20 $\pm$ 885.40	10.78 $\pm$ 6.83
Cosensitisation (SPT $\geq$ 3 mm), n (%)		
None (other than grass)	5 (31.3)	0 (0.0)
Silver birch	9 (56.3)	0 (0.0)
House dust mite (<i>D. pteronyssinus</i>)	8 (50.0)	0 (0.0)
Cat epithelia	0 (0.0)	0 (0.0)
Dog epithelia	2 (12.5)	0 (0.0)

n.a., not applicable; n.d., not detected; GPA, grass pollen allergics; NAC, non-atopic controls; SPT, skin prick test.

2.4 Rhinoconjunctivitis total symptom scores (RTSS)

All participants were asked to complete a retrospective symptom questionnaire to assess the severity of their allergic rhinoconjunctivitis symptoms for the relevant grass pollen season. Symptoms included six categories of sneezing, rhinorrhoea, itchy nose, blocked nose, itchy eyes and watery eyes. Each category was rated between 0 (no symptom) and 3 (severe symptoms), resulting in a possible total score between 0 to 18 (Rhinoconjunctivitis Total Symptom Score [RTSS]). All participants were asked to evaluate their symptoms when their allergic rhinitis was at its most severe during the grass pollen season.

2.5 Visual analogue scale (VAS)

Visual analogue scale (VAS) assessment was performed using a linear scale of 0 to 10 cm. Participants were asked to place a vertical mark on the line by assessing the severity of their symptoms during the previous grass pollen season. A mark at 0 cm denotes that they were completely symptom-free and a mark at 10 indicated having the worst possible symptoms. The value was then multiplied by 10 and rounded to the nearest whole number.

2.6 Combined symptom and medication score (CSMS)

Combined symptom and medication score (CSMS) described in the study described in Chapter 6 was obtained during the peak (weeks 23 – 25) and the whole grass pollen season (weeks 22 – 30) as recommended by European Academy of Allergy and Clinical Immunology (EAACI)²⁸⁰. Briefly, the daily symptom score (dSS) of a maximum of 18 points as described in section 2.4, was initially divided by the six symptoms, thus, resulting in a possible score between 0 to 3. Daily medication score (dMS) was rated between 0 (no medication), 1 (use of oral and/or topical antihistamines), 2 (use of intranasal corticosteroids with/without antihistamines) and 3 (use of oral corticosteroids with/without intranasal corticosteroids and

with/without antihistamines). The CSMS was then calculated by adding the daily symptom score and daily medication score, resulting in a possible total score between 0 to 6 [dSS (0-3) + dMS (0-3) = 0-6]²⁸⁰.

2.7 Isolation of human peripheral blood mononuclear cells (PBMCs) and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from 200 mL of heparinised human whole blood. Whole blood was centrifuged at 1136 g for 10 minutes to separate the plasma layer from the cell layer. The plasma layer was collected and stored at -20°C. The cell layer was diluted 1:1 with medium (RPMI 1640; Life Technologies, Paisley, UK) and layered onto 15 mL of Ficoll-Paque™PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Density gradient centrifugation was performed at 1136 g for 25 minutes at 20°C without brakes. PBMC layer was collected at the medium-Ficoll interface and washed twice with the medium prior to counting. Cells were counted using the trypan blue exclusion method on a haemocytometer, washed once and resuspended at 20×10^6 cells/mL with Recovery™ Cell Culture Freezing Medium (Life Technologies). Cells were kept in cryovials in a Mr Frosty™ freezing container at -80°C overnight before being transferred to liquid nitrogen (-196°C) for long-term cryopreservation.

2.8 Cell thawing

Cryopreserved PBMCs were taken out from liquid nitrogen and transferred in dry ice. Cells were gently warmed at 37°C in a water bath until 70% thawed. Cells were transferred into a falcon tube and diluted, dropwise, with 20 mL of media [RPMI 1640 supplemented with L-glutamine and 25 mM HEPES (Life Technologies)]. Cells were then centrifuged at 400 g for

10 minutes, resuspended in media and counted using trypan blue exclusion method on a haemocytometer, prior to cell staining and cell culture.

2.9 Cell staining by flow cytometry

2.9.1 Cell surface and intracellular staining

Cells were spun at 400 g for 5 minutes, and the supernatant was discarded. Cells were then resuspended in 100 µL of cell staining buffer (BioLegend). Henceforth, all steps were performed in the dark. 10 µL FcR blocking reagent (Miltenyi Biotec, Woking, UK) was added to block non-specific binding of antibodies and cells were incubated for 15 minutes at room temperature. Cells were then incubated with cell surface markers (Table 2.9.1) for 30 minutes at room temperature. Cells were washed with once cell staining buffer, and then with PBS (pH 7.4; Life Technologies). Staining for live cells was performed by resuspending the cells with 0.1% FVS780 (BD Biosciences, Oxford, UK) or 0.1% Zombie UV (BioLegend) in 100 µL of PBS (Life Technologies) for 10 minutes at room temperature. Cells were washed twice with cell staining buffer and fixed with 100 µL BD Cytofix/Cytoperm (BD Biosciences) for 20 minutes at 4°C. They were then washed twice with BD Perm/Wash buffer (BD Biosciences). Cells were resuspended in 75 µL of BD Perm/Wash buffer and stained with intracellular cytokine markers for 30 minutes at 4°C (Table 2.9.1). They were washed once and resuspended in 200 µL of cell staining buffer prior to acquisition.

2.9.2 Intracellular cytokine staining

In a 96-well plate, cells were washed at 400 g for 5 minutes and resuspended in 100 µL of culture medium [RPMI 1640 supplemented with L-glutamine and 25 mM HEPES, 5% heat-inactivated human AB serum (Sigma, Dorset, UK) and 1% Penicillin-Streptomycin-L-Glutamine (Life Technologies)]. Cells were stimulated with 50 ng/mL phorbol 12-myristate

13-acetate (PMA; Sigma) and 1 µg/mL ionomycin (Sigma) for 5 hours at 37°C and 5% CO₂ in the presence of 20 µg/mL monensin (BioLegend, London, UK) for the final 4 hours prior to staining.

2.9.3 Intracellular FoxP3 staining

Cells were fixed with FoxP3 Fix/Perm buffer (BioLegend) for 20 minutes at room temperature. They were then washed with FoxP3 Perm buffer (BioLegend) and resuspended in 100 µL of FoxP3 Perm buffer. Cells were stained with intracellular FoxP3 antibody for 30 minutes at 4°C. Cells were washed and resuspended with cell staining buffer prior to acquisition.

2.9.4 Transcription factors staining

Cells were resuspended in 100 µL of PBS (Life Technologies) and fixed with 1 mL of TFP Fix/Perm buffer (BD Biosciences) for 50 minutes at 4°C. Cells were kept at 4°C throughout the rest of the procedure. Cells were washed twice with TFP Perm/Wash buffer (BD Biosciences) and incubated with 1 mL of Perm Buffer III (BD Biosciences) for 20 minutes. Cells were further washed with TFP Perm/Wash buffer and stained with the cell surface and intracellular markers for 40 minutes. Cells were washed with TFP Perm/Wash buffer and resuspended in cell staining buffer prior to acquisition.

Lymphocytes were gated based on low forward- and side-scatter profiles on BD LSRFortessa™III or BD FACSCanto™II (both, BD Biosciences). All samples were analysed using FlowJo 10.5.3 (FlowJo, Tree Star, San Carlos, CA, USA).

Table 2.9.1 Antibodies used for cell staining by flow cytometry

Marker	Clone	Fluorochrome	Source (Company)
<i>Cell surface markers</i>			
CD3	UCHT1	V510	BioLegend
CD3	SP34-2	PE-Cy7	BD Biosciences
CD4	RPA-T4	BUV395	BD Biosciences
CD4	RPA-T4	APC-Cy7	BD Biosciences
CD19	SJ25C1	PE-Cy7	BioLegend
CD25	M-A251	BV510	BD Biosciences
CD27	O323	BV650	BioLegend
CD27	LG.3A10	AF700	BioLegend
CD38	HIT2	PE-Cy5	BioLegend
CD49d	9F10	BV711	BioLegend
CD63	H5C6	FITC	BioLegend
CD127	HIL-7R-M21	BB515	BD Biosciences
CD138	MI15	BV711	BD Biosciences
CD152 (CTLA-4)	L3D10	PE-Cy7	BD Biosciences
CD161	HP-3G10	BV605	BioLegend
CD185 (CXCR5)	RF8B2	BB515	BD Biosciences
CD185 (CXCR5)	RF8B2	BV421	BD Biosciences
Cd196 (CCR6)	11A9	APC-R700	BD Biosciences
CD203c	NP4D6	PerCP-Cy5.5	BioLegend
CD278 (ICOS)	DX29	BUV737	BD Biosciences
CD278 (ICOS)	DX29	PerCP-Cy5.5	BD Biosciences
CD279 (PD-1)	EH12.1	PE-Cy7	BD Biosciences
CD294 (CRTH2)	BM16	PE-Dazzle 594	BioLegend
CD294 (CRTH2)	BM16	PE	Miltenyi Biotec

CD303 (BDCA-2)	AC144	APC	Miltenyi Biotec
<i>Intracellular markers</i>			
DAO		V500	BD Biosciences
FoxP3	206D	BV421	BD Biosciences
GARP (LRRC32)	7B11	PE	BD Biosciences
IFN- γ	4S.B3	PerCP-Cy5.5	BioLegend
IgE	Ige21	FITC	eBioscience
IgG	IS11-3B2.2.3	VioBlue	Miltenyi Biotec
IL-4	8D4-8	PE	BioLegend
IL-10	JES3-9D7	BV786	BD Biosciences
IL-12/IL-35 p35	27537	PE	R&D Systems
IL-27/IL-35 EBI3	607201	APC	R&D Systems
IL-21	3A3-N2.1	AF647	BD Biosciences
SATB1	14/SATB1	AF647	BD Biosciences

2.10 Lymphocytes labelling with CellTrace Violet Cell Proliferation Kit

PBMCs were resuspended in PBS (Life Technologies) at 1×10^6 cells/mL. CellTrace Violet fluorescent dye (Life Technologies) was dissolved in DMSO as a 5 mM stock solution. 1×10^6 cells were incubated with 1 μ L of stock CellTrace dye for 20 minutes at room temperature in the dark. Cells were washed with five times the staining volume of culture medium after a 5 minutes incubation to remove free dye remaining in the solution. Cells were then resuspended in culture medium at 5×10^6 cells/mL and incubated for at least 10 minutes prior to cell culture.

2.11 *In vitro* proliferation assay

In each well, 5×10^5 cells CellTrace labelled PBMCs were cultured in culture medium in the absence or presence of stimulants, in a 96-well round-bottom plate. Cells were cultured at 37°C and 5% CO₂, protected from light for 6 days before staining and analysis by flow cytometry.

2.12 B cell isolation and culture

Naïve and memory B cells were isolated from PBMCs using EasySEP™ Human Memory B Cell Isolation Kit (STEMCELL Technologies, Cambridge, UK) according to the manufacturer's instruction. 10,000 B cells per well were stimulated with 100 ng/mL of rhCD40L (R&D Systems, Abingdon, UK), in the absence and presence of 100 ng/mL of rhIL-4 (R&D Systems), 10 ng/mL of rhIL-21 (R&D Systems) and/or 50 ng/mL of IL-10 (R&D Systems) for up to 14 days prior to performing qPCR and cell staining by flow cytometry.

2.13 Ex vivo basophil activation test

Ex vivo basophil activation test was performed as previously described¹⁵³. All steps were performed in the dark. Briefly, 100 µL of heparinised whole blood was incubated in the absence or presence of 1, 10, 33, 100, 330, 1000, 3300 and 10000 ng/mL of LPP hydrolysates candidates and immunostained with cell surface markers (Table 2.9.1) in a 37°C water bath for 15 minutes. Erythrocytes present in the whole blood were lysed using 2 mL of BD Lysing Solution (BD Biosciences) at room temperature for 10 minutes. Cells were then spun at 400 g for 5 minutes, and the supernatant was discarded. The cells were further washed once with 3 mL of PBS (Life Technologies). The resultant pellet was resuspended and fixed using BD Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4°C. Cells were then washed twice with BD Perm/Wash buffer (BD Biosciences). Intracellular basophil histamine release was quantified by incubating the cells with a V500-conjugated diamine oxidase (DAO) enzyme (BD Biosciences), for 30 minutes at 4°C. Cells were then washed once and resuspended in cell staining buffer prior to acquisition.

2.14 Quantitative PCR

RNA was extracted using RNeasy Mini Kit (Qiagen, Manchester, UK) from naïve and memory B cells following 5- and 6-days stimulation according to the manufacturer's instruction. Extracted RNA was heat-inactivated at 99°C for 2 minutes and reverse transcribed into cDNA in a 20 µL reaction containing 20 U Ribolock RNase Inhibitor (Life Technologies), 2.5 µM random hexamers (Life Technologies), 1 mM deoxynucleoside triphosphate (dNTPs) and 200 U RevertAid H Minus Reverse Transcriptase (Life Technologies) for 10 minutes at 25°C, 60 minutes at 42°C, followed by denaturation for 10 minutes at 70°C. Samples were diluted 1:1 with nuclease-free water. Quantitative PCR was performed using TaqMan Universal PCR Master Mix without uracil-N-glycosylase (UNG; Life Technologies) in a 10 µL reaction on a Viia7 real-time PCR machine (Applied Biosystems, Paisley, UK). PCR conditions consist of

initial denaturation at 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. For TaqMan gene expression assays, the following assays were used (Applied Biosystems): *BCL6*, Hs00153368_m1; *AICDA*, Hs00757808_m1; *PRDM1*, Hs00153357_m1; and *XBP1*, Hs00231936_m1. Primer pairs used for detection of germline transcript (GLT) expressions are listed in Table 2.14.1. Gene expression was normalised to housekeeping gene *18S*. All samples were run in triplicates.

Table 2.14.1 Primer sequences for the detection of germline transcript (GLT)

GLT	Primer	Sequence (5' to 3')
<i>εGLT</i>	Forward	CTGTCCAGGAACCCGACAGA
	Reverse	TGCAGCAGCGGGTCAAG
	Probe	AGGCACCAAATG

2.15 ImmunoCAP

Total IgE (tIgE) and timothy grass pollen-specific IgE (sIgE) in serum and cell culture supernatants were determined using the ImmunoCAP system (Phadia, Uppsala, Sweden) according to the manufacturer's recommended protocol.

2.16 Nasal cytokines measurements

Nasal fluid samples were analysed for IL-4, IL-21 and IL-6 using a human cytokine/chemokine magnetic bead panel 96-well plate assay (Milliplex Map Kit, Millipore, Watford, UK) and a Luminex xMAP Magpix platform (Millipore), according to the manufacturer's instructions.

2.17 Assay for transposase-accessible chromatin sequencing (ATAC-seq)

Freshly isolated PBMCs were rested overnight at 37°C and 5% CO₂ in 20 mL of RPMI 1640 supplemented with L-glutamine and 25 mM HEPES. CD4⁺ cells were isolated from PBMCs using EasySep™ Human CD4⁺ T cell Enrichment Kit (STEMCELL Technologies) according to the manufacturer's instruction. Enriched CD4⁺ T cells were then resuspended in 100 x 10⁶ cells/mL of cell staining buffer. Cells were stained with cell surface markers as outlined in section 2.9, washed and resuspended in cell staining buffer without fixation. T_{FH} and T_{FR} cells were sorted as follows: T_{FH} (CD4⁺CXCR5⁺PD-1⁺CD25⁻CD127⁺) and T_{FR} (CD4⁺CXCR5⁺PD-1⁺CD25⁺CD127⁻) using BD FACSAria (BD Biosciences). Cells were sorted into RPMI 1640 supplemented with L-glutamine and 25 mM HEPES.

Sorted cells were spun at 450 g for 5 minutes at 4°C and supernatant was discarded. Cells were further washed with 50 µL of cold PBS (Life Technologies) and the supernatant was discarded. Cells were lysed in cold lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂,

pH 7.4 and 0.01% NP-40), spun for 10 minutes at 450 g and 4°C and supernatant was discarded. Lysed cells were incubated with transposase reaction mix [Tagment DNA buffer, 5% Tn5 Transposase (Illumina Nextera; Illumina, CA, USA) and nuclease-free water] for 30 minutes at 37°C. The mixture was flicked to mix every three to five minutes to increase the fragment yield. Transposed DNA was purified using Qiagen MinElute kit (Qiagen) according to the manufacturer's instruction and eluted in 11.5 µL of Elution buffer (Qiagen). Samples were sent to Nadeau's Laboratory at Stanford University for amplification and sequencing.

2.18 ATAC-seq data analysis

ATAC-seq data analysis were performed as previously described²⁸¹ (Figure 2.18.1) using Partek® Flow® (Partek, MO, USA). Nextera adaptor sequences were trimmed from the reads using Cutadapt. Trimmed reads were aligned to hg38 reference genome using bowtie2, with standard parameters and a maximum fragment length of 2,000. Duplicate reads were removed. De-duplicated reads were then filtered for high quality (MAPQ > 30), non-mitochondrial chromosome, non-Y chromosome and properly paired reads. Peak calling was performed with MACS2 using 'macs2 callpeak-nomodel-nolambda-keep-dup-all-call-summits'. Differentially expressed genes were filtered and gene ontology (GO) enrichment and pathway enrichment were performed using Partek® Flow® (Partek) to identify the functional groups and biological context related to those genes.

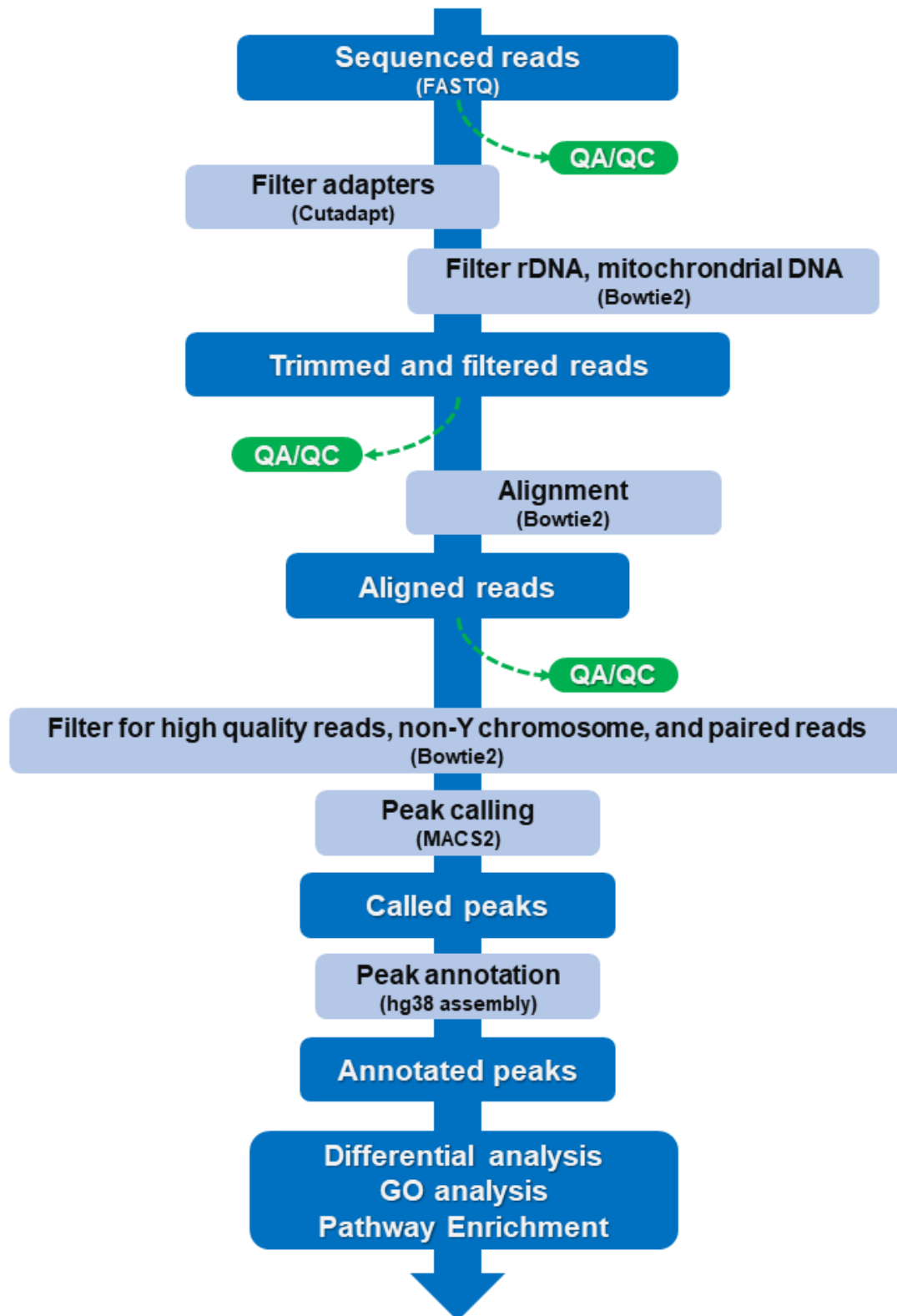


Figure 2.18.1 ATAC-seq analysis pipeline. Flowchart illustration of analysis pipeline for ATAC-seq following the sequencing of T_{FH} and T_{FR} cells. GO, gene ontology.

2.19 Statistical analysis

Statistical data analysis was performed using GraphPad Prism 7.02 (GraphPad Software, La Jolla, USA) unless stated otherwise. Non-parametric Mann-Whitney test was used for comparisons between different groups, and the non-parametric Wilcoxon signed-rank test was used for comparisons within the same group. Non-parametric ANOVA with -fdrstepup for multiple comparison correction was used for ATAC-seq analysis using Partek® Flow® (Partek). EC₅₀ analyses were performed by fitting a nonlinear regression curve with a standard slope (Hill slope of 1.0) on datasets that have been normalised to the maximal response. A *P* value of < 0.05 was considered to be statistically significant. Unless stated otherwise, all *P* values are reported to 4 decimal places.

2.20 Flow cytometry-based unbiased clustering analysis

Conventional flow cytometry scatter plots only allow visualisation of any two markers at the same time, and therefore, it is very difficult to investigate the differences between different cell subsets. Furthermore, by utilising multiple markers in flow cytometry, it has been very challenging and time-consuming to simultaneously view the various markers across different cell subsets. Various state-of-the-art unbiased visualisation tools that utilise different algorithms have been developed to handle these high-dimensional flow cytometry data such as viSNE, SPADE and FlowSOM.

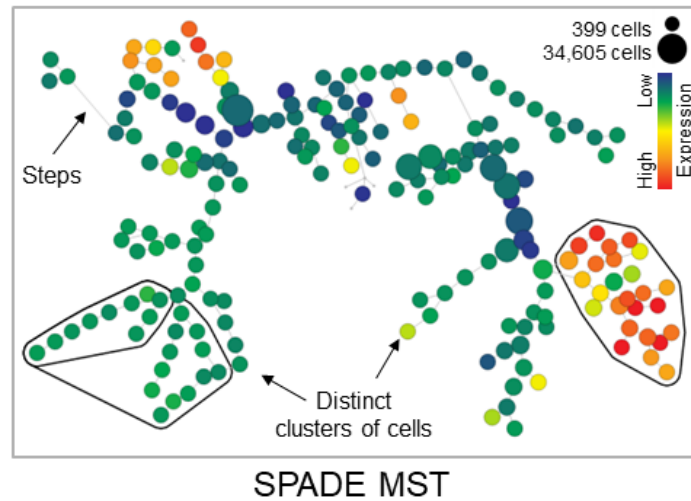
Flow cytometry-based unbiased machine-learning analyses were performed on concatenated FCS files from independent samples using Cytobank (Cytobank Inc., Santa Clara, CA, USA). viSNE (t-distributed stochastic neighbour embedding [t-SNE]-based visualisation)²⁸² analysis uses Barnes-Hut implementation of the t-SNE algorithm²⁸³ and allows a two-dimensional visualisation of the high-dimensional data. Phenotypically similar cells formed an 'island' in the

multidimensional space, and distinct cells will form distinct or separate 'islands'. viSNE analyses were run with equal event sampling.

SPADE (Spanning-tree Progression Analysis of Density-normalised Events)²⁸⁴ analysis allows hierarchical clustering of cells with similar phenotype into 'nodes' that are connected via 'steps' presented as a minimal spanning tree (MST). The number of 'steps' between two clusters were determined by the similarities between clusters of cells; distinct clusters of cells will have a higher number of 'steps' between the clusters. The size of the cluster nodes is proportional to the number of cells within the cluster. SPADE allows the simultaneous visualisation of a specific marker intensity within all the cell clusters at a time (Figure 2.20.1, A).

Another visualisation tool, FlowSOM, employs a self-organising map (SOM) that clusters cells and assigns each cluster to a metacluster (cluster of cell clusters), categorising them as one population²⁸⁵. FlowSOM data presented as an MST allows the simultaneous visualisation of various markers' intensity/expression within individual clusters of cells, thus facilitating the identification of cell subsets based on the marker expressions (Figure 2.20.1, B).

A SPADE



B FlowSOM

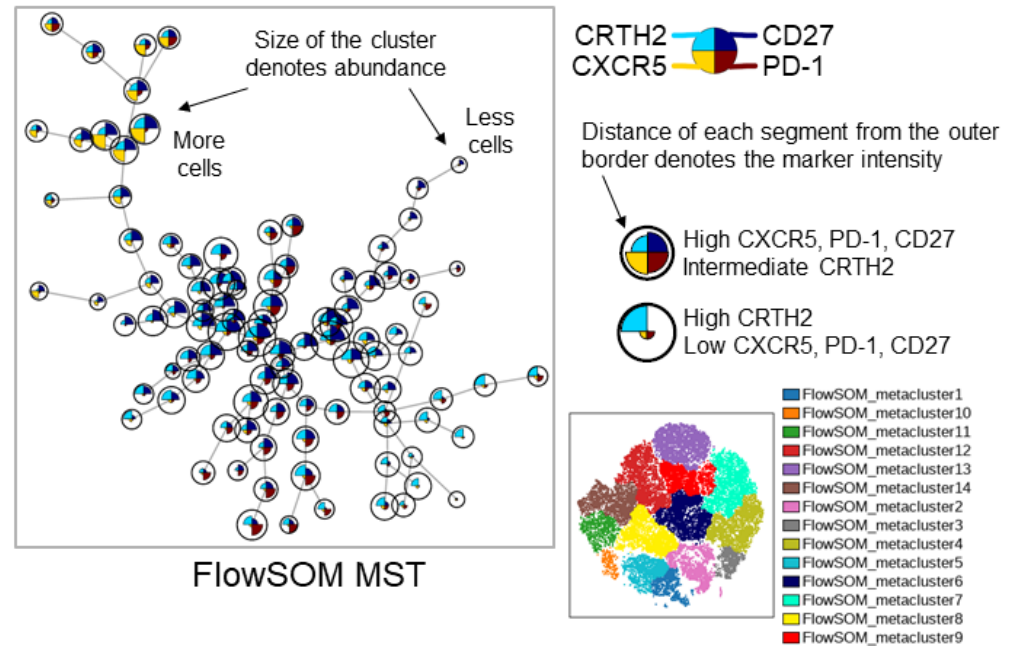


Figure 2.20.1 SPADE and FlowSOM. A summary of the differences in the images generated using SPADE and FlowSOM analysis. **A** SPADE allows hierarchical clusterings of similarly phenotyped cells into 'nodes' or clusters connected via 'steps' presented as an MST. The size of the nodes indicates cell abundance within each cluster and the colour indicates the intensity of a specific marker. **B** FlowSOM organises clusters of cells with similar phenotype into metaclusters. In a FlowSOM MST, the cluster size indicates cell abundance. The pie chart denotes the intensity of the markers, the closer the segment is to the outside border, the higher the marker expression.

CHAPTER 3 DISTINGUISHING PHENOTYPIC CHARACTERISTICS OF CIRCULATING T FOLLICULAR HELPER (T_{FH}) CELLS FROM T_H2 AND T_H2A CELLS IN SEASONAL ALLERGIC RHINITIS (SAR)

3.1 Introduction

SAR consists of a biphasic response that occurs within 15 to 20 minutes and 4 to 12 hours following allergen exposure^{12,286–289}. The former requires allergen-IgE induced cross-linking of FcεRI on mast cells and basophils 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 characterised by the expression of CCR4, CD161 and CD49d and lack the expression of CD27. They have been shown to be elevated in grass pollen allergic patients during the pollen season^{113,116}.

Moreover, other T cell subsets such as T_{FH} cells have been shown to have the capacity to promote pro-allergic responses^{161,162,270,290}. T_{FH} cells are a distinct subset of T cells within the GC that provide signals to B cells to differentiation into antibody-producing plasma cells²⁹¹. T_{FH} cells express high levels of the chemokine receptor, CXCR5, aiding its migration into the B cell follicles in the GC in response to CXCL13 gradient¹⁵⁵, and the transcription factor, BCL6²⁹². Following their initial discovery, numerous studies have also identified T_{FH} cells in the peripheral blood²³³. These circulating T_{FH} cells are a surrogate of GC T_{FH} cells and have lower *BCL6* expression¹⁶⁸. T_{FH} cells are responsible for providing B cell survival signals and plasma cell differentiation through the expression of cytokines IL-4 and IL-21²⁹¹ in a STAT3-

dependent manner²⁹³. The function of T_{FH} cells in eliciting and contributing to IgE-mediated seasonal allergic rhinitis remains to be fully determined.

This chapter aims to first, identify the phenotypic differences between T_{FH} and T_{H2} cells, as well as the recently described allergen-specific T_{H2A} cells¹¹³, using flow cytometry-based unbiased machine learning algorithms developed to visualise high-dimensional data. Secondly, to evaluate the potential impact of T_{FH} cells, through the production of their cytokines IL-4 and IL-21, on different stages of B cell differentiation within the GC *in vitro*. This includes investigating changes in GC reaction, class-switching of B cells to IgE and differentiation into antibody-producing plasma cells. Finally, to assess grass pollen-driven proliferative responses of T_{FH}, T_{H2} and T_{H2A} cells in SAR and NAC.

3.2 Results

3.2.1 T_{FH} cells are distinct from conventional T_{H2} cells

PBMCs was obtained from GPA with moderate to severe SAR (Table 2.3.1.1). Phenotypic composition of T_{FH}, T_{H2} and T_{H2A} cells in GPA was evaluated by multiparametric flow cytometry and further confirmed by visualisation tools developed to manage high-dimensional data, i.e. viSNE, SPADE and FlowSOM. CD4⁺CXCR5⁺PD-1⁺ T_{FH} cells (Figure 3.2.1.1, A) were initially interrogated by viSNE²⁸², allowing a two-dimensional visualisation whereby phenotypically similar cells form an 'island'. Here, viSNE maps of CD4⁺ cells were represented according to the expression of T_{FH}, T_{H2} and T_{H2A} cell markers, CXCR5, PD-1, CRTH2, CD27, CD49d and CD161. viSNE maps of CD4⁺ cells showed a cluster of CXCR5-expressing cells that co-expressed PD-1, which indicates a T_{FH} cell population (Figure 3.2.1.1, B, orange arrows). A proportion of CD4⁺ cells that express high CRTH2 expression (Figure 3.2.1.1, B, green arrows) showed low CD27 expression, which indicates a T_{H2} cell subset. In addition to T_{H2} cell markers, T_{H2A} cells express CD49d and CD161 and were confirmed to reside within the T_{H2} cell population (Figure 3.2.1.1, B, red arrows).

SPADE analysis connected hierarchically clustered cells of similar phenotype with 'steps' in an MST. The more 'steps' between any two clusters, the distinct they are. Several MSTs were presented, each displaying the median intensity of T_{FH}, T_{H2} and T_{H2A} cell markers. Here, it was confirmed that cluster nodes of CXCR5-expressing cells that co-express PD-1 (T_{FH} cells, orange arrows) and T_{H2} and T_{H2A} cell clusters that express low CD27 and high CRTH2 (T_{H2} cells, green arrows) expression were separated into different branches, highlighting the distinct phenotype between these cell subsets (Figure 3.2.1.1, C).

Furthermore, an unbiased approach of FlowSOM²⁸⁵ that allows grouping of cell subsets into metaclusters (cluster of cell clusters of similar phenotype) confirmed that T_{FH} and T_{H2} and

T_H2A cell subsets formed distinct metaclusters, indicated by the different colours (orange and green arrows, respectively; Figure 3.2.1.1, D). Furthermore, overlaid viSNE maps of T_{FH} and T_H2 cell subsets showed the two cell subsets were in different islands, with T_H2A cells as a part of the T_H2 cell subsets (T_{FH} cells, orange; T_H2 cells, green; T_H2A cells, red; Figure 3.2.1.1, E).

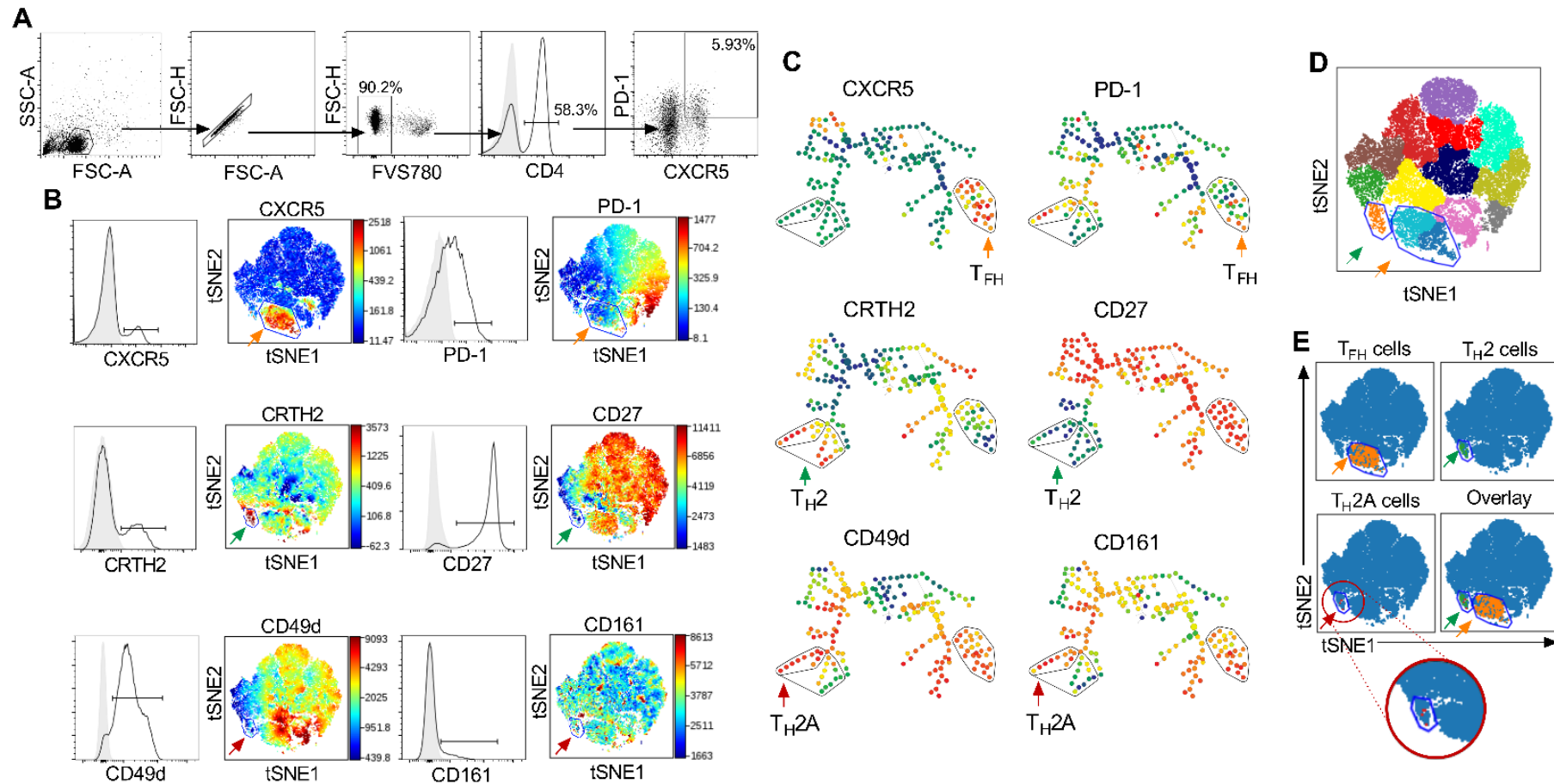


Figure 3.2.1.1 T_{FH} cells are a distinct population from T_{H2} and T_{H2A} cells. **A** Gating strategy to identify T_{FH} ($CD4^+CXCR5^+PD-1^+$) cells in peripheral blood. **B** viSNE maps showing a proportion of $CD4^+$ cells expressing CXCR5, PD-1, CRTH2, CD27, CD49d and CD161. **C** SPADE MSTs of $CD4^+$ cells that expressed markers for T_{FH} , T_{H2} ($CD4^+CRTH2^+CD27^-$) and T_{H2A} ($CRTH2^+CD27^+CD161^+CD49d^+$) cells. **D** FlowSOM analysis showing T_{FH} and T_{H2} cells as two separate metaclusters. **E** Overlaid viSNE maps of T_{FH} , T_{H2} and T_{H2A} cells. T_{H2A} cells were within the T_{H2} cell population. Orange, green and red events indicate T_{FH} , T_{H2} and T_{H2A} cells, respectively. Colours in viSNE maps and SPADE MSTs indicate the median expression of labelled cell markers; red indicates high expression and green indicates low expression. Orange, green and red arrows indicate T_{FH} , T_{H2} and T_{H2A} cell populations, respectively.

The median expression of CXCR5, PD-1, CCRTH2, CD27 CD49d and CD161 was also investigated in T_{FH}, T_{H2} and T_{H2A} cells. T_{FH} cells expressed significantly higher expression of CXCR5 ($P < 0.001$), PD-1 ($P < 0.01$) and CD27 ($P < 0.001$) compared to T_{H2} and T_{H2A} cell subsets. In contrast, T_{FH} cells expressed lower expression of CCRTH2 compared to T_{H2} and T_{H2A} cells (all, $P < 0.001$). T_{FH} and T_{H2} cells were shown to expressed significantly lower expression of CD49d (all, $P < 0.001$) and CD161 (all, $P < 0.001$) compared to T_{H2A} cells (Figure 3.2.1.2, A). These observations were consistent in 13 GPA patients (Figure 3.2.1.2, B).

It has been established that T_{H2} cells have the ability to secrete IL-4 cytokines²⁹⁴ and T_{FH} cells have been previously reported to secrete IL-21 cytokines^{165,295}. Here, it was observed that T_{H2} and T_{H2A} cells constitute a larger proportion of IL-4-producing cells compared to T_{FH} cells, which express lower IL-4 expression compared to T_{H2} and T_{H2A} cells (T_{FH} cells, 25%; T_{H2} and T_{H2A} cells, 75%; Figure 3.2.1.2, C and D). In contrast, T_{FH} cells constitute a higher proportion of IL-21-expressing cells compared to the T_{H2} and T_{H2A} cells, with T_{FH} cells showing higher IL-21 expression compared to the latter (T_{FH} cells, 58%; T_{H2} and T_{H2A} cells, 42%; Figure 3.2.1.2, C and D). Collectively, these data highlight the phenotypic differences between T_{FH}, T_{H2} and T_{H2A} cells.

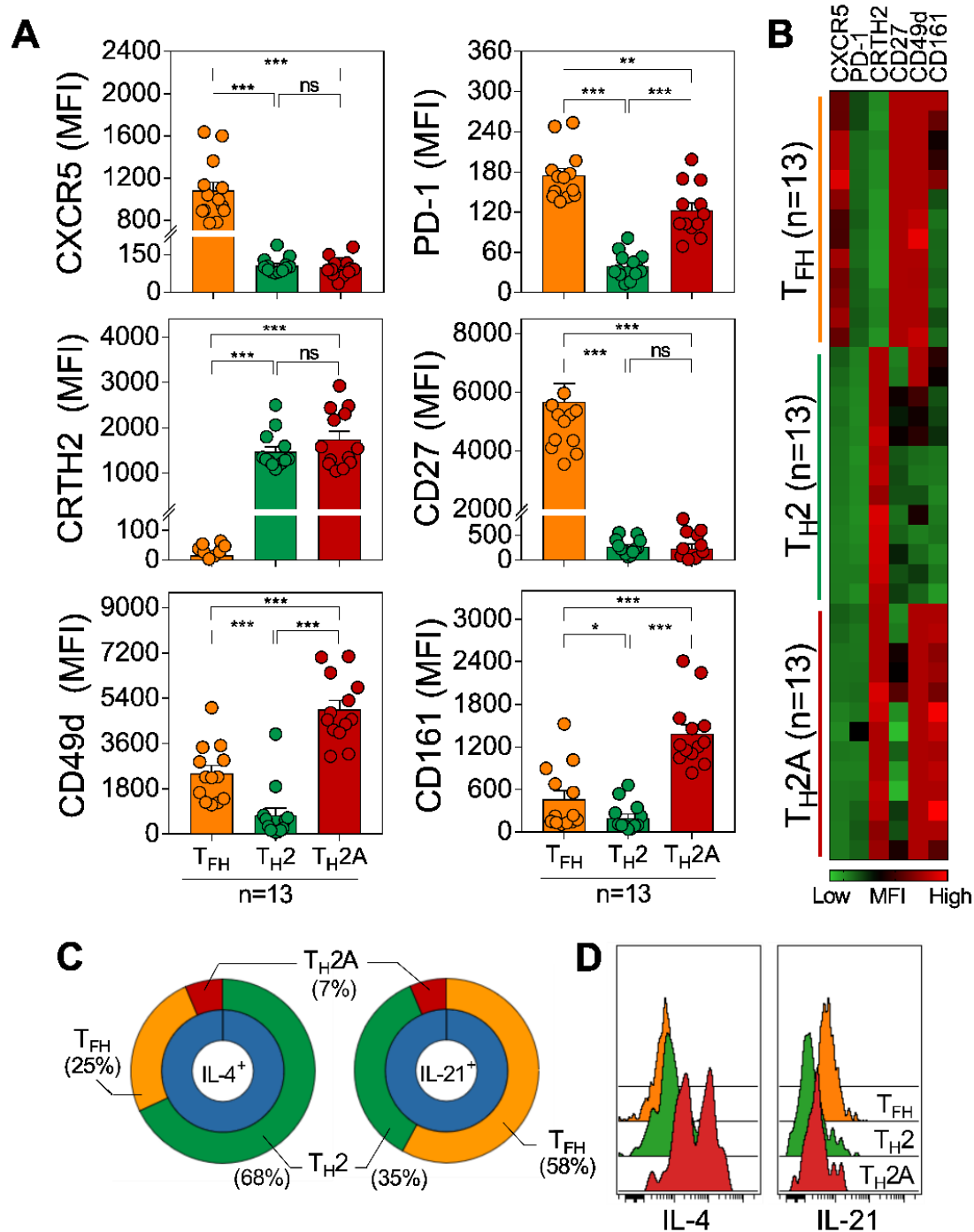


Figure 3.2.1.2 Differential expression of cell surface markers of T_{FH} , T_{H2} and T_{H2A} cell subsets. **A** MFI of CXCR5, PD-1, CRTH2, CD27, CD49d and CD161 in T_{FH} , T_{H2} and T_{H2A} cells. **B** Heatmap of CXCR5, PD-1, CRTH2, CD27, CD49d and CD161 MFI in T_{FH} , T_{H2} and T_{H2A} cells of individual GPA patients ($n = 13$). Red indicates high expression and green indicates low expression of the markers. **C** Proportions of T_{FH} , T_{H2} and T_{H2A} cells within $IL-4^+$ and $IL-21^+$ $CD4^+$ cells. **D** Differences in the expression of IL-4 and IL-21 in T_{FH} , T_{H2} and T_{H2A} cells. Error bars represent SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney test.

