Acknowledgments

I am very honoured to have been given this opportunity by Professor Stephen Durham to work in such a world class research facility, the knowledge and experience I have gained will prove invaluable for me in my future science career. I am also indebted to Dr Mohamed Shamji for all his supervision and experience given to me from the fascinating world of clinical immunology. I acknowledge and extend my sincere warmest gratitude to Dr. Henrique Girão for his unwavering support. I would like to thank Dr. Tomokatzu Matsouka for his support both in and out of the lab, without his patient teachings I would never have mastered the techniques implemented in this thesis. I would also like to thank Rachel Yan and Andrea Goldstone for the hard work at the Royal Brompton hospital, for providing all the necessary patients for my research. For some of the technical support that I received in the lab, I would like to show my appreciation to Amy Switzer, Orla McMahon and Miss Rebecca Parkin. To Janice Layhadi, James Charlesworth and Alan Perera for their friendship and support given me throughout my project. Finally, I would like to extend my gratitude to Jinjin Zhan, Clara Wo, Esther Steveling, Gilda Varrichi, Liliana Fuentes, Mimi Poon, Natalia-Klimowska, Guy Scadding, Mongkol Lao-aray, Faris Mustafa and for those hugs, chocolates, lunch and chill out sessions.

I dedicate all my work, achievements and success to my mother Maria.

All this would not be possible without her support.

Abstract

Introduction: Allergic Rhinitis affects ¼ of the population in developed countries. Sublingual grass pollen-specific immunotherapy (SLIT) involves immunomodulation of Th2 responses and the induction of IL-10+ Tregs (Tr1). IL-35-producing regulatory T cells (iTR35) have been recently reported as a novel subset of regulatory T cells with modulatory properties. We hypothesized that IL-35 suppresses grass pollen-driven Th2 responses following ex-vivo allergen stimulation, and induces iTR35 cells following grass pollen-SLIT. We further hypothesized that IL-35 suppresses an in vitro Th2 inflammatory response, induced by epithelial derived cytokines.

Methods: T effector cells (CD4+CD25-) obtained from grass pollen allergics (n=12) were purified and enriched from peripheral blood mononuclear cells by magnetic separation. CD4+CD25- T cells were co-cultured with irradiated antigen-presenting cells using 5ug/mL of *Phleum pratense* in the presence/absence 10ng/mL of recombinant human IL-35: Fc. T cell proliferative responses were measured by 3H-thymidine incorporation. Cytokine protein levels were assessed by Luminex MagPix assay. Proportion of FoxP3, IL-10 and iTR35 was determined in non-atopics (NA, n=12), untreated allergics (SAR, n=12) and SLIT-treated patients (SLIT, n=7). DCs obtained from SAR patients with seasonal allergic rhinitis (n=14) were primed with TSLP, IL-25, IL-33 or all three cytokines in the presence of 5ug/mL of *Phleum pratense* for 24 hour. Primed DCs were co-cultured with naive T cells for 6 days at 1:10 ratio. Proliferative responses of naive T cells were measured by 3H tritiated thymidine incorporation and cytokine analysis by Luminex MagPix assay.

Results: IL-35 significantly suppressed *Phleum pratense*-driven CD4+CD25- T cell proliferative responses (n=12; $p<0.0009$). This suppression was associated with reduced IL-4

(p=0.0001), IL-5 (p=0.0001), IL-9 (p=0.0001), IL-13 (p=0.0009) and an increase in IFN-γ (p<0.