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

Having demonstrated that T_{FH} cells express IL-4 and IL-21, the role of IL-4 and IL-21 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 described²⁹². However, the effect of IL-21 on CSR, particularly to IgE, and the synergistic effect of IL-4 and IL-21 has yet to be fully elucidated and remains controversial. T_{FH} and B cell interaction leads to a GC reaction, marked by the upregulation of *BCL6* expression, and the production of IL-4 and IL-21. The latter leads to B cell proliferation and class-switching, indicated by upregulation of *AICDA* expression. A proportion of these B cells then undergo further differentiation into plasmablasts and plasma cells, denoted by an increase in the expression of *PRDM1* and *XBP1*. To identify the effect of IL-4 and IL-21 on the different stages of B cell development, the expression of *BCL6*, *AICDA*, *PRDM1* and *XBP1* were investigated by qPCR.

IL-4 stimulation resulted in an upregulation of *BCL6* (1.47-fold, $P = 0.0244$) and *AICDA* in naïve B cells on day 5 (445.04-fold, $P = 0.001$), which remained upregulated on day 6 (*AICDA*, 271.60-fold, $P = 0.001$) compared to CD40L alone. *AICDA* expression was significantly upregulated in response to IL-21 stimulation on day 5 (58.03-fold, $P = 0.0039$), relative to CD40L alone (Figure 3.2.2.1, A). In the presence of IL-4, IL-21 downregulated *BCL6* (day 5, 2.07-fold, $P = 0.0002$; day 6, 2.76-fold, $P = 0.0001$) and *AICDA* expression (day 5, 2.10-fold, $P = 0.0034$; day 6, 2.91-fold, $P = 0.0001$). A similar trend was observed in memory B cells; however, it was not statistically significant, with the exception on day 6, *AICDA* expression was significantly upregulated in response to dual IL-4/IL-21 compared to CD40L alone (12.88-fold, $P = 0.0238$; Figure 3.2.2.1, A). No significant differences were observed between GPA and NAC (Table 3.2.2.1 and Table 3.2.2.2).

A significant upregulation of *PRDM1* was observed in response to stimulation with IL-21 and dual IL-4/IL-21 in naïve B cells on days 5 (IL-21, 11.58-fold, $P = 0.0039$; IL-4/IL-21, 12.70-fold, $P < 0.001$) and 6 (IL-21, 12.97-fold, $P = 0.0039$; IL-4/IL-21, 22.63-fold, $P < 0.001$) and to a lesser extent with IL-4 (day 5, 1.69-fold, $P = 0.0020$; day 6, 3.12-fold, $P = 0.0029$) in naïve B cells, relative to CD40L alone (Figure 3.2.2.1, B). Although a similar trend was noted in memory B cells, a significant upregulation of *PRDM1* was only observed in response to stimulation with dual IL-4/IL-21 on days 5 (7.64-fold, $P = 0.0357$) and 6 (6.46-fold, $P = 0.0238$) compared to CD40L alone (Figure 3.2.2.1, B). No significant differences were observed in the expression of *PRDM1* between GPA and NAC (Table 3.2.2.3).

XBP1 expression was upregulated in naïve B cells in response to both IL-4 and IL-21 as well as dual IL-4/IL-21 on days 5 (IL-4, 12.07-fold, $P = 0.0010$; IL-21, 7.42-fold, $P = 0.0039$; IL-4/IL-21, 12.88-fold, $P < 0.001$) and 6 (IL-4, 14.12-fold, $P = 0.0010$; IL-21, 8.96-fold, $P = 0.0039$; IL-4/IL-21, 23.28-fold, $P < 0.0001$), relative to CD40L alone (Figure 3.2.2.1, C). A similar trend was observed in memory B cells, however, a significant upregulation of *XBP1* was only observed in response to dual IL-4/IL-21 stimulation on days 5 (14.26-fold, $P = 0.0357$) and 6 (10.23-fold, $P = 0.0238$), compared to CD40L alone (Figure 3.2.2.1, C). No differences were observed in *XBP1* expression between GPA and NAC (Table 3.2.2.4).

Table 3.2.2.1 *BCL6* expression following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
BCL6 expression	Day 5															
	CD40L	9	0.11	0.07	0.02	NA	NA	NA	2	0.09	0.05	0.03	NA	NA	NA	NA
	CD40L+IL-4	9	0.15	0.05	0.02	0.0547	NA	NA	5	0.15	0.06	0.03	NA	NA	NA	NA
	CD40L+IL-21	7	0.09	0.04	0.01	0.8125	0.0229	NA	2	0.06	0.03	0.02	NA	0.1905	NA	NA
	CD40L+IL-4+IL-21	8	0.07	0.04	0.01	0.1996	0.0078	0.2188	5	0.08	0.03	0.01	0.8571	0.0625	NA	NA
	Day 6															
	CD40L	9	0.10	0.05	0.02	NA	NA	NA	2	0.08	0.03	0.02	NA	NA	NA	0.7273
	CD40L+IL-4	9	0.13	0.05	0.02	0.2500	NA	NA	5	0.14	0.04	0.02	NA	NA	NA	0.6064
	CD40L+IL-21	7	0.06	0.02	0.01	0.0313	0.0052	NA	2	0.08	0.05	0.03	NA	0.1905	NA	0.6667
	CD40L+IL-4+IL-21	9	0.05	0.02	0.01	0.0056	0.0039	0.0469	5	0.05	0.03	0.01	0.3810	0.0625	NA	0.7972
Memory B cells																
BCL6 expression	Day 5															
	CD40L	2	0.02	0.01	0.00	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	0.05	0.00	0.00	NA	NA	NA	2	0.07	0.03	0.02	NA	NA	NA	0.6667
	CD40L+IL-21	1	0.01	0.00	0.00	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	3	0.02	0.01	0.01	0.8000	NA	NA	2	0.02	0.00	0.00	NA	NA	NA	0.8000
	Day 6															
	CD40L	2	0.02	0.00	0.00	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	0.05	0.00	0.00	NA	NA	NA	2	0.09	0.06	0.04	NA	NA	NA	0.3333
	CD40L+IL-21	1	0.01	0.00	0.00	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	4	0.02	0.00	0.00	>0.9999	NA	NA	2	0.02	0.00	0.00	NA	NA	NA	0.8000

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Table 3.2.2.2 AICDA expression following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
A/CDA expression	Day 5															
	CD40L	9	0.01	0.01	0.00	NA	NA	NA	2	0.00	0.00	0.00	NA	NA	NA	0.5818
	CD40L+IL-4	9	3.35	2.76	0.92	0.0039	NA	NA	5	1.90	1.03	0.46	NA	NA	NA	0.0829
	CD40L+IL-21	7	0.46	1.11	0.42	0.0156	0.0052	NA	2	0.06	0.08	0.05	NA	0.0952	NA	0.8889
	CD40L+IL-4+IL-21	8	1.43	0.79	0.28	<0.0001	0.0391	0.4375	5	1.22	0.78	0.35	0.0952	0.1250	NA	0.6216
	Day 6															
	CD40L	9	0.01	0.01	0.00	NA	NA	NA	2	0.01	0.01	0.01	NA	NA	NA	0.7273
	CD40L+IL-4	9	3.20	1.23	0.41	0.0039	NA	NA	5	2.35	1.09	0.49	NA	NA	NA	0.1469
	CD40L+IL-21	7	0.23	0.55	0.21	0.1563	0.0002	NA	2	0.05	0.06	0.04	NA	0.0952	NA	0.8889
CD40L+IL-4+IL-21	9	1.02	0.66	0.22	<0.0001	0.0039	0.1563	5	0.96	0.51	0.23	0.0952	0.0625	NA	>0.9999	
Memory B cells																
A/CDA expression	Day 5															
	CD40L	2	0.11	0.14	0.10	NA	NA	NA	1	0.01	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	1.88	0.34	0.24	NA	NA	NA	2	0.68	0.36	0.25	NA	NA	NA	0.3333
	CD40L+IL-21	1	0.09	0.00	0.00	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	3	0.98	0.50	0.29	0.2000	NA	NA	2	0.43	0.38	0.27	NA	NA	NA	0.4000
	Day 6															
	CD40L	2	0.08	0.08	0.06	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	1.72	0.06	0.04	NA	NA	NA	2	1.35	0.71	0.50	NA	NA	NA	>0.9999
	CD40L+IL-21	1	0.05	0.00	0.00	NA	NA	NA	1	0.01	0.00	0.00	NA	NA	NA	NA
CD40L+IL-4+IL-21	4	0.90	0.34	0.17	0.1333	NA	NA	2	0.59	0.23	0.16	NA	NA	NA	0.2667	

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Table 3.2.2.3 *PRDM1* expression following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
PRDM1 expression	Day 5															
	CD40L	9	0.04	0.03	0.01	NA	NA	NA	2	0.03	0.02	0.01	NA	NA	NA	>0.9999
	CD40L+IL-4	9	0.08	0.05	0.02	0.0078	NA	NA	5	0.05	0.01	0.00	NA	NA	NA	0.5185
	CD40L+IL-21	7	0.58	0.52	0.20	0.0156	0.0418	NA	2	0.07	0.05	0.03	NA	>0.9999	NA	0.3333
	CD40L+IL-4+IL-21	8	0.58	0.47	0.17	<0.0001	0.0078	0.5625	5	0.40	0.15	0.07	0.0952	0.0625	NA	0.7242
	Day 6															
	CD40L	9	0.03	0.02	0.01	NA	NA	NA	2	0.03	0.02	0.01	NA	NA	NA	0.7273
	CD40L+IL-4	9	0.11	0.11	0.04	0.0117	NA	NA	5	0.07	0.03	0.01	NA	NA	NA	0.6993
	CD40L+IL-21	7	0.49	0.52	0.20	0.0156	0.1738	NA	2	0.09	0.09	0.06	NA	>0.9999	NA	0.3333
	CD40L+IL-4+IL-21	9	0.80	0.54	0.18	<0.0001	0.0039	0.1094	5	0.54	0.18	0.08	0.0952	0.0625	NA	0.5185
Memory B cells																
PRDM1 expression	Day 5															
	CD40L	2	0.09	0.02	0.02	NA	NA	NA	1	0.07	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	0.16	0.04	0.03	NA	NA	NA	2	0.13	0.06	0.04	NA	NA	NA	0.6667
	CD40L+IL-21	1	0.86	0.00	0.00	NA	NA	NA	1	0.59	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	3	0.63	0.41	0.24	0.2000	NA	NA	2	0.66	0.06	0.04	NA	NA	NA	0.8000
	Day 6															
	CD40L	2	0.12	0.04	0.03	NA	NA	NA	1	0.07	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	0.19	0.03	0.02	NA	NA	NA	2	0.41	0.26	0.18	NA	NA	NA	0.3333
	CD40L+IL-21	1	0.49	0.00	0.00	NA	NA	NA	1	2.01	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	4	0.72	0.28	0.14	0.1333	NA	NA	2	0.51	0.10	0.07	NA	NA	NA	0.5333

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Table 3.2.2.4 *XBP1* expression following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
Day 5																
XBP1 expression	CD40L	9	0.09	0.03	0.01	NA	NA	NA	2	0.08	0.06	0.04	NA	NA	NA	0.5818
	CD40L+IL-4	9	1.22	0.44	0.15	0.0039	NA	NA	5	0.83	0.37	0.17	NA	NA	NA	0.1469
	CD40L+IL-21	7	0.81	0.67	0.25	0.0156	0.1416	NA	2	0.15	0.10	0.07	NA	0.0952	NA	0.2222
	CD40L+IL-4+IL-21	8	1.25	0.56	0.20	<0.0001	0.8438	0.3125	5	0.98	0.35	0.16	0.0952	0.0625	NA	0.6216
	Day 6															
	CD40L	9	0.07	0.03	0.01	NA	NA	NA	2	0.06	0.03	0.02	NA	NA	NA	0.5818
	CD40L+IL-4	9	1.06	0.24	0.08	0.0039	NA	NA	5	0.78	0.24	0.11	NA	NA	NA	0.0599
	CD40L+IL-21	7	0.74	0.75	0.28	0.0156	0.4698	NA	2	0.15	0.11	0.08	NA	0.0952	NA	0.3333
	CD40L+IL-4+IL-21	9	1.73	0.59	0.20	<0.0001	0.0195	0.0781	5	1.33	0.54	0.24	0.0952	0.0625	NA	0.2398
Memory B cells																
Day 5																
XBP1 expression	CD40L	2	0.09	0.02	0.01	NA	NA	NA	1	0.07	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	0.51	0.08	0.05	NA	NA	NA	2	0.36	0.04	0.03	NA	NA	NA	0.3333
	CD40L+IL-21	1	1.44	0.00	0.00	NA	NA	NA	1	0.86	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	3	1.38	0.42	0.24	0.2000	NA	NA	2	1.03	0.12	0.08	NA	NA	NA	0.4000
	Day 6															
	CD40L	2	0.16	0.04	0.03	NA	NA	NA	1	0.06	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	0.58	0.09	0.06	NA	NA	NA	2	0.57	0.19	0.13	NA	NA	NA	>0.9999
	CD40L+IL-21	1	1.34	0.00	0.00	NA	NA	NA	1	0.76	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	4	1.37	0.43	0.22	0.1333	NA	NA	2	1.17	0.12	0.09	NA	NA	NA	0.8000

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Following stimulation of naïve and memory B cells with IL-4, IL-21 and dual IL-4/IL-21 for 7 and 14 days, CD38⁺CD27^{hi} plasmablasts, IgE⁺ plasmablasts, IgE⁺ B and IgG⁺ B cells were enumerated by flow cytometry (Figure 3.2.2.2). There was an increasing proportion of CD38⁺CD27^{hi} plasmablasts observed in response to stimulation with IL-4, IL-21 and dual IL-4/IL-21 in naïve and memory B cells (Figure 3.2.2.3, A). Moreover, representative viSNE maps illustrated that there was little to no CD38-expressing cells when stimulated with CD40L alone. However, there was a higher proportion of CD38-expressing cells following stimulation with IL-4, IL-21 and dual IL-4/IL-21 (Figure 3.2.2.3, B). FlowSOM analysis was performed and represented as an MST to allow simultaneous visualisation of various marker's intensity within individual cell clusters. The data demonstrated that clusters of cells that express high CD38 and CD27 expression (indicative of plasmablasts population) were more abundant when stimulated with IL-4 and IL-21 compared to CD40L alone (Figure 3.2.2.3, C). This observation was consistent for all cell clusters within the metacluster for day 7 and 14 (Figure 3.2.2.3, D). When compared to CD40L alone, IL-21 and dual IL-4/IL-21 stimulation of naïve B cells resulted in significantly higher proportions of CD38⁺CD27^{hi} plasmablasts on day 7 (IL-21, 18.97-fold, $P = 0.0078$; IL-4/IL-21, 32.51-fold, $P = 0.0003$) and 14 (IL4/IL-21, 6.08-fold, $P = 0.0056$; Figure 3.2.2.3, E). Similarly, the proportion of plasmablasts were significantly higher following stimulation of memory B cells with IL-4 (day 7, 2.01-fold, $P = 0.0488$; day 14, 4.78-fold, $P = 0.0039$), IL-21 (day 7, 7.64-fold, $P = 0.0098$; day 14, 12.15-fold, $P = 0.0020$) and dual IL-4/IL-21 (day 7, 12.54-fold; day 14, 16.15-fold; both, $P < 0.0001$), compared to CD40L alone (Figure 3.2.2.3, E).

viSNE maps illustrated that the high CD38-expressing cells (plasmablasts) were also shown to co-express IgE (Figure 3.2.2.4, A). The proportion these IgE⁺ plasmablasts was significantly higher in response to dual IL-4/IL-21 stimulation of naïve B cells on day 7 (12.70-fold, $P = 0.0057$) but was lost on day 14, despite showing a trend of increasing proportions of IgE⁺ plasmablasts (2.32-fold), compared to CD40L alone (Figure 3.2.2.4, B). In memory B cell

culture, a significant increase in IgE⁺ plasmablasts was observed in response to IL-21 (3.76-fold, $P = 0.0137$) and dual IL-4/IL-21 (9.37-fold, $P = 0.0030$) on day 7 and in response to stimulation with IL-4 (3.98-fold, $P = 0.0078$), IL-21 (5.83-fold, $P = 0.0098$) and dual IL-4/IL-21 (9.80-fold, $P = 0.0001$) on day 14, compared to CD40L alone (Figure 3.2.2.4, B). No significant differences were observed between GPA and NAC (Table 3.2.2.5 and Table 3.2.2.6).

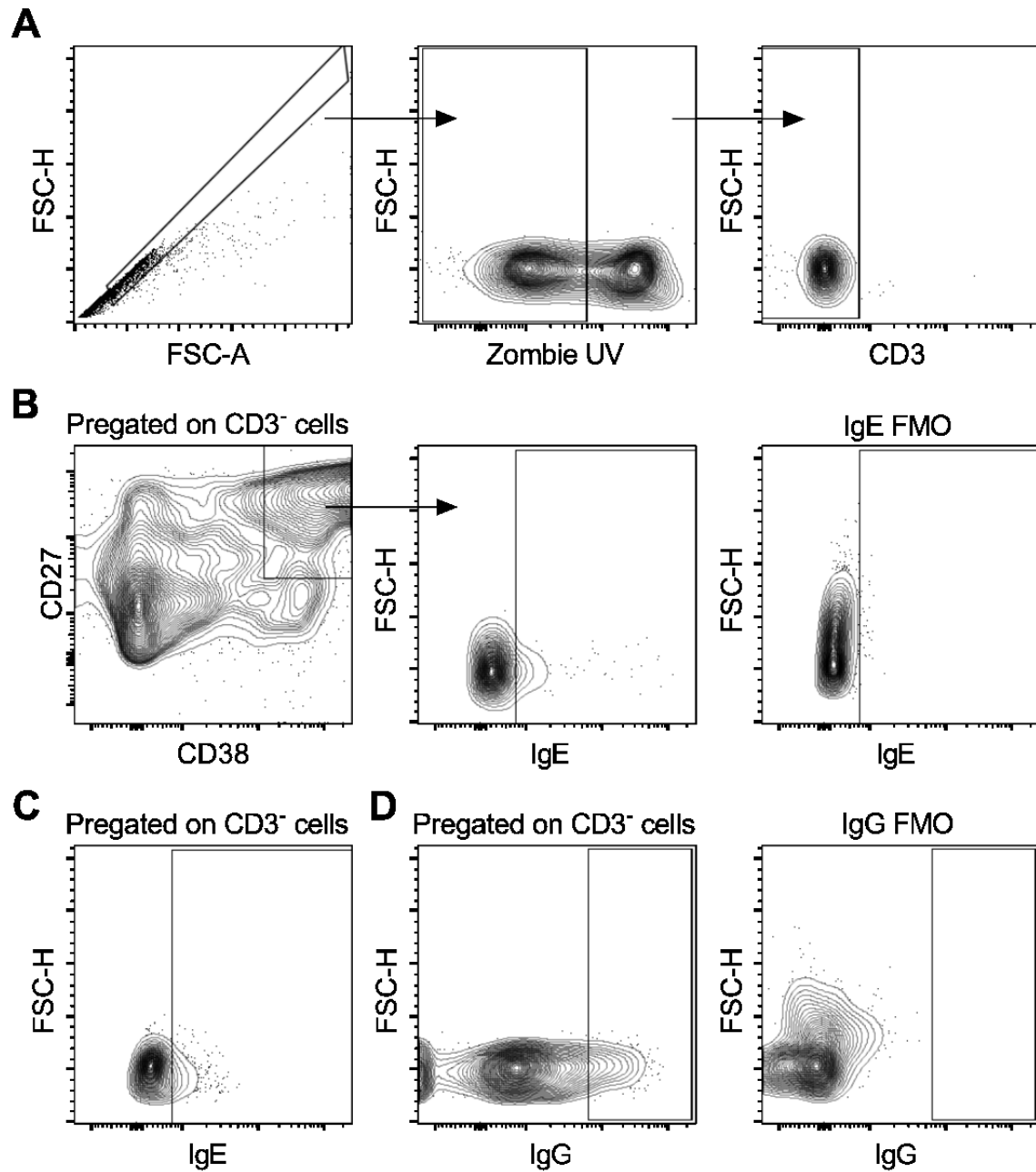


Figure 3.2.2.2 B cell subsets gating strategy following stimulation of isolated naïve and memory B cells with IL-4 and IL-21, in the presence of CD40L. A Doublets were excluded and live cells were discriminated using Zombie UV dye. B cells were gated on the basis of live single cells that lack the expression of CD3. **B** Plasmablasts were defined as CD3⁻ cells that expressed CD38 and high CD27 expression (left panel). IgE⁺ plasmablasts were gated within plasmablasts population (middle panel). **C** IgE⁺ B cells; and **D** IgG⁺ B cells (left panel) were gated within CD3⁻ cells.

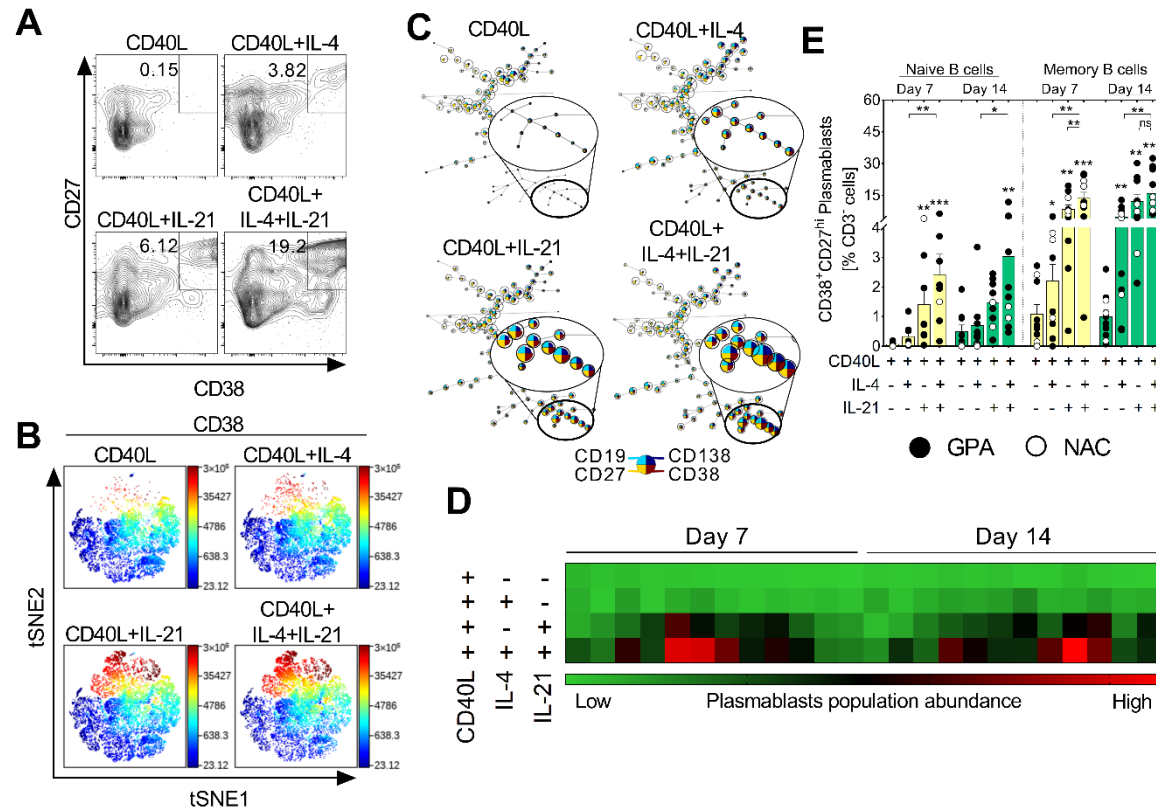


Figure 3.2.2.3 Dual IL-4 and IL-21 stimulation drive differentiation of naive and memory B cells into plasmablasts. **A** Differentiation of memory B cells into CD38⁺CD27^{hi} plasmablasts as observed by conventional flow cytometry analysis; and **B** unbiased viSNE analysis. **C** Unbiased FlowSOM analyses showing clusters of CD38⁻ and CD27⁻ expressing B cells. Colours in the individual clusters indicate the intensity of CD27 (yellow), CD38 (red), CD19 (aqua) and CD138 (blue). Marker intensity is visualised as the distance of each segment from the border of each cluster node and its size indicates the cluster abundance. **D** Heatmap of the population abundance of individual clusters. Red indicates high abundance and green indicates a low abundance of the cell cluster. **E** Proportions of CD38⁺CD27^{hi} plasmablasts following stimulations of naïve ($n = 7$) and memory B cells ($n = 7$). Colours in viSNE maps indicate the median expression of labelled cell markers; red indicates high expression and green indicates low expression. Closed circles indicate GPA and open circles indicate NAC. Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic controls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Wilcoxon signed-rank test and Mann-Whitney test.

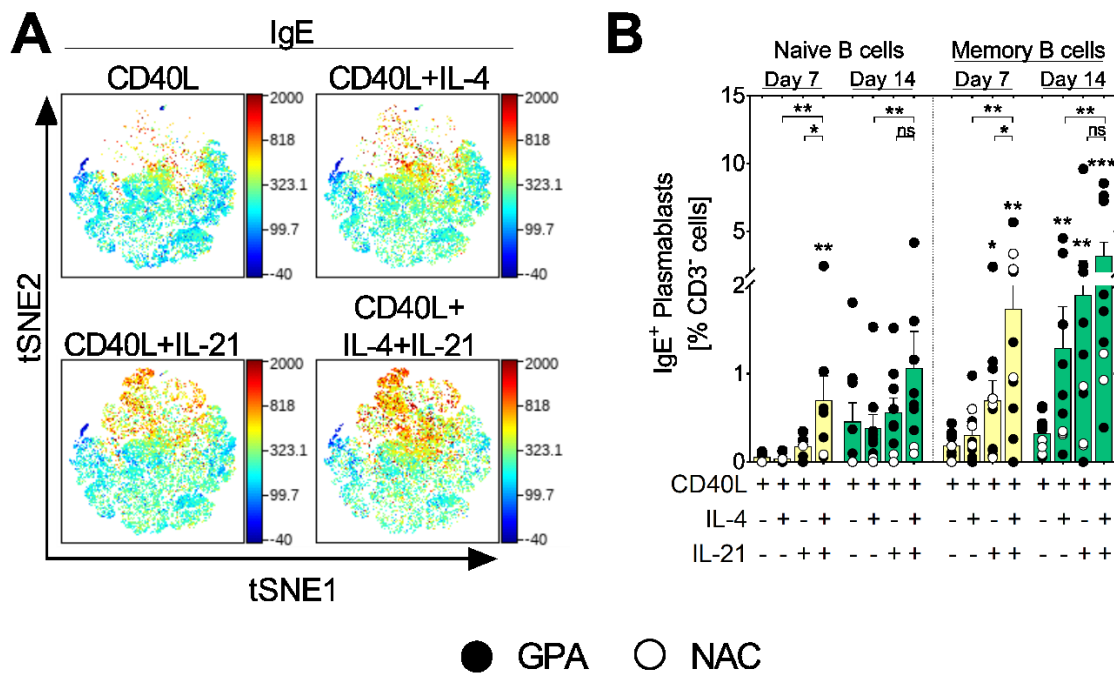


Figure 3.2.2.4 Dual IL-4 and IL-21 stimulation drive differentiation of naïve and memory B cells into IgE-expressing plasmablasts. **A** viSNE maps showing CD38-expressing cells co-express IgE. **B** The proportion of IgE⁺ plasmablasts following stimulations of naïve and memory B cells. Colours in viSNE maps indicate the median expression of labelled cell markers; red indicates high expression and green indicates low expression. Closed circles indicate GPA and open circles indicate NAC. Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic controls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Wilcoxon signed-rank test and Mann-Whitney test.

Table 3.2.2.5 The proportion of CD38⁺CD27^{hi} plasmablasts following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition		GPA							NAC							P value GPA vs NAC	
		n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
						CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																	
CD38 ⁺ CD27 ^{hi} Plasmablasts	Day 7																
	CD40L	7	0.09	0.07	0.03	NA	NA	NA	1	0.00	0.00	0.00	NA	NA	NA	NA	
	CD40L+IL-4	7	0.37	0.41	0.16	0.2188	NA	NA	1	0.06	0.00	0.00	NA	NA	NA	NA	
	CD40L+IL-21	7	1.05	1.08	0.41	0.0156	0.2086	NA	1	4.02	0.00	0.00	NA	NA	NA	NA	
	CD40L+IL-4+IL-21	7	2.56	2.03	0.77	0.0012	0.0156	0.0469	1	1.50	0.00	0.00	NA	NA	NA	NA	
	Day 14																
	CD40L	7	0.64	0.69	0.26	NA	NA	NA	2	0.02	0.03	0.02	NA	NA	NA	0.1389	
	CD40L+IL-4	7	0.91	1.12	0.42	>0.9999	NA	NA	2	0.02	0.03	0.02	NA	NA	NA	0.0556	
	CD40L+IL-21	7	1.58	0.81	0.31	0.1563	0.1649	NA	2	1.07	0.58	0.41	NA	0.3333	NA	0.5000	
	CD40L+IL-4+IL-21	7	3.57	3.98	1.50	0.0262	0.0469	0.1094	2	1.15	0.27	0.19	0.3333	NA	NA	0.5000	
	Memory B cells																
	CD38 ⁺ CD27 ^{hi} Plasmablasts	Day 7															
CD40L		7	1.20	0.89	0.34	NA	NA	NA	3	0.86	1.36	0.78	NA	NA	NA	0.3500	
CD40L+IL-4		7	1.96	1.89	0.71	0.3750	NA	NA	3	2.79	1.59	0.92	0.2500	NA	NA	0.4167	
CD40L+IL-21		7	8.03	7.42	2.81	0.0781	0.0973	NA	3	9.18	4.15	2.39	0.2500	0.1000	NA	0.6667	
CD40L+IL-4+IL-21		7	12.32	9.25	3.50	0.0023	0.0313	0.0156	3	17.10	6.13	3.54	0.1000	0.2500	0.2500	0.5167	
Day 14																	
CD40L		7	1.01	0.85	0.32	NA	NA	NA	3	0.95	0.70	0.40	NA	NA	NA	>0.9999	
CD40L+IL-4		7	5.06	4.88	1.85	0.0313	NA	NA	3	4.07	2.22	1.28	0.2500	NA	NA	>0.9999	
CD40L+IL-21		7	14.23	11.04	4.17	0.0156	0.0973	NA	3	7.11	3.84	2.22	0.2500	0.4000	NA	0.5167	
CD40L+IL-4+IL-21	7	18.17	11.79	4.46	0.0006	0.0156	0.2188	3	11.17	3.16	1.82	0.1000	0.2500	0.5000	0.8333		

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Table 3.2.2.6 The proportion of IgE⁺CD38⁺CD27^{hi} plasmablasts following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
Day 7																
IgE ⁺ CD38 ⁺ CD27 ^{hi} Plasmablasts	CD40L	7	0.06	0.06	0.02	NA	NA	NA	1	0.00	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	7	0.04	0.06	0.02	0.7500	NA	NA	1	0.03	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-21	7	0.18	0.15	0.06	0.1563	0.1544	NA	1	0.18	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	7	0.79	0.79	0.30	0.0064	0.0156	0.0313	1	0.09	0.00	0.00	NA	NA	NA	NA
	Day 14															
	CD40L	7	0.59	0.67	0.25	NA	NA	NA	2	0.00	0.00	0.00	NA	NA	NA	0.2778
	CD40L+IL-4	7	0.48	0.49	0.18	0.6875	NA	NA	2	0.02	0.03	0.02	NA	NA	NA	0.0556
	CD40L+IL-21	7	0.71	0.45	0.17	0.2969	0.2593	NA	2	0.04	0.06	0.04	NA	>0.9999	NA	0.0556
CD40L+IL-4+IL-21	7	1.33	1.31	0.50	0.2465	0.0156	0.3750	2	0.13	0.05	0.04	0.3333	NA	NA	0.0556	
Memory B cells																
Day 7																
IgE ⁺ CD38 ⁺ CD27 ^{hi} Plasmablasts	CD40L	7	0.24	0.18	0.07	NA	NA	NA	3	0.06	0.10	0.06	NA	NA	NA	0.1583
	CD40L+IL-4	7	0.27	0.35	0.13	>0.9999	NA	NA	3	0.40	0.21	0.12	0.2500	NA	NA	0.3500
	CD40L+IL-21	7	0.79	0.83	0.31	0.0781	0.1369	NA	3	0.47	0.37	0.21	0.2500	0.7000	NA	0.6667
	CD40L+IL-4+IL-21	7	1.55	1.94	0.73	0.0781	0.0313	0.1094	3	2.17	1.20	0.69	0.1000	0.2500	0.2500	0.2667
	Day 14															
	CD40L	7	0.39	0.20	0.08	NA	NA	NA	3	0.17	0.08	0.05	NA	NA	NA	0.1833
	CD40L+IL-4	7	1.71	1.63	0.61	0.0469	NA	NA	3	0.33	0.02	0.01	0.2500	NA	NA	0.1167
	CD40L+IL-21	7	2.53	3.22	1.22	0.0313	0.6200	NA	3	0.42	0.38	0.22	0.5000	0.7000	NA	0.1833
CD40L+IL-4+IL-21	7	4.11	3.51	1.33	0.0070	0.0156	0.4688	3	1.03	0.17	0.10	0.1000	0.2500	0.2500	0.1083	

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

When the expression of ϵ germline transcript (ϵGLT) was assessed in naïve and memory B cells, a significant upregulation of ϵGLT expression was observed in response to IL-4 compared to CD40L alone in naïve B cells (day 5, 11.20-fold, $P = 0.0010$; day 6, 5.72-fold, $P = 0.0020$), but not in response to stimulation with IL-21 and dual IL-4/IL-21 in naïve B cells. No significant changes were observed in memory B cells (Figure 3.2.2.5, A). Despite the significant changes observed at mRNA level in naïve B cells, the proportion of total IgE⁺ cells showed no significant differences in response to IL-4, IL-21 and dual IL-4/IL-21 stimulations, compared to CD40L alone, although a trend of higher IgE⁺ cells was observed in response to dual IL-4/IL-21 stimulation. However, in the presence of IL-4, IL-21 stimulation increases the proportion of IgE⁺ cells (3.44-fold, $P = 0.0078$) compared to IL-4 alone (Figure 3.2.2.5, B). In memory B cells, the proportions of total IgE⁺ cells were significantly higher in response to stimulation with IL-4 (day 7, 2.31-fold, $P = 0.0391$; day 14, 2.45-fold, $P = 0.0039$) and dual IL-4/IL-21 (day 7, 3.64-fold, $P = 0.0057$; day 14, 2.76-fold, $P = 0.0089$) compared to CD40L alone. In addition, the proportion of IgE⁺ cells was also significantly higher in response to IL-21 (1.53-fold, $P = 0.0391$) on day 7 but was lost on day 14, although the proportion remained high compared to CD40L alone (Figure 3.2.2.5, B). No significant differences were observed between GPA and NAC (Table 3.2.2.7 and Table 3.2.2.8).

The tIgE levels in culture supernatants were also measured. There was a significantly higher tIgE level observed in response to IL-4 (day 7, 1.67-fold, $P = 0.0078$; day 14, 2.12-fold, $P = 0.0005$) and dual IL-4/IL-21 (day 7, 2.02-fold, $P = 0.0005$; day 14, 4.97-fold, $P < 0.0001$) stimulation in naïve B cells, and to a lesser extent when stimulated with IL-21 alone (day 7, 1.04-fold, $P = 0.0313$; day 14, 1.03-fold, $P = 0.0078$) compared to CD40L alone (Figure 3.2.2.5, C). A similar observation was noted in memory B cells. tIgE level significantly increased following stimulation of memory B cells with IL-4 (day 7, 1.09-fold, $P = 0.0020$; day 14, 4.89-fold, $P = 0.0005$) and dual IL-4/IL-21 (day 7, 1.29-fold, $P = 0.0058$; day 14, 6.19-fold, $P < 0.0001$), and to a lesser extent when stimulated with IL-21 alone (day 7,

1.03-fold; day 14, 1.01-fold; both, $P = 0.0313$), compared to CD40L alone (Figure 3.2.2.5, C). In addition to IgE⁺ cells, IgG⁺ cells were also enumerated. The proportions of IgG⁺ cells were higher in response to stimulation of naïve B cells with IL-21 (2.03-fold, $P = 0.0391$) and dual IL-4/IL-21 (4.14-fold, $P = 0.0083$) on day 14. IgG⁺ cells were significantly higher following stimulation of memory B cells with IL-4 (day 7, 2.19-fold; day 14, 2.65-fold; both, $P = 0.0020$), IL-21 (day 7, 2.50-fold; day 14, 3.34-fold; both, $P = 0.0020$) and dual IL-4/IL-21 (day 7, 3.34-fold, $P = 0.0029$; day 14, 4.64-fold, $P = 0.0001$; Figure 3.2.2.5, D). No significant differences were observed between GPA and NAC (Table 3.2.2.9 and Table 3.2.2.10).

Table 3.2.2.7 ϵ GLT expression following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							<i>P</i> value GPA vs NAC	
	n	mean	SD	SEM	<i>P</i> value vs			n	mean	SD	SEM	<i>P</i> value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
<i>Naïve B cells</i>																
<i>ε</i> GLT expression	<i>Day 5</i>															
	CD40L	9	54.40	59.44	19.81	NA	NA	NA	2	16.06	16.71	11.82	NA	NA	NA	0.4364
	CD40L+IL-4	9	659.20	744.50	248.20	0.0039	NA	NA	5	301.30	218.30	97.65	NA	NA	NA	0.3636
	CD40L+IL-21	7	63.80	155.20	58.66	0.3750	0.0021	NA	2	5.74	7.25	5.12	NA	0.0952	NA	0.8889
	CD40L+IL-4+IL-21	8	130.00	138.20	48.86	0.2766	0.0078	0.4375	5	67.86	53.61	23.98	0.1905	0.0625	NA	0.7242
	<i>Day 6</i>															
	CD40L	9	46.15	75.49	25.16	NA	NA	NA	2	89.42	116.90	82.63	NA	NA	NA	0.7273
	CD40L+IL-4	9	330.20	289.30	96.42	0.0078	NA	NA	5	270.80	237.90	106.40	NA	NA	NA	0.7972
	CD40L+IL-21	7	48.64	122.30	46.22	0.3750	0.0079	NA	2	11.42	15.44	10.92	NA	0.0952	NA	0.6667
	CD40L+IL-4+IL-21	9	66.41	67.53	22.51	0.2581	0.0078	0.3750	5	83.96	47.64	21.31	>0.9999	0.1875	NA	0.3636
<i>Memory B cells</i>																
<i>ε</i> GLT expression	<i>Day 5</i>															
	CD40L	2	0.19	0.18	0.12	NA	NA	NA	1	0.01	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	13.81	2.60	1.84	NA	NA	NA	2	65.30	87.84	62.11	NA	NA	NA	>0.9999
	CD40L+IL-21	1	0.02	0.00	0.00	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	3	11.22	11.74	6.78	0.2000	NA	NA	2	15.63	20.98	14.84	NA	NA	NA	>0.9999
	<i>Day 6</i>															
	CD40L	2	0.06	0.01	0.00	NA	NA	NA	1	0.02	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	2	10.77	2.69	1.91	NA	NA	NA	1	12.98	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-21	1	0.02	0.00	0.00	NA	NA	NA	1	0.00	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	4	5.26	5.57	2.78	NA	NA	NA	2	15.07	18.43	13.03	NA	NA	NA	0.5333

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Table 3.2.2.8 The proportion of IgE⁺ cells following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
Day 7																
IgE ⁺ cells	CD40L	7	2.01	1.67	0.63	NA	NA	NA	1	2.03	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	7	0.98	0.68	0.26	0.1563	NA	NA	1	0.45	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-21	7	2.06	1.69	0.64	0.8125	0.2086	NA	1	0.36	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	7	3.52	3.18	1.20	0.4027	0.0156	0.2969	1	0.46	0.00	0.00	NA	NA	NA	NA
Day 14																
IgE ⁺ cells	CD40L	7	3.54	2.95	1.12	NA	NA	NA	2	1.30	1.00	0.71	NA	NA	NA	0.5000
	CD40L+IL-4	7	3.28	2.39	0.90	>0.9999	NA	NA	2	1.32	1.09	0.77	NA	NA	NA	0.2222
	CD40L+IL-21	7	6.70	5.53	2.09	0.4688	0.0530	NA	2	2.49	0.54	0.39	NA	0.3333	NA	0.0556
	CD40L+IL-4+IL-21	7	6.81	5.89	2.23	0.2086	0.0781	0.6875	2	1.90	1.32	0.94	0.6667	NA	NA	0.2222
Memory B cells																
Day 7																
IgE ⁺ cells	CD40L	6	1.98	1.56	0.64	NA	NA	NA	3	0.48	0.54	0.31	NA	NA	NA	0.1667
	CD40L+IL-4	7	3.32	2.30	0.87	0.0938	NA	NA	3	3.63	3.92	2.26	0.5000	NA	NA	>0.9999
	CD40L+IL-21	7	2.28	2.00	0.76	0.2188	0.3829	NA	3	2.22	1.73	1.00	0.2500	>0.9999	NA	0.8333
	CD40L+IL-4+IL-21	7	5.31	5.29	2.00	0.1375	0.2188	0.0156	3	5.55	4.01	2.32	0.1000	0.2500	0.2500	0.6667
Day 14																
IgE ⁺ cells	CD40L	7	3.24	1.44	0.55	NA	NA	NA	3	3.16	0.60	0.34	NA	NA	NA	>0.9999
	CD40L+IL-4	7	9.71	5.98	2.26	0.0156	NA	NA	3	3.62	0.71	0.41	0.5000	NA	NA	0.1167
	CD40L+IL-21	7	5.36	3.99	1.51	0.0313	0.0973	NA	3	2.58	0.82	0.47	0.5000	0.2000	NA	0.1167
	CD40L+IL-4+IL-21	7	10.73	7.01	2.65	0.0262	0.9375	0.0156	3	4.55	1.30	0.75	0.2000	0.2500	0.2500	0.2667

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Table 3.2.2.9 Total IgE (tIgE) levels in culture supernatants following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
Day 7																
tIgE (kU/L)	CD40L	10	15.19	5.10	1.61	NA	NA	NA	2	15.50	0.99	0.70	NA	NA	NA	0.7576
	CD40L+IL-4	10	28.08	41.14	13.01	0.2246	NA	NA	3	15.77	1.38	0.80	NA	NA	NA	0.5734
	CD40L+IL-21	10	14.09	3.04	0.96	0.5449	0.8102	NA	3	16.83	1.30	0.75	NA	0.4000	NA	0.2168
	CD40L+IL-4+IL-21	10	34.83	40.06	12.67	0.1276	0.0645	0.0020	3	16.57	3.72	2.15	>0.9999	>0.9999	>0.9999	0.7832
	Day 14															
	CD40L	10	15.47	7.45	2.36	NA	NA	NA	4	15.65	1.18	0.59	NA	NA	NA	0.3736
	CD40L+IL-4	10	39.18	57.96	18.33	0.0273	NA	NA	4	16.70	1.64	0.82	0.1250	NA	NA	0.6134
	CD40L+IL-21	10	14.44	4.32	1.37	0.9238	0.3622	NA	4	15.33	1.36	0.68	0.6250	0.3143	NA	0.4735
CD40L+IL-4+IL-21	10	98.71	176.00	55.65	0.0630	0.0059	0.0039	4	22.95	10.77	5.39	0.2000	0.2500	0.2500	0.6354	
Memory B cells																
Day 7																
tIgE (kU/L)	CD40L	9	17.02	6.32	2.11	NA	NA	NA	5	13.74	1.98	0.89	NA	NA	NA	0.2003
	CD40L+IL-4	9	16.71	4.77	1.59	0.8203	NA	NA	5	14.70	1.40	0.63	0.0625	NA	NA	0.3117
	CD40L+IL-21	9	15.33	2.44	0.81	0.3789	0.7460	NA	5	14.10	2.07	0.93	0.5625	0.8413	NA	0.4191
	CD40L+IL-4+IL-21	9	21.67	11.63	3.88	0.4235	0.0508	0.0273	5	15.00	2.44	1.09	0.4127	>0.9999	0.5000	0.4376
	Day 14															
	CD40L	9	16.82	2.07	0.69	NA	NA	NA	6	13.70	1.74	0.71	NA	NA	NA	0.0004
	CD40L+IL-4	10	112.10	243.50	76.99	0.1250	NA	NA	6	16.00	2.74	1.12	0.0313	NA	NA	0.2294
	CD40L+IL-21	9	15.59	1.00	0.33	0.0391	0.0789	NA	5	14.46	1.69	0.76	0.1875	0.3290	NA	0.2977
CD40L+IL-4+IL-21	10	141.50	305.40	96.56	0.6169	0.3223	0.0195	6	20.63	8.49	3.47	0.0238	0.8438	0.3125	0.3530	

GPA, grass pollen allergics; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

Table 3.2.2.10 The proportion of IgG⁺ cells following stimulation of naïve and memory B cells with CD40L, IL-4 and IL-21 in GPA and NAC

Parameter/Condition	GPA							NAC							P value GPA vs NAC	
	n	mean	SD	SEM	P value vs			n	mean	SD	SEM	P value vs				
					CD40L	CD40L+ IL-4	CD40L+ IL-21					CD40L	CD40L+ IL-4	CD40L+ IL-21		
Naïve B cells																
Day 7																
IgG ⁺ cells	CD40L	7	0.57	0.90	0.34	NA	NA	NA	1	0.88	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4	7	0.74	0.76	0.29	0.5781	NA	NA	1	0.90	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-21	7	1.22	1.35	0.51	0.1563	0.6200	NA	1	2.87	0.00	0.00	NA	NA	NA	NA
	CD40L+IL-4+IL-21	7	2.69	2.77	1.05	0.0699	0.0781	0.1094	1	1.55	0.00	0.00	NA	NA	NA	NA
	Day 14															
	CD40L	7	0.70	0.60	0.23	NA	NA	NA	2	0.92	0.67	0.48	NA	NA	NA	0.6667
	CD40L+IL-4	7	1.09	1.26	0.48	>0.9999	NA	NA	2	0.65	0.29	0.21	NA	NA	NA	0.8333
	CD40L+IL-21	7	1.49	1.14	0.43	0.1563	0.4330	NA	2	1.58	0.58	0.41	NA	0.3333	NA	0.8889
CD40L+IL-4+IL-21	7	3.64	3.99	1.51	0.0122	0.0156	0.0313	2	1.15	0.15	0.11	>0.9999	NA	NA	0.3333	
Memory B cells																
Day 7																
IgG ⁺ cells	CD40L	7	4.98	5.50	2.08	NA	NA	NA	3	1.51	1.32	0.76	NA	NA	NA	0.1833
	CD40L+IL-4	7	8.54	6.94	2.62	0.0156	NA	NA	3	8.90	8.01	4.63	0.2500	NA	NA	>0.9999
	CD40L+IL-21	7	10.43	9.68	3.66	0.0156	0.8048	NA	3	8.47	2.98	1.72	0.2500	0.7000	NA	0.8333
	CD40L+IL-4+IL-21	7	13.18	12.36	4.67	0.0530	0.0781	0.0313	3	13.13	2.39	1.38	0.1000	0.5000	0.2500	0.3833
	Day 14															
	CD40L	7	3.40	2.56	0.97	NA	NA	NA	3	2.12	0.28	0.16	NA	NA	NA	0.2667
	CD40L+IL-4	7	9.36	6.54	2.47	0.0156	NA	NA	3	4.73	0.93	0.54	0.2500	NA	NA	0.2667
	CD40L+IL-21	7	11.82	6.86	2.59	0.0156	0.6200	NA	3	5.92	1.70	0.98	0.2500	0.4000	NA	0.1833
CD40L+IL-4+IL-21	7	16.43	9.58	3.62	0.0041	0.0156	0.0469	3	8.22	0.76	0.44	0.1000	0.2500	0.2500	0.3833	

GPA, grass pollen allergies; NAC, non-atopic healthy controls. Bolded values represent significant *P* values.

3.2.3 Higher allergen-driven proliferation of pro-allergic T_{FH} cell subsets in allergics compared to healthy controls

Having shown the effect of T_{FH} cell cytokines IL-4 and IL-21 on B cell maturation and antibody production, the role of T_{FH} cell subsets in grass pollen-induced allergy was investigated. The proliferation of T_{FH}, T_{H2} and T_{H2A} cell subsets was assessed by CellTrace Violet (Figure 3.2.3.1, A). A concentration-dependent increase in the proliferation of IL-4⁺ T_{FH} cells was observed in GPA but not in NAC (Figure 3.2.3.1, B). The proportion of proliferated IL-4⁺ T_{FH} cells were significantly higher in GPA at 10 ($P = 0.0390$) and 25 $\mu\text{g/mL}$ ($P = 0.0369$) compared to NAC (Figure 3.2.3.1, B). Furthermore, there was a concentration-dependent increase of IL-21⁺ T_{FH} cells in GPA but not NAC and was significantly higher in GPA at 1 $\mu\text{g/mL}$ ($P = 0.0485$) compared to NAC (Figure 3.2.3.1, C). T_{H2} cells showed increased proliferation in response to increasing concentration of Phl p in GPA and were significantly higher in GPA at 1 $\mu\text{g/mL}$ ($P = 0.0106$) compared to NAC (Figure 3.2.3.1, D). Similarly, there was a concentration-dependent increase in proliferation of T_{H2A} cells in GPA, which was significantly higher in GPA at 0.1 ($P = 0.0391$) and 1 $\mu\text{g/mL}$ ($P = 0.0113$) compared to NAC (Figure 3.2.3.1, E).

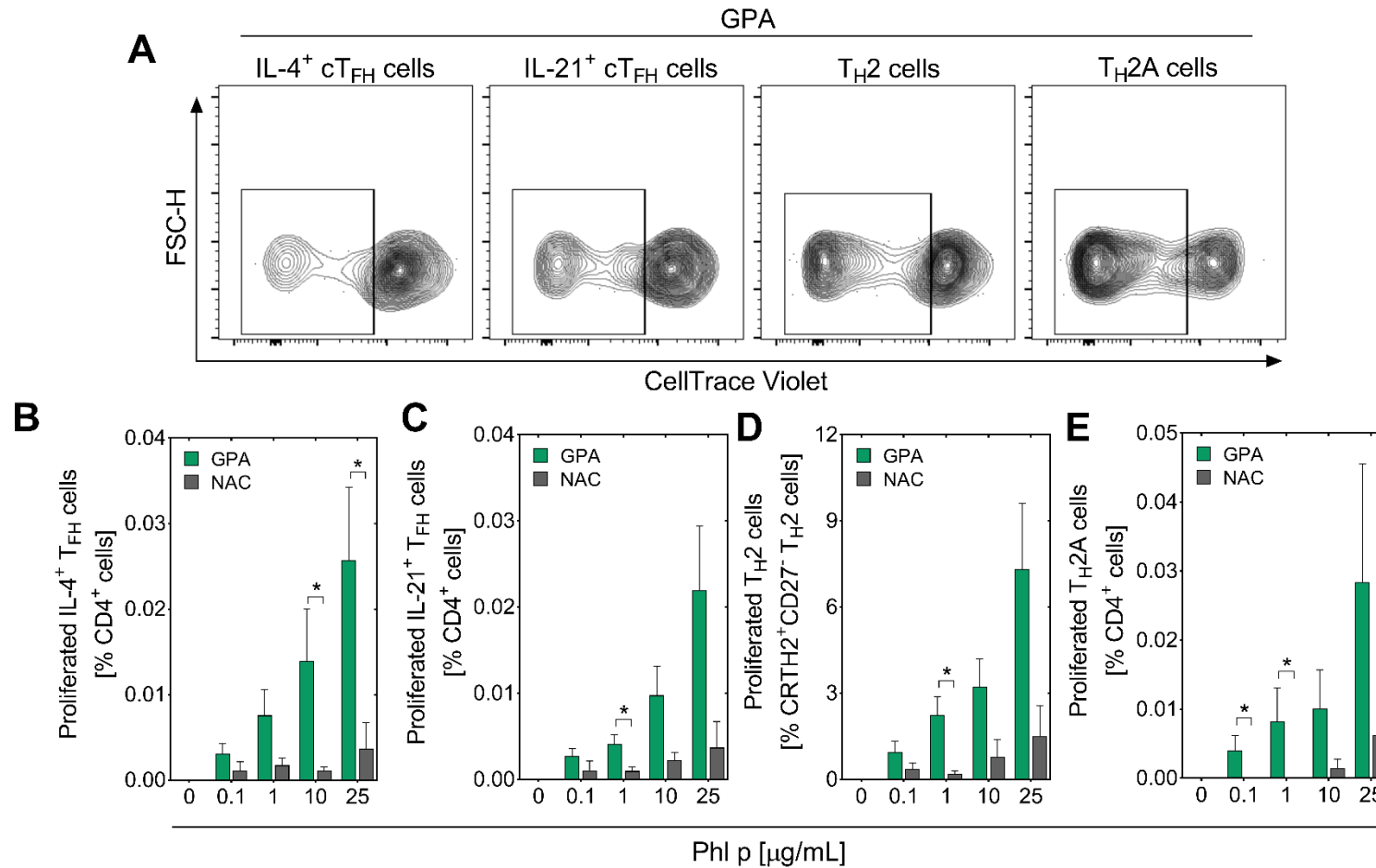


Figure 3.2.3.1 Allergen-driven proliferation of pro-allergic T_{FH}, T_H2 and T_H2A cell subsets. **A** Representative plots illustrating proliferated IL-4⁺ and IL-21⁺ T_{FH}, T_H2 and T_H2A cell subsets following stimulation with Phl p in GPA as assessed by CellTrace Violet. Proportions of proliferated **B** IL-4⁺ T_{FH}; **C** IL-21⁺ T_{FH}; **D** T_H2; and **E** T_H2A cell subsets in response to stimulation with 0, 0.1, 1, 10 and 25 μg/mL of Phl p in GPA (n = 16) and NAC (n = 6). Graphs are represented as a change from baseline (unstimulated, 0 μg/mL). Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic healthy controls; Phl p, *Phleum pratense* (timothy grass). *P < 0.05, Mann-Whitney test.

3.3 Summary

In this chapter, I confirmed that T_{FH} cells were distinct from T_H2 and T_H2A cells using tools that utilise mathematical algorithms, i.e. viSNE, SPADE and FlowSOM. T_{FH} cells expressed IL-21 in addition to T_H2 cytokine, IL-4 which is in agreement with previous studies²⁹¹. The functions of IL-4 in driving antibody production by B cells have been previously established²⁹². However, the dual effect of IL-4 and IL-21 in human have not been fully elucidated. Here, I showed that dual IL-4 and IL-21 results in the downregulation of *BCL6* and upregulation of *AICDA*, indicating that in the presence of both IL-4 and IL-21, B cells have undergone germinal centre reaction and is undergoing class-switching. Furthermore, IL-21 alone and dual IL-4 and IL-21 stimulation results in the upregulation of *PRDM1* and *XBPI*. In the presence of IL-4 alone, only *XBPI* expression was upregulation. Dual IL-4 and IL-21 stimulation for 7 and 14 days also resulted in the highest abundance of plasmablasts and IgE⁺ plasmablasts in naïve and memory B cells. Despite the increase in IgE⁺ plasmablasts and IgE⁺ cells, no significant change was observed in ϵ GLT expression. Interestingly, the proportion of IgG⁺ cells were also higher following stimulation of IL-4, IL-21 and dual IL-4/IL-21. Furthermore, there were higher proportions of proliferated pro-allergic IL-4⁺ T_{FH} , IL-21⁺ T_{FH} , T_H2 and T_H2A cell subsets in patients with SAR compared to NAC. Collectively, this chapter demonstrated that T_{FH} and T_H2 cells are distinct populations and therefore likely to play a distinct role in driving allergic inflammation by interacting with GC B cells.

CHAPTER 4 EPIGENETIC CHANGES IN T FOLLICULAR CELLS FOLLOWING ALLERGEN-SPECIFIC SCIT AND SLIT

4.1 Introduction

The hallmark of SAR is the type I hypersensitivity reaction following exposure to sensitising allergen that is mediated by IgE. Findings from Chapter 3 have revealed that T_{FH} cell cytokines IL-4 and IL-21 play a role in driving B cell development and antibody-producing plasma cell differentiation. These data strongly suggest the potential role of T_{FH} cells in the pathophysiology of SAR. Previous studies have reported the role of T_{FH} cells in perennial allergy, mainly in house dust mite allergy and peanut allergy in a murine study²⁹⁶. However, there has not been a report on the role of T_{FH} cells in SAR.

SAR is an IgE-mediated disease associated with the inflammation of the nasal mucosa resulting upon the exposure to prevalent common aeroallergens²⁹⁷. Near 20% do not respond to conventional treatments and in those patients, grass pollen SCIT and SLIT is effective. Currently, SCIT and SLIT are the only disease-modifying treatments that confer long-term clinical benefits and allergen tolerance^{34–37}, as well as improvement in the quality of life⁸. Immune mechanisms of tolerance induction following SCIT and SLIT are associated with the induction of B regulatory cells, immune deviation from T_H2 to T_H1 cell responses and deletion of T_H2 and T_H2A cells. Moreover, FoxP3⁺ T_{reg} cells and inducible IL-10⁺ and IL-35⁺ T_{reg} cells have been reported to contribute towards tolerance induction following AIT^{87,92,144}. Additionally, B and T_{FH} cell interaction is tightly regulated by T_{FR} cells to prevent the development of autoimmunity and/or allergy. The effect of grass pollen-SCIT and SLIT on T_{FH} and T_{FR} cells that are important in modulating IgE-mediated pro-allergic response remains to be elucidated.

This chapter aims to enumerate pro-allergic T_{FH} and T_{FR} cell subsets in peripheral blood of GPA and patients treated with SCIT and SLIT, in a cross-sectional, controlled, proof-of-concept study of SCIT and SLIT. NAC were recruited as controls. Furthermore, the underlying epigenetic changes in GPA, NAC, SCIT and SLIT is investigated in T_{FH} and T_{FR} cells by ATAC-seq.

4.2 Results

4.2.1 Dysregulation of pro-allergic T_{FH} cells in GPA and restored following allergen-specific SCIT and SLIT

In a cross-sectional study of AIT, NAC, GPA, SCIT- and SLIT-treated patients were recruited (Table 2.3.1.2). NAC did not exhibit any clinical symptoms. RTSS of GPA was significantly higher compared to NAC ($P < 0.0001$). Patients treated with SCIT and SLIT showed lower RTSS compared to untreated GPA ($P < 0.0001$ and 0.0104 , respectively; Figure 4.2.1.1, A), suggestive of clinical efficacy of SCIT and SLIT. Moreover, SCIT exhibited lower RTSS compared to SLIT ($P = 0.0444$).

Activated T_{FH} cells have previously been reported to express ICOS^{298,299}. Here, the proportion of activated ICOS⁺ T_{FH} cells were significantly higher in GPA compared to NAC (2.53-fold, $P = 0.0001$) and were lower in SCIT (1.83-fold, $P = 0.0027$) and SLIT (1.53-fold, $P = 0.0793$) compared to untreated GPA (Figure 4.2.1.1, B). Moreover, IL-4⁺ T_{FH} cells were significantly higher in GPA compared to NAC (1.44-fold, $P < 0.0001$). SCIT- (1.39-fold, $P = 0.0006$) and SLIT- (1.49-fold, $P = 0.0001$) treated patients showed significantly lower IL-4⁺ T_{FH} cells compared to GPA (Figure 4.2.1.1, C). Similarly, IL-21-producing T_{FH} cells were significantly higher in GPA compared to NAC (1.41-fold, $P < 0.0001$) and were significantly lower in SCIT (1.58-fold, $P < 0.0001$) and SLIT (1.50-fold, $P = 0.0051$) compared to untreated GPA (Figure 4.2.1.1, D). Dual IL-4⁺IL-21⁺ T_{FH} cells were also significantly higher in GPA compared to NAC (3.04-fold, $P < 0.0001$) and were significantly lower in SCIT (3.41-fold, $P < 0.0001$) and SLIT (2.31-fold, $P = 0.0031$) compared to GPA (Figure 4.2.1.1, E). No significant differences were observed between SCIT- and SLIT-treated patients.

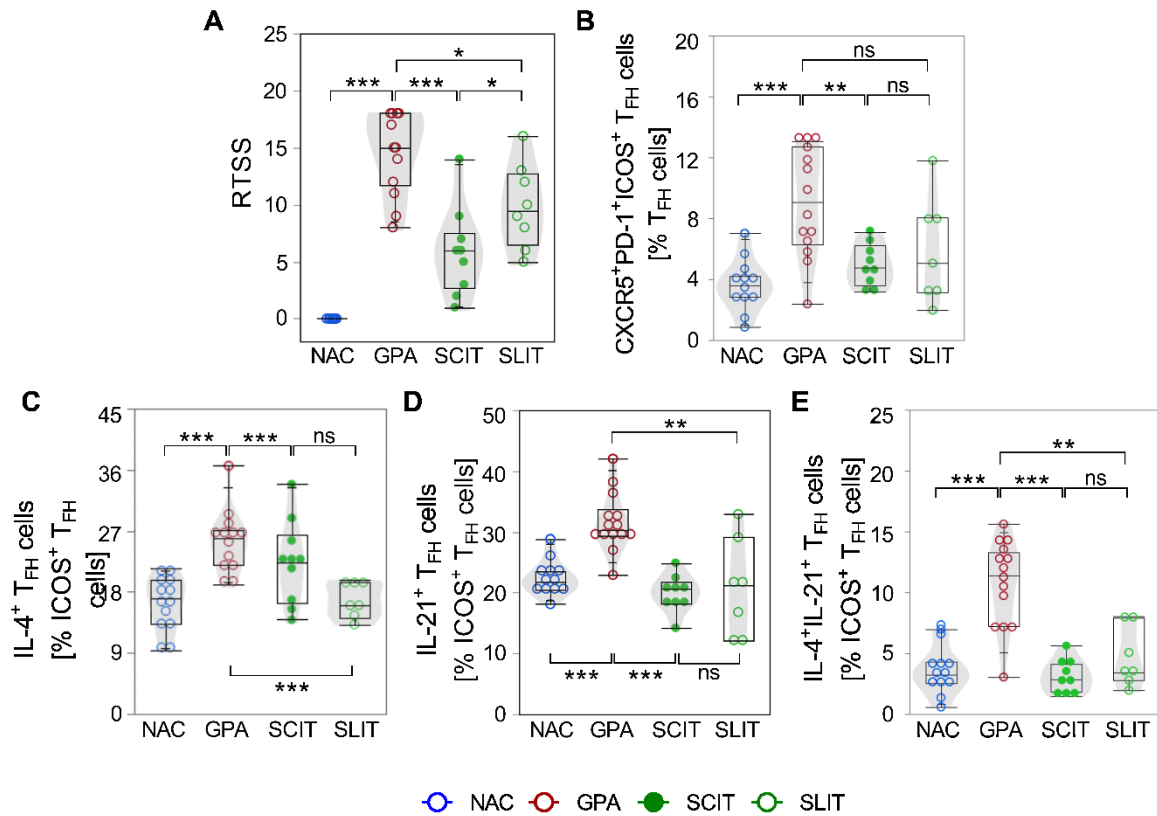


Figure 4.2.1.1 Dysregulation of T_H cells in GPA compared to NAC and SCIT- and SLIT-treated patients. **A** Rhinoconjunctivitis total symptom score (RTSS) of NAC (n = 12), GPA (n = 14), SCIT (n = 10) and SLIT (n = 8). Proportions of **B** activated ICOS⁺ T_H; **C** IL-4⁺ T_H; **D** IL-21⁺ T_H; and **E** dual IL-4⁺IL-21⁺ T_H cells, in NAC, GPA, SCIT and SLIT. Error bars represent SEM. NAC, non-atopic healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients; SLIT, sublingual immunotherapy-treated patients; ns, not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, Mann-Whitney test.

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

IL-4, IL-21 and IL-6 levels were measured in nasal fluid collected from NAC, GPA, SCIT and SLIT groups who underwent intranasal grass pollen allergen challenge. Nasal fluid samples were collected prior to the allergen challenge and 1, 2, 3, 5 and 8 hours post-challenge. A time-course induction of IL-4 was observed in nasal fluid in GPA following nasal allergen challenge but not in NAC, SCIT and SLIT (Figure 4.2.2.1, A). IL-4 levels were significantly higher in GPA compared to NAC at 3 (2.75-fold, $P = 0.0113$), 5 (2.82-fold, $P = 0.0028$) and 8 (2.07-fold, $P = 0.0327$) hours. IL-4 levels were also significantly higher in GPA compared to SCIT at 5 (2.22-fold, $P = 0.0435$) hours and SLIT at 3 (3.13-fold, $P = 0.0152$), 5 (3.28-fold, $P = 0.0111$) and 8 (2.51-fold, $P = 0.0264$) hours (Figure 4.2.2.1, A). IL-21 levels were significantly higher at 3 (2.60-fold, $P = 0.0195$) and 5 (3.21-fold, $P = 0.0078$) hours compared to baseline in GPA. No significant changes were observed in NAC, SCIT and SLIT (Figure 4.2.2.1, B). IL-21 levels were significantly higher in GPA compared to NAC at 2 (4.72-fold, $P = 0.0073$), 3 (8.05-fold, $P < 0.0001$), 5 (10.74-fold, $P < 0.0001$) and 8 (5.87-fold, $P = 0.0052$) hours. Furthermore, IL-21 levels were significantly higher in GPA compared to SCIT at 3 (4.58-fold, $P = 0.0391$) hours and SLIT at 5 (4.23-fold, $P = 0.0041$) hours (Figure 4.2.2.1, B). There was an induction of IL-6 following nasal allergen challenge and was significantly higher at 1 (5.95-fold, $P = 0.0005$), 2 (4.03-fold, $P = 0.0005$), 3 (3.88-fold, $P = 0.0005$) and 8 (1.89-fold, $P = 0.0024$) hours in GPA compared to baseline. No significant differences were observed between the groups (Figure 4.2.2.1, C).

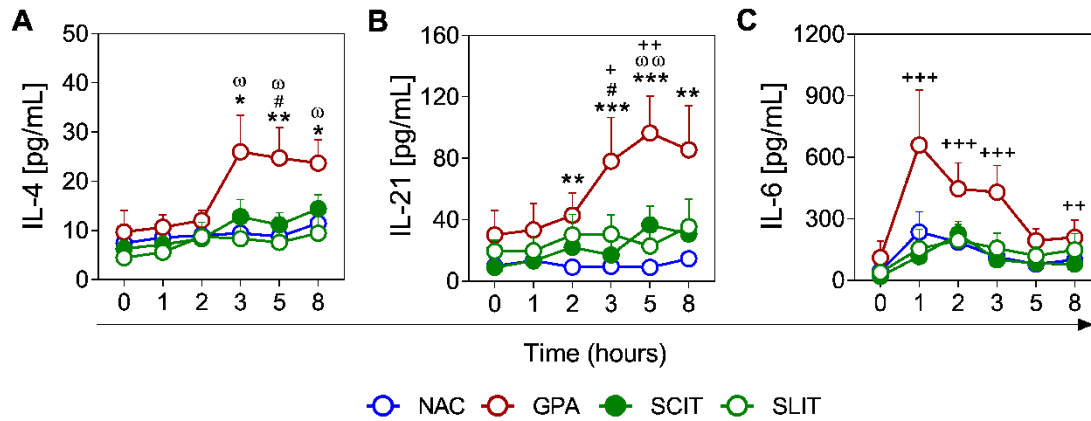


Figure 4.2.2.1 Induction of IL-4, IL-21 and IL-6 in GPA but not NAC, SCIT and SLIT. Time-course measurement of nasal fluid **A** IL-4; **B** IL-21; and **C** IL-6, in NAC (blue; n = 12), GPA (red; n = 14), SCIT (green, closed circle; n = 10) and SLIT (green, open circle; n = 8) following nasal allergen 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 time-points of GPA compared to baseline (0 hours). Error bars represent SEM. NAC, non-atopic healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients; SLIT, sublingual immunotherapy-treated patients. *, #, ω, + $P < 0.05$, **, ωω, ++ $P < 0.01$, ***, +++ $P < 0.001$, Mann-Whitney and Wilcoxon paired signed-rank tests.

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

The underlying epigenetic modifications in sorted T_{FH} cells (Figure 4.2.3.1) of GPA, NAC, SCIT and SLIT were further investigated by assay for transposase-accessible chromatin sequencing (ATAC-seq). The key players in ATAC-seq, Tn5 transposases, bind to and excise accessible chromatin regions and simultaneously ligates DNA sequencing adapters onto the fragments. The resulting tagged DNA fragments were then amplified and sequenced to identify accessible (open) chromatin regions (Figure 4.2.3.2, A). There were 567 regions were observed to be more accessible in GPA compared to NAC, 715 regions compared to SCIT and 604 regions compared to SLIT (Figure 4.2.3.2, B). In total, there were 680 differentially accessible regions between NAC, GPA, SCIT and SLIT ($P < 0.05$; Figure 4.2.3.2, C). Noticeably, GPA showed open chromatin regions that were less accessible in NAC, SCIT and SLIT. Regions of accessible chromatin in SCIT overlapped with open chromatin regions in NAC. Interestingly, SCIT and SLIT have different accessible chromatin regions (Figure 4.2.3.2, C).

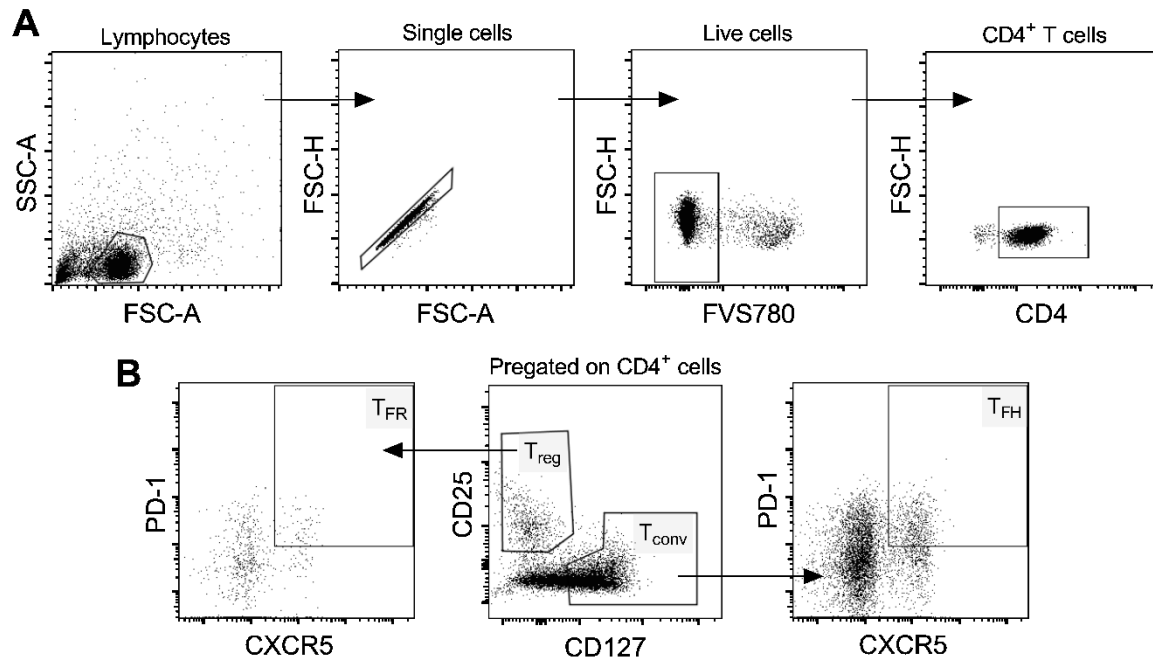


Figure 4.2.3.1 Gating strategy to sort T_{FH} and T_{FR} cells for ATAC-seq. **A** Lymphocytes were gated on the basis of low forward (FSC) and side (SSC) scatter and doublets were excluded. Live cells were discriminated using FVS780 dye and T cells were identified as CD4-expressing cells. **B** CXCR5⁺PD-1⁺ T_{FH} cells were sorted from T_{conv} (CD127⁺CD25^{lo}) cell population and CXCR5⁺PD-1⁺ T_{FR} cells were sorted from T_{reg} (CD127⁻CD25⁺) cell population for ATAC-seq analysis. T_{reg}, T regulatory cells; T_{conv}, T conventional cells.

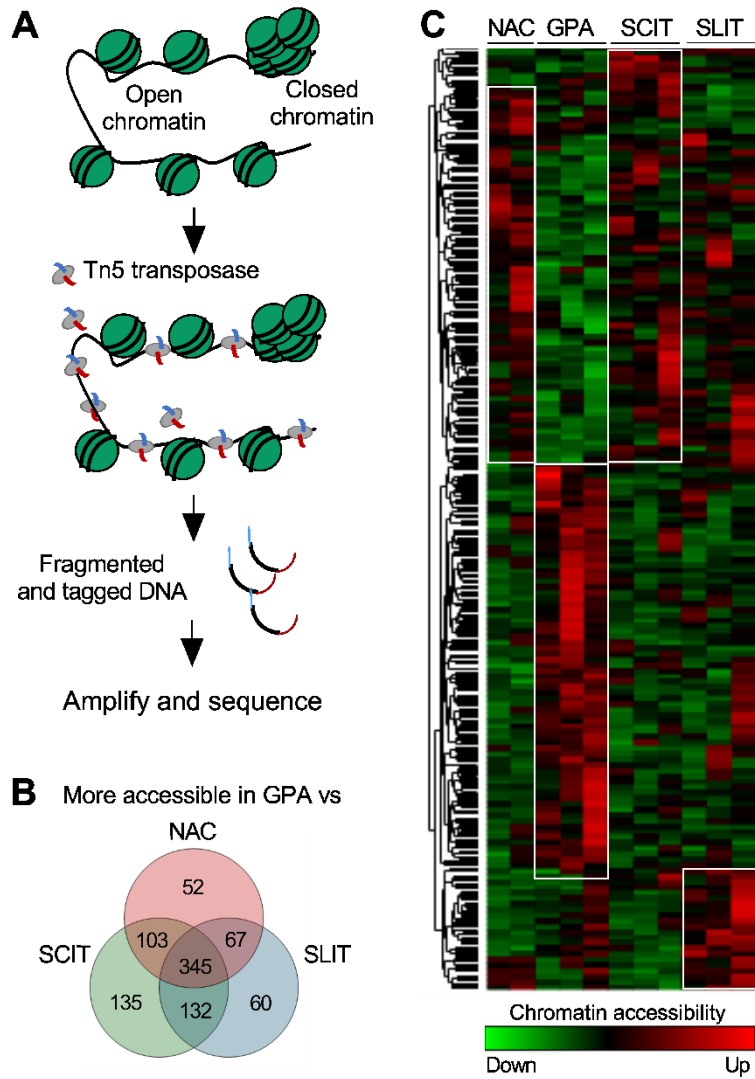
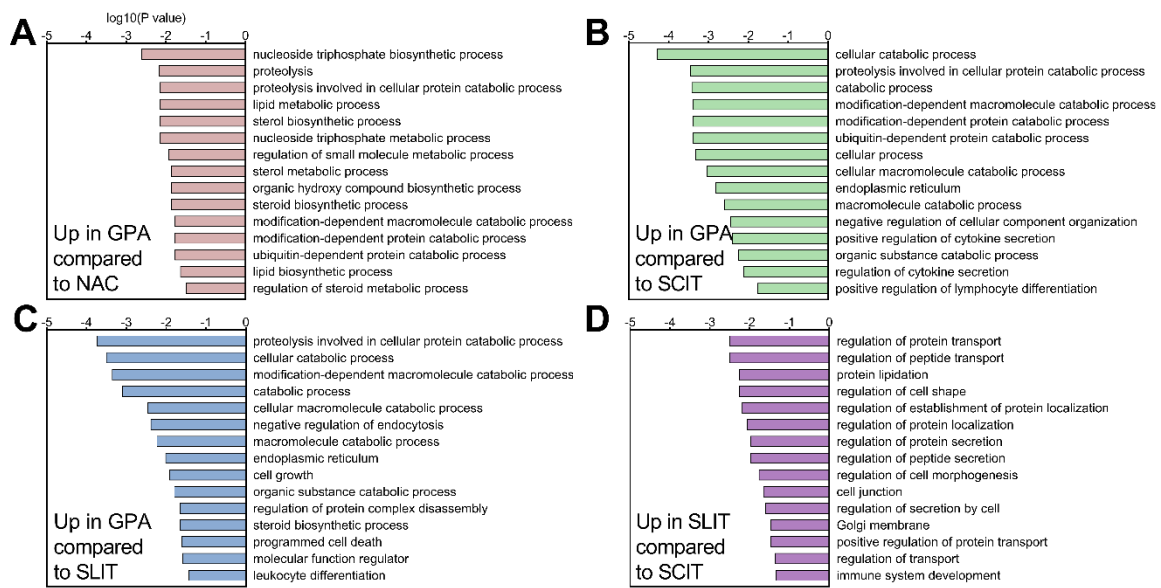


Figure 4.2.3.2 Differentially accessible regions in T_{FH} cells of GPA, NAC, SCIT and SLIT. **A** A schematic of ATAC-seq mechanism to identify accessible chromatin regions using Tn5 transposase. **B** Number of accessible regions in T_{FH} cells of GPA compared to NAC, SCIT and SLIT. **C** Heatmap of the accessible chromatin landscape in NAC ($n = 2$), GPA ($n = 3$), SCIT ($n = 3$) and SLIT ($n = 3$). Colour represents the intensity of chromatin accessibility. Each row represents individual regions and each column represents individual patients.

The nearest gene associated with the differentially accessible regions were identified and gene ontology (GO) analysis was performed. Gene ontology analysis provides information on the functions of genes concerning molecular function, cellular component and biological processes. These include activities resulting from a gene product, localisation of the gene and other biological processes resulting from the molecular activities³⁰⁰. The regions that were more accessible in T_{FH} cells of GPA compared to NAC were associated with genes that belong to the GO terms associated with various metabolic processes (Figure 4.2.3.3, A). 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 (Figure 4.2.3.3, B and C). Accessible regions in SLIT compared to SCIT were mainly associated with the regulation of protein transport and secretion (Figure 4.2.3.3, D).

Pathway enrichment analysis was also performed. Pathway analysis provides information to identify the functional groups and biological context related to a group of genes. Unlike GO analysis, pathway analysis takes into account the dynamics and interactions between the different genes that contribute to a biological pathway. The regions that were more accessible in GPA compared to NAC were associated with various signalling pathways (Figure 4.2.3.3, E). Accessible regions in GPA compared to SCIT and SLIT includes various signalling and metabolic pathways as well as tight junctions (Figure 4.2.3.3, F and G). Pathways that were associated with more accessible regions in SLIT compared to SCIT includes signalling pathways as well as epigenetics processes (Figure 4.2.3.3, H). It was noted that genes associated with the RAS-MAPK signalling pathway, the gene encoding for CACN and G $\beta\gamma$ proteins were more accessible in the T_{FH} cells of GPA compared to SLIT and SCIT. These leads to the activation of Ras and recruitment of Raf and results in the cell growth, proliferation and differentiation³⁰¹ (Figure 4.2.3.4).

GO terms



Pathways

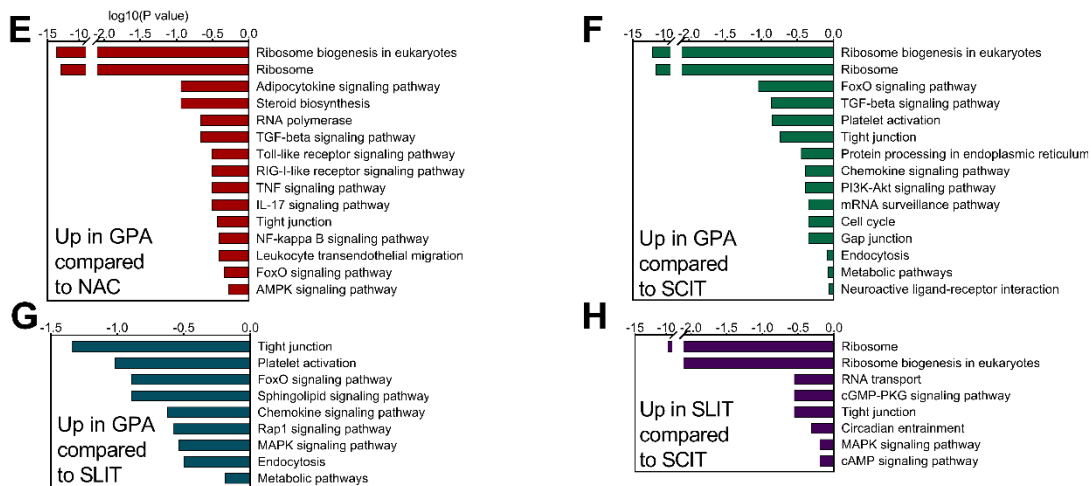


Figure 4.2.3.3 GO and pathway enrichment analysis for significantly accessible regions of T_{FH} cells in GPA, NAC, SCIT and SLIT. GO enrichment analysis of biological processes for significantly accessible regions in **A** GPA compared to NAC; **B** GPA compared to SCIT; **C** GPA compared to SLIT; **D** SLIT compared to SCIT. Pathway enrichment analysis for significantly accessible regions in **E** GPA compared to NAC; **F** GPA compared to SCIT; **G** GPA compared to SLIT; **H** 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.

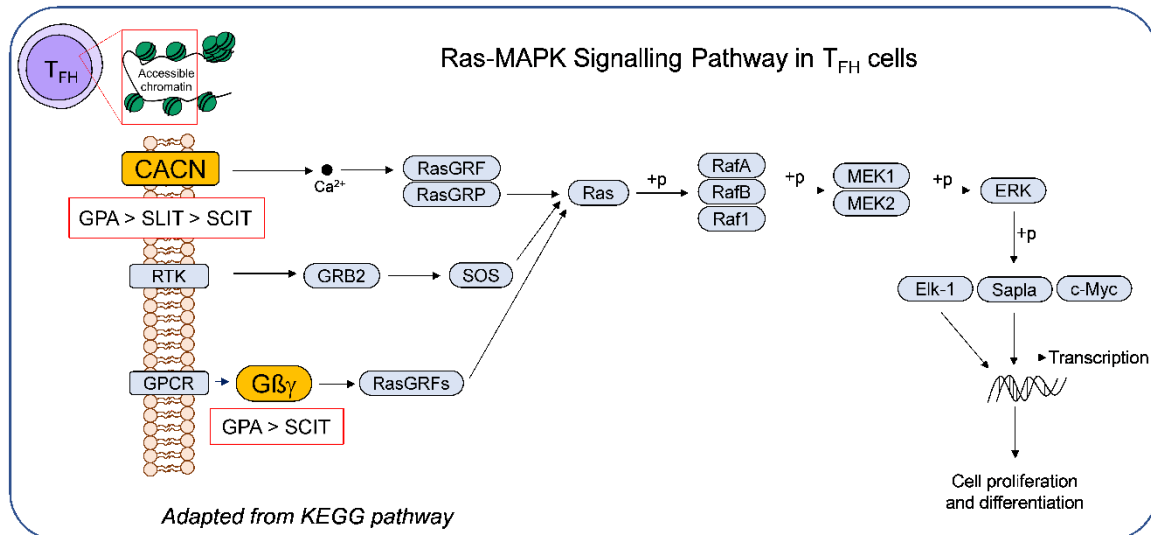


Figure 4.2.3.4 Accessible chromatin regions associated with genes encoding proteins in the Ras-MAPK signalling pathway in T_{FH} cells. Highly accessible gene regions in GPA, SCIT and SLIT, that are associated with Ras and MAPK signalling pathways in T_{FH} cells, indicated in orange. Figure adapted from the KEGG pathway database for MAPK signalling pathway (hsa04010) and Ras signalling pathway (hsa04014).

4.2.4 Induction of T_{FR} and IL-10⁺ T_{FH} cells 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 CTLA-4²²⁸. In the same cohort of patients from the cross-sectional study, suppressive CTLA-4⁺ T_{FR} cells were enumerated. CTLA-4⁺ T_{FR} cells were significantly lower in GPA compared to NAC (1.37-fold, $P = 0.0001$) and were significantly higher in SCIT (1.29-fold, $P = 0.0020$) and SLIT (1.30-fold, $P = 0.0045$) compared to GPA (Figure 4.2.4.1, A). Furthermore, IL-10⁺ T_{FH} cells were significantly lower in GPA compared to NAC (3.42-fold, $P < 0.0001$) and were significantly higher in SCIT (3.20-fold, $P = 0.0003$) and SLIT (3.42-fold, $P < 0.0001$) compared to GPA (Figure 4.2.4.1, B).

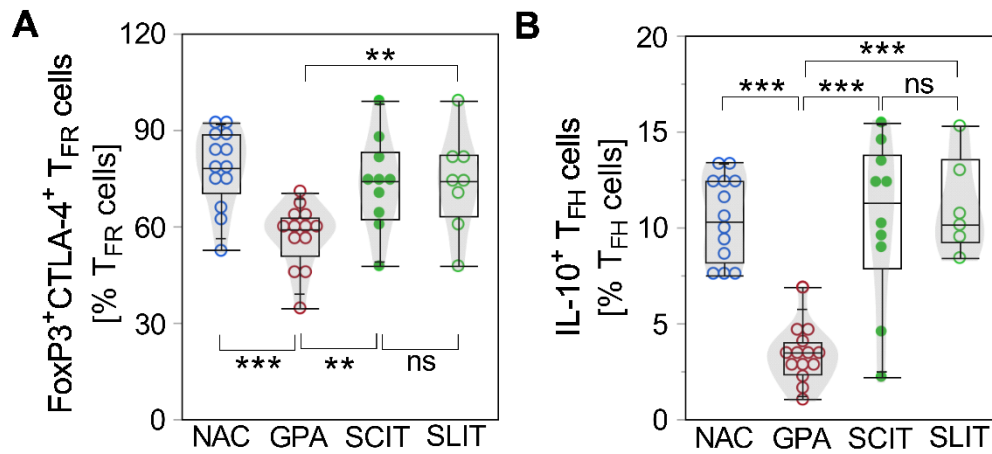


Figure 4.2.4.1 Induction of suppressive T_{FR} and IL-10⁺ T_{TH} cells following SCIT and SLIT. **A** Proportions of CTLA-4⁺FoxP3⁺ T_{FR} cells; and **B** IL-10⁺ T_{TH} cells, in NAC (n = 12), GPA (n = 14), SCIT (n = 10) and SLIT (n = 8). Error bars represent SEM. NAC, non-atopic healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients; SLIT, sublingual immunotherapy-treated patients; ns, not significant. ***P* < 0.01, ****P* < 0.001, Mann-Whitney test.

4.2.5 IL-10 drives plasmablasts differentiation in the presence of IL-4 and IL-21

To assess the effect of IL-10 on B cell differentiation, naïve and memory B cells stimulated with CD40L, IL-4 and IL-21 were cultured in the absence and presence of IL-10. IL-10 stimulation resulted in a significant upregulation in *PRDM1* (day 5, 1.51-fold, $P = 0.0049$; day 6, 1.35-fold, $P = 0.0012$) and *XBP1* (day 5, 1.27-fold, $P = 0.0024$; day 6, 1.24-fold, $P = 0.0002$) expression in the naïve B cells in the presence of both IL-4 and IL-21 (Figure 4.2.5.1, A and B). Although a similar trend was observed in memory B cells, no significant differences were observed (Figure 4.2.5.1, A and B). The proportion of plasmablasts were significantly higher in the presence of IL-10 in memory (day 7, 1.44-fold; day 14, 1.39-fold; both, $P = 0.0039$), but not naïve B cells when stimulated with IL-4 and IL-21 (Figure 4.2.5.1, C). IL-10 stimulation resulted in a significantly higher proportion of IgE⁺ cells in naïve B cells in the presence of both IL-4 and IL-21 (day 7, 1.30-fold, $P = 0.0469$; day 14, 1.18-fold, $P = 0.0273$). In contrast, there was a significant reduction in IgE⁺ cells following stimulation with IL-10, in the presence of IL-4 and IL-21 in memory B cells (day 7, 1.23-fold, $P = 0.0098$), although significance was lost at day 14 (Figure 4.2.5.1, D). Furthermore, IL-10 stimulation resulted in a significantly higher proportion of IgG⁺ cells (day 7, 1.91-fold $P = 0.0195$; day 14, 1.92-fold, $P = 0.0039$) in the presence of IL-4 in memory B cells (Figure 4.2.5.1, E).

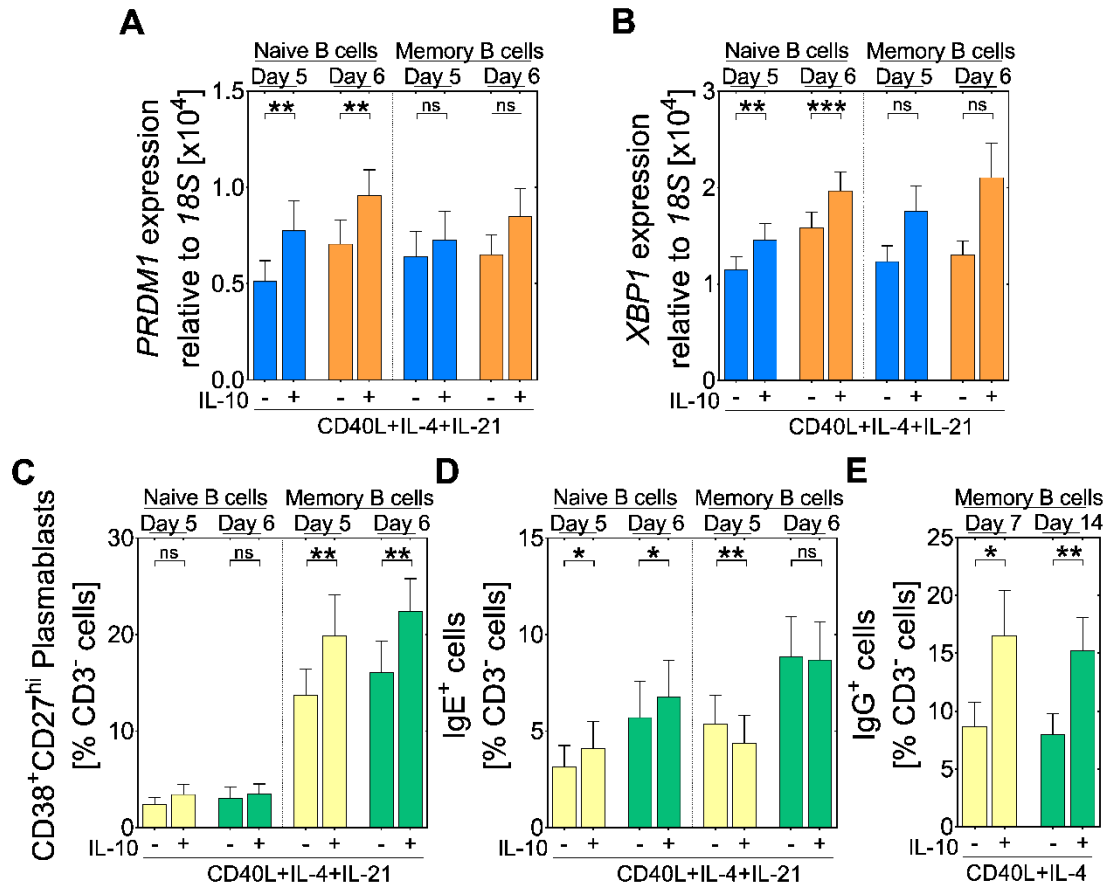


Figure 4.2.5.1 IL-10 drives plasmablasts differentiation in the presence of IL-4 and IL-21. Effect of IL-10 on the relative **A** *PRDM1*; and **B** *XBP1* expressions in naïve and memory B cells stimulated with CD40L, IL-4 and IL-21. **C** Proportions of CD38⁺CD27^{hi} plasmablasts; **D** IgE⁺; and **E** IgG⁺ cells following stimulations of naïve (n = 7) and memory B cell (n = 7). All qPCR data were normalised to 18S. Error bars represent SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, Wilcoxon paired signed-rank test.

4.2.6 Altered epigenetic landscape of T_{FR} cells in GPA, NAC, SCIT and SLIT

ATAC-seq was carried out to identify differentially accessible regions in sorted T_{FR} cells (Figure 4.2.3.1) in GPA and following SCIT and SLIT. There were 141 regions identified that were more accessible in T_{FR} cells in GPA compared to NAC, 184 regions compared to SCIT and 139 regions compared to SLIT (Figure 4.2.6.1, A). 221 differentially accessible regions in T_{FR} cells were observed between NAC, GPA, SCIT and SLIT (Figure 4.2.6.1, B). 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 (Figure 4.2.6.2, A). Various catabolic processes were associated with accessible regions in SCIT (Figure 4.2.6.2, B) and various cellular and biological processes were associated with accessible regions in SLIT (Figure 4.2.6.2, C) 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 (Figure 4.2.6.2, D). Pathway analysis on regions that were more accessible in SCIT compared to GPA was associated with metabolic processes and pathways (Figure 4.2.6.2, E). Furthermore, regions that were more accessible in SCIT compared to SLIT were associated with various signalling and metabolic pathways (Figure 4.2.6.2, F). It was observed that genes associated with the RAS-MAPK signalling pathway, that encodes for receptor tyrosine kinases (RTK) were more accessible in the T_{FR} cells of SCIT compared to SLIT, but not in GPA. These results in the recruitment of guanine nucleotide exchange factors such as SOS that interacts with and activates Ras protein, leading to the recruitment of Raf and results in the cell growth, proliferation and differentiation³⁰¹ (Figure 4.2.6.3).

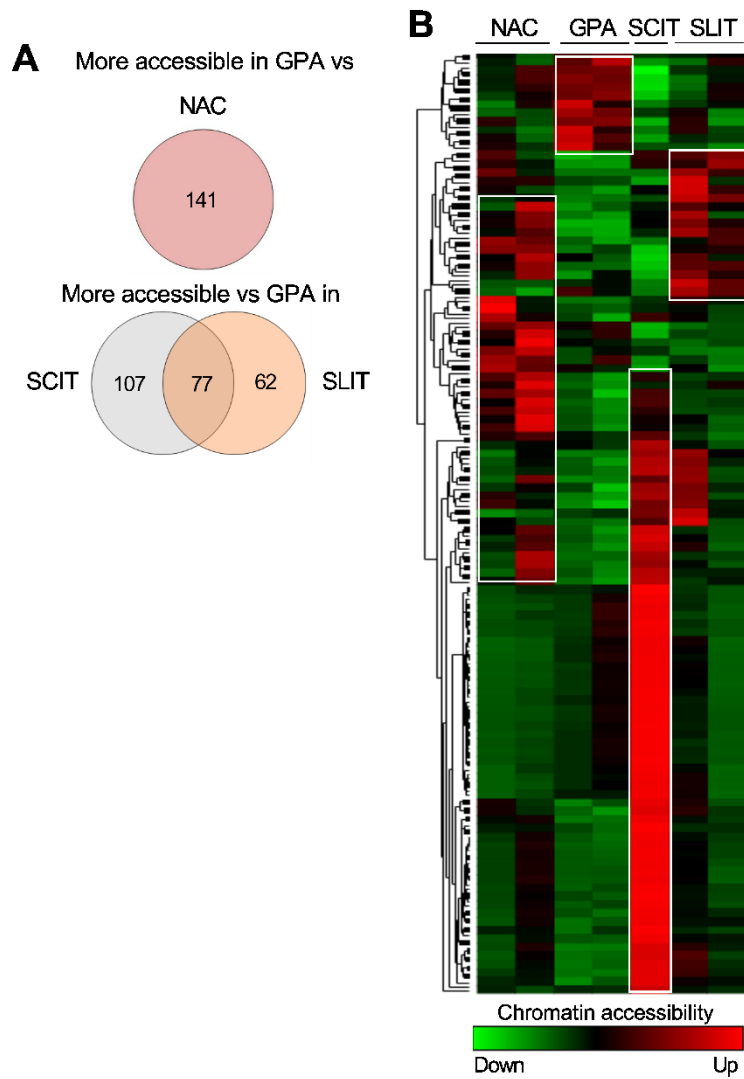


Figure 4.2.6.1 Differentially accessible regions in T_{FR} cells of GPA, NAC, SCIT and SLIT.
A Number of accessible regions in T_{FR} cells of GPA compared to NAC, SCIT and SLIT.
B Heatmap of accessible chromatin landscape in NAC ($n = 2$), GPA ($n = 2$), SCIT ($n = 1$) and SLIT ($n = 2$). Colour represents the intensity of chromatin accessibility. Each row represents individual regions.

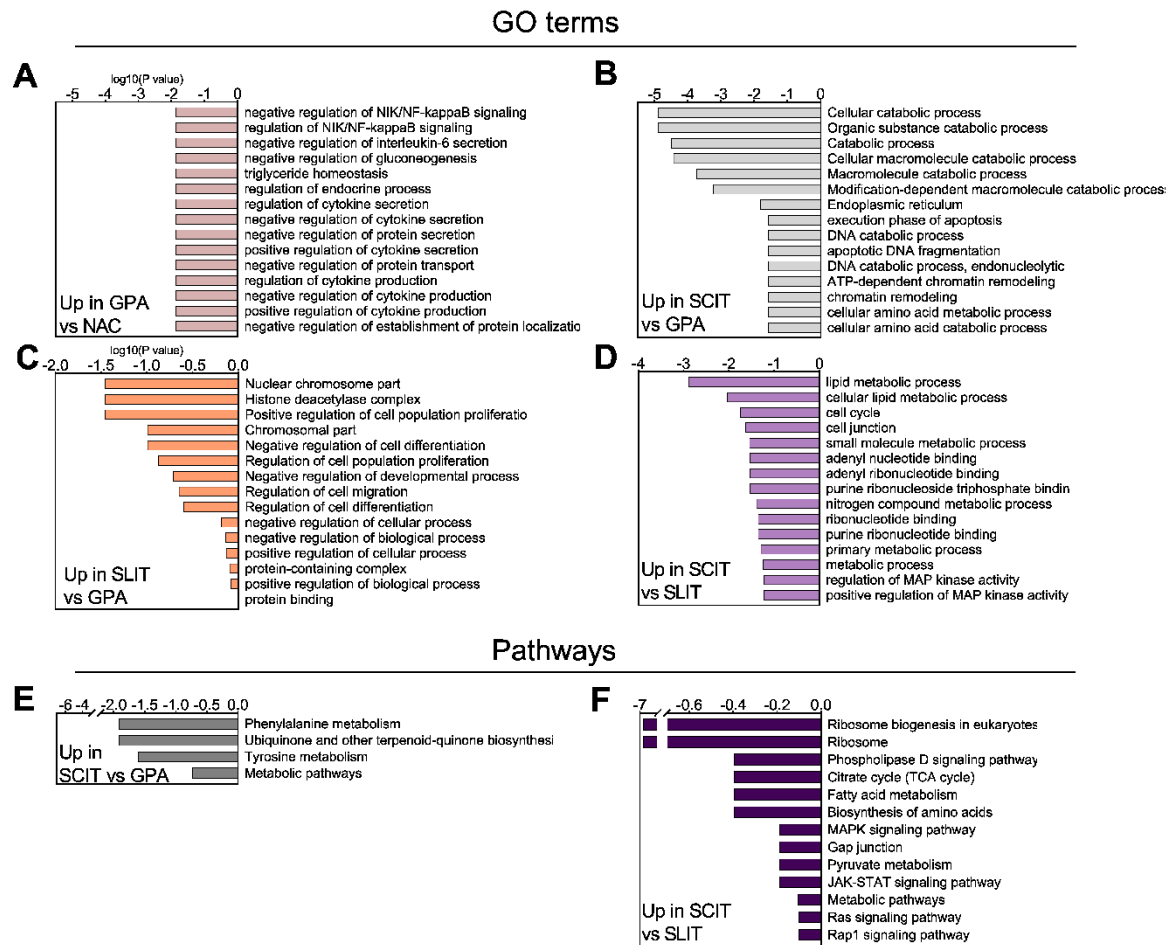


Figure 4.2.6.2 GO and pathway enrichment analysis for significantly accessible regions of T_{FR} cells in GPA, NAC, SCIT and SLIT. GO enrichment analysis of significantly accessible regions in T_{FR} cells of **A** GPA compared to NAC; and **B** SCIT compared to GPA; **C** SLIT compared to GPA; and **D** SCIT compared to SLIT. Pathway enrichment analysis for significantly accessible regions in **E** SCIT compared to GPA; and **F** SCIT compared to SLIT. NAC, non-atopic healthy controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy-treated patients; SLIT, sublingual immunotherapy-treated patients; GO, gene ontology.

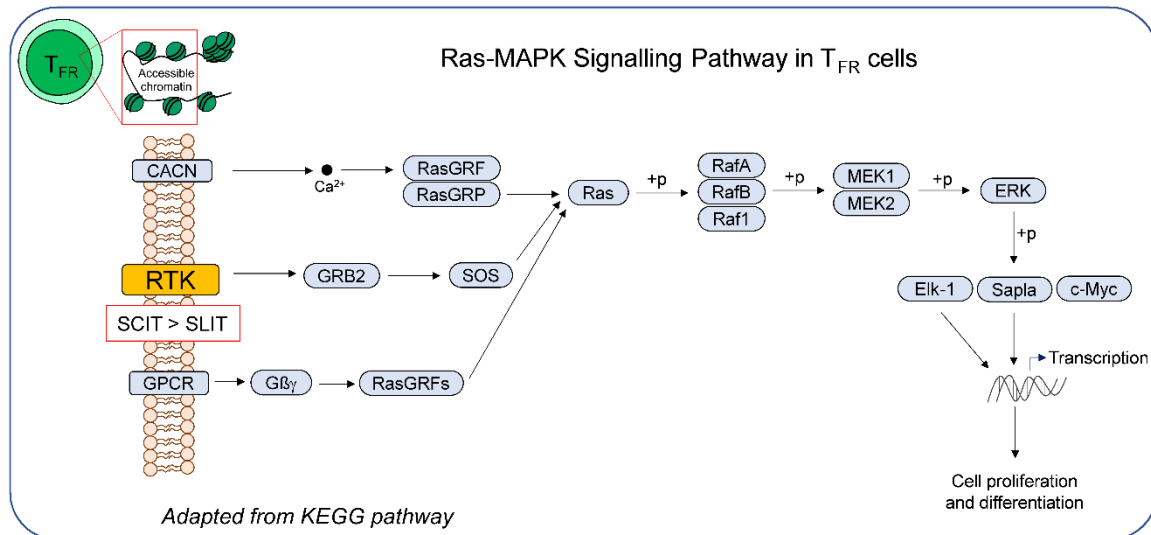


Figure 4.2.6.3 Accessible chromatin regions associated with genes encoding proteins in the Ras-MAPK signalling pathway in T_{FR} cells. Highly accessible gene regions in GPA, SCIT and SLIT, that are associated with Ras and MAPK signalling pathways in T_{FR} cells, indicated in orange. Figure adapted from the KEGG pathway database for MAPK signalling pathway (hsa04010) and Ras signalling pathway (hsa04014).

4.3 Summary

In this chapter, it was demonstrated that both SCIT and SLIT were clinically effective in patients with SAR, which is in corroboration with previous clinical studies¹¹². Pro-allergic, activated and IL-4- and IL-21-producing T_{FH} cells in patients treated with SCIT and SLIT were significantly lower compared to GPA. There was a local induction of IL-4 and IL-21 in GPA following nasal allergen challenge which was inhibited in SCIT or SLIT. Furthermore, IL-6 was induced as early as one hour in GPA, which was attenuated in SCIT and SLIT. Suppressive T_{FR} cells and IL-10-producing T_{FH} cells were induced in SCIT- and SLIT-treated patients, which is in corroboration with previous studies that have shown induction of T_{FR} cells following AIT in HDM allergic patients²⁷¹ and immunotherapy using allergen peptides in GPA³⁰². Furthermore, previously studied mechanism of AIT have been associated with the induction of IL-10⁸⁸ 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 in naïve B cells. Furthermore, IgG⁺ cells were higher in the presence of IL-10 and IL-4 in memory B cells, but not in naïve B cells. It was 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. These include various lipid metabolic pathways, protein transport, cellular communications and cytokine production and secretion. Regions that were more accessible in SCIT were mainly associated with molecular functions, suggesting the role of epigenetic modification in the immune tolerance induction following SCIT. However, there was an aberrant chromatin accessibility pattern observed in the T_{FR} cells of SCIT, which is potentially due to a technical error. As there is only one sample in the group, it was not feasible to determine whether the signal is an anomaly. In summary, T_{FH} and T_{FR} cell subsets were dysregulated in patients with SAR, and were modulated following SCIT and SLIT, which was underscored by changes in the chromatin landscape.

CHAPTER 5 PRE-CLINICAL EVALUATION OF AN ALTERNATIVE IMMUNOTHERAPY APPROACH USING PEPTIDE HYDROLYSATES

5.1 Introduction

AIT is effective in patients with SAR and involves repeated administration of high-dose allergen either SCIT or SLIT⁶⁸. Immune mechanism of tolerance induction following SCIT and SLIT are associated with the induction of protective IgG₄ antibodies and T_{reg} cells, and immune deviation from T_H2 to T_H1 responses and deletion of T_H2 cells. However, SCIT and SLIT have previously been associated with risks of adverse effects, including anaphylaxis in some patients⁴¹. Unlike conventional SCIT and SLIT using purified whole allergen extracts³⁰³, peptide immunotherapy has been proposed to be a safer alternative approach. Peptide immunotherapy involves the administration of short, synthetic peptides, as opposed to using the whole allergen, but is still representative of the whole allergen. Due to their short and linear structure, they are unable to bind to IgE and cross-link with FcεRI on the surface of basophils and mast cells and therefore are unable to elicit an allergic response through the release of mediators such as histamine, thus reduces the likelihood of anaphylaxis³⁰⁴.

This chapter aims to evaluate the allergenicity of four linearised grass pollen peptide candidates ranging from 1 to 10 kDa in size as a potential treatment for SAR. The peptides were generated by hydrolysis of purified allergen extract, resulting in highly purified peptidic fragments of ryegrass (*Lolium perenne* peptides, LPP). Allergenicity of LPP candidates compared to unmodified grass pollen native protein will be assessed using *ex vivo* basophil activation test. The effect of LPP candidates and unmodified grass pollen native protein on IL-4⁺ and IL-21⁺ T_{FH} cells and T_H2 and T_H2A cell responses will also be investigated.

5.2 Results

5.2.1 Basophil hyporesponsiveness in response to stimulation with peptide hydrolysates

The allergenicity of LPP candidates (peptide 1, 2, 3 and 4) compared to grass pollen native protein was firstly investigated by performing *ex vivo* basophil activation test. Basophils are the surrogate cells for mast cells in the peripheral blood and have been used as a biomarker for FcεRI-mediated allergic response. The activation of basophils was investigated through the expression of CD63 and CD203c (Figure 5.2.1.1). A concentration-dependent increase in CD63⁺ basophils was observed in response to stimulation with native protein and LPP candidates in GPA (Figure 5.2.1.2, A and B). When compared to native protein, a rightward shift in the curve was observed following stimulation with LPP candidates. The proportion of CD63⁺ basophils were significantly higher when stimulated with native protein compared to the LPP candidates at 1, 10, 33, 100 and 330 ng/mL (Table 5.2.1.1) in GPA (Figure 5.2.1.2, B). No significant differences were observed between the LPP candidates. No responses were observed in NAC (Figure 5.2.1.2, C; Table 5.2.1.1).

The concentration of native protein or LPP candidates required to elicit 50% of the maximal basophil activation (EC₅₀) was estimated by performing a nonlinear regression curve fit on a normalised basophil activation to the maximal response and with a standard slope (Hill slope = 1.0). All LPP candidates have a reduced potency to elicit CD63⁺ basophil activation when compared to native protein, as represented by a rightward shift in the curve compared to the native protein (Figure 5.2.1.2, D, left). Peptide 1 was observed to be the least potent, followed by peptide 2, 4 and 3, which was significant when compared to the native protein (peptides 1 and 2, $P < 0.0001$; peptide 3, $P = 0.0003$; peptide 4, $P = 0.0004$; Figure 5.2.1.2, D; Table 5.2.1.2).

Similarly, a concentration-dependent increase in CD203c^{bright} basophils was also observed following stimulation with native protein and LPP candidates in GPA (Figure 5.2.1.2, E and F) but not NAC (Figure 5.2.1.2, E and G). A rightward shift in the curve was observed following stimulation with LPP candidates compared to native protein. The proportion of activated CD203c^{bright} basophils were significantly higher at 1, 10, 33, 100 and 330 ng/mL in GPA when stimulated with native protein compared to the LPP candidates (Figure 5.2.1.2, F; Table 5.2.1.3). No significant differences were observed between the LPP candidates. No responses were observed in NAC (Figure 5.2.1.2, G; Table 5.2.1.3).

EC₅₀ analysis of CD203c^{bright} basophils was also performed. A rightward shift in the EC₅₀ curve was observed following stimulation with LPP candidates compared to native protein, indicating the lower potency of LPP candidates compared to native protein in eliciting CD203c^{bright} basophil response (Figure 5.2.1.2, H, left). Peptide 2 was the least potent, followed by peptide 1, 3 and 4, which was significant when compared to native protein (peptides 1 and 2, $P < 0.0001$; peptides 3 and 4, $P = 0.0003$; Figure 5.2.1.2, H; Table 5.2.1.4).

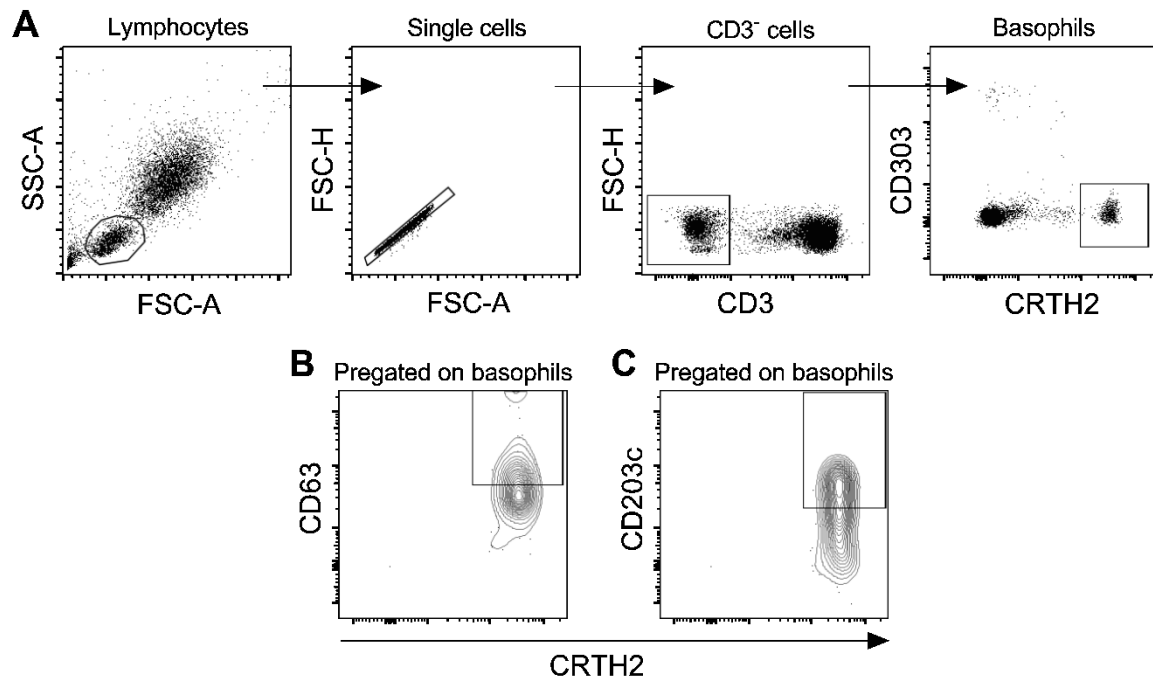


Figure 5.2.1.1 Gating strategy to identify activated basophils. **A** Lymphocytes were gated on the basis of low forward (FSC) and side (SSC) scatter and doublets were excluded. Basophils were defined as CD3⁻CD303⁻CRTH2⁺ cells. Basophil activation was assessed by the expression of **B** CD63; and **C** CD203c, within the basophil population.

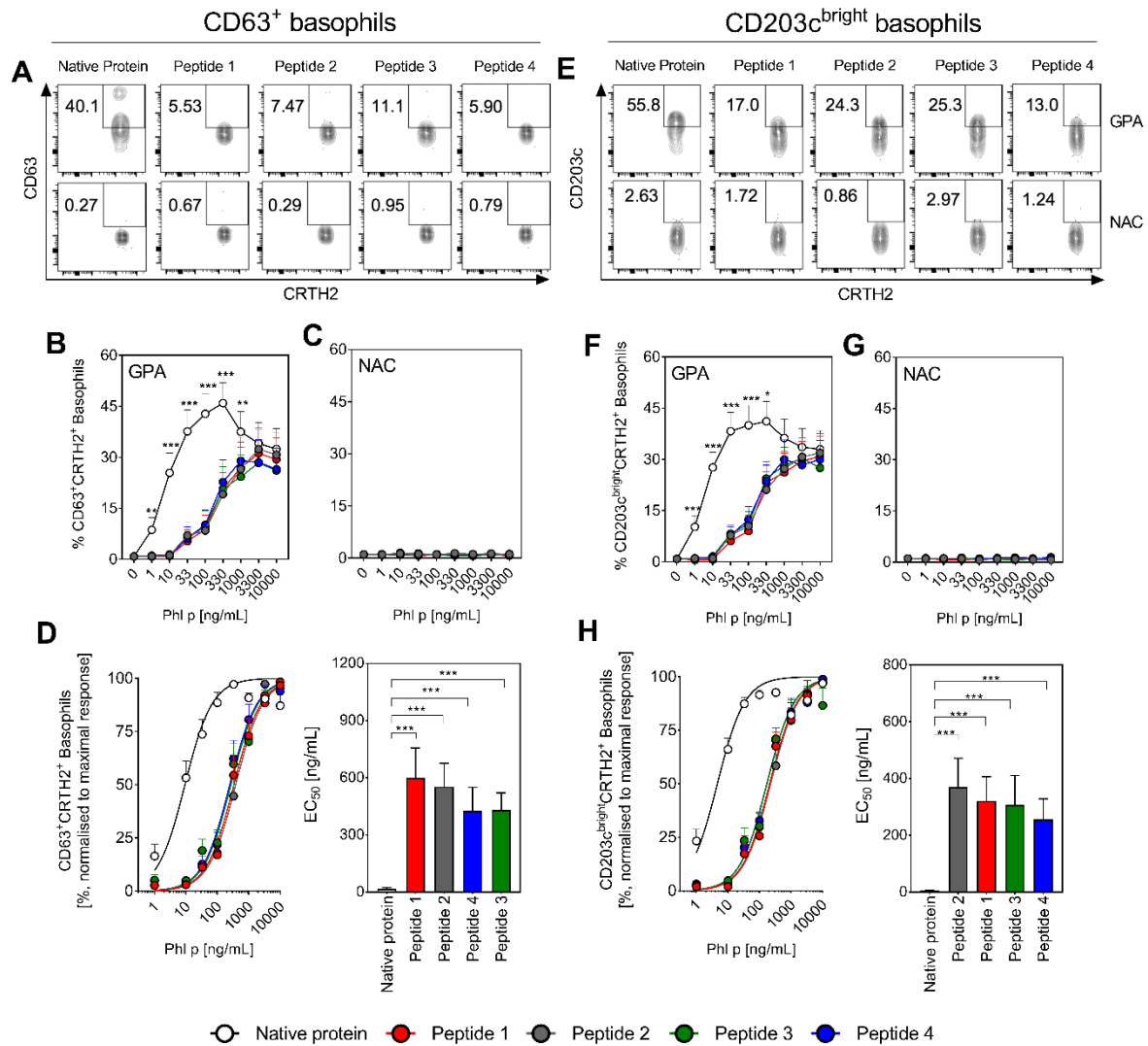


Figure 5.2.1.2 Basophil hyporesponsiveness following stimulation with LPP candidates. **A** Representative plots illustrating basophil activation (CD63⁺CRTH2⁺) elicited by 100 ng/mL of the native protein and LPP candidates in GPA (top panels) and NAC (bottom panels). Dose-response of CD63⁺ basophils in **B** GPA (n = 16); and **C** NAC (n = 6), following stimulation with various concentrations of the native protein and LPP candidates. **D** EC₅₀ analysis of GPA as represented as dose-response curves (left panel) and bar charts (right panel). **E-H** Similarly, for CD203c^{bright}CRTH2⁺ basophils. Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic healthy controls. ***P* < 0.01, ****P* < 0.001, Mann-Whitney test and Kruskal-Wallis test with Dunn's multiple comparison test for EC₅₀ analysis versus native protein.

Table 5.2.1.1 Statistical analysis of CD63⁺ basophil response following stimulation with native protein and LPP candidates in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					P value GPA vs NAC
		n	mean	SEM	P value vs		n	mean	SEM	P value vs		
					Native protein	Baseline				Native protein	Baseline	
0 (Baseline)	Native protein	16	0.88	0.08	NA	NA	6	1.04	0.19	NA	NA	0.3765
	Peptide 1	16	0.88	0.08	>0.9999	NA	6	1.04	0.19	>0.9999	NA	0.3765
	Peptide 2	16	0.88	0.08	>0.9999	NA	6	1.04	0.19	>0.9999	NA	0.3765
	Peptide 3	16	0.88	0.08	>0.9999	NA	6	1.04	0.19	>0.9999	NA	0.3765
	Peptide 4	16	0.88	0.08	>0.9999	NA	6	1.04	0.19	>0.9999	NA	0.3765
1	Native protein	16	8.69	3.56	NA	0.0007	6	1.13	0.32	NA	>0.9999	0.0387
	Peptide 1	13	0.80	0.16	0.0010	0.4143	6	0.87	0.26	0.5152	0.5625	0.7161
	Peptide 2	16	0.94	0.32	0.0002	0.2461	6	1.04	0.27	0.9372	>0.9999	0.4397
	Peptide 3	16	0.98	0.36	0.0002	0.1261	6	1.09	0.19	0.8182	0.6875	0.1192
	Peptide 4	16	1.13	0.30	0.0019	0.8102	6	1.16	0.26	>0.9999	0.8438	0.4402
10	Native protein	16	25.43	5.73	NA	<0.0001	6	0.94	0.29	NA	>0.9999	0.0007
	Peptide 1	16	0.99	0.25	<0.0001	0.8493	6	1.51	0.45	0.3290	0.5625	0.2909
	Peptide 2	16	1.20	0.33	<0.0001	0.8108	6	1.28	0.34	0.4848	0.6875	0.6267
	Peptide 3	15	1.48	0.63	<0.0001	0.4293	6	1.14	0.23	0.6991	>0.9999	0.2927
	Peptide 4	16	1.24	0.43	<0.0001	0.6968	6	1.23	0.24	0.5887	0.4375	0.2546
33	Native protein	16	37.65	6.31	NA	<0.0001	6	0.81	0.16	NA	0.3125	<0.0001
	Peptide 1	16	5.38	2.99	<0.0001	0.0149	6	0.74	0.22	0.9372	0.3125	0.0725
	Peptide 2	16	7.01	4.14	<0.0001	0.2068	6	1.21	0.27	0.1797	0.4375	0.9285
	Peptide 3	16	6.19	2.54	<0.0001	0.0049	6	1.35	0.24	0.0931	0.2188	0.4402
	Peptide 4	16	6.22	3.32	<0.0001	0.0558	6	1.10	0.18	0.3939	0.6563	0.5794
100	Native protein	16	42.82	5.98	NA	<0.0001	6	1.11	0.25	NA	>0.9999	<0.0001
	Peptide 1	16	8.78	4.28	<0.0001	0.1262	6	1.02	0.30	0.8182	0.8438	0.6028
	Peptide 2	16	8.45	3.70	<0.0001	0.0040	6	1.05	0.28	0.9372	>0.9999	0.1835
	Peptide 3	16	9.77	4.44	<0.0001	0.0125	6	0.92	0.19	0.5887	0.6250	0.1297
	Peptide 4	16	10.16	4.39	<0.0001	0.2468	6	1.13	0.32	0.8182	0.8438	0.4490
330	Native protein	16	45.94	5.95	NA	<0.0001	6	0.82	0.29	NA	0.4688	<0.0001
	Peptide 1	16	20.81	6.36	0.0043	0.0003	6	0.79	0.16	0.8182	0.2188	<0.0001
	Peptide 2	16	19.15	6.39	0.0007	<0.0001	6	1.37	0.40	0.4177	0.6875	0.0032
	Peptide 3	15	20.73	6.46	0.0009	0.0007	6	0.80	0.21	0.9805	0.0938	0.0059
	Peptide 4	16	22.71	6.52	0.0082	0.0002	6	0.91	0.24	0.8593	0.8438	0.0016

PhI p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					<i>P</i> value GPA vs NAC
		n	mean	SEM	<i>P</i> value vs		n	mean	SEM	<i>P</i> value vs		
					Native protein	Baseline				Native protein	Baseline	
1000	Native protein	16	37.53	5.95	NA	<0.0001	6	0.94	0.17	NA	0.8438	<0.0001
	Peptide 1	14	26.88	7.66	0.1628	0.0002	6	1.06	0.22	>0.9999	0.8125	0.0009
	Peptide 2	16	26.57	6.40	0.1458	<0.0001	6	1.13	0.26	0.5887	>0.9999	0.0032
	Peptide 3	16	24.33	6.48	0.0656	0.0002	6	0.66	0.20	0.3095	0.1563	0.0008
	Peptide 4	16	28.97	7.11	0.1930	<0.0001	6	0.84	0.25	0.5887	0.4375	0.0023
3300	Native protein	16	34.08	6.22	NA	<0.0001	6	1.09	0.46	NA	>0.9999	<0.0001
	Peptide 1	16	31.17	6.97	0.5330	<0.0001	6	1.09	0.26	0.5887	>0.9999	0.0003
	Peptide 2	16	32.41	6.38	0.9186	<0.0001	6	1.28	0.22	0.3939	>0.9999	0.0036
	Peptide 3	16	28.34	6.67	0.3657	<0.0001	6	1.07	0.20	0.4848	>0.9999	0.0011
	Peptide 4	16	28.57	6.65	0.4850	0.0002	6	1.06	0.38	0.9372	>0.9999	0.0011
10000	Native protein	16	32.47	5.96	NA	<0.0001	6	0.83	0.17	NA	0.5625	<0.0001
	Peptide 1	16	29.39	6.41	0.6894	<0.0001	6	0.71	0.32	0.9394	0.5625	0.0005
	Peptide 2	16	30.75	6.38	0.8019	<0.0001	6	1.16	0.42	0.8528	0.8438	<0.0001
	Peptide 3	16	26.10	6.23	0.3180	<0.0001	6	1.03	0.18	0.3095	>0.9999	0.0011
	Peptide 4	16	26.52	6.23	0.4967	<0.0001	6	1.17	0.33	0.5887	0.5625	0.0002

GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs native protein and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significant values.

Table 5.2.1.2 Native protein and LPP candidates EC₅₀ to elicit CD63⁺ basophil activation in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	16.79 ± 6.65	1.00
Peptide 1	599.00 ± 156.70***	35.68
Peptide 2	553.00 ± 122.40***	32.94
Peptide 3	445.50 ± 100.50***	26.53
Peptide 4	461.20 ± 139.50***	27.47

Statistical significance versus native protein. *** $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test.

Table 5.2.1.3 Statistical analysis of CD203c^{bright} basophils response following stimulation with native protein and LPP candidates in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					P value GPA vs NAC
		n	mean	SEM	P value vs		n	mean	SEM	P value vs		
					Native protein	Baseline				Native protein	Baseline	
0 (Baseline)	Native protein	16	0.91	0.11	NA	NA	6	1.10	0.17	NA	NA	0.3384
	Peptide 1	16	0.91	0.11	>0.9999	NA	6	1.10	0.17	>0.9999	NA	0.3384
	Peptide 2	16	0.91	0.11	>0.9999	NA	6	1.10	0.17	>0.9999	NA	0.3384
	Peptide 3	16	0.91	0.11	>0.9999	NA	6	1.10	0.17	>0.9999	NA	0.3384
	Peptide 4	16	0.91	0.11	>0.9999	NA	6	1.10	0.17	>0.9999	NA	0.3384
1	Native protein	16	10.29	3.25	NA	0.0002	6	1.18	0.33	NA	0.5625	0.0077
	Peptide 1	16	0.62	0.18	<0.0001	0.0435	6	0.93	0.46	0.4177	0.4375	0.8721
	Peptide 2	16	1.09	0.26	0.0001	0.8110	6	1.11	0.37	0.9805	>0.9999	0.8433
	Peptide 3	16	0.75	0.13	<0.0001	0.2362	6	0.98	0.14	0.6190	0.4375	0.3023
	Peptide 4	16	1.08	0.22	0.0002	0.7333	6	0.99	0.23	0.6991	0.8438	0.9017
10	Native protein	16	27.67	4.43	NA	<0.0001	6	0.84	0.13	NA	0.5625	<0.0001
	Peptide 1	16	0.66	0.18	<0.0001	0.1970	6	0.73	0.22	0.6991	0.0625	0.5521
	Peptide 2	16	1.04	0.17	<0.0001	0.5532	6	1.10	0.31	0.9372	>0.9999	0.9575
	Peptide 3	16	1.62	0.41	<0.0001	0.2688	6	1.15	0.21	0.0649	>0.9999	0.7326
	Peptide 4	16	1.41	0.31	<0.0001	0.4871	6	1.14	0.35	0.4848	0.8438	0.8162
33	Native protein	16	38.25	5.56	NA	<0.0001	6	1.29	0.29	NA	0.8438	0.0003
	Peptide 1	16	6.10	2.31	<0.0001	0.0006	6	0.72	0.18	0.2403	0.2188	0.0142
	Peptide 2	16	7.76	3.01	<0.0001	0.0109	6	0.73	0.15	0.1320	0.2500	0.0163
	Peptide 3	16	8.26	2.47	0.0001	0.0017	6	1.05	0.21	0.6991	>0.9999	0.0205
	Peptide 4	16	7.77	2.56	<0.0001	0.0003	6	1.03	0.23	0.6991	0.8438	0.0163
100	Native protein	16	39.99	5.90	NA	<0.0001	6	0.92	0.24	NA	0.6250	<0.0001
	Peptide 1	16	9.06	3.41	<0.0001	0.0040	6	0.89	0.37	0.6991	0.6875	0.0801
	Peptide 2	16	10.51	3.18	0.0002	0.0002	6	1.04	0.28	0.9372	0.8438	0.0099
	Peptide 3	16	11.16	3.51	0.0004	0.0003	6	0.89	0.32	>0.9999	0.6875	0.0125
	Peptide 4	16	12.39	3.78	0.0003	0.0005	6	0.68	0.17	0.6991	0.2188	0.0163
330	Native protein	16	41.14	5.90	NA	<0.0001	6	1.22	0.33	NA	>0.9999	0.0001
	Peptide 1	16	23.21	4.82	0.0184	0.0003	6	0.82	0.15	0.3939	0.0313	0.0164
	Peptide 2	16	21.13	5.21	0.0228	<0.0001	6	0.93	0.26	0.8182	0.9063	0.0032
	Peptide 3	15	24.41	4.92	0.0437	0.0004	6	0.86	0.29	0.3939	0.6250	0.0067
	Peptide 4	16	23.67	4.68	0.0378	0.0003	6	0.98	0.27	0.6991	0.8438	0.0111

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					<i>P</i> value GPA vs NAC
		n	mean	SEM	<i>P</i> value vs		n	mean	SEM	<i>P</i> value vs		
					Native protein	Baseline				Native protein	Baseline	
1000	Native protein	16	36.26	5.61	NA	<0.0001	6	1.35	0.35	NA	0.6875	<0.0001
	Peptide 1	14	26.21	5.66	0.1759	0.0006	6	0.99	0.22	0.6991	0.6875	0.0083
	Peptide 2	16	27.29	5.07	0.1682	0.0002	6	0.97	0.21	0.6991	0.4375	0.0100
	Peptide 3	16	28.25	5.34	0.3180	0.0002	6	0.66	0.22	0.1320	0.0938	0.0018
	Peptide 4	16	29.99	5.73	0.4394	<0.0001	6	0.93	0.26	0.2403	0.8438	0.0043
3300	Native protein	16	33.63	5.35	NA	<0.0001	6	1.13	0.19	NA	>0.9999	<0.0001
	Peptide 1	16	29.50	5.76	0.6090	<0.0001	6	1.12	0.42	0.6991	0.8438	0.0011
	Peptide 2	16	30.74	5.53	0.6353	<0.0001	6	0.94	0.38	0.6147	0.5625	0.0001
	Peptide 3	16	30.33	5.62	0.4846	<0.0001	6	0.91	0.20	0.5887	0.4375	0.0032
	Peptide 4	16	28.36	5.11	0.4176	<0.0001	6	0.75	0.35	0.2403	0.3438	0.0011
10000	Native protein	16	32.98	5.42	NA	<0.0001	6	0.93	0.17	NA	0.5625	<0.0001
	Peptide 1	16	30.92	5.82	0.6894	<0.0001	6	1.02	0.30	0.8182	>0.9999	0.0002
	Peptide 2	16	31.93	5.66	0.8308	<0.0001	6	1.41	0.35	0.3095	0.4375	<0.0001
	Peptide 3	16	27.52	5.30	0.3859	<0.0001	6	1.17	0.23	0.4848	0.8750	<0.0001
	Peptide 4	16	30.02	5.49	0.7450	<0.0001	6	0.70	0.15	0.3939	0.1563	<0.0001

GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs native protein and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significant values.

Table 5.2.1.4 Native protein and LPP candidates EC₅₀ to elicit CD203c^{bright} basophil activation in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	5.79 ± 1.23	1.00
Peptide 1	321.20 ± 86.60***	55.47
Peptide 2	369.80 ± 101.60***	63.91
Peptide 3	307.70 ± 103.20***	53.14
Peptide 4	256.4 ± 71.59***	44.28

Statistical significance versus native protein. *** $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test.

5.2.2 Histamine-releasing basophil hyporesponsiveness in response to stimulation with peptide hydrolysates

Following basophil activation, the release of intracellular histamine was quantified using a fluorochrome-conjugated DAO enzyme. DAO binds to intracellular histamine within the vesicles in basophils, and therefore, there will be a loss of DAO expression following histamine release (Figure 5.2.2.1). DAO has previously been shown to be a gold standard tool for the measurement of intracellular histamine release at a single-cell level¹⁵³.

A concentration-dependent increase in DAO⁺CD63⁺ basophil histamine release was observed following stimulation with native protein and LPP candidates in GPA (Figure 5.2.2.2, A and B) but not NAC (Figure 5.2.2.2, A and C). A rightward shift in the curve was observed following stimulation with LPP candidates compared to native protein. The proportion of histamine release by activated (DAO⁺CD63⁺) basophils was significantly higher at 10, 33, 100 and 330 ng/mL following stimulation with native protein compared to LPP candidates in GPA (Figure 5.2.2.2, B; Table 5.2.2.1). No significant differences were observed between the LPP candidates (Table 5.2.2.1). No basophil histamine release was observed in NAC (Figure 5.2.2.2, C; Table 5.2.2.1).

A rightward shift in the EC₅₀ curve was observed following stimulation with LPP candidates compared to native protein, indicating the lower potency of LPP candidates compared to native protein in eliciting DAO⁺CD63⁺ basophil histamine release (Figure 5.2.2.2, D, left). Peptide 1 was the least potent, followed by peptide 2, 3 and 4, which was significant when compared to native protein (peptide 1, $P < 0.0001$; peptide 2, $P = 0.0003$; peptide 3, $P = 0.0017$; and peptide 4, $P = 0.0032$; Table 5.2.2.2).

There was a concentration-dependent increase in DAO-CD203c^{bright} basophil histamine release observed following stimulation with native protein and LPP candidates in GPA (Figure 5.2.2.2, E and F), but not NAC (Figure 5.2.2.2, E and G). A rightward shift in the curve was observed following stimulation with LPP candidates compared to native protein. The proportion of basophil histamine release (DAO-CD203c^{bright}) was significantly higher following stimulation with native protein compared to LPP candidates at 10, 33, 100 and 330 ng/mL (Figure 5.2.2.2, F; Table 5.2.2.3). No significant differences were observed between the LPP candidates (Table 5.2.2.3) and no basophil histamine release was observed in NAC (Figure 5.2.2.2, G; Table 5.2.2.3)

A rightward shift in the EC₅₀ curve was observed following stimulation with LPP candidates compared to native protein, implying the lower potency of LPP candidates compared to native protein in eliciting DAO-CD203c^{bright} basophil histamine release (Figure 5.2.2.2, H, left). Peptide 1 was the least potent, followed by peptide 2, 3 and 4, which was significant when compared to native protein (peptide 1, $P = 0.0004$; peptide 2, $P = 0.0009$; peptide 3, $P = 0.0021$; and peptide 4, $P = 0.0092$; Table 5.2.2.4).

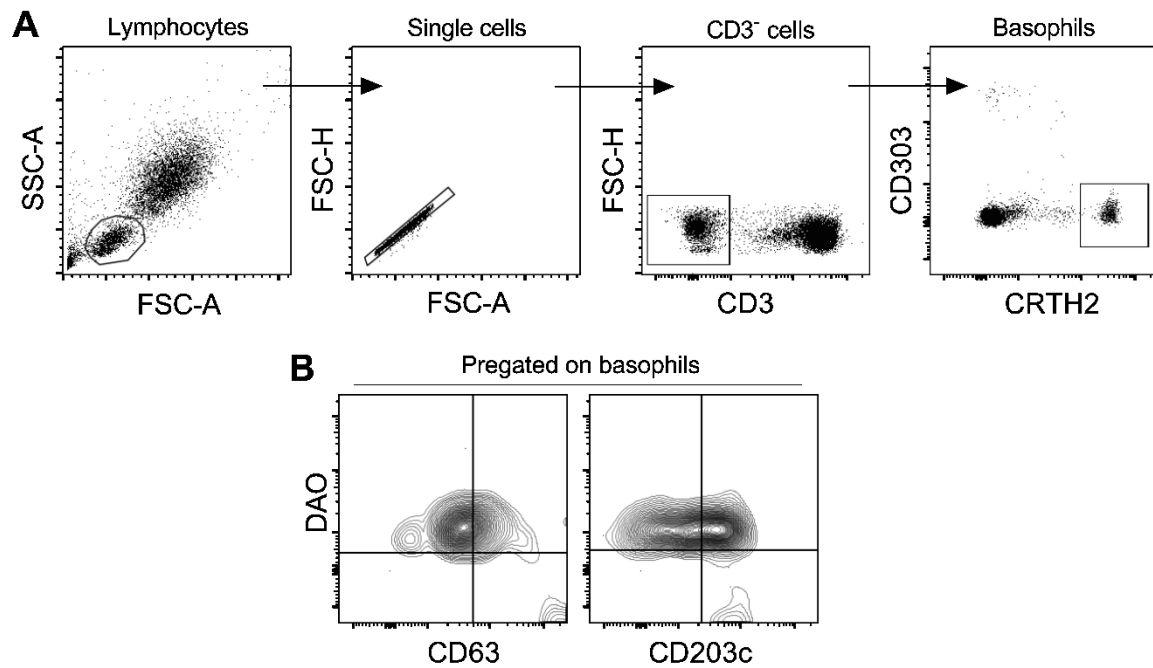


Figure 5.2.2.1 Gating strategy to enumerate basophil histamine release. **A** Lymphocytes were gated on the basis of low forward (FSC) and side (SSC) scatter and doublets were excluded. Basophils were defined as CD3⁻CD303⁺CRTH2⁺ cells. **B** Intracellular histamine release by activated CD63⁺ and CD203c^{bright} basophils was quantified by the loss of DAO expression.

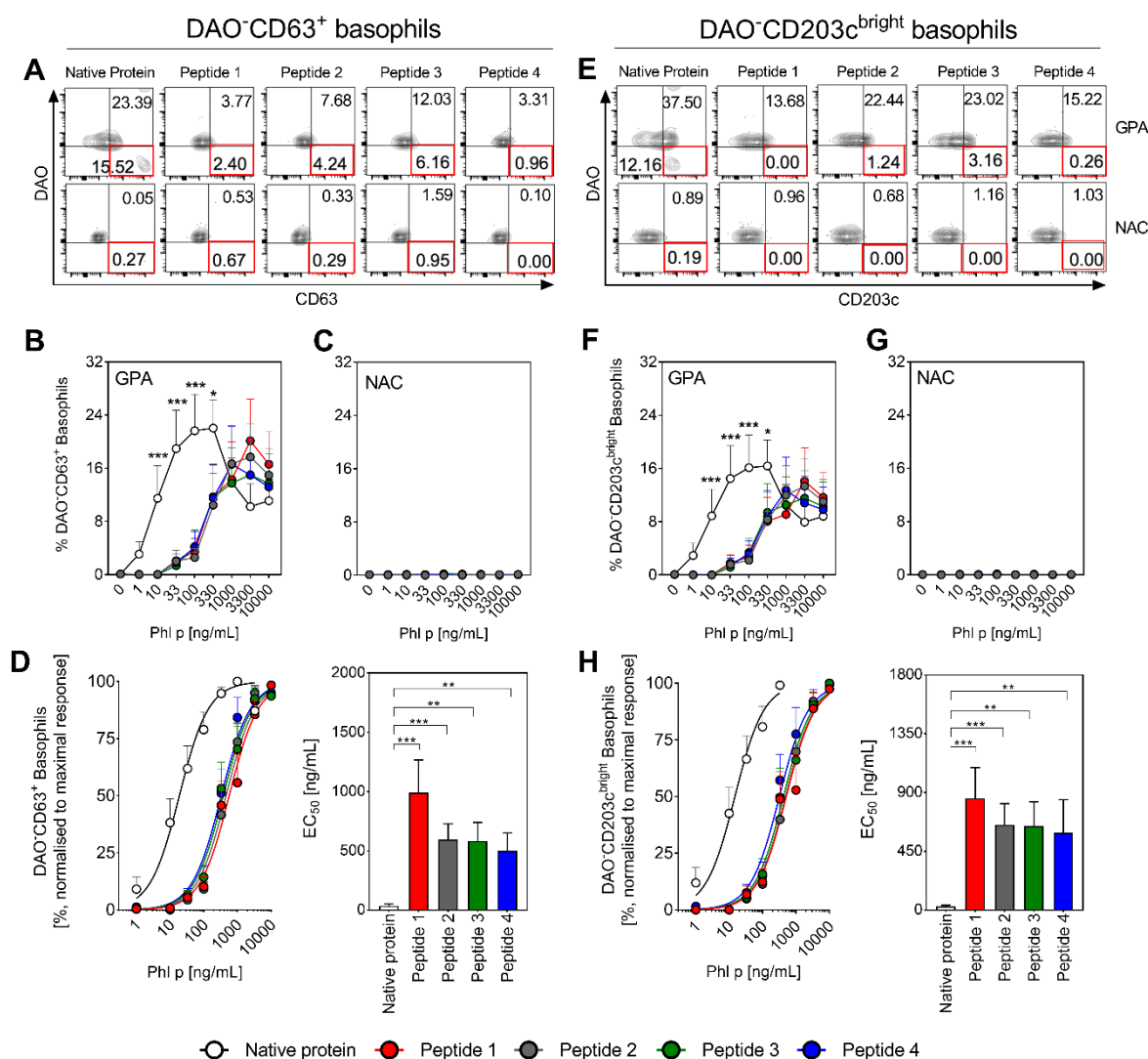


Figure 5.2.2.2 Lower basophil histamine release following stimulation with LPP candidates. **A** Representative plots illustrating histamine released following basophil activation (DAO⁻CD63⁺) elicited by 100 ng/mL of the native protein and LPP candidates in GPA (top panel) and NAC (bottom panel). Dose-response of DAO⁻CD63⁺ basophils in **B** GPA (n = 16); and **C** NAC (n = 6), following stimulation with various concentrations of the native protein and LPP candidates. **D** EC₅₀ analysis of GPA as represented as dose-response curves (left panel), and bar charts (right panel). **E-H** Similarly, for DAO⁻CD203c^{bright} basophils. Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic healthy controls. ***P* < 0.01, ****P* < 0.001, Mann-Whitney test and Kruskal-Wallis test with Dunn's multiple comparison test for EC₅₀ analysis versus native protein.

Table 5.2.2.1 Statistical analysis of DAO⁺CD63⁺ basophil response following stimulation with native protein and LPP candidates in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					<i>P</i> value GPA vs NAC
		n	mean	SEM	<i>P</i> value vs		n	mean	SEM	<i>P</i> value vs		
					Native protein	Baseline				Native protein	Baseline	
0 (Baseline)	Native protein	16	0.11	0.05	NA	NA	6	0.10	0.07	NA	NA	0.7179
	Peptide 1	16	0.11	0.05	>0.9999	NA	6	0.10	0.07	>0.9999	NA	0.7179
	Peptide 2	16	0.11	0.05	>0.9999	NA	6	0.10	0.07	>0.9999	NA	0.7179
	Peptide 3	16	0.11	0.05	>0.9999	NA	6	0.10	0.07	>0.9999	NA	0.7179
	Peptide 4	16	0.11	0.05	>0.9999	NA	6	0.10	0.07	>0.9999	NA	0.7179
1	Native protein	16	3.09	1.89	NA	0.2686	6	0.12	0.08	NA	>0.9999	0.4315
	Peptide 1	16	0.05	0.03	0.0558	0.1465	6	0.04	0.04	0.4545	0.6250	>0.9999
	Peptide 2	16	0.03	0.02	0.0266	0.0840	6	0.05	0.04	0.8485	0.6250	0.6045
	Peptide 3	16	0.01	0.01	0.0024	0.0020	6	0.06	0.04	0.8485	0.6250	0.1169
	Peptide 4	16	0.07	0.03	0.1622	0.8164	6	0.12	0.10	>0.9999	>0.9999	>0.9999
10	Native protein	16	11.49	4.89	NA	0.0002	6	0.03	0.02	NA	0.5000	0.0014
	Peptide 1	16	0.04	0.03	<0.0001	0.0059	6	0.05	0.05	>0.9999	0.6250	0.8442
	Peptide 2	16	0.03	0.02	<0.0001	0.0469	6	0.08	0.08	>0.9999	0.8750	>0.9999
	Peptide 3	16	0.01	0.01	<0.0001	0.0059	6	0.16	0.10	0.6970	>0.9999	0.3356
	Peptide 4	16	0.05	0.03	<0.0001	0.4004	6	0.04	0.04	>0.9999	0.6250	0.9171
33	Native protein	16	18.96	5.81	NA	<0.0001	6	0.08	0.05	NA	0.8750	0.0004
	Peptide 1	16	2.10	1.54	<0.0001	0.6992	6	0.03	0.03	0.4545	0.7500	0.3231
	Peptide 2	16	2.04	1.63	<0.0001	0.1310	6	0.00	0.00	0.4545	0.2500	0.0215
	Peptide 3	16	1.36	0.82	<0.0001	0.1577	6	0.10	0.07	0.8485	0.8750	0.3345
	Peptide 4	16	1.85	1.24	0.0001	0.0635	6	0.11	0.07	0.6970	0.8750	0.3354
100	Native protein	16	21.65	5.46	NA	<0.0001	6	0.15	0.10	NA	>0.9999	<0.0001
	Peptide 1	16	3.45	2.01	<0.0001	0.1414	6	0.08	0.05	0.8485	>0.9999	0.3566
	Peptide 2	16	2.58	1.33	<0.0001	0.0313	6	0.03	0.03	0.4242	0.2500	0.0425
	Peptide 3	16	4.23	2.51	<0.0001	0.0394	6	0.21	0.12	0.5671	0.6250	0.5765
	Peptide 4	16	4.11	2.41	<0.0001	0.0381	6	0.13	0.06	0.8485	0.6250	0.3086
330	Native protein	16	22.06	4.19	NA	<0.0001	6	0.08	0.06	NA	0.8750	<0.0001
	Peptide 1	16	11.73	4.78	0.0147	0.0009	6	0.02	0.02	0.7273	0.5000	0.0073
	Peptide 2	16	10.46	4.79	0.0064	0.0001	6	0.07	0.05	0.8485	0.5000	0.0021
	Peptide 3	15	11.62	5.06	0.022	0.0015	6	0.18	0.13	0.8485	0.8750	0.0311
	Peptide 4	16	11.52	4.94	0.024	0.0005	6	0.06	0.04	>0.9999	0.5000	0.0050

PhI p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					<i>P</i> value GPA vs NAC
		n	mean	SEM	<i>P</i> value vs		n	mean	SEM	<i>P</i> value vs		
					Native protein	Baseline				Native protein	Baseline	
1000	Native protein	16	13.89	3.63	NA	<0.0001	6	0.11	0.07	NA	0.2500	<0.0001
	Peptide 1	15	14.31	5.69	0.4174	0.0006	6	0.16	0.11	0.9242	0.5000	0.0048
	Peptide 2	16	16.70	5.71	0.8598	0.0002	6	0.07	0.04	0.8485	0.6250	0.0047
	Peptide 3	16	13.75	5.36	0.4393	0.0001	6	0.04	0.04	0.4242	0.6250	0.0009
	Peptide 4	16	16.61	5.74	0.7590	<0.0001	6	0.06	0.06	0.4242	0.2500	0.0006
3300	Native protein	16	10.25	3.39	NA	<0.0001	6	0.08	0.04	NA	0.8750	<0.0001
	Peptide 1	16	20.13	6.27	0.4394	0.0001	6	0.00	0.00	0.1818	0.2500	0.0007
	Peptide 2	16	17.70	5.03	0.4618	0.0001	6	0.12	0.08	>0.9999	0.8750	0.0029
	Peptide 3	16	15.03	4.96	0.7449	0.0001	6	0.08	0.08	0.5455	0.8750	0.0009
	Peptide 4	16	14.97	5.07	0.6353	<0.0001	6	0.07	0.03	0.7835	0.8750	0.0003
10000	Native protein	16	11.11	3.23	NA	<0.0001	6	0.12	0.08	NA	>0.9999	<0.0001
	Peptide 1	16	16.60	4.98	0.6089	0.0001	6	0.09	0.09	>0.9999	0.7500	0.0009
	Peptide 2	16	14.97	3.95	0.5831	<0.0001	6	0.13	0.06	0.6212	0.6250	<0.0001
	Peptide 3	16	13.72	4.51	>0.9999	<0.0001	6	0.09	0.07	0.8485	0.8750	0.0007
	Peptide 4	16	13.18	3.79	0.9778	<0.0001	6	0.14	0.07	0.8485	0.8750	0.0005

GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs native protein and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significant values.

Table 5.2.2.2 Native protein and LPP candidates EC₅₀ to elicit DAO⁺CD63⁺ basophil histamine release in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	36.18 ± 15.85	1.00
Peptide 1	990.40 ± 277.00***	27.37
Peptide 2	597.40 ± 131.20***	16.51
Peptide 3	582.90 ± 156.40**	16.10
Peptide 4	499.30 ± 151.60**	13.80

Statistical significance versus native protein. ** $P < 0.01$ and *** $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test.

Table 5.2.2.3 Statistical analysis of DAO-CD203c^{bright} basophil response following stimulation with native protein and LPP candidates in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					<i>P</i> value GPA vs NAC
		n	mean	SEM	<i>P</i> value vs		n	mean	SEM	<i>P</i> value vs		
					Native protein	Baseline				Native protein	Baseline	
0 (Baseline)	Native protein	16	0.09	0.05	NA	NA	6	0.06	0.04	NA	NA	0.9061
	Peptide 1	16	0.09	0.05	>0.9999	NA	6	0.06	0.04	>0.9999	NA	0.9061
	Peptide 2	16	0.09	0.05	>0.9999	NA	6	0.06	0.04	>0.9999	NA	0.9061
	Peptide 3	16	0.09	0.05	>0.9999	NA	6	0.06	0.04	>0.9999	NA	0.9061
	Peptide 4	16	0.09	0.05	>0.9999	NA	6	0.06	0.04	>0.9999	NA	0.9061
1	Native protein	16	2.93	1.87	NA	0.2832	6	0.00	0.00	NA	0.5000	0.1094
	Peptide 1	16	0.01	0.01	0.0198	0.1250	6	0.02	0.02	>0.9999	0.7500	>0.9999
	Peptide 2	16	0.01	0.01	0.0068	0.1094	6	0.01	0.01	>0.9999	0.5000	>0.9999
	Peptide 3	16	0.01	0.01	0.0111	0.0313	6	0.00	0.00	>0.9999	0.5000	>0.9999
	Peptide 4	16	0.05	0.03	0.0869	0.5234	6	0.05	0.05	>0.9999	>0.9999	0.7703
10	Native protein	16	8.88	4.00	NA	0.0002	6	0.00	0.00	NA	0.5000	0.0007
	Peptide 1	16	0.03	0.03	<0.0001	0.0625	6	0.00	0.00	>0.9999	0.5000	0.5844
	Peptide 2	16	0.01	0.01	<0.0001	0.0313	6	0.04	0.04	>0.9999	>0.9999	0.7922
	Peptide 3	16	0.00	0.00	<0.0001	0.0625	6	0.11	0.07	0.4545	0.6250	0.0649
	Peptide 4	16	0.04	0.02	<0.0001	0.8125	6	0.00	0.00	>0.9999	0.5000	0.3356
33	Native protein	16	14.49	4.95	NA	0.0002	6	0.05	0.04	NA	>0.9999	0.0003
	Peptide 1	16	1.80	1.33	<0.0001	0.4688	6	0.00	0.00	0.4545	0.5000	0.2663
	Peptide 2	16	1.60	1.35	<0.0001	0.1406	6	0.00	0.00	0.4545	0.5000	0.0384
	Peptide 3	16	1.16	0.75	0.0001	0.1133	6	0.00	0.00	0.4545	0.5000	0.0656
	Peptide 4	16	1.54	1.00	0.0003	0.1270	6	0.06	0.05	0.8485	>0.9999	0.1979
100	Native protein	16	16.13	4.90	NA	<0.0001	6	0.02	0.02	NA	0.5000	<0.0001
	Peptide 1	16	2.72	1.76	<0.0001	0.1094	6	0.04	0.04	>0.9999	>0.9999	0.3414
	Peptide 2	16	2.23	1.15	<0.0001	0.0391	6	0.00	0.00	>0.9999	0.5000	0.0391
	Peptide 3	16	3.37	2.09	0.0002	0.1367	6	0.15	0.06	0.1061	0.2500	0.8481
	Peptide 4	16	3.13	1.97	0.0001	0.0654	6	0.04	0.03	0.7273	>0.9999	0.2135
330	Native protein	16	16.39	3.91	NA	<0.0001	6	0.02	0.02	NA	0.5000	<0.0001
	Peptide 1	16	8.07	3.61	0.0224	0.0034	6	0.00	0.00	>0.9999	0.5000	0.0005
	Peptide 2	16	8.34	4.08	0.0239	0.0007	6	0.02	0.02	>0.9999	0.5000	0.0073
	Peptide 3	15	9.41	4.29	0.0259	0.0015	6	0.05	0.05	>0.9999	>0.9999	0.0058
	Peptide 4	16	8.79	3.86	0.0376	0.0012	6	0.04	0.03	0.4545	>0.9999	0.0133

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					<i>P</i> value GPA vs NAC
		n	mean	SEM	<i>P</i> value vs		n	mean	SEM	<i>P</i> value vs		
					Native protein	Baseline				Native protein	Baseline	
1000	Native protein	16	10.47	3.16	NA	<0.0001	6	0.10	0.05	NA	0.6250	<0.0001
	Peptide 1	14	9.10	4.18	0.2038	0.0034	6	0.00	0.00	0.1818	0.5000	0.0093
	Peptide 2	16	11.99	4.42	0.6089	0.0001	6	0.00	0.00	0.1818	0.5000	0.0007
	Peptide 3	16	10.56	4.21	0.5207	0.0001	6	0.04	0.04	0.303	>0.9999	0.0009
	Peptide 4	16	12.72	5.00	0.5830	0.0001	6	0.02	0.02	0.3030	0.5000	0.0009
3300	Native protein	16	7.95	3.07	NA	<0.0001	6	0.03	0.03	NA	0.7500	<0.0001
	Peptide 1	16	14.11	5.01	0.2580	0.0001	6	0.00	0.00	>0.9999	0.5000	0.0007
	Peptide 2	16	13.35	4.16	0.6352	0.0002	6	0.05	0.04	0.7273	>0.9999	0.0050
	Peptide 3	16	11.55	4.16	0.4849	0.0001	6	0.00	0.00	>0.9999	0.5000	0.0007
	Peptide 4	16	10.81	4.03	0.6090	<0.0001	6	0.00	0.00	>0.9999	0.5000	0.0002
10000	Native protein	16	8.81	3.03	NA	<0.0001	6	0.05	0.05	NA	>0.9999	<0.0001
	Peptide 1	16	11.68	3.75	0.6352	0.0001	6	0.04	0.04	>0.9999	>0.9999	0.0009
	Peptide 2	16	11.04	3.35	0.8014	<0.0001	6	0.07	0.03	0.5455	0.7500	<0.0001
	Peptide 3	16	10.29	3.70	0.6889	0.0001	6	0.05	0.03	>0.9999	>0.9999	0.0004
	Peptide 4	16	9.82	3.38	0.6755	<0.0001	6	0.02	0.02	>0.9999	0.7500	0.0002

GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs native protein and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significant values.

Table 5.2.2.4 Native protein and LPP candidates EC₅₀ to elicit DAO⁺CD203c^{bright} basophil activation in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	26.40 ± 10.15	1.00
Peptide 1	855.70 ± 235.90***	32.41
Peptide 2	652.90 ± 161.10***	24.73
Peptide 3	643.30 ± 186.90**	24.37
Peptide 4	592.70 ± 252.20**	22.45

Statistical significance versus native protein. ** $P < 0.01$ and *** $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test.

5.2.3 Attenuation of T_{FH} cell subsets' proliferation in response to LPP candidates

The effect of LPP candidates on the proliferation of T_{FH} cell subsets was further investigated (Figure 5.2.3.1) in the same cohort of patients. There was a concentration-dependent increase in the proliferation of T_{FH} cells observed in GPA, but not NAC when stimulated with various concentration of native protein (Figure 5.2.3.2, A). There was no increase in proliferation of T_{FH} cells observed in GPA following stimulation with LPP candidates (Figure 5.2.3.2, B). No proliferation of T_{FH} cells was observed in NAC following stimulation with native protein and LPP candidates (Figure 5.2.3.2, C). A concentration-dependent increase in the proliferation of activated ICOS⁺ T_{FH} cells was observed in GPA, but not in NAC following stimulation with native protein (Figure 5.2.3.2, D). No significant changes were observed in the proportion of proliferated ICOS⁺ T_{FH} cells following stimulation with LPP candidates in GPA (Figure 5.2.3.2, E) and NAC (Figure 5.2.3.2, F). The proportion of proliferated ICOS⁺ T_{FH} cells was significantly higher following stimulation with native protein compared to LPP candidates at 1 (peptide 1, $P = 0.0409$; peptide 3, $P = 0.0328$), 10 (peptide 1, $P = 0.0070$; peptide 2, $P = 0.0269$), 25 (peptide 4, $P = 0.0494$) and 50 $\mu\text{g/mL}$ (peptide 2, $P = 0.0309$; peptide 4, $P = 0.0127$; Figure 5.2.3.2, E). No significant differences were observed in NAC (Figure 5.2.3.2, F).

A concentration-dependent increase in the proliferation of IL-4⁺ T_{FH} cells was observed following stimulation with native protein in GPA, but not NAC (Figure 5.2.3.2, G). Furthermore, LPP candidates elicit a concentration-dependent increase in the proliferation of IL-4⁺ T_{FH} cells in GPA, but to a lower capacity compared to native protein (Figure 5.2.3.2, H). The proportion of proliferated IL-4⁺ T_{FH} cells were significantly higher in GPA when stimulated with native protein compared to LPP candidates at 1 (peptide 1, $P = 0.0244$; peptide 2, $P = 0.0154$) and 10 $\mu\text{g/mL}$ (peptide 2, $P = 0.0190$). There was no proliferation of IL-4⁺ T_{FH} cells following stimulation with native protein and LPP candidates in NAC (Figure 5.2.3.2, I).

Similarly, there was a concentration-dependent increase in the proliferation of IL-21⁺ T_{FH} cells following stimulation with native protein in GPA but not NAC (Figure 5.2.3.2, J). A concentration-dependent increase of proliferated IL-21⁺ T_{FH} cells was observed following stimulation with LPP candidates in GPA, however, this was to a lower extent when compared to native protein (Figure 5.2.3.2, K). The proportion of proliferated IL-21⁺ T_{FH} cells was significantly higher when stimulated with native protein compared to peptide 2 at 1 µg/mL ($P = 0.0480$). Although a trend was observed, no other significant changes were observed. No changes in the proliferation of IL-21⁺ T_{FH} cells was observed following stimulation with native protein and LPP candidates in NAC (Figure 5.2.3.2, L).

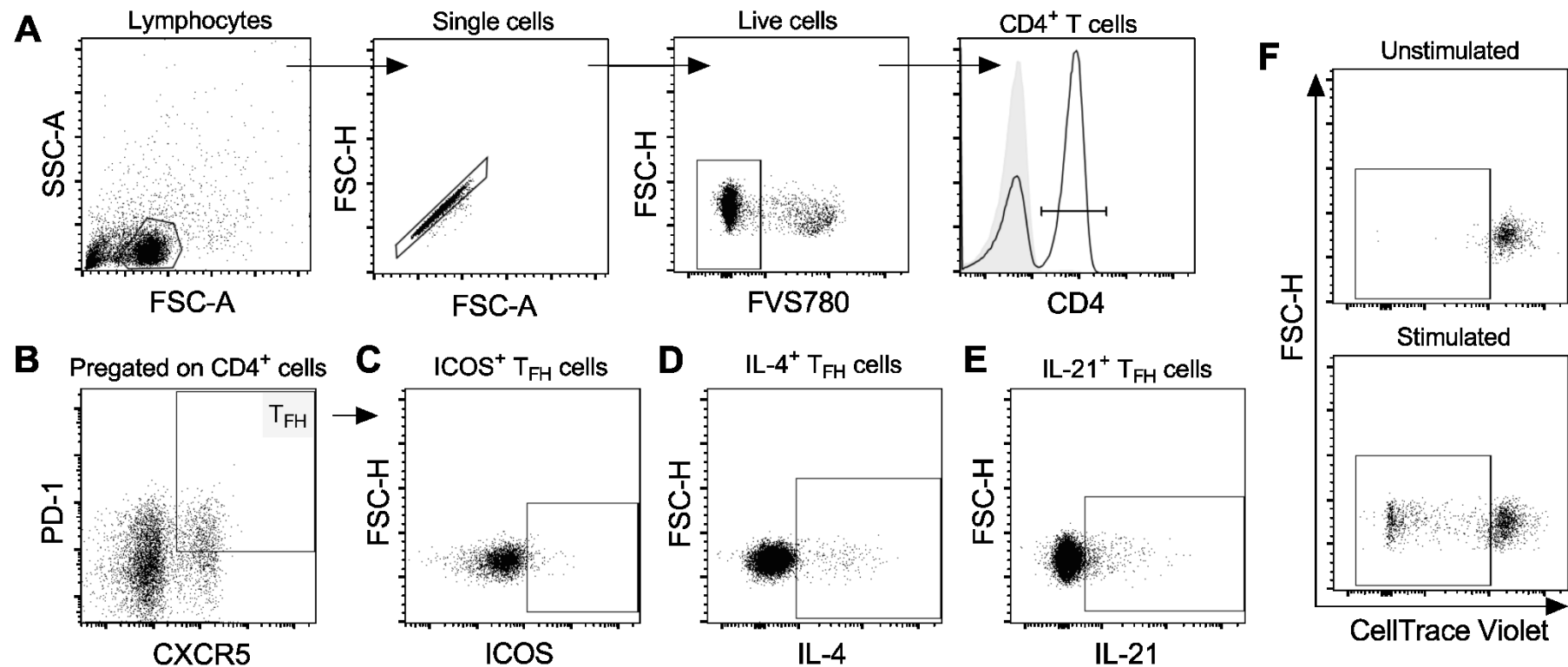


Figure 5.2.3.1 Gating strategy to identify proliferated T_{FH} cell subsets. **A** Lymphocytes were gated on the basis of low forward (FSC) and side (SSC) scatter. Doublets were excluded. Live cells were discriminated using FVS780 dye and T cells were defined as CD4⁺ cells. **B** T_{FH} cells are defined as CD4⁺ cells that expressed CXCR5 and PD-1, and **C** ICOS⁺; **D** IL-4⁺; and **E** IL-21⁺ T_{FH} cell subsets that expressed the respective markers. **F** Proliferating T_{FH} cell subsets were assessed using CellTrace Violet dye, of which proliferated cells showed lower CellTrace Violet expression as a result of cell division.

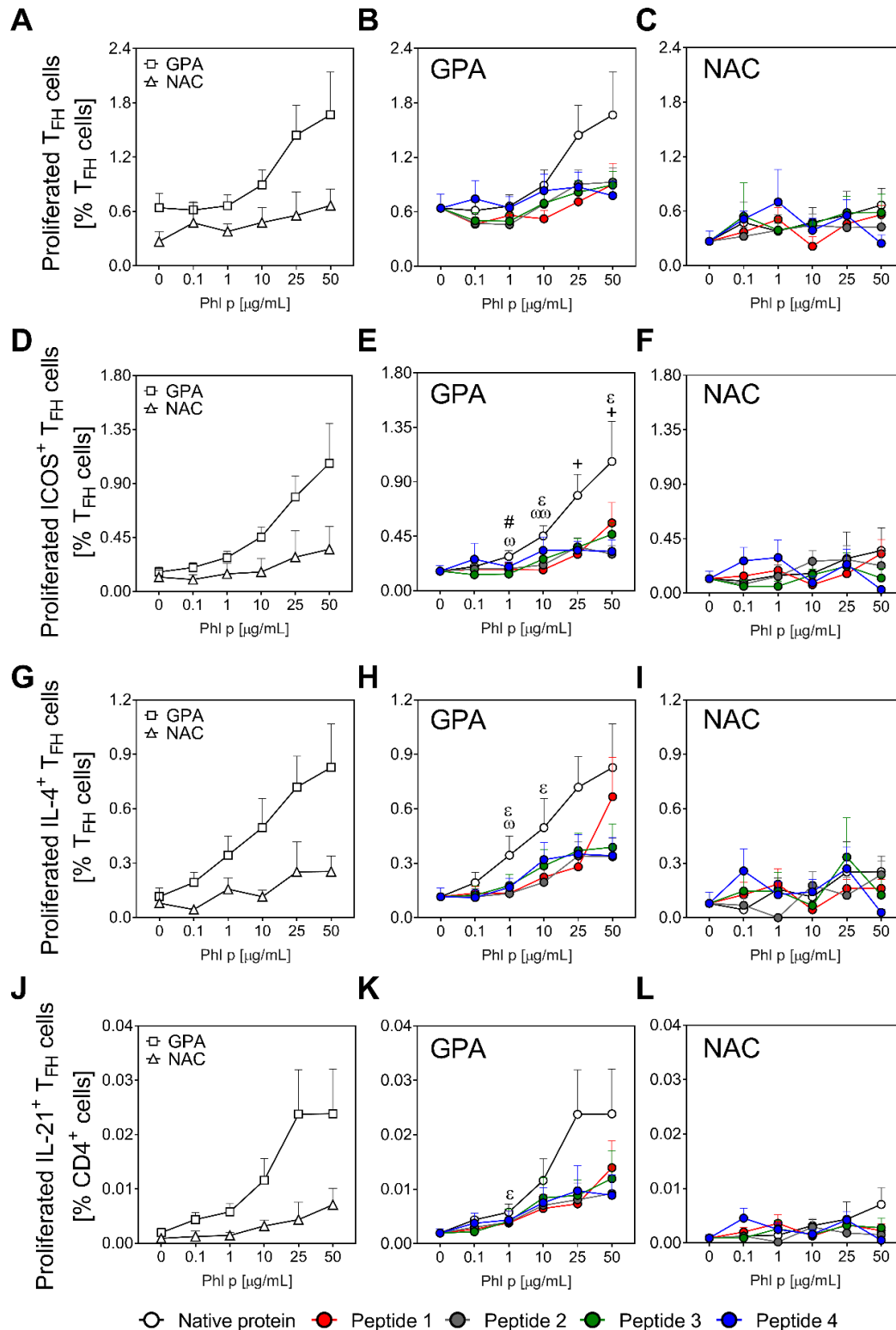


Figure 5.2.3.2 Lower proliferation of T_{FH} cell subsets in GPA following stimulation with LPP candidates compared to native protein. The proportion of proliferated **A-C** T_{FH} (CD4⁺CXCR5⁺PD-1⁺); **D-F** ICOS⁺ T_{FH} ; **G-I** IL-4⁺ T_{FH} ; and **J-L** IL-21⁺ T_{FH} cells, following stimulation with native protein in GPA (n = 16) and NAC (n = 6); and native protein and LPP candidates in GPA and NAC. Statistical significance for grass pollen native protein versus LPP candidates is represented as ω for peptide 1, ε for peptide 2, # for peptide 3 and + for peptide 4. Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic healthy controls. ω, ε, #, + $P < 0.05$ and ωω $P < 0.01$, Mann-Whitney test.

5.2.4 Reduced proportion of pro-allergic T_H2, T_H2A and T_H17 cell subsets following stimulation with LPP candidates

The effect of LPP candidates on the proliferation of pro-inflammatory T_H2, T_H2A and T_H17 cells were also investigated in the same cohort of patients (Figure 5.2.4.1). A concentration-dependent increase in the proliferation of T_H2 cells was observed following stimulation with native protein in GPA, but not NAC (Figure 5.2.4.2, A). In GPA, a trend of lower proliferation of T_H2 cells was observed following stimulation with LPP candidates compared to native protein (Figure 5.2.4.2, B). No significant response was observed in NAC when stimulated with native protein and LPP candidates (Figure 5.2.4.2, C). Similarly, there was a concentration-dependent increase in the proliferation of T_H2A cells observed in GPA, but not NAC (Figure 5.2.4.2, D). The proportion of proliferated T_H2A cells following stimulation with native protein were significantly higher in GPA compared to NAC at 1 µg/mL ($P = 0.0400$). A trend of an increasing proliferation of T_H2A cells was observed in GPA following stimulation with increasing concentration of native protein, which was observed to a lesser extent following stimulation with LPP candidates (Figure 5.2.4.2, E). No response was observed in NAC (Figure 5.2.4.2, F). Furthermore, a concentration-dependent increase in the proliferation of T_H17 cells was observed in GPA and to a lesser extent in NAC (Figure 5.2.4.2, G). The proportion of proliferated T_H17 cells following stimulation with native protein was significantly higher in GPA compared to NAC at 25 µg/mL ($P = 0.0266$). A trend of higher proliferation of T_H17 cells was observed in GPA following stimulation with native protein compared to LPP candidates (Figure 5.2.4.2, H). The proportion of proliferated T_H17 cells were significantly higher in GPA when stimulated with native protein compared to LPP candidates at 25 (peptide 1, $P = 0.0194$; peptide 2, $P = 0.0216$; peptide 3, $P = 0.0096$; peptide 4, $P = 0.0152$) and 50 µg/mL (peptide 2, $P = 0.0139$; peptide 3, $P = 0.0282$; peptide 4, $P = 0.0131$). No significant changes were observed in response to stimulation with native protein and LPP candidates in NAC (Figure 5.2.4.2, I).

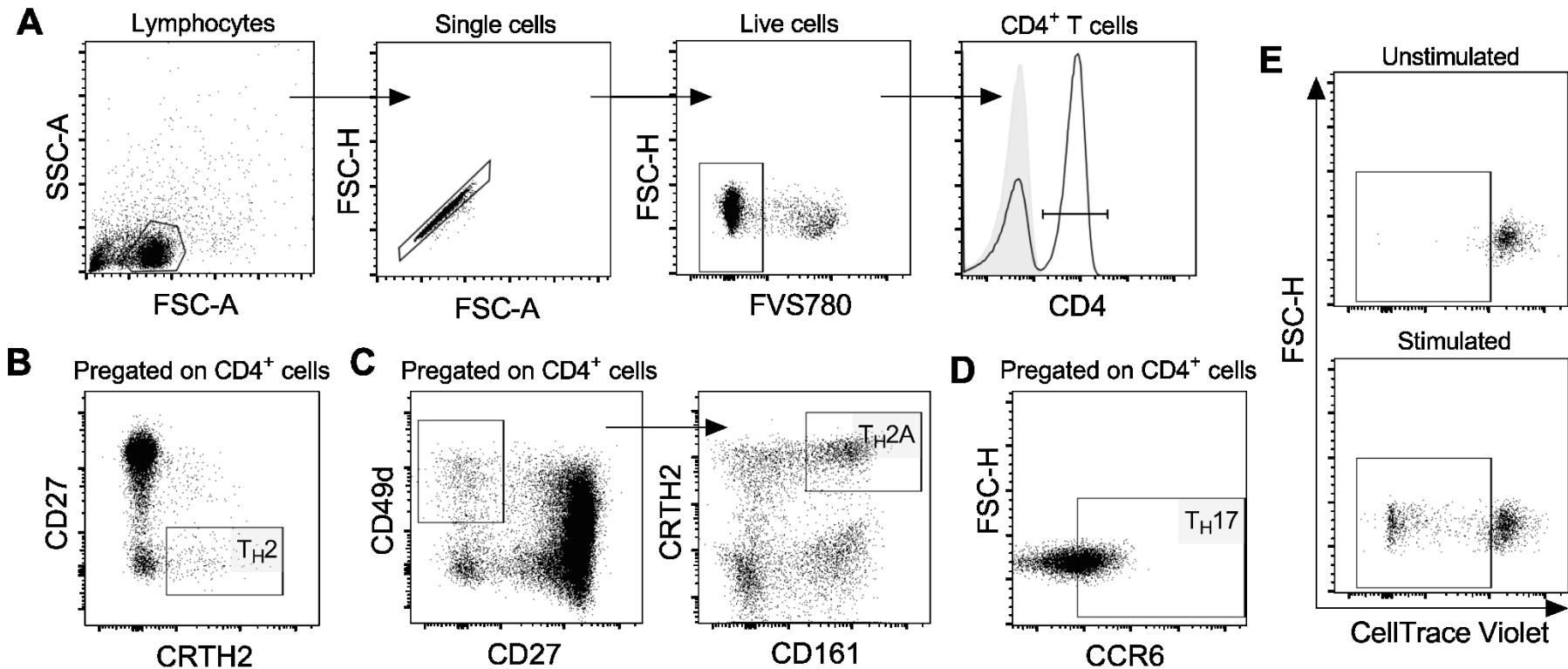


Figure 5.2.4.1 Gating strategy to identify proliferated TH₂, TH₂A and TH₁₇ cell subsets. **A** Lymphocytes were gated on the basis of low forward (FSC) and side (SSC) scatter and doublets were excluded. Live cells were discriminated using FVS780 dye and T cells were identified as CD4⁺-expressing cells. **B** TH₂ cells were defined as CD4⁺ cells that expressed CRTH2 and lacked CD27 expression. **C** TH₂A cells were defined as CD4⁺CD27⁻CRTH2⁺CD49⁺CD161⁺ cells. **D** TH₁₇ were gated within CD4⁺ that expressed CCR6⁺. **E** Proliferating T cell subsets were assessed using CellTrace Violet dye, of which proliferated cells showed lower CellTrace Violet expression as a result of cell division.

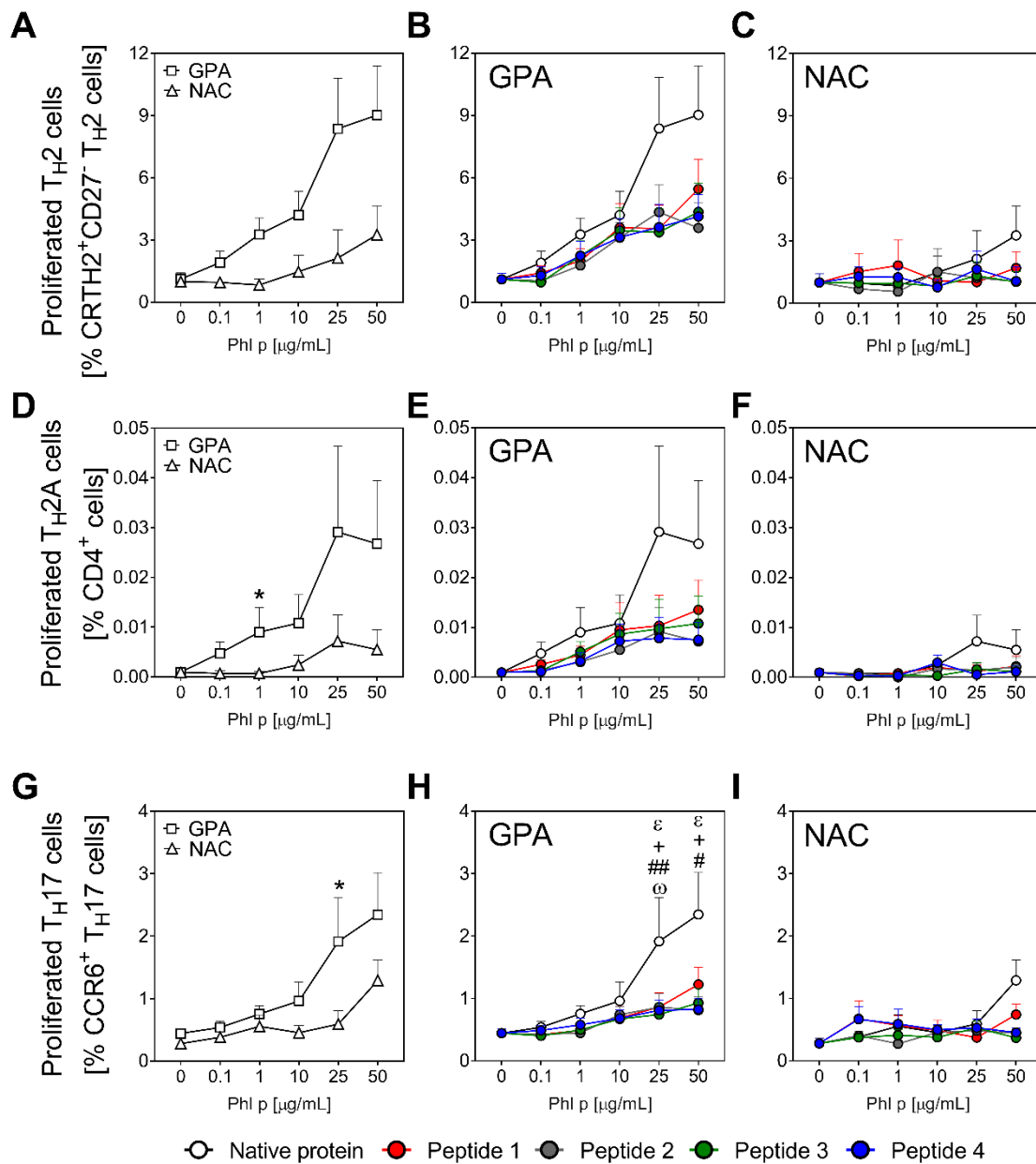


Figure 5.2.4.2 Lower proliferation of T_H17 cells in GPA following stimulation with LPP candidates compared to native protein. The proportion of proliferated **A-C** T_H2 (CD4⁺CRTH2⁺CD27⁻); **D-F** T_H2A (CD4⁺CRTH2⁺CD27⁻CD161⁺CD49d⁺); and **G-I** T_H17 (CD4⁺CCR6⁺) cells, following stimulation with native protein in GPA and NAC, and native protein and LPP candidates in GPA (n = 16) and NAC (n = 6). Statistical significance for grass pollen native protein versus LPP candidates is represented as ω for peptide 1, ϵ for peptide 2, # for peptide 3 and * for peptide 4. Error bars represent SEM. GPA, grass pollen allergics; NAC, non-atopic healthy controls. *, ω , ϵ , #, * P < 0.05 and ## P < 0.01, Mann-Whitney test.

5.3 Summary

This chapter assessed the allergenicity of peptide (LPP) candidates, as an alternative approach of immunotherapy of using the whole allergen. Through allergenicity assay that measures basophil responsiveness and immunogenicity assay that measures the modulation of T cell responses by LPP candidates, their safety and immune potency were thoroughly investigated. All LPP candidates (peptides 1, 2, 3 and 4) were hypoallergenic and possessed a lower potency in eliciting the activation of basophils, as measure by the expression of basophil activation markers, CD63 and CD203c, compared to grass pollen native protein (whole allergen). As basophil activation does not necessarily lead to histamine release, basophil histamine release was also quantified by utilising DAO as an endpoint readout. All LPP candidates showed reduced ability and lower potency in inducing basophil histamine release compared to grass pollen native protein. Furthermore, LPP candidates induced less proliferation of T_{FH} cell subsets, including activated ($ICOS^+$), IL-4- and IL-21-producing T_{FH} cells, as well as T_H2 , allergen-specific T_H2A and T_H17 cells, compared to grass pollen native protein. Native protein and LPP candidates did not induce the proliferation of T_{FH} , T_H2 , T_H2A and T_H17 cell subsets in NAC. In summary, this chapter reported the hypoallergenicity of LPP candidates. Furthermore, these findings suggest that unlike the whole allergen, allergen peptides lack the ability to elicit pro-allergic T cell responses, highlighting their advantages and potential as a safer alternative approach compared to conventional AIT using the whole allergen.

CHAPTER 6 ATTENUATION OF PRO-ALLERGIC RESPONSES FOLLOWING A SHORT-COURSE OF *LOLIUM* *PERENNE* PEPTIDE IMMUNOTHERAPY: A RDBPC TRIAL STUDY

6.1 Introduction

SCIT and SLIT are effective and have been shown to modulate the immune system and induced immune tolerance in allergic patients⁶⁸. Despite that, they have been reported to induce anaphylaxis in some patients⁴¹. Furthermore, both SCIT and SLIT need to be administered for at least three years to confer clinical benefits³¹, which resulted in poor patient compliance. Therefore, there is a need to develop an alternative therapeutic strategy with reduced adverse events whilst maintaining efficiency and improving compliance. The administration of short synthetic peptidic fragments is deemed to be a safer alternative as they do not cross-link with IgE, reducing the risks of adverse events. Furthermore, peptide immunotherapy may be administered for a shorter amount of period, thus will improve patient compliance.

Chapter 5 illustrated the hypoallergenicity of four peptide candidates generated from hydrolysis of purified ryegrass (*Lolium perenne*) allergen extracts (LPP). One of the candidate peptides (peptide 1) was further developed for subcutaneous immunotherapy administration²⁷⁹ to treat SAR. Both Phl p (timothy grass) and *Lolium perenne* (Lol p) belong to the subfamily of Pooideae and shared an extensive cross-reactivity between its members²⁷⁷, and thus justifies the use of LPP as a treatment for grass pollen allergy. Dose-escalation³⁰⁵ and dose-finding³⁰⁶ studies were performed. Eight subcutaneous injections of LPP over four clinic visits in 3 weeks to a cumulative dose of 170 µg were identified to be optimal in inducing clinical benefits^{306,307}.

The efficacy of LPP treatment was further evaluated in a prospective, multicentre, randomised, double-blind placebo-controlled (RDBPC) phase III trial study³⁰⁸. The phase III study provided safety data and showed improvement in symptoms, quality of life and a reduction in grass pollen conjunctival provocation test (CPT) scores³⁰⁸.

This chapter aims to investigate the effect of LPP treatment on a sub-study site in Belgium, whereby 32 patients (Table 2.3.3.1) were randomised into receiving LPP (21 patients) and PL (11 patients). LPP treatment were administered prior to the grass pollen season and blood samples were collected at V2 before the treatment period. Blood samples were also collected during follow-up visits before the grass pollen season at V6 and following the pollen season at V8 (Figure 6.2.1.1, A). The allergenicity of LPP treatment in inducing basophil responsiveness is assessed through *ex vivo* basophil activation test, and IL-4⁺, IL-21⁺, IL-4⁺IL-21⁺ T_{FH}, FoxP3⁺ T_{FR}, FoxP3⁺ T_{reg} and inducible IL-10⁺ and IL-35⁺ T cell responses in LPP-treated and PL will also be investigated. The data described in this chapter has been previously published in The Journal of Allergy and Clinical Immunology³⁰².

6.2 Results

6.2.1 Reduction of symptom and medication scores following LPP immunotherapy

The clinical results of the phase III study have been reported³⁰⁸. In Belgium, daily CSMS was significantly reduced in LPP-treated patients during the peak pollen season (weeks 23 – 25; -35.1%, $P = 0.0251$; Figure 6.2.1.1, B and C) and the entire grass pollen season (weeks 22 – 30; -53.7-fold, $P = 0.0314$; Figure 6.2.1.1, B and D) compared to PL. RTSS was lower in LPP- compared to PL-treated group during the peak pollen season (-27.4%; Figure 6.2.1.1, E and F). Moreover, RTSS was significantly lower following LPP treatment compared to PL during the peak and the entire pollen season (-57.0%, $P = 0.0194$; Figure 6.2.1.1, E and G).

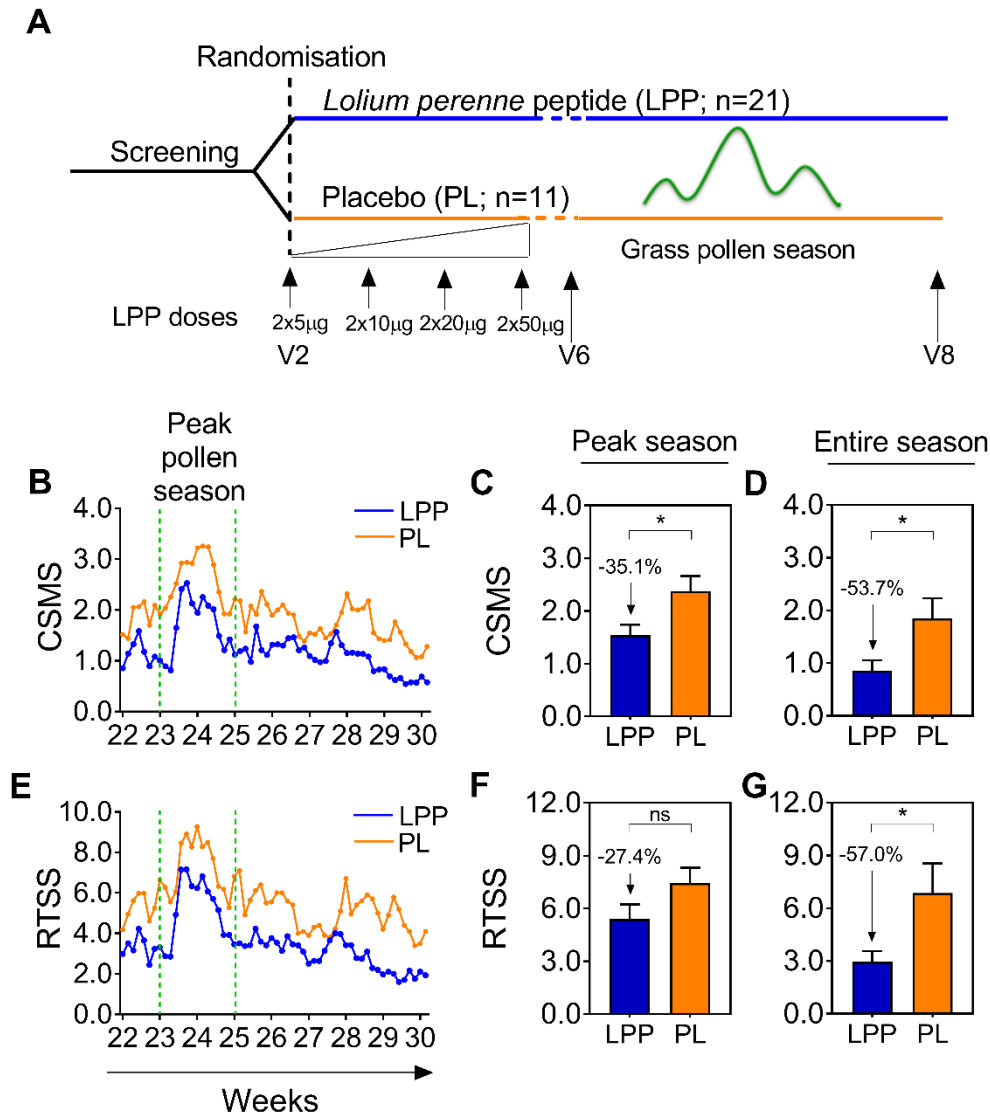


Figure 6.2.1.1 Reduced symptoms and medication scores following LPP immunotherapy but not PL. **A** 32 patients were randomised into receiving LPP (n = 21) and placebo (PL; n = 11) for a sub-study site in Belgium. LPP treatment was administered prior to the grass pollen season between January 2016 and April 2016. Patients were followed-up prior to the grass pollen season at V6 (between March and April 2016) and following the grass pollen season at V8 (August 2016). Blood samples were collected at V2 (before the treatment), V6 and V8. **B** Daily CSMS of LPP- and PL- treated patients measured during the entire grass pollen season. CSMS of LPP- and PL-treated patients measured during **C** the peak pollen season (weeks 23 – 25); and **D** the entire grass pollen season (weeks 22 – 30). **E** Daily RTSS of LPP- and PL- treated patients measured during the entire grass pollen season. RTSS of LPP- and PL-treated patients measured during **F** the peak pollen season; and **G** the entire grass pollen season. Error bars represent SEM. Green dotted lines illustrating the peak grass pollen season. CSMS, combined symptom and medication scores; RTSS, rhinoconjunctivitis total symptom scores; LPP, *Lolium perenne* peptide; PL, placebo. * $P < 0.05$, Mann-Whitney test.

6.2.2 Basophil hyporesponsiveness following LPP immunotherapy

The effect of LPP treatment in inducing FcεRI-mediated allergic response was investigated by assessing the activation of basophils through the expression of CD63 and CD203c. At V2, there was a concentration-dependent increase in CD63⁺ and CD203c^{bright} basophil response observed in LPP- and PL-treated groups (Figure 6.2.2.1). In LPP-treated group, a rightward shift in the curve and a reduction in the maximal CD63⁺ basophil response was observed at V6 and V8, compared to V2. The proportion of CD63⁺ basophils were significantly lower at V6 and V8, compared to V2 at 1, 3, 10, 100 and 330 ng/mL (Figure 6.2.2.1, B, left; Table 6.2.2.1). No significant differences were observed in PL (Figure 6.2.2.1, B, right; Table 6.2.2.1). Similarly, the maximal CD203c^{bright} basophil response was lower and a rightward shift in the curve was noted in LPP-treated groups at V6 and V8, compared to V2. The proportion of CD203c^{bright} basophils were significantly lower at 1, 3, 10, 33, 100 and 330 ng/mL at V6 and V8, compared to V2 in LPP- (Figure 6.2.2.1, C, left; Table 6.2.2.2) but not PL- (Figure 6.2.2.1, C, right; Table 6.2.2.2) treated group.

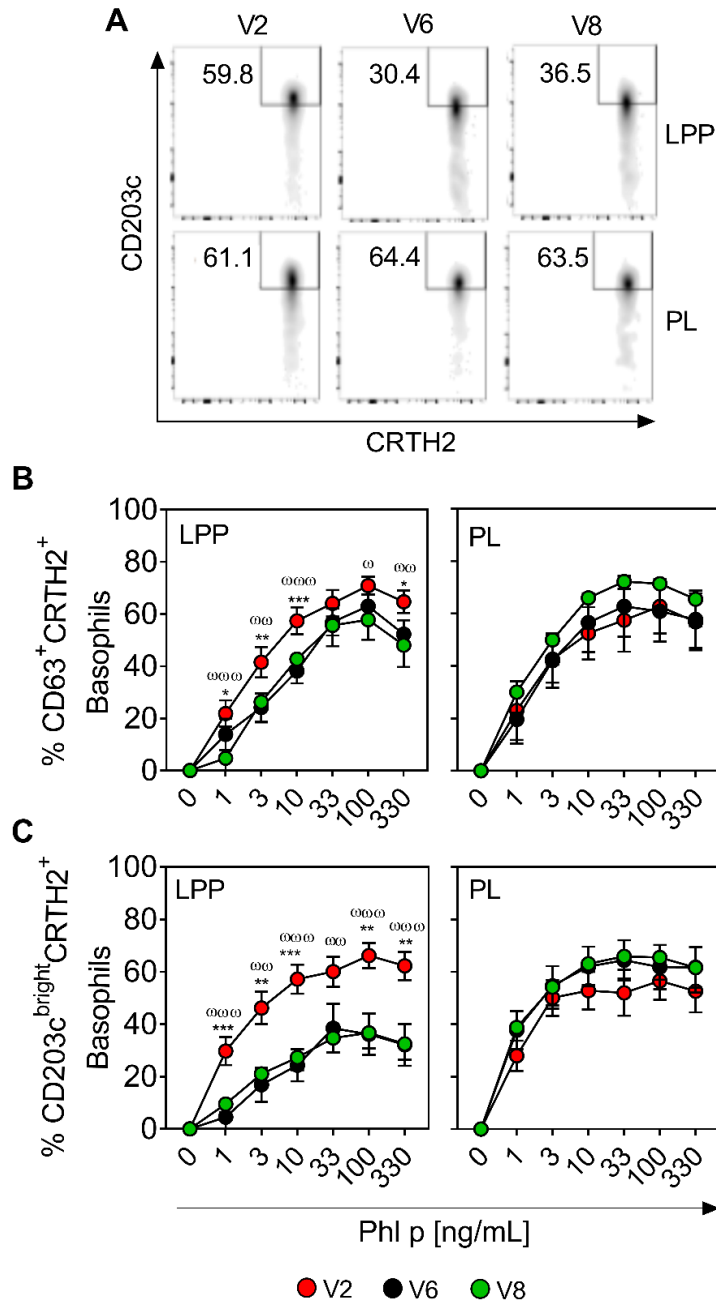


Figure 6.2.2.1 Reduction in basophil responsiveness following LPP immunotherapy, but not PL. **A** Representative plots illustrating basophil activation in LPP- and PL-treated groups at V2, V6 and V8. Dose-response of **B** CD63⁺ basophils; and **C** CD203c^{bright} basophils, in LPP- (left panels; n = 21) and PL- (right panels; n = 11) treated groups at V2 (before the treatment), V6 (after the treatment) and V8 (after the pollen season). Statistical significance against V2 is represented as * for V6 and ω for V8. Error bars represent SEM. LPP, *Lolium perenne* peptide; PL, placebo; V, visit. ω , * P < 0.05, $\omega\omega$, ** P < 0.01 and $\omega\omega\omega$, *** P < 0.001, Mann-Whitney test.

Table 6.2.2.1 Statistical analysis of CD63⁺ basophil response in LPP- and PL-treated groups

Phl p Concentration [ng/mL]	Visit	LPP				PL				<i>P</i> value LPP vs PL
		n	mean	SEM	<i>P</i> value vs V2	n	mean	SEM	<i>P</i> value vs V2	
0	V2	21	0.00	0.00	NA	11	0.00	0.00	NA	>0.9999
	V6	21	0.00	0.00	NA	11	0.00	0.00	NA	>0.9999
	V8	21	0.00	0.00	NA	11	0.00	0.00	NA	>0.9999
1	V2	21	21.89	5.04	NA	11	22.99	11.24	NA	0.8148
	V6	21	13.89	5.95	0.0101	11	19.60	9.15	0.7646	0.1330
	V8	21	4.71	5.99	0.0002	11	30.01	7.84	0.4131	0.0122
3	V2	21	41.55	5.85	NA	11	42.59	8.96	NA	0.9378
	V6	21	24.18	5.43	0.0025	11	42.06	10.41	0.8311	0.1330
	V8	21	26.30	7.83	0.0069	11	49.95	9.46	>0.9999	0.0738
10	V2	21	57.44	5.17	NA	11	52.46	10.00	NA	0.8759
	V6	21	38.19	4.75	<0.0001	11	56.54	11.21	0.2402	0.0807
	V8	21	42.70	5.38	0.0002	11	66.01	9.03	0.5771	0.0155
33	V2	21	64.10	5.09	NA	11	57.45	11.95	NA	0.5060
	V6	21	56.92	5.16	0.1571	11	62.75	11.59	0.0830	0.1932
	V8	21	55.64	7.92	0.0760	11	72.25	8.29	0.0703	0.0064
100	V2	21	70.87	3.37	NA	11	62.75	10.32	NA	0.8148
	V6	21	63.03	4.49	0.0958	11	60.83	11.57	0.7646	0.3673
	V8	21	57.83	7.66	0.0101	11	71.40	8.92	0.4131	0.0175
330	V2	21	64.72	4.30	NA	11	56.86	10.84	NA	0.7846
	V6	21	52.27	5.35	0.0195	11	57.74	10.95	>0.9999	0.2713
	V8	21	48.05	8.31	0.0033	11	65.49	10.22	0.7002	0.0417

Phl p, *Phleum pratense*; LPP, *Lolium perenne* peptide; PL, placebo; V, visit. *P* value LPP vs PL, Mann-Whitney test; *P* value vs V2, Wilcoxon paired signed-rank test. Bolded values represent significant values.

Table 6.2.2.2 Statistical analysis of CD203c^{bright} basophil response in LPP- and PL-treated groups

Phl p Concentration [ng/mL]	Visit	LPP				PL				<i>P</i> value LPP vs PL
		n	mean	SEM	<i>P</i> value vs V2	n	mean	SEM	<i>P</i> value vs V2	
0	V2	21	0.00	0.00	NA	11	0.00	0.00	NA	>0.9999
	V6	21	0.00	0.00	NA	11	0.00	0.00	NA	>0.9999
	V8	21	0.00	0.00	NA	11	0.00	0.00	NA	>0.9999
1	V2	21	29.73	5.32	NA	11	27.96	5.77	NA	0.7846
	V6	21	4.50	6.11	<0.0001	11	37.73	7.29	0.4131	0.0009
	V8	21	9.54	3.01	<0.0001	11	38.82	8.32	0.1748	0.0020
3	V2	21	46.16	6.18	NA	11	50.10	6.84	NA	>0.9999
	V6	21	16.88	6.46	0.0063	11	54.66	7.50	0.9658	0.0019
	V8	21	21.00	4.05	0.0012	11	54.21	8.25	0.8311	0.0014
10	V2	21	57.19	5.57	NA	11	52.82	7.17	NA	0.3267
	V6	21	24.35	6.13	<0.0001	11	62.02	7.58	0.2061	0.0005
	V8	21	27.26	4.75	<0.0001	11	63.07	8.03	0.4131	0.0006
33	V2	21	60.05	5.75	NA	11	52.04	8.76	NA	0.3673
	V6	21	38.46	9.30	0.0646	11	64.42	7.69	0.1230	0.0417
	V8	21	34.71	5.46	0.0022	11	65.92	8.37	0.0537	0.0023
100	V2	21	66.19	4.78	NA	11	56.57	7.20	NA	0.1135
	V6	21	36.15	7.88	0.0019	11	61.85	8.44	0.5195	0.0417
	V8	21	36.72	5.96	<0.0001	11	65.52	8.56	0.2783	0.0081
330	V2	21	62.27	5.39	NA	11	52.62	8.06	NA	0.2078
	V6	21	32.12	7.98	0.0016	11	61.73	7.77	0.1748	0.0339
	V8	21	32.49	6.10	<0.0001	11	61.64	9.59	0.2402	0.0339

Phl p, *Phleum pratense*; LPP, *Lolium perenne* peptide; PL, placebo; V, visit. *P* value LPP vs PL, Mann-Whitney test; *P* value vs V2, Wilcoxon paired signed-rank test. Bolded values represent significant values.

6.2.3 Blunting of grass pollen sIgE and attenuation of IL-4- and IL-21-producing T_{FH} cells following LPP immunotherapy, but not placebo

Serum sIgE was measured in LPP- and PL-treated groups. An increase in serum grass pollen sIgE was observed at V6 from V2 in LPP- but not in PL-treated group. However, the differences in grass pollen sIgE between V8 (after the grass pollen season) and V6 (before the pollen season) which indicates the changes following exposure to naturally occurring allergen during the season was significantly higher in PL- compared to LPP-treated group ($P = 0.0004$; Figure 6.2.3.1, A).

Furthermore, there was a significantly lower proportion of IL-4⁺ T_{FH} cells at V6 in LPP- compared to PL-treated group ($P = 0.0030$) that persisted at V8 ($P = 0.0040$; Figure 6.2.3.1, B). IL-21⁺ T_{FH} cells were lower following LPP immunotherapy at V6 ($P = 0.0030$) and V8 ($P = 0.0020$) compared to PL-treated group (Figure 6.2.3.1, C). Similarly, less dual IL-4⁺IL-21⁺ T_{FH} cells were observed LPP-treated group compared to PL at V6 ($P = 0.004$) and V8 ($P = 0.0100$; Figure 6.2.3.1, D). On the contrary, IFN- γ -producing T_{FH} cells were induced following LPP treatment and were significantly higher compared to PL-treated group at V6 ($P = 0.0300$) and V8 ($P = 0.0100$; Figure 6.2.3.1, E).

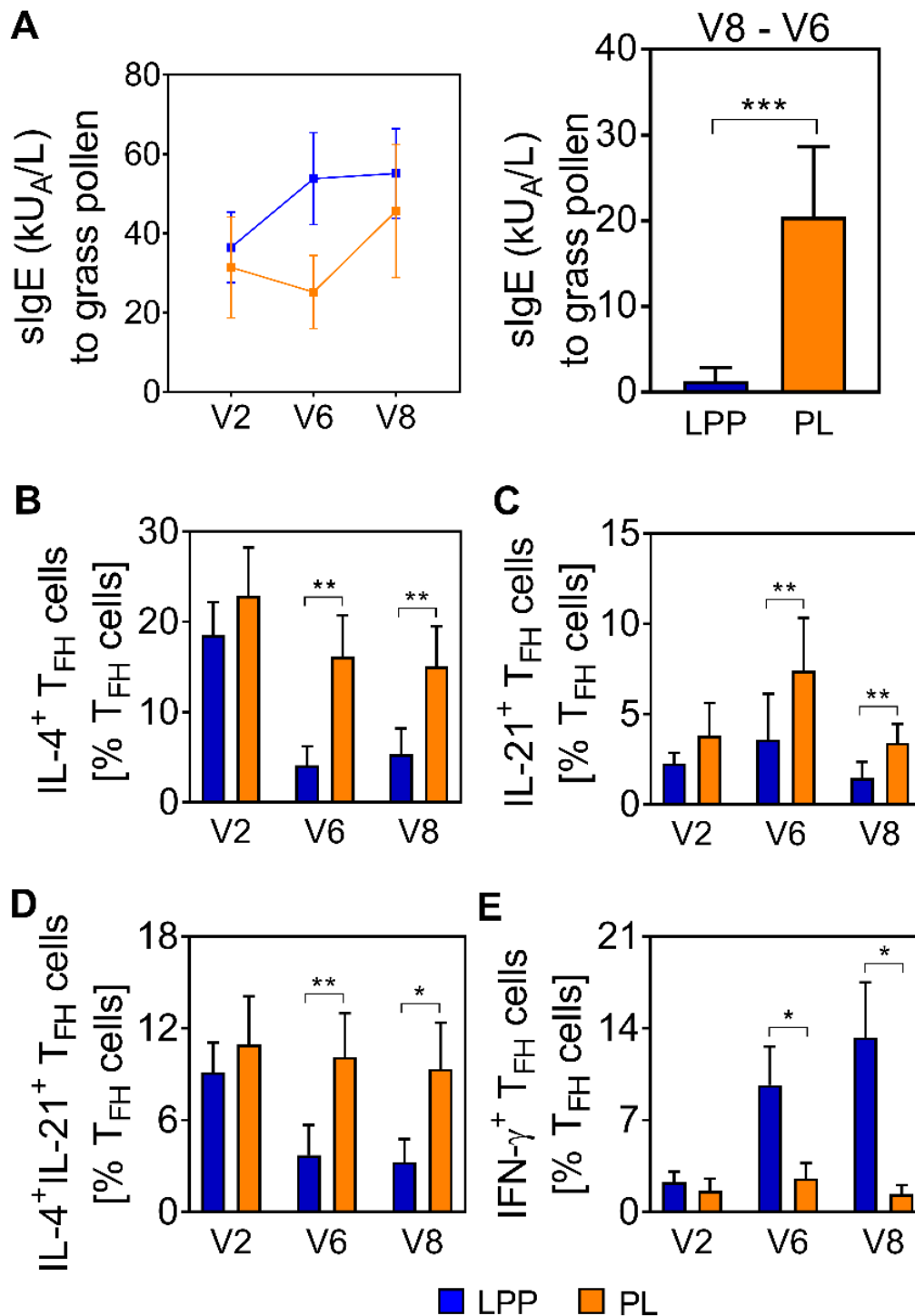


Figure 6.2.3.1 Lower pro-inflammatory T_{FH} cell subsets following LPP immunotherapy, but not PL. **A** Grass pollen sIgE in the serum of LPP- (n = 21) and PL- (n = 11) treated patients at V2, V6 and V8 (left panel), and the difference of serum sIgE levels between V8 and V6 in LPP- and PL-treated groups (right panel). Proportions of **B** IL-4⁺ T_{FH}; **C** IL-21⁺ T_{FH}; **D** IL-4⁺IL-21⁺ T_{FH}; and **E** IFN-γ⁺ T_{FH} cell subsets in LPP- and PL-treated groups at V2 (before the treatment), V6 (after the treatment period) and V8 (after the grass pollen season). Error bars represent SEM. LPP, *Lolium perenne* peptide; PL, placebo; V, visit. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001, Mann-Whitney test.

6.2.4 Induction of T_{FR} and T_{reg} cells following LPP immunotherapy

T_{FR} cells were enumerated in the same cohort of patients (Figure 6.2.4.1, A). The proportion of FoxP3⁺ T_{FR} cells were induced following LPP treatment and was significantly higher compared to PL-treated group at V6 and V8 (both, $P = 0.0040$; Figure 6.2.4.1, B). Furthermore, suppressive CTLA-4⁺ T_{FR} cells were significantly higher in LPP-treated group at V6 ($P = 0.0010$) and persisted until V8 ($P = 0.002$) when compared to PL (Figure 6.2.4.1, C). In addition to T_{FR} cells, naturally occurring T_{reg} cells were also investigated (Figure 6.2.4.2, A). There was an induction of FoxP3⁺ T_{reg} cells in LPP- but not PL-treated group. An induction of FoxP3⁺ T_{reg} cells was observed in LPP treated group at V6 and was significant compared to PL ($P = 0.0300$). Although not statistically significant, there was a trend of higher FoxP3⁺ T_{reg} cells shown at V8 in LPP- compared to PL-treated group (Figure 6.2.4.2, B). Although FoxP3 has been well known to be a marker of T_{reg} cells, FoxP3 is also expressed in activated T cells³⁰⁹. Previous studies have identified GARP as well as repression of SATB1 to be associated with the suppressive functions of T_{reg} cells. Here, GARP⁺ T_{reg} cells were induced following LPP treatment and were significantly higher compared to PL-treated group at V6 ($P = 0.0300$) that remained elevated at V8 ($P = 0.0100$; Figure 6.2.4.2, C). Moreover, there was a significantly higher proportion of T_{reg} cells that suppressed SATB1 expression in LPP- compared to PL-treated group at V6 ($P = 0.0020$) and persisted following the pollen season at V8 ($P = 0.0100$; Figure 6.2.4.2, D). Similarly, inducible IL-35-producing T_{reg} (iT_{R35}) and IL-10⁺ T_{reg} cell subsets were induced following LPP treatment and were significantly higher in LPP- compared to PL-treated group at V6 (iT_{R35}, $P = 0.01$; IL-10⁺ T_{reg}, $P = 0.0004$) and V8 (IL-10⁺ T_{reg}, $P = 0.0010$; Figure 6.2.4.2, E – G).

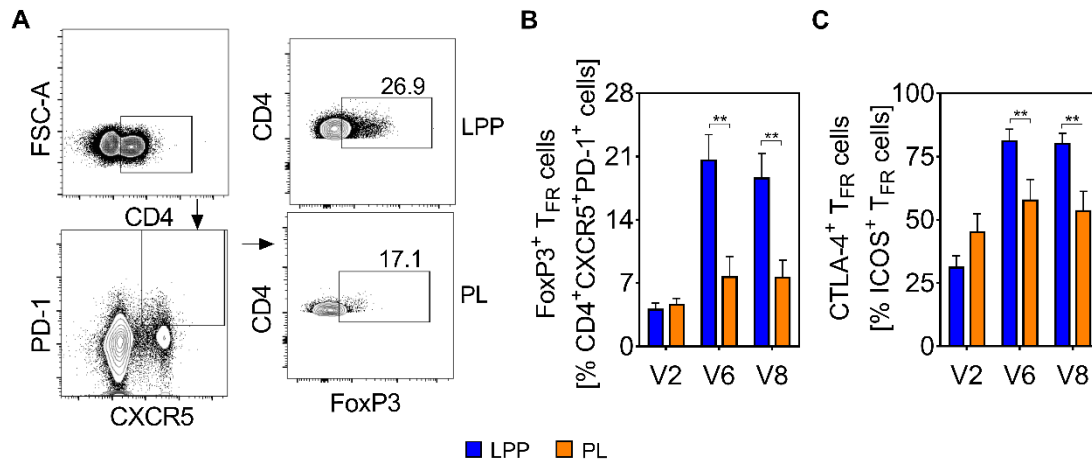


Figure 6.2.4.1 Induction of T_{FR} cell subsets following LPP treatment. **A** Representative plots of T_{FR} cells in LPP- (n = 21) and PL- (n = 11) treated groups. The proportion of **B** FoxP3⁺ T_{FR} (CD4⁺CXCR5⁺PD-1⁺FoxP3⁺); and **C** CTLA-4⁺ T_{FR} cell subsets in LPP- and PL-treated groups before the treatment (V2), after the treatment period (V6) and after the pollen season (V8). Error bars represent SEM. LPP, *Lolium perenne* peptide; PL, placebo; V, visit. *P < 0.05, **P < 0.01 and ***P < 0.001, Mann-Whitney test.

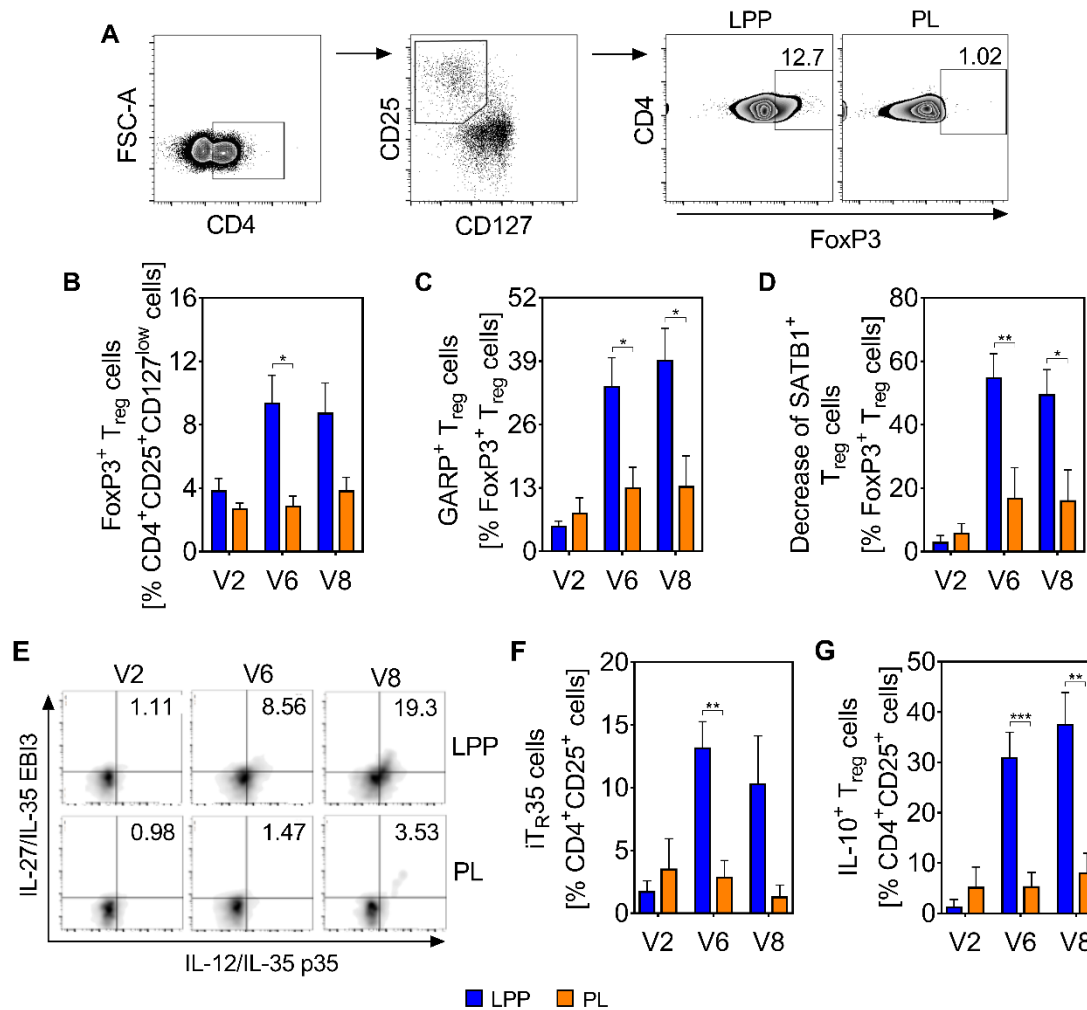


Figure 6.2.4.2 Induction of T_{reg} cell subsets following LPP treatment. **A** Representative plots of T_{reg} cells in LPP- (n = 21) and PL- (n = 11) treated groups. The proportion of **B** FoxP3⁺ T_{reg}; **C** GARP⁺ T_{reg}; and **D** SATB1⁺ T_{reg} cell subsets in LPP- and PL-treated groups at before the treatment (V2), after the treatment period (V6) and after the pollen season (V8). **E** Representative plots of iT_{reg}35 cells (CD4⁺CD25⁺EBI3⁺p35⁺) in LPP- and PL-treated groups. The proportion of inducible **F** iT_{reg}35; and **G** IL-10⁺ T_{reg} cell subsets in LPP- and PL-treated groups at V2, V6 and V8. Error bars represent SEM. LPP, *Lolium perenne* peptide; PL, placebo; V, visit. *P < 0.05, **P < 0.01 and ***P < 0.001, Mann-Whitney test.

6.3 Summary

This chapter demonstrated the findings from a sub-study site of a phase III RDBPC study to assess the mechanistic aspects of peptide immunotherapy. A 3-week course of LPP immunotherapy over four clinical visits was shown to be safe and clinically effective in reducing symptoms during the grass pollen season. LPP-treated patients demonstrated basophil hyporesponsiveness following the treatment prior to the pollen season (V6) that persisted after the pollen season (V8). A blunting of grass pollen sIgE was also observed in LPP but not PL-treated group. Pro-allergic IL-4⁺, IL-21⁺ and dual IL-4⁺IL-21⁺ T_{FH} cells were attenuated following LPP treatment but not in PL-treated group. Interestingly, IFN- γ -producing T_{FH} cells were higher in LPP- compared to PL-treated group during (V6) and after (V8) the pollen season. There were induction of regulatory T_{FR} and suppressive CTLA-4⁺ T_{FR} cell subsets following LPP treatment that persisted until after the grass pollen season. Suppressive Treg and inducible iT_{R35} and IL-10⁺ Treg cell subsets were higher in LPP-treated group compared to PL. Overall, these findings further illustrated the safety and efficacy of a short-course LPP immunotherapy that persisted throughout the grass pollen season, which is associated with the modulation of pro-allergic T_{FH} cells and the induction of regulatory T_{FR} and Treg cell subsets.

CHAPTER 7 NOVEL APPROACHES OF ALLERGEN-SPECIFIC IMMUNOTHERAPY USING DEPIGMENTED AND POLYMERISED ALLERGEN EXTRACT

7.1 Introduction

The challenges in grass pollen immunotherapy administration have always been associated with the safety of the treatment and patient adherence. This warrant a need for continuous development of novel immunotherapy approaches. Allergoids, the term coined for allergen extracts subjected to chemical modifications, is thought to be a safer alternative. Allergoids maintain their clinical efficacy despite the modifications introduced to the allergen. The chemical modifications prevent the allergen from forming IgE-allergen complexes and cross-linking with FcεRI on the surface of basophils and mast cells. This leads to the inability to elicit an allergic response through the release of mediators such as histamine, thus providing a greater safety profile compared to conventional SCIT and SLIT. The use of allergoids has been reported to treat birch pollen and HDM allergy^{59,60,64,65}, where allergoids was demonstrated to be clinically effective in reducing symptoms and medication scores, induced IgG₄, in the active-treated group compared to placebo. These changes were observed to be comparable to that of conventional SCIT and SLIT using whole allergen extracts.

This chapter aims to investigate the allergenicity of depigmented (DPG Phl p) and polymerised (DPG-POL Phl p) grass pollen (Phl p) allergoids. Allergenicity of DPG Phl p and DPG-POL Phl p is assessed using *ex vivo* basophil activation test. IL-4⁺ and IL-21⁺ T_{FH} cells and T_H2 and T_H2A cell responses in response to *in vitro* stimulation with the allergoids are investigated.

7.2 Results

7.2.1 Basophil hyporesponsiveness in response to stimulation with DPG-POL Phl p compared to DPG Phl p and native protein

The allergenicity of DPG-POL Phl p compared to DPG Phl p and grass pollen native protein was firstly investigated by performing *ex vivo* basophil activation test. To evaluate the FcεRI-mediated allergic response. The activation of basophils was investigated through the expression of CD63 and CD203c. A concentration-dependent increase in CD63⁺ basophil activation was observed in response to native protein, DPG Phl p and DPG-POL Phl p in GPA (Figure 7.2.1.1, A and B) but not NAC (Figure 7.2.1.1, A and C). There was a rightward shift in the curve following stimulation with DPG-POL Phl p, compared to DPG Phl p and native protein in GPA (Figure 7.2.1.1, B). The proportion of CD63⁺ basophils were significantly higher when stimulated with native protein and DPG Phl p compared to the DPG-POL Phl p at 1, 10, 33, and 100 ng/mL (Table 7.2.1.1) in GPA (Figure 7.2.1.1, B). No basophil activation was elicited following stimulation with negative control and in NAC (Figure 7.2.1.1, B and C; Table 7.2.1.1). EC₅₀ analysis of activated CD63⁺ basophils revealed that DPG-POL Phl p has a reduced potency as represented by a shift towards the right in the curve, compared to DPG Phl p ($P = 0.0001$) and native protein ($P = 0.0015$; Figure 7.2.1.1, D and E; Table 7.2.1.2). No significant differences were observed between native protein and DPG Phl p.

Similarly, there was a concentration-dependent increase of CD203c^{bright} basophils following stimulation with native protein, DPG Phl p and DPG-POL Phl p in GPA (Figure 7.2.1.1, F and G) but not NAC (Figure 7.2.1.1, F and H). A shift to the right in the curve was observed in response to DPG-POL Phl p stimulation compared to native protein and DPG Phl p in GPA (Figure 7.2.1.1, G). The proportion of CD203c^{bright} basophils were significantly higher when stimulated with native protein and DPG Phl p compared to the DPG-POL Phl p at 1, 10, 33, and 100 ng/mL (Table 7.2.1.3) in GPA (Figure 7.2.1.1, G). No response was observed

following negative control stimulation and in NAC (Figure 7.2.1.1, G and H). EC₅₀ analysis of CD203c^{bright} basophils showed reduced potency of DPG-POL Phl p compared to native protein ($P = 0.0117$) and DPG Phl p ($P = 0.0018$), as shown as a rightward shift in the EC₅₀ curve (Figure 7.2.1.1, I and J; Table 7.2.1.4). No significant differences were noted between native protein and DPG Phl p.

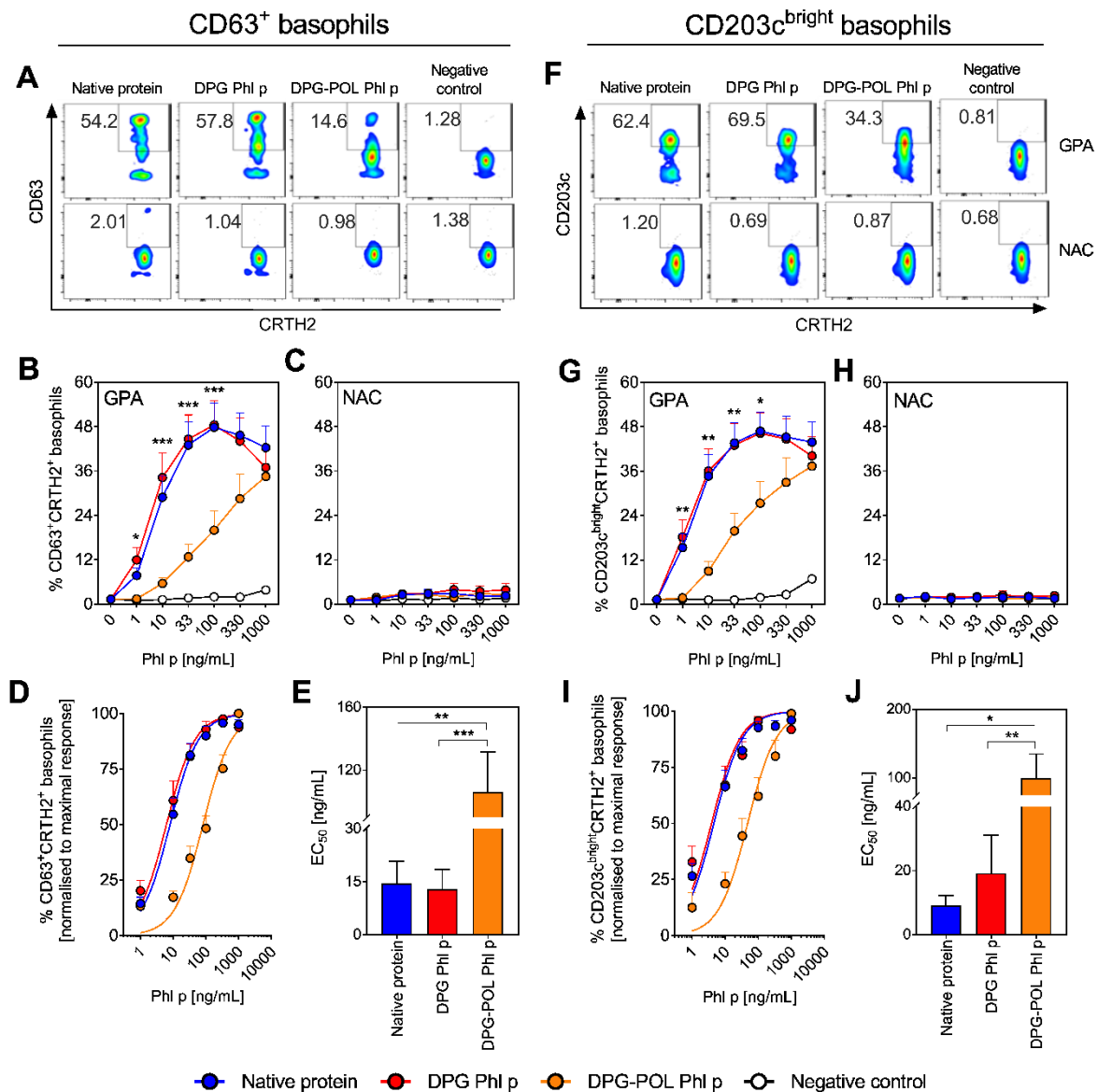


Figure 7.2.1.1 Reduced basophil activation following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein. **A** Representative plots illustrating basophil activation (CD63⁺CRTH2⁺) in the absence and presence of native protein, DPG Phl p, DPG-POL Phl p and a negative control in GPA (top panels) and NAC (bottom panels). Dose-response of CD63⁺ basophils in **B** GPA (n = 16); and **C** NAC (n = 8), following stimulation with various concentrations of the native protein, DPG and DPG-POL Phl p and negative control. **D** EC₅₀ analysis of GPA as represented as dose-response curves; and **E** bar charts. **F-J** Similarly, for CD203c^{bright}CRTH2⁺ basophils. Error bars represent SEM. DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids; GPA, grass pollen allergics; NAC, non-atopic healthy controls. **P < 0.01 and ***P < 0.001, Kruskal-Wallis test with Dunn's multiple comparison test for EC₅₀ analysis versus DPG-POL Phl p.

Table 7.2.1.1 Statistical analysis of CD63⁺ basophil response following stimulation with native protein, DPG Phl p and DPG-POL Phl p in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					P value GPA vs NAC
		n	mean	SEM	P value vs		n	mean	SEM	P value vs		
					DPG-POL Phl p	Baseline				DPG-POL Phl p	Baseline	
0 (Baseline)	Native protein	16	1.29	0.11	>0.9999	NA	8	1.13	0.20	>0.9999	NA	0.7417
	DPG Phl p	16	1.29	0.11	>0.9999	NA	8	1.13	0.20	>0.9999	NA	0.7417
	DPG-POL Phl p	16	1.29	0.11	NA	NA	8	1.13	0.20	NA	NA	0.7417
	Negative control	16	1.29	0.11	>0.9999	NA	8	1.13	0.20	>0.9999	NA	0.7417
1	Native protein	16	7.71	1.96	0.0176	0.0155	8	1.07	0.36	0.2786	0.8438	0.0107
	DPG Phl p	13	11.91	3.35	0.0010	0.0009	8	1.35	0.28	0.6255	0.8203	0.0031
	DPG-POL Phl p	16	1.41	0.28	NA	0.8158	8	1.90	0.57	NA	0.5469	0.5910
	Negative control	16	1.01	0.12	0.5916	0.0413	8	0.92	0.21	0.1949	0.4609	0.7755
10	Native protein	16	28.89	5.89	0.0005	<0.0001	8	2.56	0.78	0.7984	0.1484	0.0001
	DPG Phl p	16	34.22	6.65	0.0002	<0.0001	8	2.73	0.82	0.5054	0.0234	0.0009
	DPG-POL Phl p	16	5.57	1.63	NA	0.0076	8	2.77	1.02	NA	0.1484	0.2144
	Negative control	16	1.21	0.15	0.0077	0.7820	8	1.38	0.41	0.3823	0.6406	0.9761
33	Native protein	16	43.01	6.15	0.0006	<0.0001	8	2.83	1.11	0.6454	0.1094	<0.0001
	DPG Phl p	16	44.61	6.53	0.0006	<0.0001	8	2.96	0.98	0.7984	0.4375	<0.0001
	DPG-POL Phl p	16	12.77	3.45	NA	0.0006	8	2.57	0.78	NA	0.0781	0.0192
	Negative control	16	1.65	0.29	0.0007	0.2069	8	1.07	0.24	0.0830	>0.9999	0.3265
100	Native protein	16	47.83	6.47	0.0008	<0.0001	8	2.88	1.14	0.3282	0.4609	<0.0001
	DPG Phl p	16	48.43	6.65	0.0009	<0.0001	8	3.92	1.68	0.1304	0.0078	<0.0001
	DPG-POL Phl p	16	19.99	5.18	NA	<0.0001	8	1.83	0.97	NA	0.8438	0.0017
	Negative control	16	1.94	0.46	0.0005	0.2918	8	1.71	0.68	0.7001	0.8438	0.7765
330	Native protein	16	45.64	5.99	0.0615	<0.0001	8	2.12	0.67	0.5737	0.3125	<0.0001
	DPG Phl p	16	44.10	6.28	0.1100	<0.0001	8	3.46	1.53	0.9364	0.0781	<0.0001
	DPG-POL Phl p	16	28.51	6.53	NA	<0.0001	8	3.00	1.15	NA	0.0391	0.0045
	Negative control	16	1.88	0.47	<0.0001	0.2918	8	1.20	0.30	0.1374	>0.9999	0.4162
1000	Native protein	16	42.30	5.89	0.3226	<0.0001	8	2.29	1.10	0.6454	0.3828	<0.0001
	DPG Phl p	16	36.94	5.58	0.7240	<0.0001	8	3.82	1.78	0.8785	0.1484	<0.0001
	DPG-POL Phl p	16	34.50	6.99	NA	<0.0001	8	2.41	0.84	NA	0.0469	0.0004
	Negative control	16	3.82	1.17	0.0001	0.0110	8	1.65	0.43	0.6454	0.1641	0.2144

DPG, depigmented; DPG-POL, depigmented and polymerised; GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs DPG-POL Phl p and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significance.

Table 7.2.1.2 EC₅₀ of the native protein, DPG Phl p and DPG-POL Phl p to elicit CD63⁺ basophil activation in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	14.52 ± 6.24**	1.00
DPG Phl p	13.00 ± 5.42***	0.90
DPG-POL Phl p	106.20 ± 25.64	7.31

DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids. Statistical significance versus DPG-POL Phl p. ** $P < 0.01$ and *** $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test.

Table 7.2.1.3 Statistical analysis of CD203c^{bright} basophil response following stimulation with native protein, DPG Phl p and DPG-POL

Phl p in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					<i>P</i> value GPA vs NAC
		n	mean	SEM	<i>P</i> value vs		n	mean	SEM	<i>P</i> value vs		
					DPG-POL Phl p	Baseline				DPG-POL Phl p	Baseline	
0 (Baseline)	Native protein	16	1.33	0.09	>0.9999	NA	8	1.57	0.24	>0.9999	NA	0.881
	DPG Phl p	16	1.33	0.09	>0.9999	NA	8	1.57	0.24	>0.9999	NA	0.881
	DPG-POL Phl p	16	1.33	0.09	NA	NA	8	1.57	0.24	NA	NA	0.881
	Negative control	16	1.33	0.09	>0.9999	NA	8	1.57	0.24	>0.9999	NA	0.881
1	Native protein	16	15.35	3.81	0.0057	0.0002	8	2.06	0.86	0.9823	0.8438	0.0230
	DPG Phl p	13	18.17	4.60	0.0015	0.0002	8	2.05	0.82	0.9357	0.6406	0.0089
	DPG-POL Phl p	16	1.78	0.25	NA	0.1392	8	1.74	0.57	NA	0.6406	0.5800
	Negative control	16	1.39	0.22	0.1425	0.7197	8	1.68	0.27	0.5540	0.8438	0.3421
10	Native protein	16	34.66	5.81	0.0003	<0.0001	8	1.39	0.45	0.5054	0.4609	<0.0001
	DPG Phl p	16	36.03	5.98	0.0019	0.0002	8	1.87	0.78	0.8200	0.9453	0.0007
	DPG-POL Phl p	16	8.97	2.61	NA	0.0027	8	1.68	0.51	NA	>0.9999	0.0395
	Negative control	16	1.24	0.14	0.0045	0.4874	7	1.62	0.25	0.3357	0.2969	0.1353
33	Native protein	16	43.60	5.55	0.0014	<0.0001	8	1.78	0.73	0.5737	0.8438	<0.0001
	DPG Phl p	16	43.00	5.78	0.0050	<0.0001	8	1.98	0.53	0.7209	0.3672	<0.0001
	DPG-POL Phl p	16	19.86	4.79	NA	0.0017	8	2.05	0.71	NA	0.4609	0.0324
	Negative control	16	1.18	0.17	0.0011	0.2260	8	1.68	0.56	0.9043	0.9453	0.8810
100	Native protein	16	46.76	5.26	0.0216	<0.0001	8	1.82	0.56	0.5054	0.6406	<0.0001
	DPG Phl p	16	46.23	5.52	0.0325	<0.0001	8	2.35	1.24	0.6671	0.8438	0.0002
	DPG-POL Phl p	16	27.34	5.93	NA	0.0002	8	1.53	0.64	NA	0.1953	0.0022
	Negative control	16	1.87	0.35	0.0003	0.1630	8	2.52	1.43	0.7195	0.9453	0.5681
330	Native protein	16	45.21	5.74	0.1964	<0.0001	8	1.93	0.86	>0.9999	>0.9999	<0.0001
	DPG Phl p	16	44.66	5.56	0.1355	<0.0001	8	2.12	0.77	0.6454	0.4609	<0.0001
	DPG-POL Phl p	16	32.96	6.53	NA	0.0002	8	1.40	0.34	NA	0.6406	0.0007
	Negative control	16	2.67	0.77	0.0002	0.2312	8	1.43	0.45	0.8785	0.5781	0.3576
1000	Native protein	16	43.82	5.52	0.4737	<0.0001	8	1.55	0.33	0.9395	0.6406	<0.0001
	DPG Phl p	16	40.10	5.20	0.7804	<0.0001	8	2.31	0.99	0.7984	0.3828	<0.0001
	DPG-POL Phl p	16	37.33	6.34	NA	0.0002	8	2.01	0.85	NA	0.6406	0.0007
	Negative control	16	6.90	2.53	0.0004	0.0076	8	1.46	0.26	0.9043	>0.9999	0.1391

DPG, depigmented; DPG-POL, depigmented and polymerised; GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs DPG-POL Phl p and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significance.

Table 7.2.1.4 EC₅₀ of the native protein, DPG Phl p and DPG-POL Phl p to elicit CD203c^{bright} basophil activation in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	9.05 ± 3.16*	1.00
DPG Phl p	19.19 ± 11.87**	2.12
DPG-POL Phl p	99.32 ± 35.65	10.97

DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids. Statistical significance versus DPG-POL Phl p. * $P < 0.05$ and ** $P < 0.01$, Kruskal-Wallis test with Dunn's multiple comparison test.

7.2.2 Reduced histamine release from activated basophils following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein

A concentration-dependent increase in DAO⁺CD63⁺ basophil histamine release was observed following stimulation with native protein, DPG Phl p and DPG-POL Phl p in GPA (Figure 7.2.2.1, A and B) but not NAC (Figure 7.2.2.1, A and C). There was a rightward shift in the curve observed in response to DPG-POL Phl p compared to DPG Phl p and native protein in GPA (Figure 7.2.2.1, B). The proportion of DAO⁺CD63⁺ basophils were significantly higher when stimulated with native protein and DPG Phl p compared to the DPG-POL Phl p at 1, 10, 33, and 100 ng/mL (Table 7.2.2.1) in GPA (Figure 7.2.2.1, B). No response was observed following stimulation with negative control (Figure 7.2.2.1, B) and in NAC (Figure 7.2.2.1, C). A shift to the right in the EC₅₀ curve was observed following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein (Figure 7.2.2.1, D), indicating the lower potency of DPG-POL Phl p in eliciting DAO⁺CD63⁺ basophil histamine release compared to DPG Phl p ($P < 0.0001$) and native protein ($P = 0.0003$; Figure 7.2.2.1, E; Table 7.2.2.2).

Similarly, there was a concentration-dependent increase in DAO⁺CD203c^{bright} basophil histamine release observed in response to stimulation with native protein, DPG Phl p and DPG-POL Phl p in GPA (Figure 7.2.2.1, F and G) but not NAC (Figure 7.2.2.1, F and H). A rightward shift in the curve following stimulation with DPG-POL Phl p was observed compared to native protein and DPG Phl p in GPA (Figure 7.2.2.1, G). The proportion of DAO⁺CD203c^{bright} basophils were significantly higher when stimulated with native protein and DPG Phl p compared to the DPG-POL Phl p at 1, 10, 33, and 100 ng/mL (Table 7.2.2.3) in GPA (Figure 7.2.2.1, G). No changes were observed in response to negative control and NAC (Figure 7.2.2.1, G and H). The EC₅₀ curve was shifted to the right following stimulation with DPG-POL Phl p (Figure 7.2.2.1, I), indicating the reduced potency of DPG-POL Phl p compared to native protein ($P = 0.0080$) and DPG Phl p ($P = 0.0018$; Figure 7.2.2.1, J; Table 7.2.2.4). No significant differences were observed between native protein and DPG Phl p.

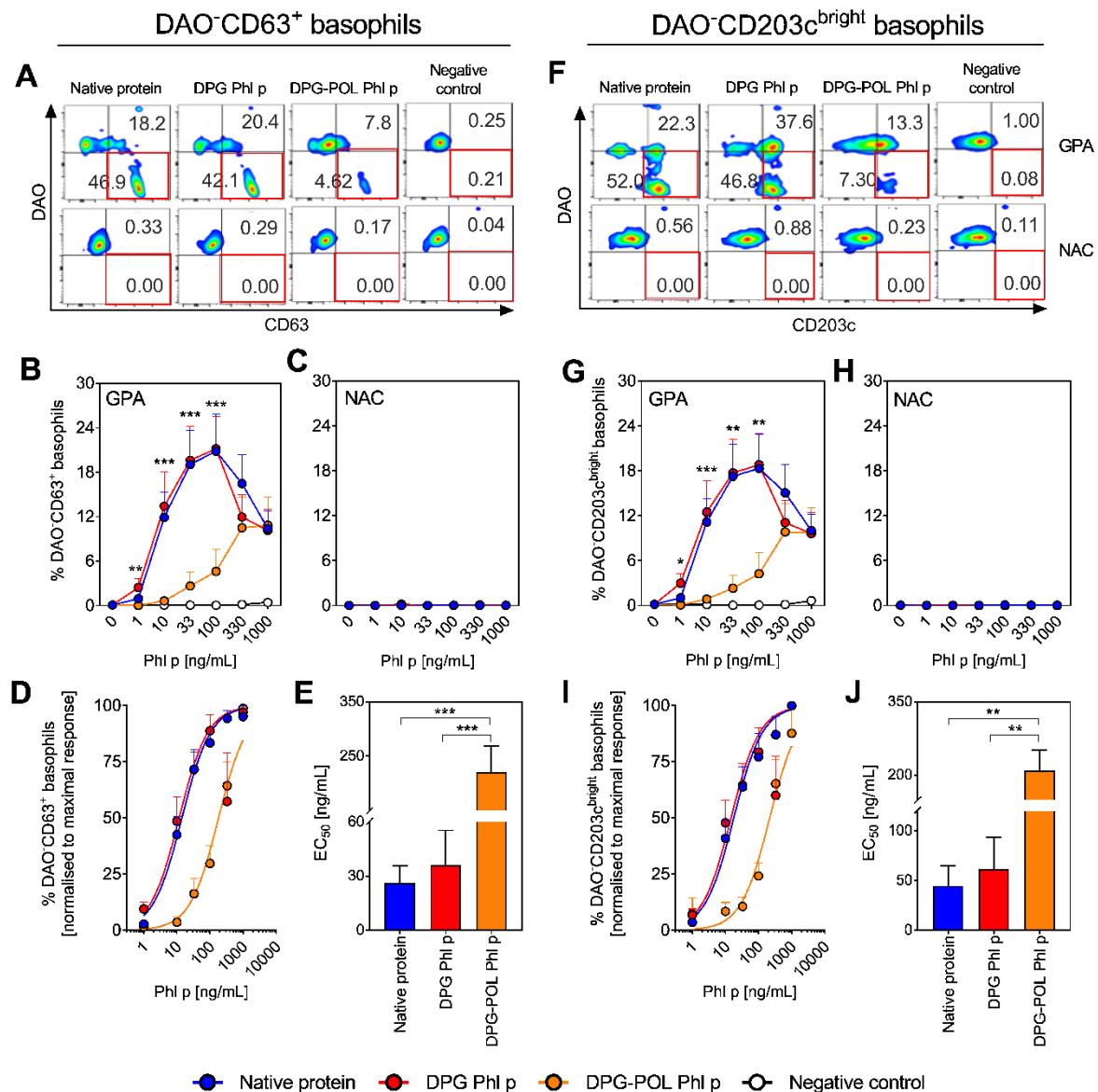


Figure 7.2.2.1 Lower basophil histamine release following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein. **A** Representative plots illustrating basophil histamine release following activation (DAO-CD63⁺) in the absence and presence of native protein, DPG Phl p, DPG-POL Phl p and a negative control in GPA (top panels) and NAC (bottom panels). Dose-response of DAO-CD63⁺ basophils in **B** GPA (n = 16); and **C** NAC (n = 8), following stimulation with various concentrations of the native protein, DPG and DPG-POL Phl p and negative control. **D** EC₅₀ analysis of GPA as represented as dose-response curves; and **E** bar charts. **F-J** Similarly, for DAO-CD203c^{bright} basophils. Error bars represent SEM. DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids; GPA, grass pollen allergics; NAC, non-atopic healthy controls. ****P* < 0.001, Kruskal-Wallis test with Dunn's multiple comparison test for EC₅₀ analysis versus DPG-POL Phl p.

Table 7.2.2.1 Statistical analysis of DAO⁺CD63⁺ basophil response following stimulation with native protein, DPG Phl p and DPG-POL

Phl p in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					P value GPA vs NAC
		n	mean	SEM	P value vs		n	mean	SEM	P value vs		
					DPG-POL Phl p	Baseline				DPG-POL Phl p	Baseline	
0 (Baseline)	Native protein	16	0.08	0.05	>0.9999	NA	8	0.03	0.02	>0.9999	NA	0.7644
	DPG Phl p	16	0.08	0.05	>0.9999	NA	8	0.03	0.02	>0.9999	NA	0.7644
	DPG-POL Phl p	16	0.08	0.05	NA	NA	8	0.03	0.02	NA	NA	0.7644
	Negative control	16	0.08	0.05	>0.9999	NA	8	0.03	0.02	>0.9999	NA	0.7644
1	Native protein	16	0.94	0.50	0.0039	0.0342	8	0.00	0.00	>0.9999	0.5000	0.0075
	DPG Phl p	13	2.44	1.18	0.0021	0.0186	7	0.03	0.03	>0.9999	>0.9999	0.0199
	DPG-POL Phl p	16	0.02	0.01	NA	0.3125	8	0.00	0.00	NA	0.5000	0.4519
	Negative control	16	0.01	0.01	0.6663	0.0625	8	0.02	0.02	>0.9999	>0.9999	0.8419
10	Native protein	16	11.92	3.39	<0.0001	<0.0001	8	0.01	0.01	0.4667	>0.9999	<0.0001
	DPG Phl p	16	13.39	4.67	0.0003	0.0001	7	0.17	0.13	0.5692	0.7500	0.0011
	DPG-POL Phl p	16	0.64	0.45	NA	0.3594	8	0.04	0.03	NA	0.2500	0.4239
	Negative control	16	0.07	0.03	0.6726	0.8438	8	0.00	0.00	0.4667	0.5000	0.1304
33	Native protein	16	19.04	4.63	0.0001	<0.0001	8	0.04	0.03	0.4667	0.7500	<0.0001
	DPG Phl p	16	19.61	4.62	0.0001	<0.0001	7	0.04	0.03	0.7333	0.5000	<0.0001
	DPG-POL Phl p	16	2.64	1.86	NA	0.0312	8	0.01	0.01	NA	0.5000	0.0113
	Negative control	16	0.06	0.04	0.0116	0.8125	8	0.03	0.03	>0.9999	>0.9999	0.5995
100	Native protein	16	20.84	5.01	0.0002	<0.0001	8	0.06	0.03	0.2000	0.7500	<0.0001
	DPG Phl p	16	21.17	4.33	0.0006	<0.0001	7	0.06	0.03	0.3231	0.8750	<0.0001
	DPG-POL Phl p	16	4.63	2.94	NA	0.0081	8	0.01	0.01	NA	>0.9999	0.0029
	Negative control	16	0.05	0.02	0.0029	0.8438	8	0.00	0.00	>0.9999	0.2500	0.1038
330	Native protein	16	16.46	3.89	0.1034	<0.0001	8	0.02	0.01	0.3538	0.2500	<0.0001
	DPG Phl p	16	11.95	2.99	0.2540	<0.0001	7	0.09	0.04	0.2821	0.7500	<0.0001
	DPG-POL Phl p	16	10.50	4.11	NA	0.0002	8	0.04	0.02	NA	>0.9999	0.0019
	Negative control	16	0.15	0.05	0.0006	0.3652	8	0.06	0.04	>0.9999	0.5000	0.2051
1000	Native protein	16	10.39	2.40	0.5967	<0.0001	8	0.02	0.01	0.4974	>0.9999	<0.0001
	DPG Phl p	16	10.15	2.84	0.4510	<0.0001	7	0.05	0.02	0.7846	0.1250	<0.0001
	DPG-POL Phl p	16	10.84	3.80	NA	0.0001	8	0.05	0.03	NA	0.8750	0.0002
	Negative control	16	0.42	0.14	0.0005	0.0669	8	0.00	0.00	0.2000	>0.9999	0.0075

DPG, depigmented; DPG-POL, depigmented and polymerised; GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs DPG-POL Phl p and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significance.

Table 7.2.2.2 EC₅₀ of the native protein, DPG Phl p and DPG-POL Phl p to elicit DAO⁺CD63⁺ basophil activation in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	26.12 ± 9.49***	1.00
DPG Phl p	36.14 ± 19.17***	1.38
DPG-POL Phl p	219.80 ± 48.36	8.42

DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids. Statistical significance versus DPG-POL Phl p. *** $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test.

Table 7.2.2.3 Statistical analysis of DAO-CD203c^{bright} basophil response following stimulation with native protein, DPG Phl p and DPG-POL Phl p in GPA and NAC

Phl p Concentration [ng/mL]	Protein/Peptide	GPA					NAC					P value GPA vs NAC
		n	mean	SEM	P value vs		n	mean	SEM	P value vs		
					DPG-POL Phl p	Baseline				DPG-POL Phl p	Baseline	
0 (Baseline)	Native protein	16	0.18	0.07	>0.9999	NA	8	0.06	0.04	>0.9999	NA	0.2726
	DPG Phl p	16	0.18	0.07	>0.9999	NA	8	0.06	0.04	>0.9999	NA	0.2726
	DPG-POL Phl p	16	0.18	0.07	NA	NA	8	0.06	0.04	NA	NA	0.2726
	Negative control	16	0.18	0.07	>0.9999	NA	8	0.06	0.04	>0.9999	NA	0.2726
1	Native protein	16	1.02	0.48	0.0003	0.0291	8	0.01	0.01	>0.9999	0.5000	0.0002
	DPG Phl p	13	3.01	1.28	0.0379	0.0378	8	0.04	0.03	0.4667	>0.9999	0.1039
	DPG-POL Phl p	16	0.08	0.05	NA	0.1543	8	0.00	0.00	NA	0.5000	0.2622
	Negative control	16	0.17	0.09	0.6221	0.5469	8	0.01	0.01	>0.9999	0.5000	0.2852
10	Native protein	16	11.15	3.16	<0.0001	<0.0001	8	0.00	0.00	>0.9999	0.5000	<0.0001
	DPG Phl p	16	12.49	4.22	0.0002	<0.0001	8	0.03	0.03	>0.9999	>0.9999	<0.0001
	DPG-POL Phl p	16	0.85	0.46	NA	0.2958	8	0.01	0.01	NA	0.5000	0.0070
	Negative control	16	0.13	0.06	0.0594	0.5410	8	0.00	0.00	>0.9999	0.5000	0.0996
33	Native protein	16	17.27	4.26	0.0013	0.0002	8	0.03	0.03	>0.9999	>0.9999	0.0002
	DPG Phl p	16	17.72	4.50	0.0001	<0.0001	8	0.04	0.03	0.4667	>0.9999	<0.0001
	DPG-POL Phl p	16	2.32	1.70	NA	0.0457	8	0.00	0.00	NA	0.5000	0.0075
	Negative control	16	0.05	0.03	0.0041	0.0352	8	0.01	0.01	>0.9999	0.5000	0.6838
100	Native protein	16	18.31	4.64	0.0043	0.0002	8	0.08	0.04	0.2000	0.7500	0.0004
	DPG Phl p	16	18.81	4.03	0.0016	<0.0001	8	0.06	0.03	0.2	>0.9999	<0.0001
	DPG-POL Phl p	16	4.25	2.80	NA	0.0151	8	0.00	0.00	NA	0.5000	0.0001
	Negative control	16	0.07	0.05	<0.0001	0.0293	8	0.02	0.01	0.4667	0.5000	0.8233
330	Native protein	16	15.06	3.75	0.1078	<0.0001	8	0.05	0.04	>0.9999	>0.9999	<0.0001
	DPG Phl p	16	11.08	2.96	0.3412	<0.0001	8	0.03	0.02	>0.9999	0.5000	<0.0001
	DPG-POL Phl p	16	9.85	3.79	NA	0.0003	8	0.02	0.02	NA	0.5000	<0.0001
	Negative control	16	0.14	0.04	<0.0001	0.6221	8	0.04	0.04	>0.9999	>0.9999	0.0645
1000	Native protein	16	10.00	2.25	0.4284	<0.0001	8	0.05	0.04	0.7846	>0.9999	<0.0001
	DPG Phl p	16	9.62	2.83	0.5146	<0.0001	8	0.01	0.01	0.3231	0.5000	<0.0001
	DPG-POL Phl p	16	9.65	3.39	NA	0.0003	8	0.04	0.02	NA	>0.9999	<0.0001
	Negative control	16	0.64	0.31	0.0003	0.3021	8	0.01	0.01	0.4462	0.5000	0.0462

DPG, depigmented; DPG-POL, depigmented and polymerised; GPA, grass pollen allergics; NAC, non-atopic healthy controls. *P* value vs DPG-

POL Phl p and GPA vs NAC, Mann-Whitney test; *P* value vs baseline, Wilcoxon paired signed-rank test. Bolded values represent significance.

Table 7.2.2.4 EC₅₀ of the native protein, DPG Phl p and DPG-POL Phl p to elicit DAO-CD203c^{bright} basophil activation in GPA

Sample	EC ₅₀ [ng/mL], mean ± SEM	Fold change (normalised to native protein)
Native protein	44.65 ± 20.26**	1.00
DPG Phl p	61.52 ± 32.08**	1.38
DPG-POL Phl p	209.10 ± 42.59	4.68

DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids. Statistical significance versus DPG-POL Phl p. ** $P < 0.01$, Kruskal-Wallis test with Dunn's multiple comparison test.

7.2.3 Lower IL-4⁺ and IL-21⁺ T_{FH} cell proliferation following stimulation with DPG-POL

Phl p compared to DPG Phl p and native protein

The effect of DPG-POL Phl p, DPG Phl p and native protein on the proliferation of T_{FH} cell subsets were investigated. There was a concentration-dependent increase in the proliferation of IL-4⁺ T_{FH} cells following stimulation with native protein and DPG Phl p in GPA, and to a lesser extent in response to DPG-POL Phl p stimulation and negative control (Figure 7.2.3.1, A). The proportion of proliferated IL-4⁺ T_{FH} cells were significantly reduced following stimulation with DPG-POL Phl p compared to the native protein at 1 µg/mL ($P = 0.0043$; Figure 7.2.3.1, A). No significant changes in the proportion of proliferated IL-4⁺ T_{FH} cells were observed in NAC (Figure 7.2.3.1, B). Similarly, a concentration-dependent increase in the proliferation of IL-21⁺ T_{FH} cells was observed following stimulation with native protein and DPG Phl p, and to a lesser extent following stimulation with DPG-POL Phl p and negative control (Figure 7.2.3.1, C). The proportion of proliferated IL-21⁺ T_{FH} cells were significantly lower in response to DPG-POL Phl p compared to native protein at 0.03 µg/mL ($P = 0.0411$) and 1 µg/mL ($P = 0.0043$; Figure 7.2.3.1, C). No significant response was observed in NAC following stimulation with native protein, DPG Phl p, DPG-POL Phl p and negative control (Figure 7.2.3.1, D).

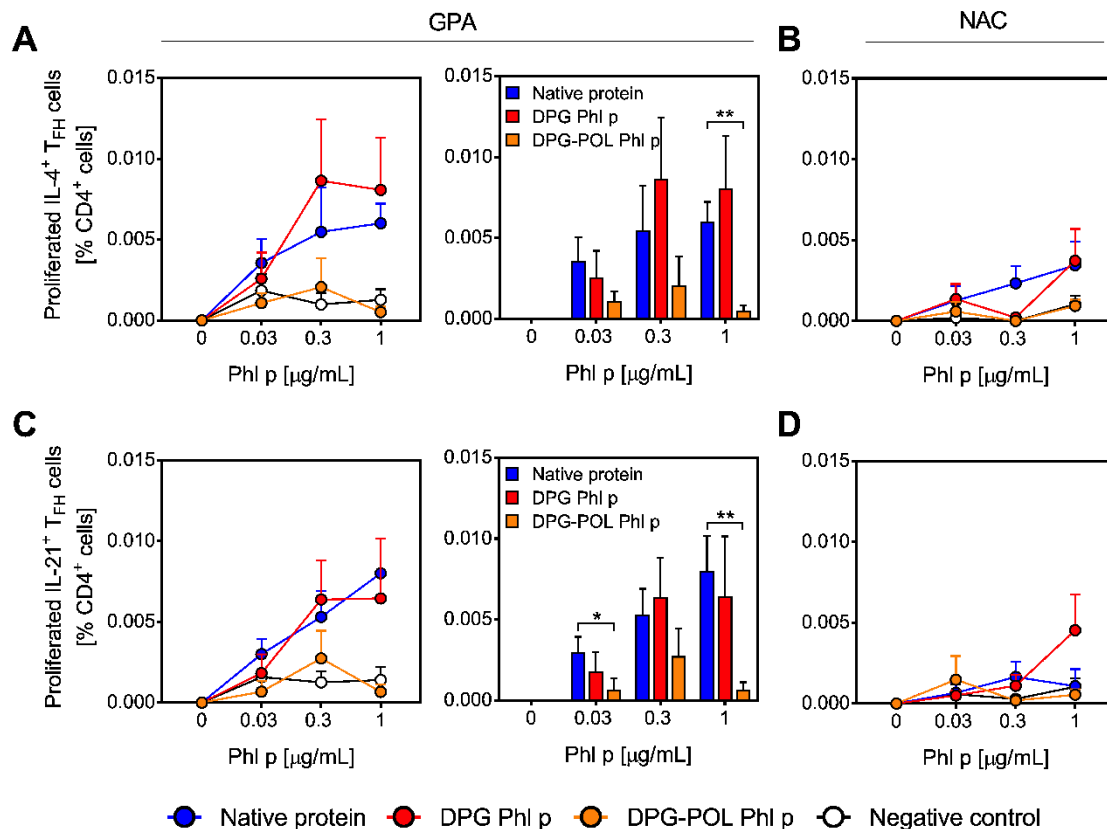


Figure 7.2.3.1 Lower proliferation of IL-4⁺ and IL-21⁺ T_{FH} cell subsets following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein. The proportion of proliferated IL-4⁺ T_{FH} (CD4⁺CXCR5⁺PD-1⁺) cells in **A** GPA (n = 6) represented as line and bar graphs; and **B** NAC (n = 6), in response to stimulation with varying concentrations of the native protein, DPG Phl p and DPG-POL Phl p. The proportion of proliferated IL-21⁺ T_{FH} cells in **C** GPA represented as a line and bar graphs; and **D** NAC, following stimulation with varying concentrations of the native protein, DPG Phl p and DPG-POL Phl p. Error bars represent SEM. DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids; GPA, grass pollen allergics; NAC, non-atopic healthy controls. **P* < 0.05 and ***P* < 0.01, Mann-Whitney test.

7.2.4 Reduced T_H2 and T_H2A cell responses following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein

There was a trend of a concentration-dependent increase in the proliferation of T_H2 cells following stimulation with native protein, DPG Phl p, DPG-POL Phl p and negative control in GPA (Figure 7.2.4.1, A). Although a trend of lower proliferation of T_H2 cells was observed following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein in GPA, this was not statistically significant (Figure 7.2.4.1, B). No significant differences were observed in NAC (Figure 7.2.4.1, B). Levels of IL-5 and IL-13 cytokines in culture supernatants were also evaluated using multiplex. A trend of higher IL-5 levels in culture supernatants was observed following stimulation with native protein and DPG Phl p compared to DPG-POL Phl p and negative control in GPA but not NAC (Figure 7.2.4.1, C). Furthermore, there was a trend of higher IL-13 levels in culture supernatant in response to stimulation with native protein and DPG Phl p compared to DPG-POL Phl p and negative control in GPA which was observed to be lower than in NAC (Figure 7.2.4.1, D).

A concentration-dependent increase in the proliferation of T_H2A cells was also observed following stimulation with native protein and DPG Phl p, and to a lesser extent following stimulation with DPG-POL Phl p and negative control (Figure 7.2.4.2, A). The proportion of proliferated T_H2A cells were significantly lower in response to DPG-POL Phl p compared to native protein at 0.3 µg/mL ($P = 0.0152$) and 1 µg/mL ($P = 0.0303$) and compared to DPG Phl p at 0.3 µg/mL ($P = 0.0022$) and 1 µg/mL ($P = 0.0152$; Figure 7.2.3.1, C). No significant response was observed in NAC following stimulation with native protein, DPG Phl p, DPG POL Phl p and negative control (Figure 7.2.4.2, B).

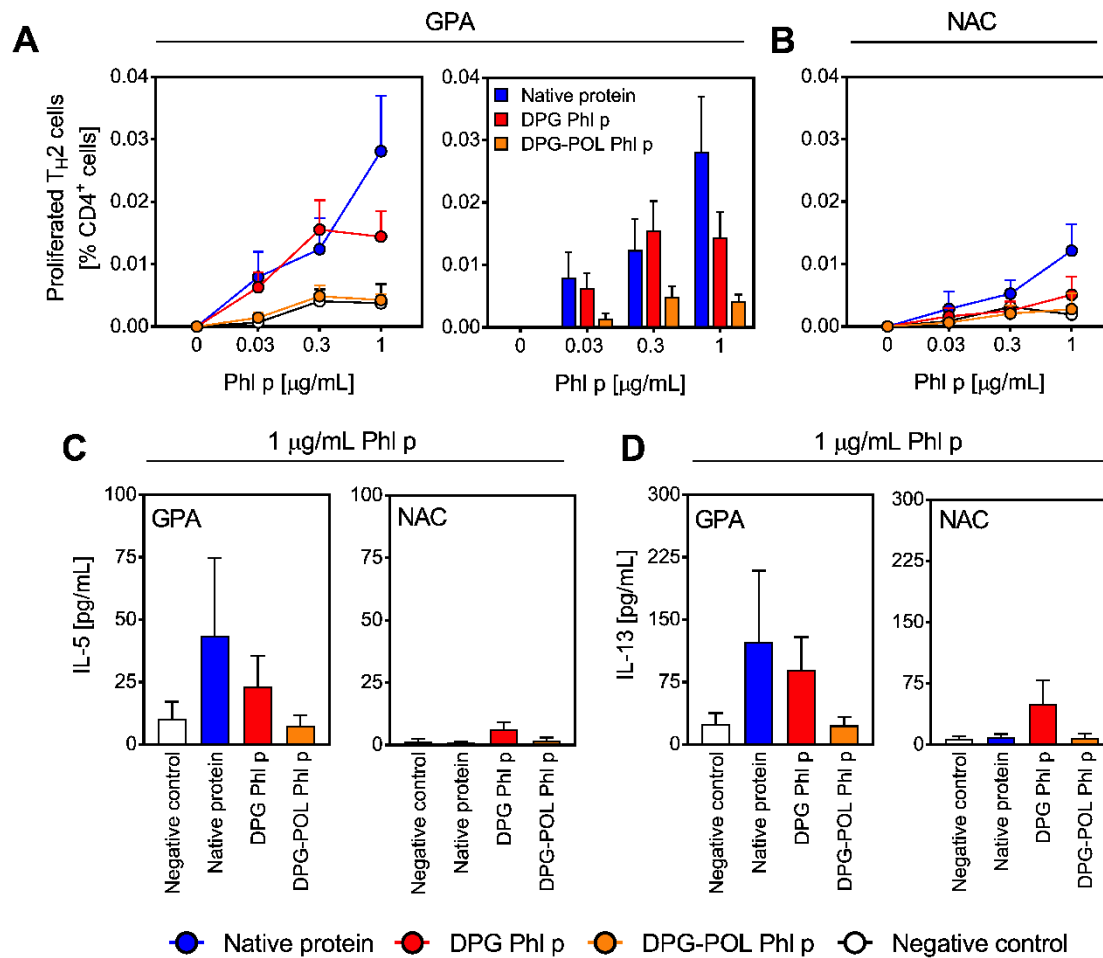


Figure 7.2.4.1 Lower proliferation of TH2 cells following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein. The proportion of proliferated TH2 (CD4⁺CRTH2⁺CD27⁻) cells in **A** GPA (n = 6) represented as a line and bar graphs; and **B** NAC (n = 6), in response to stimulation with varying concentrations of the native protein, DPG Phl p and DPG-POL Phl p. Levels of **C** IL-5; and **D** IL-13, in culture supernatant in GPA and NAC, following stimulation with 1 µg/mL of the native protein, DPG Phl p and DPG-POL Phl p. Error bars represent SEM. DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids; GPA, grass pollen allergics; NAC, non-atopic healthy controls.

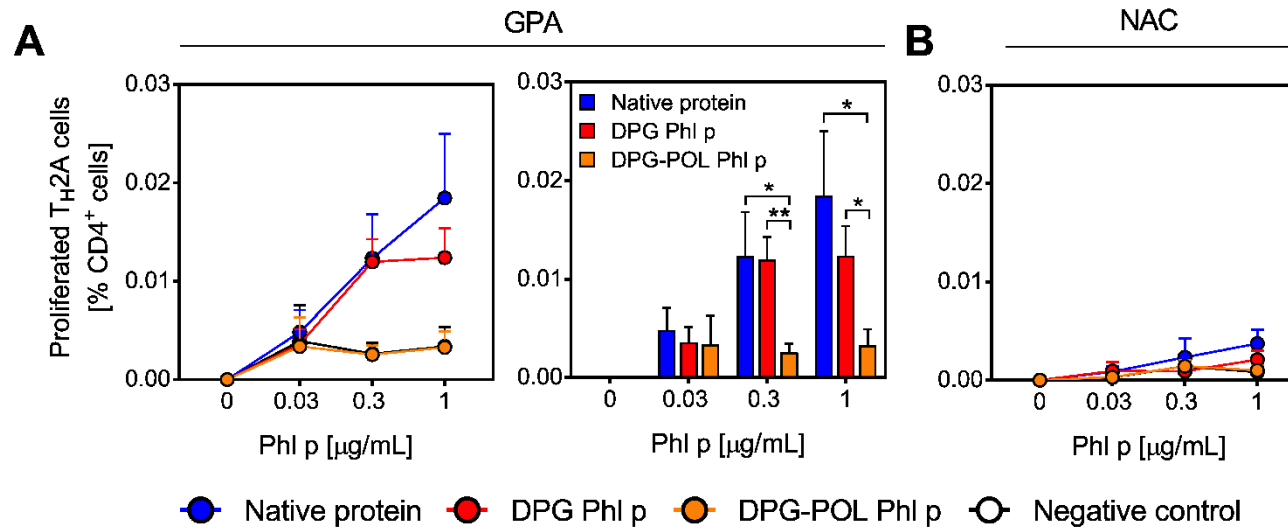


Figure 7.2.4.2 Lower proliferation of TH₂A cells following stimulation with DPG-POL Phl p compared to native protein and DPG Phl p. The proportion of proliferated TH₂A (CD4⁺CRTH2⁺CD27⁻CD49d⁺CD161⁺) cells in **A** GPA (n = 6) represented as a line and bar graphs; and **B** NAC (n = 6), in response to stimulation with varying concentrations of the native protein, DPG Phl p and DPG-POL Phl p. Error bars represent SEM. DPG, depigmented allergoids; DPG-POL, depigmented and polymerised allergoids; GPA, grass pollen allergics; NAC, non-atopic healthy controls. **P* < 0.05 and ***P* < 0.01, Mann-Whitney test.

7.2.5 Attenuation of T_{FH}, T_{H2} and T_{H2A} cell responses following stimulation with DPG-POL Phl p compared to DPG Phl p as illustrated by unbiased analyses

The effect of DPG Phl p and DPG-POL Phl p on T cell subsets in GPA were further evaluated using unbiased visualisation tools. viSNE maps of CD4⁺ illustrated a modest increase in CXCR5 and PD-1 expression, which indicates T_{FH} cell population (Figure 7.2.5.1, A, black arrows) in response to stimulation with 1 µg/mL of DPG Phl p compared to DPG-POL Phl p. Furthermore, the IL-4 expression within the T_{H2} cell populations (Figure 7.2.5.1, A, brown arrows) showed subtle reduction following stimulation with DPG-POL Phl p compared to DPG Phl p. Furthermore, FlowSOM analysis was performed and the data was represented as an MST to allow simultaneous visualisation of various marker's intensity within individual cell clusters and their abundance. FlowSOM analyses identified two different metaclusters of T_{FH} cells, both of which express CXCR5 with varying PD-1 expression (Figure 7.2.5.1, B and C, red and blue). Metacluster of T_{H2} cells was also identified which showed high CRTH2 expression and lacks CD27 expression (Figure 7.2.5.1, B and C, purple). One cluster of cells within the T_{H2} cell metacluster showed expression of CD49d and CD161 and was therefore identified as a T_{H2A} cell metacluster (Figure 7.2.5.1, B and C, green). T_{FH} cell metaclusters 1 and 2 showed higher abundance when stimulated with 1 µg/mL of DPG Phl p compared to DPG Phl p in GPA (Figure 7.2.5.1, D). Similarly, T_{H2} and T_{H2A} cell metaclusters were more abundant when stimulated with DPG Phl p compared to DPG-POL Phl p in GPA (Figure 7.2.5.1, E and F).

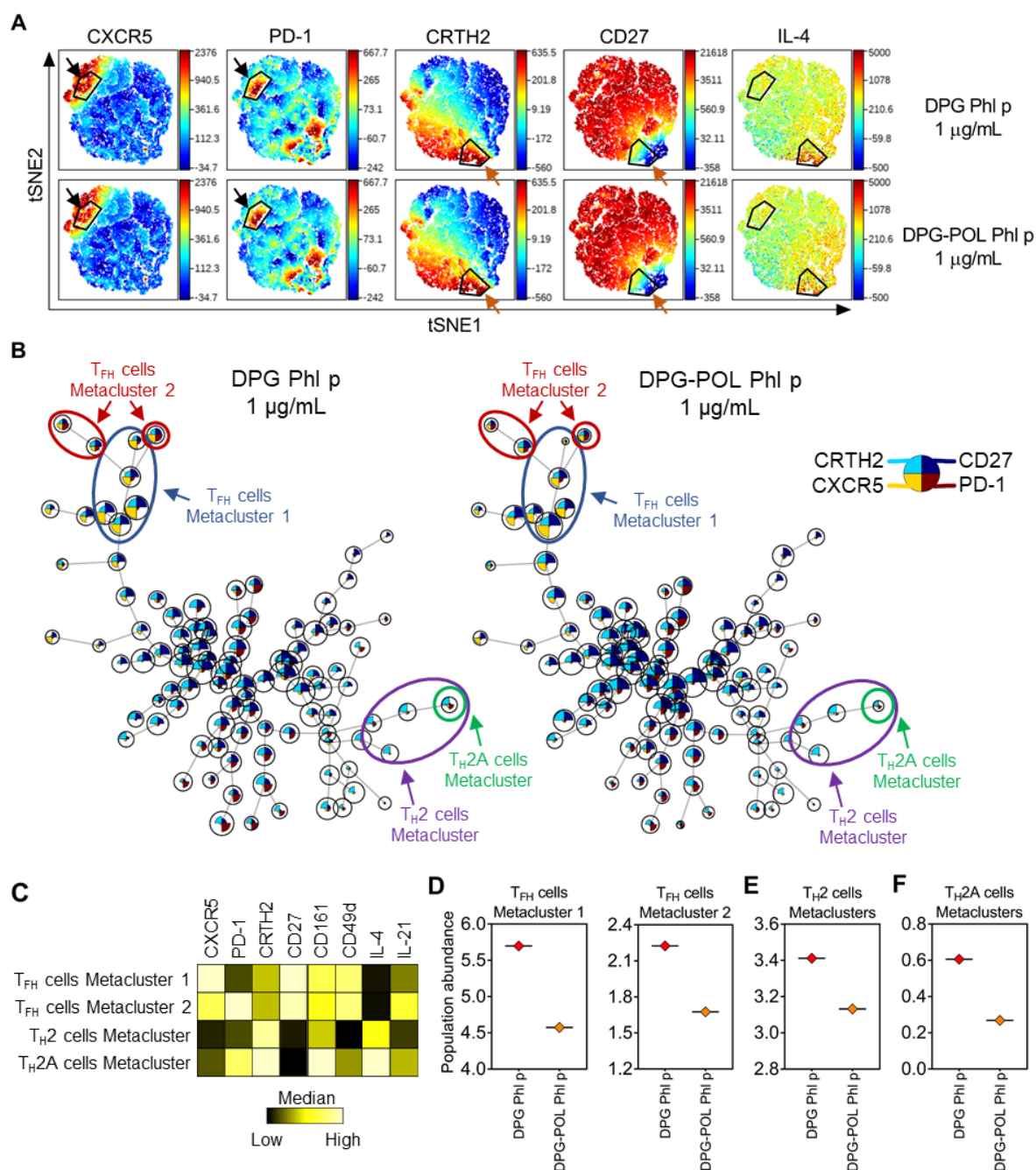


Figure 7.2.5.1 Lower T cell subsets following stimulation with DPG-POL Phl p compared to DPG Phl p in GPA. **A** viSNE maps illustrating the expression of CXCR5, PD-1, CRTH2, CD27 and IL-4 in CD4⁺ cells following stimulation with 1 μ g/mL of DPG Phl p and DPG-POL Phl p in GPA. **B** FlowSOM star plots showing clusters of cells with the respective expression of CXCR5, PD-1, CRTH2 and CD27. Metaclusters of T_{FH}, T_H2 and T_H2A cell subsets were identified based on the expression of CXCR5, PD-1, CRTH2 and CD27. Colours in individual clusters indicate the intensity of CXCR5 (yellow), PD-1 (red), CRTH2 (aqua) and CD27 (blue). Marker intensity is visualised as the distance of each segment from the border of each node and its size indicates the cluster abundance. **C** Heatmap illustrating the median expression of indicated markers within individual metaclusters. Black represents a low median expression and yellow/white indicates high median expression. Population abundance of metaclusters of **D** T_{FH}; **E** T_H2; and **F** T_H2A cell subsets following stimulation with 1 μ g/mL of DPG Phl p and DPG-POL Phl p in GPA.

7.3 Summary

This chapter investigated the allergenicity of a novel immunotherapy approach using depigmented and polymerised allergoids. The safety and allergenicity of native protein, DPG Phl p and DPG-POL Phl p were assessed by measuring basophil activation and histamine release. DPG Phl p showed no differences in eliciting basophil response and histamine release when compared to the native protein. However, DPG-POL Phl p demonstrated a reduced capacity in inducing basophil activation, suggesting its hypoallergenic nature compared to native protein and DPG Phl p. EC₅₀ analysis also illustrated the reduced potency of DPG-POL Phl p in eliciting basophil activation and histamine release, compared to DPG Phl p and unmodified native protein. Moreover, DPG-POL Phl p induced less proliferation of IL-4⁺ and IL-21⁺ T_{FH} cells compared to unmodified native protein and DPG Phl p in GPA. Similarly, less proliferation of T_H2 and T_H2A cells was observed in response to DPG-POL Phl p compared to native protein and DPG Phl p. Unbiased FlowSOM analysis showed reductions in the abundance of T_{FH}, T_H2 and T_H2A cell metaclusters when stimulated with DPG-POL Phl p compared to DPG Phl p which complements the findings by flow cytometry. In summary, these findings demonstrated the potential of using modified allergoids (DPG-POL Phl p) as a novel alternative for allergen immunotherapy that is both safe and able to modulate T cell responses.

CHAPTER 8 DISCUSSION

8.1 T_{FH} cells are distinct from T_H2 cells and promote class-switching and plasma cell differentiation via IL-4 and IL-21

Chapter 3 describes the phenotypic characteristics of T_{FH} cells and their potential to promote B cell class-switching and proliferation. For the first time, using computational tools that utilise mathematical algorithms, T_{FH} cells were shown to represent a subset of $CD4^+$ T cells distinct from T_H2 and T_H2A cells, capable of secreting IL-4 and IL-21. IL-4 stimulation of B cells induced class-switching, which was inhibited in the presence of IL-21. Nonetheless, IL-4, IL-21 and dual IL-4/IL-21 stimulation promoted differentiation of plasmablasts, IgE^+ cells and IgE secretion, of which the latter promote pro-allergic IgE -mediated response.

T_H2 cells are known as primary cells that drive allergic inflammation. However, in the GC, T_{FH} cells interact with GC B cells, promoting allergic inflammation. Using unbiased machine learning algorithms and visualisation tools, T_{FH} , T_H2 and T_H2A cells were phenotyped according to their surface markers. These analyses identified T_{FH} cells as a distinct population of $CD4^+$ cells from T_H2 and T_H2A cells, and therefore, potentially play a distinct role in driving allergic inflammation. In addition, T_{FH} cells expressed IL-21 in addition to T_H2 cytokine, IL-4 which is in agreement with the previous studies²⁹¹.

The functions of IL-4 in driving antibody production by B cells have been previously established²⁹². However, the effect of IL-21 on CSR, particularly for IgE production, and the synergistic effect of IL-4 and IL-21 in human have not been fully elucidated and remained controversial. Chapter 3 illustrated that IL-4 stimulation resulted in the upregulation of *BCL6*, a marker of GC response, and *AICDA*, a critical component of B cell class-switching and

somatic hypermutation pathways in B cells. However, the combined additive effect of IL-4 and IL-21 downregulated the expression of *BCL6* and *AICDA* 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 T_{FH} cell migration to the B cell follicle, while IL-4 is heightened following the migration of T_{FH} cells into the B cell follicle^{310,311}. Furthermore, the distinct localisation of IL-21⁺ and IL-4⁺ T_{FH} cells within the B cell follicle highlights the differential roles of IL-21⁺ and IL-4⁺ T_{FH} cells. IL-21⁺ T_{FH} cells are localised close to the dark zone, suggesting their role in promoting somatic hypermutation and affinity maturation. Meanwhile, IL-4⁺ T_{FH} cells are predominantly located distal to the dark zone and are crucial in the class-switching of GC B cells²¹⁶. While the effect of IL-4 stimulation on IgE class-switching has been explored, the role of IL-21 on CSR has not been fully elucidated. Despite the inhibitory effect of IL-21 observed on CSR, a higher proportion of IgE⁺ cells was observed in the presence of dual IL-4/IL-21 stimulation. This suggests the potential role of IL-21 in driving differentiation to IgE⁺ cells occurs post-CSR. Previous studies investigating the effect of IL-21 on IgE production have yielded contradicting results. Studies performed on IL-21R-deficient mice reported aberrant IgE production and that rIL-21 administration suppressed IgE production^{312,313}. Despite having similar proportions of proliferated T and B cells with wild type mice, IL-21R^{-/-} mice demonstrated higher serum IgE following immunisation with ovalbumin³¹³. On the other hand, other studies have stimulated sorted human B cells from PBMCs and spleen with IL-21 in the presence of anti-CD40, IL-4 and IL-13 and showed elevated IgE levels in culture supernatants^{314,315}, which is consistent with the findings observed in Chapter 3. Interestingly, following stimulation of human PBMCs with IL-21 in the presence of PHA to induce T cell activation, IL-4-induced IgE production was significantly inhibited³¹⁵. These differences are potentially due to the different experimental models used in these studies. Furthermore, whilst stimulation with IL-21 and dual IL-4/IL-21 upregulated the expression of both *PRDM1* and *XBP1*, stimulation with IL-4 alone resulted in the upregulation of *XBP1* expression only. Previous studies have shown that *PRDM1* expression is essential

for the initiation of plasma cell differentiation^{316,317}. *XBP1* is expressed further downstream and is crucial for the unfolded protein response (UPR) that is essential for the secretory function of plasma cells^{215,318}. This, therefore, indicates that while IL-4 is crucial for the UPR, IL-21 is essential for both the initiation stage of plasma cell differentiation and antibody-secretory function of plasma cells. This was confirmed as dual IL-4 and IL-21 stimulation for 7 and 14 days resulted in the highest abundance of plasmablasts and IgE⁺ plasmablasts in naïve and memory B cells. Interestingly, the proportion of IgG⁺ cells were also higher following stimulation of IL-4, IL-21 and dual IL-4/IL-21 in memory B cells. This is in corroboration with previous studies that have shown an increase in IgG-producing B cells following stimulation of human splenic B cells with IL-4^{319,320} and IL-21 in porcine B cells³²¹. Stimulation of human splenic B cells induced high IgG₄-producing cells when stimulated with IL-4 in the presence of irradiated EL4 cells to activate the B cells and PMA for 7 days³²⁰. IL-21 stimulation of splenic B cells isolated from porcine resulted in a marked increase in IgG-producing B cells following culture of 48 to 72 hours³²¹. There was little to no evidence of dual IL-4/IL-21 stimulation on IgG-producing B cells. However, it is speculated that IL-21 is playing a supporting role in the development of antibody-producing B cells. Collectively, these findings suggest that the attenuation of IL-21 expression may be essential for an optimal GC response and B cell CSR, which was then upregulated during plasma cell differentiation stage. This further highlights the differential role of T_{FH} cell cytokines IL-4 and IL-21 in regulating the different stages of B cell differentiation and clonal expansion, leading to a pro-allergic IgE-mediated response. Furthermore, grass pollen (Phl p)-driven proliferation of IL-4⁺ and IL-21⁺ T_{FH} cells was observed in GPA, but not NAC, suggesting the role of T_{FH} cells in the pathophysiology of SAR.

8.2 Dysregulation of T_{FH} and T_{FR} cells, IL-4, IL-21 and IL-6 in GPA and modulation following SCIT and SLIT

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 intranasal allergen challenge which was inhibited in SCIT or SLIT. This observation was consistent with the elevated IL-4⁺ and IL-21⁺ T_{FH} cells in GPA, which were lower in SCIT- and SLIT-treated groups, from the same patient cohort. Furthermore, this suggests that the increased T_{FH} cells in GPA are associated with the production of IgE, subsequently, resulting in allergic inflammation. Previous studies have also reported the correlation of T_{FH} cells with serum IgE in patients with house dust mite allergy^{271,322}. Serum IgE in patients with house dust mite allergy has been shown to correlate with the proportion of T_{FH} cells in peripheral blood²⁷¹. Moreover, as T_{FH} cells are crucial for GC responses and promote antibody-producing B cells, dysregulation of T_{FH} cells has been implicated in the development of autoimmune diseases³²³. Increased T_{FH} cells have been detected in patients with autoimmune diseases including RA²⁶⁰ and pSS²⁵⁸. Moreover, patients with untreated IgG₄-related disease (IgG₄-RD) have been reported to have higher proportions of circulating T_{FH} cells and serum IgG and IgG₄ compared to healthy controls³²⁴. Furthermore, immunohistochemistry staining revealed higher CXCR5-expressing T_{FH} cells in the tissues of the gland in patients with IgG₄-RD compared to controls that expressed IL-21, BCL6 and IgG₄. These T_{FH} cells demonstrated heightened capacity in inducing B cell differentiation into antibody-secreting plasma cells, thus resulting in the development of the disease³²⁴. These findings highlighted the important role of T_{FH} cells in maintaining the 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 mice¹⁸⁴. IL-6 upregulates BCL6 expression and IL-21 secretion^{160,179}. The autocrine role of secreted IL-21 further promotes the induction of BCL6 expression, which is crucial for T_{FH} cell differentiation. IL-6 and IL-21 signalling induce STAT1 and STAT3 expression and result in

the expression of T_{FH} cell markers, including CXCR5, PD-1 and ICOS^{325,326}. The early induction of IL-6 in GPA potentially leads 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-21³²⁷ 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.

In Chapter 4, I have confirmed that both SCIT and SLIT were clinically effective in patients with SAR, which is in corroboration with previous clinical studies^{32–35,112}. For the first time, I showed that there was dysregulation of T_{FH} cells and induction of suppressive T_{FR} cells and IL-10-producing T_{FH} cells following grass pollen SCIT and SLIT. These changes were underscored by changes in the chromatin landscape.

The involvement of T_{reg} cells in immune tolerance induction following AIT has been well studied^{76,92}, and studies investigating the role of T_{FR} cells have only recently emerged. T_{FR} cells have the capacity to disrupt the crosstalk between T_{FH} and B cell to prevent the development of autoimmune diseases and allergy^{222,224,328}. Here, T_{FR} cells were shown to be induced following SCIT and SLIT, which is in corroboration with previous studies that have shown induction of T_{FR} cells following AIT in HDM allergic patients²⁷¹ and grass pollen peptide immunotherapy in GPA³⁰². Furthermore, previously studied mechanism of AIT have been associated with the induction of IL-10⁸⁸ from allergen-specific T cells. As described in 0, 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 in naïve B cells. Furthermore, IgG⁺ 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 differential role of IL-10 on allergen-inexperienced (naïve) and allergen-experienced B cells, where it may play a protective role when interacting with the latter.

It was speculated that the changes observed in peripheral blood were underscored by changes in the chromatin landscape. ATAC-seq was performed and differentially accessible chromatin regions were observed between GPA and NAC, SCIT and SLIT. Regions that were more accessible in T_{FH} 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^{329,330} and leads to the upregulation of lipid synthesis, consistent with the observation that showed accessible chromatin regions associated with lipid biosynthesis, consequently, contributes to 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 T cell survival and proliferation^{331,332}. Moreover, 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 signalling³³³, which was consistently more accessible in GPA compared to NAC, SCIT and SLIT. Furthermore, there was higher chromatin accessibility in regions associated with PI3K-Akt, MAPK and AMPK signalling in GPA. These pathways are known to play a role in glucose homeostasis³³³, a process that is crucial for cell differentiation and growth. Regions associated with toll-like receptor (TLR) signalling pathway was also more accessible in GPA compared to NAC. To complement this, higher T_{FH} cells and their cytokine-producing subsets was observed in GPA compared to NAC, SCIT and SLIT. Not only that these findings validate the previous study highlighting the role of TLR signalling in promoting T cell responses and enhancing cytokine production^{334,335}, but also suggest that the higher proportion of T_{FH} cells observed in GPA could be due to the predisposition for T_{FH} cell differentiation and cytokine production in GPA. This, however, will need 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 proliferation³³⁶, suggesting that T_{FH} cells in SLIT were more active compared to SCIT. This is in conjunction with the modest differences in IL-4⁺IL-21⁺ T_{FH} cells between SLIT and SCIT observed in the cohort. Interestingly, chromatin regions associated ribosome biogenesis and MAPK signalling were also shown to be more accessible in SLIT compared to SCIT. However, these regions were more accessible in GPA compared to SLIT. These highlights that these pathways, which is crucial for cell differentiation and growth through increase metabolism of macromolecules³³³ were higher in SLIT compared to SCIT, although not as high as in GPA. The above molecular mechanisms underscore the potential differences in the mechanism of SCIT and SLIT. Collectively, these data suggest that accessible regions of T_{FH} cells are mostly related to cell growth and cytokine secretion, which was observed to be higher in GPA compared to SCIT and SLIT, both in the epigenetics and protein levels.

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^{329,330,337} were attenuated in GPA. These findings coincide with the 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^{329,330,332}. 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. 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 T_{FH} and T_{FR} cells provides 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. Altogether, highly accessible regions of T_{FR} cells are predominantly associated with cell survival and growth, which was higher following SCIT and SLIT, and thus, suggests that these were inhibited in GPA, potentially resulting in the dysregulation of T_{FR} cells in GPA.

8.3 *Lolium perenne* peptide hydrolysates have lower allergenicity compared to native protein whilst maintaining immunogenicity *in vitro*

Chapter 5 assessed the allergenicity of LPP candidates, as an alternative approach of conventional immunotherapy that utilises the whole allergen. All LPP candidates (peptides 1, 2, 3 and 4) were hypoallergenic and possessed a lower potency in eliciting the activation of basophils, compared to grass pollen native protein (whole allergen). Moreover, all LPP candidates showed reduced ability in inducing basophil histamine release compared to the native protein. The proliferation of T_{FH} cell subsets, including activated (ICOS⁺), IL-4- and IL-21-producing T_{FH} cells, as well as T_H2, allergen-specific T_H2A and T_H17 cells, were lower following stimulation with LPP candidates compared to native protein. Neither the native protein nor the LPP candidates elicit a response in NAC.

Although very effective, SCIT and SLIT pose a risk of inducing adverse events, including anaphylaxis, in some patients⁴¹. Various studies have explored the use of peptides as a safer

alternative to conventional SCIT and SLIT that use the whole allergen^{43,44,52}. Despite being unable to bind to IgE and cross-link with FcεRI on the surface of mast cells and basophils due to their short structure, short synthetic peptides have been shown to induce allergen-specific T_H2 hyporesponsiveness (anergy)⁴². ASIT Biotech S.A. has developed an approach to generate linear peptide candidates from *Lolium perenne* (LPP) as a potential treatment for SAR, and their allergenicity and immunogenicity were evaluated following stimulation with timothy grass pollen allergen (Phl p).

Allergenicity was investigated by measuring basophil activation and histamine release, which is critical in inducing the type I hypersensitivity reaction. Basophils are the surrogate cells for mast cells in the peripheral blood and have been used to evaluate FcεRI-mediated allergic response. It is extremely crucial to determine the reactivity of LPP candidates to assess their safety and immune potency. The study showed that all four LPP candidates were hypoallergenic and possessed a lower potency in inducing basophil activation and histamine release in GPA, compared to native protein (whole allergen). LPP candidates required approximately 30- and 50-times higher concentration compared to the native protein to achieve 50% of the maximal CD63⁺ and CD203c^{bright} basophil response, respectively. Basophil activation, however, does not necessarily lead to histamine release. Previous studies have reported the use of DAO as a gold standard surrogate biomarker of basophil histamine release^{153,338,339}. DAO binds tightly to its substrate histamine within the vesicles and the loss of DAO expression is, therefore, proportional to histamine release. This study demonstrated that approximately 20- and 25-times higher concentration of LPP candidates were required to elicit 50% of the maximal DAO-CD63⁺ and DAO-CD203c^{bright} basophil histamine release compared to the native protein. It was also observed that the basophil activation curve in response to native protein is almost bell-shaped. This is speculated to be due to the oversaturation of the binding of allergen-IgE complexes to FcεRI on basophils⁵⁷. Collectively, these findings demonstrated the superior safety profile of LPP candidates over whole

allergens. As basophils are the surrogate cells for mast cells and are crucial for the degranulation and release of inflammatory mediators upon allergen exposure, the lower potency of LPP candidates in eliciting basophil response and histamine release highlights that LPP is less likely to induce anaphylaxis.

T cell responses are crucial in driving allergic inflammation. The involvement of T_H2 and T_H17 cells in the pathophysiology of inflammation have been well described. In the context of allergy, T_{FH} cells are critical in driving the production of antibodies by B cells. In the same study, the immunogenicity of LPP candidates was investigated following an *in vitro* stimulation of PBMCs. LPP candidates induced lower allergen-driven proliferation of $ICOS^+$, $IL-4^+$ and $IL-21^+$ T_{FH} cell subsets in GPA, compared to the native protein. These data suggested that compared to native protein, LPP candidates have a lower capacity to elicit T cell responses through the activation of T_{FH} cells, which potentially leads to dampening of the synergistic effect of IL-4 and IL-21 on IgE production by B cells. Similarly, LPP candidates induced lower proliferation of T_H2 , T_H2A and T_H17 cells in GPA compared to the native protein. T_H2 and T_H17 are well known to be the key players of allergic inflammation. In the same patient cohort, there was a higher proportion of regulatory B (B_{reg}) cells following stimulation with LPP candidates compared to native protein (data not shown), suggesting that whilst being hypoallergenic, LPP candidates maintained their immunogenicity. This potentially contributes to the dampening of the pro-allergic T cell responses observed following stimulation with LPP candidates. Collectively, these findings suggested that in contrast to native protein, LPP candidates are hypoallergenic and lacks the ability to elicit pro-allergic T cell responses. This, therefore, results in the dampening of the T cell-mediated IgE production, thus resulting in reduced IgE-mediated response. This includes the formation of allergen-IgE complexes that ultimately leads to the degranulation of basophils and mast cells, thus, highlighting their potential as a safer alternative approach of immunotherapy.

8.4 Short-course LPP immunotherapy is clinically effective, attenuates T_{FH} cells and induces T_{FR} and T_{reg} cells *in vivo*

Having shown the hypoallergenicity of LPP candidates whilst maintaining their immunogenicity, one of the LPP candidate was further developed for a subcutaneous immunotherapy administration as a short-course treatment for SAR^{279,302,307,308}. Both Lol p and Phl p belong to the subfamily Pooideae, which has been demonstrated to display an extensive cross-reactivity among members of the same family²⁷⁷. Another study also reported that 30-90% of the sequences of Lol p and Phl p 1 are homologous, which contributes to their cross-reactivity²⁷⁸, thus justifying the use of Phl p in the assays and to treat SAR. Dose-escalation and dose-finding studies identified that administration of a cumulative dose of 170 µg of LPP in eight subcutaneous injections over four clinical visits within 3 weeks was optimal inducing clinical benefit^{306,307}. An RDBPC phase III trial study was then performed to further evaluate the efficacy of LPP treatment, which reported safety data and clinical improvements, quality of life and reduction in CPT scores following LPP treatment³⁰⁸. Chapter 6 described the findings from a sub-study site of a phase III RDBPC study to assess the mechanistic aspects of LPP immunotherapy. Basophil responsiveness and T cell responses were evaluated in LPP- and PL-treated groups, before the treatment (V2), after the treatment before (V6) and after (V8) the grass pollen season.

LPP treatment administered as a short-course over four clinical visits demonstrated clinical efficacy as measured by CSMS and RTSS during the pollen season. LPP-treated group demonstrated a marked reduction of CSMS and RTSS of more than 50% compared to PL-treated group during the pollen season. LPP-treated groups exhibited reduced basophil responsiveness following the LPP immunotherapy that persisted throughout the grass pollen season. In contrast to conventional SCIT and SLIT which takes about 6 to 12 months to achieve basophil hyporesponsiveness, LPP immunotherapy is capable of inducing a quicker

response. An increase in Phl p sIgE was also observed following the LPP treatment and persisted throughout the grass pollen season. This is consistent with previous studies that have shown elevated IgE levels following immunotherapy treatment⁸⁶. However, the magnitude of increase in sIgE levels in LPP after the pollen season was lower in LPP- compared to PL-treated group, suggesting that the IgE production by B cells following natural exposure to grass pollen allergen during the season was blunted in LPP- but not PL-treated group. Nevertheless, despite the increase in sIgE levels, LPP-treated group showed clinical improvements compared to PL-treated group. Moreover, the level of IL-4⁺, IL-21⁺ and dual IL-4⁺IL-21⁺ T_{FH} cells were attenuated in LPP- compared to PL-treated group following the LPP treatment that persisted throughout the entire grass pollen season. As T_{FH} cells play a role in providing help to B cell through IL-4 and IL-21, consequently inducing IgE production from B cells, the lower proportion of T_{FH} cell subsets may contribute to the blunting of IgE production throughout the grass pollen season, thus improving clinical symptoms in LPP-treated groups.

Suppressive T_{FR} and T_{reg} cells were also induced in LPP-treated group following LPP treatment that persisted throughout the entire grass pollen season. These findings are in corroboration with previous studies that have reported elevated levels of T_{FR} and T_{reg} cells following conventional immunotherapy^{8,76,87,271}. In this study, CTLA-4⁺ T_{FR} cells were induced in LPP- but not PL-treated group. T_{FR} cells have previously been reported to suppress T_{FH} and B cell interaction by cell-to-cell contact through the expression of CTLA-4²²⁸, suggesting that the T_{FR} cells induced following LPP may suppress T_{FH}-B cell interaction, potentially resulting in the blunting of IgE production by B cell following allergen exposure. Furthermore, FoxP3⁺ T_{reg} cells were higher in LPP- compared to PL-treated group following the treatment and remained high after the grass pollen season. Although FoxP3 has been a well-established marker for T_{reg} cells, the expression of FoxP3 in activated T cells has been previously reported³⁰⁹. This leads to studies that explored at markers for T_{reg} cells. Activated T_{reg} cells have been shown to express high expression of GARP expression³⁴⁰. In addition, FoxP3

negatively regulates the expression of SATB1 and have been shown to play a role in the development of T_{reg} cells^{137,138}. The expression of GARP and repression of SATB1, therefore, indicates a subset of T_{reg} cells that are suppressive. In this study, T_{reg} cells that expressed GARP and repressed SATB1 expression were induced in LPP-, but not PL-treated groups following LPP treatment, that persisted throughout the grass pollen season.

Collectively, this study has shown that the administration of short-course LPP immunotherapy dampens the pro-inflammatory T_{FH} cell responses whilst inducing T_{FR} and T_{reg} cells following a 3-weeks administration of LPP. This suggested that peptide immunotherapy may share a similar mechanism of immune tolerance observed in conventional SCIT and SLIT, which can only be achieved when given for at least three years^{31,341}. In addition, as peptide immunotherapy can be given as a short-course, this may improve patient adherence which has been reported to be only 25% for SCIT and 12.5% for SLIT³⁴². There has been limited evidence on long-term tolerance induction following short-course immunotherapy treatments. A phase IIb study that performed in cat allergic patients to investigate the effect of short-course peptide immunotherapy using major cat allergen, Fel d 1, referred to as Cat-PAD, demonstrated persistent tolerance to cat allergen for up to two years following cessation of treatment⁴³. However, the phase III study showed a strong placebo effect and no significant changes were observed following immunotherapy. This may be due to the patients having continuous exposure to cat and thus may potentially develop protective IgG antibodies, although the findings are yet to be published. Other short-course immunotherapy approach using monophosphoryl lipid (MPL)-adjuvanted allergoids and intralymphatic immunotherapy have been reported to induce IgG antibodies and induced long-term tolerance. These studies highlight the potential of inducing long-term immunological tolerance following short-course immunotherapy treatments. A booster injection prior to the pollen season has previously been reported to improve clinical symptoms in GPA during the pollen season and therefore could also be given to further assessed clinical and immunological endpoints.

8.5 Allergoids induced basophil hyporesponsiveness whilst dampening T_{FH}, T_H2 and T_H2A cell responses *in vitro*

Chapter 7 investigated the allergenicity and immunogenicity of a novel immunotherapy approach using depigmented and polymerised allergoids. DPG-POL Phl p demonstrated a reduced capacity and potency in inducing basophil activation and histamine release compared to DPG Phl p and native protein. DPG Phl p showed no differences in eliciting basophil response and histamine release when compared to the native protein. Moreover, DPG-POL Phl p induced less proliferation of IL-4⁺ and IL-21⁺ T_{FH} cells compared to unmodified native protein and DPG Phl p in GPA. Similarly, less proliferation of T_H2 and T_H2A cells was observed in response to DPG-POL Phl p compared to native protein and DPG Phl p and was confirmed using unbiased FlowSOM analysis.

Conventional immunotherapy involved administration of whole allergen extract in incremental doses to prevent allergic reactions, where tolerance is built with each incremental dose. This results in the build-up phase lasting for 6 – 12 weeks, and potentially longer in patients with a severe allergy. This is due to conformational epitopes of the whole allergen that were processed by APCs and presentation to TCR. The use of chemically modified allergens, termed allergoids, have been proposed to reduce the risk of an allergic reaction during the administration of immunotherapy. These allergoids underwent polymerisation and depigmentation, resulting in the deactivation of the enzymatic activity and removal of pigments. This subsequently increases the solubility and allows the administration of higher allergen dose during immunotherapy⁵⁷. Allergoids are unable to bind to IgE and cross-linking FcεRI on mast cells and basophils and therefore were deemed to be a safer alternative to conventional SCIT and SLIT. Laboratorios LETI S.L.U. generated DPG Phl p and DPG-POL Phl p allergoids from native protein (Phl p) and their allergenicity and immunogenicity were evaluated. The study revealed that DPG Phl p and native protein showed similar basophil

response and histamine release. DPG-POL Phl p demonstrated hypoallergenicity and was less potent in inducing basophil activation and histamine release in GPA, compared to DPG Phl p and native protein. Approximately 7- and 11-times higher concentration of DPG-POL Phl p was required to elicit 50% of the maximal CD63⁺ and CD203c^{bright} basophil response, respectively, compared to native protein and DPG Phl p. In addition, compared to native protein and DPG Phl p, DPG-POL Phl p required approximately 8- and 5-times higher concentration to elicit 50% of the maximal basophil histamine release.

The immunogenicity of DPG Phl p and DPG-POL Phl p was also investigated by assessing the proliferation of T_{FH}, T_{H2} and T_{H2A} cell subsets. DPG-POL Phl p induced lower allergen-driven proliferation of IL-4⁺ and IL-21⁺ T_{FH} cells compared to DPG Phl p and native protein. DPG Phl p and native protein display similar profile in eliciting proliferation of IL-4⁺ and IL-21⁺ T_{FH} cells. These data suggest that unlike DPG Phl p and native protein, DPG-POL have a lower capacity in providing help to B cells, potentially leading to lower IgE production by B cells. Similarly, DPG-POL Phl p elicited lower proliferation of T_{H2} and T_{H2A} cells compared to DPG Phl p and native protein. Moreover, IL-5 and IL-13 levels were higher following stimulation with DPG Phl p and native protein compared to DPG-POL Phl p, both of which have been associated with allergic inflammation and T_{H2} cell responses. Of note, the negative control used in the same study provided by Laboratorios LETI S.L.U was generated from *Salsoia kali*. The negative control elicited low proliferation of T_{FH}, T_{H2} and T_{H2A} cells and basophil activation and histamine release at higher concentrations, suggesting potential sensitisation to *Salsoia kali* in the participants. The study was performed in a geographic area in which the prevalence of sensitisation to *Salsoia kali* was low. No SPT was performed against *Salsoia kali*, and therefore, it is not feasible to determine whether the participants were sensitised to *Salsoia kali*. Together, these findings suggest that DPG-POL Phl p is hypoallergenic and lacks the ability to elicit pro-allergic T cell responses, highlighting their advantages as a potentially safer alternative to conventional immunotherapy. These assays

also demonstrated the similar profiles of DPG Phl p and native protein, suggesting that the polymerisation step following depigmentation is crucial to achieving hypoallergenicity. Several clinical studies have demonstrated the success of using depigmented and polymerised allergoids in patients with HDM, birch and grass pollen allergy^{59,64,343,344}. These studies reported safety data and improved clinical symptoms in patients following at least a year of treatment. To date, there are very limited studies on the immunological effects of allergoids in allergic patients. There is, therefore, a need to evaluate the immunological endpoints in a large study.

8.6 Summary and conclusions

The data presented in this thesis confirmed that T_{FH} cells represent a subset of $CD4^+$ T cells that are distinct from T_H2 and T_H2A cells, following computational analysis with viSNE, SPADE and FlowSOM that utilise mathematical algorithms. T_{FH} cells have the capacity to secrete IL-4 and IL-21 that drives GC response, B cell CSR and differentiation into antibody-producing plasma cells, as well as IgE^+ cells, which are key in driving IgE-mediated allergic inflammation. Furthermore, the proliferation of T_{FH} cell subsets was observed following stimulation with grass pollen allergen in GPA, but not NAC, highlighting the role of T_{FH} cells in the pathophysiology of SAR.

A proof-of-concept, controlled, cross-sectional study of grass pollen SCIT and SLIT, for the first time, showed the dysregulation of T_{FH} cells in GPA and was modulated following SCIT and SLIT. Moreover, T_{FR} cells were induced following SCIT and SLIT. These were underscored by changes in the chromatin accessibility landscape of T_{FH} and T_{FR} cells. Highly accessible regions in T_{FH} cells were associated with cell proliferation and differentiation, which was higher in GPA compared to SCIT and SLIT. In contrast, accessible regions of T_{FR} cells associated with cell survival and growth were higher in SCIT and SLIT, and therefore, were inhibited in GPA. Additionally, $IL10^+$ T_{FH} cells were induced following SCIT and SLIT. IL-10, in the presence of IL-4 and IL-21 drives proliferation of IgE^+ cells from naïve B cells, and IgG^+ cells from memory B cells, thus suggest its differential role in modulating antibody responses by B cells.

Despite being very effective, conventional SCIT and SLIT using whole allergens pose a risk in inducing adverse events including anaphylaxis. This thus warrants alternative approaches that provide superior safety profile, and yet are able to elicit the immune response. The use of peptide immunotherapy has been explored. The allergenicity and immunogenicity of LPP

candidates were presented in this thesis. LPP candidates displayed hypoallergenicity as assessed by basophil activation test and reduced proliferation of pro-allergic T_{FH} , T_H2 and T_H2A cell subsets, compared to the native protein. This was then validated in a phase III RDBPC trial study whereby the LPP treatment was administered as a 3-week short-course treatment over four clinical visits prior to the pollen season. LPP-treated patients showed a marked decrease in the CSMS and RTSS of greater than 50%, compared to PL, and accompanied by the blunting of seasonal Phl p sIgE, highlighting the clinical efficacy of LPP immunotherapy. Furthermore, LPP-treated patients demonstrated basophil hypoallergenicity and attenuated the proportion of IL-4⁺, IL-21⁺ and dual IL-4⁺IL-21⁺ T_{FH} cells, compared to PL. An induction of FoxP3⁺ T_{FR} and T_{reg} cells, as well as T_R1 and iT_R35 cells, was observed following LPP treatment.

The use of allergoids as an alternative treatment for SAR was also described in this thesis. DPG-POL Phl p demonstrated lower allergenicity and potency compared to DPG Phl p and native protein as measured by basophil activation test and histamine release. Additionally, DPG-POL Phl p induced lower proliferation of T_{FH} , T_H2 and T_H2A cells. This was then confirmed with unbiased FlowSOM analysis that illustrated lower abundance of T_{FH} , T_H2 and T_H2A cell metaclusters following stimulation with DPG-POL Phl p compared to DPG Phl p and native protein.

In conclusion, the studies described in this thesis explored the roles of T_{FH} and T_{FR} cells as a distinct CD4⁺ T cell subset in the pathophysiology of SAR. Conventional grass pollen SCIT and SLIT are effective and associated with modulations of T_{FH} and T_{FR} cells that was accompanied by epigenetic changes. Alternative approaches of immunotherapy using peptides and allergoids demonstrated reduced allergenicity and were associated with attenuation of T_{FH} cells and induction of T_{FR} cells. This, therefore, will result in the disruption

of cognate T_{FH} and B cell interactions and therefore, the production of antibodies, which is critical in the development of IgE-mediated allergic inflammation.

8.7 Limitations

The current study highlights the importance of T_{FH} cells in the pathophysiology of SAR and T_{FR} cells in immune tolerance induction following AIT in a cross-sectional study of SCIT and SLIT. The main limitation of this study is the lack of samples from a prospective SCIT and SLIT study, that will enable investigation of the time-course reduction of T_{FH} cells and the induction of T_{FR} cells throughout the treatment. Although this was performed in the study using LPP, as described in Chapter 6, this could not be achieved with the SCIT and SLIT study. The roles of the individual cell types could be further investigated by sorting T_{FH} and T_{FR} cells, as well as T_H2 and T_H2A cells from PBMCs. A co-culture experiment of T_{FH} , T_H2 and T_H2A cells could be performed in the absence or presence of T_{FR} cells to assess the inhibitory effect of T_{FR} cells in both GPA and NAC, by measuring cell proliferation by flow cytometry and antibody levels in culture supernatants. However, the main challenge of the study is the small proportions of T_{FH} , T_{FR} , T_H2 and T_H2A cells present in peripheral blood. A huge amount of PBMCs is required to obtain a sufficient number of cells for the culture experiments, which was not feasible. Equally, a huge number of sorted T_{FH} and T_{FR} cells is required for ATAC-seq experiments. Due to the low frequency of sorted circulating T_{FR} cells, sufficient number of cells were obtained from only one sample from the SCIT-treated group. A higher sample number is required to improve the reliability of the dataset and to determine whether or not the aberrant signal observed was an anomaly. Furthermore, as the current study involves the enumeration of T_{FH} and T_{FR} cells in circulating peripheral blood, this potentially highlights the difference of these circulating cells from their GC counterparts. Previous studies have shown that although circulating T_{FH} and T_{FR} cells showed differences from GC T_{FH} and T_{FR} cells, including the lower expression of BCL6¹⁶⁸, these circulating T_{FH} and T_{FR} cells represent memory counterparts of the GC T_{FH} and T_{FR} cells with higher potency following re-exposure to an antigen²³³. This, therefore, justifies the investigation on circulating T_{FH} and T_{FR} cells as surrogate cells in the peripheral blood. As the number of T_{FH} and T_{FR} cells are low in the peripheral blood, the use of nasal biopsies to obtain sufficient T_{FH} and T_{FR} cells for co-culture experiments is an

alternative approach. However, nasal biopsies collection was not a part of the clinical studies performed in this thesis, and thus, collection was not feasible. Moreover, as the studies covered in this thesis are focusing on T cell subsets in allergic patients, it is paramount to identify the allergen specificity of these cells. This could be achieved by performing peptide-MHC II tetramer staining with intracellular CD154 assays to sort out the allergen-specific T cells^{113,345}. However, this was not feasible due to the low cell number present in the peripheral blood. Furthermore, the significant genes observed in this study were a combination of known and unknown genes. This, therefore, limits the current understanding of the implication of those genes. More recently, a new subset of T_{FH} cells, T_{FH}13 cells have been reported, that secretes IL-4, IL-5 and IL-13, of which the latter has the capacity to induce anaphylactic IgE responses^{346,347}. Flow cytometry panels used in this thesis allows enumeration of the different subsets of T cells, including T_{FH}, IL-4⁺ and IL-21⁺ T_{FH}, T_H2, T_H2A and T_H17 cells within one panel. The limitation of the flow cytometry instruments is that there is a limit on the number of filters that can be used at one time. Thus, the identification of T_{FH}13 cells could not be performed.

8.8 Future studies

This study reported the attenuation of T_{FH} cell subsets and induction of T_{FR} cells following conventional SCIT and SLIT in a proof-of-concept, cross-sectional study of SCIT and SLIT. An important follow-up study would be to validate these findings in a large, prospective RDBPC study, from a single centre, that investigates the specific time-points, before, during and after the treatment period. The mechanistic endpoints can then be investigated as the time-course effect of SCIT and SLIT on the proportions of T_{FH} and induction of T_{FR} cells. The enumeration of the recently described $T_{FH}13$ cells could also be performed on future projects, to investigate the functions of $T_{FH}13$ cells in GPA and NAC and following SCIT and SLIT, as well as in studies that investigate the alternative approaches of immunotherapy. Furthermore, differential chromatin accessibility landscape was observed in GPA, NAC, SCIT and SLIT. A transcriptomic study on the same group of patients could be performed to complement the ATAC-seq data to assess whether the chromatin accessibility leads to gene expression. Recent advancements in the genomics study open up opportunities to perform transcriptomics and ATAC-seq at a single-cell level. Due to the small number of T_{FH} and T_{FR} cells in peripheral blood, performing single-cell assays would improve the resolution of the data. Additionally, RNA-seq could also be performed in parallel with protein expression at a single-cell level (cellular indexing of transcriptome and epitopes by sequencing [CITE-seq])³⁴⁸ to assess the differences in gene and protein expression and thus facilitate the identification of T_{FH} and T_{FR} cells in GPA, NAC, SCIT and SLIT. This will then allow identification of potential gene signatures that potentially distinguish responders from non-responders. Similarly, single-cell transcriptomics could be performed in a prospective AIT study to identify gene signatures for desensitisation at 12 months, clinical efficacy and tolerance at 24 to 36 months. Single-cell immune profiling on T_{FH} and T_{FR} cells could also be performed to investigate T cell receptors and antigen specificity in parallel with gene and protein expression following treatment with allergoids and peptides. GO and pathway analyses identify the functional groups and biological context related to the genes. Further analysis could also be performed on the

enrichment dataset such as using the generalised supply-demand analysis to study the kinetics of a cellular system and identify essential pathways that could be experimentally blocked to determine the effect on the functions of T_{FH} and T_{FR} cells. More importantly, the main goal of immunotherapy treatments is the long-term tolerance induction following discontinuation of the treatment. Although it has been shown with conventional SCIT and SLIT, the tolerance endpoint for the studies on alternative approaches using peptides and allergoids that were discussed in this thesis, however, has not been investigated. An extension of these clinical trials to determine the allergen tolerance in actively treated patients at one- or two-years following cessation of treatment is thus of utmost importance. This additional mechanistic endpoint would enumerate the proportions of T_{FR} and T_{reg} cells and the levels of blocking IgG₄ antibodies could be measured to determine the persistence of regulatory cells and antibodies following discontinuation of the treatment, thus leading to long-term tolerance.

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APPENDIX

Table A1 Summary of patient demographics recruited for the studies described in Chapter 3, Chapter 4, Chapter 5 and Chapter 7

Characteristics	NAC (n = 36)	GPA (n = 61)	SCIT (n = 13)	SLIT (n = 11)
Sex, n (%)				
Male	10 (27.8)	36 (59.0)	11 (84.6)	3 (27.3)
Female	26 (72.2)	25 (41.08)	2 (15.4)	8 (72.8)
Age (y), mean $\pm$ SD	42 $\pm$ 13	38 $\pm$ 12	46 $\pm$ 13	39 $\pm$ 13
Disease duration (y), mean $\pm$ SD	n.a.	17.98 $\pm$ 9.01	18.80 $\pm$ 9.45	33.50 $\pm$ 15.63
Phl p sIgE (kU _A /L), mean $\pm$ SD	n.d.	34.07 $\pm$ 35.39	29.60 $\pm$ 33.36	30.83 $\pm$ 36.32
Total IgE (kU/L), mean $\pm$ SD	14.10 $\pm$ 15.62	356.5 $\pm$ 607.7	383.50 $\pm$ 590.80	151.60 $\pm$ 166.70
Cosensitisation (SPT $\geq$ 3 mm), n (%)				
None (other than grass)	0 (0.0)	18 (29.5)	3 (23.1)	5 (45.5)
Silver birch	0 (0.0)	30 (49.2)	10 (76.9)	4 (36.4)
House dust mite	0 (0.0)	29 (47.5)	7 (53.8)	3 (27.3)
Cat epithelia	0 (0.0)	19 (31.1)	8 (61.5)	4 (36.4)
Dog epithelia	0 (0.0)	16 (26.2)	2 (15.4)	4 (36.4)

n.a., not applicable; n.d., not detected; NAC, non-atopic controls; GPA, grass pollen allergics; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy.

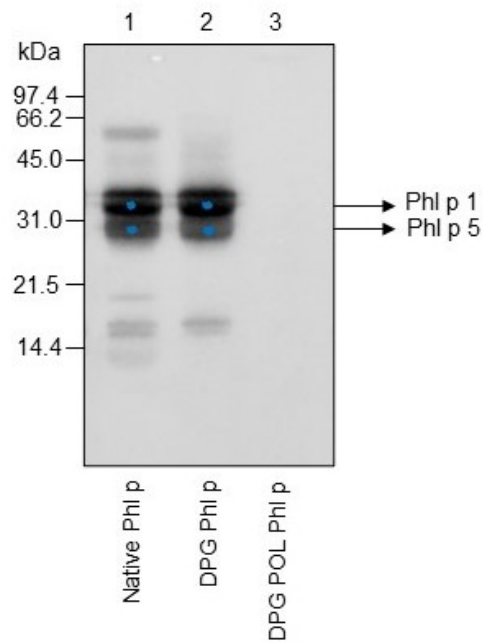


Figure A7.2.5.1 IgE immunoblot. Western blot image provided by Laboratorios LETI S.L.U. (Spain) who performed the assay, illustrating IgE-reactive protein bands following incubation of native protein (lane 1), DPG Phl p (lane 2) and DPG-POL Phl p (lane 3) with a pool of sera obtained from GPA.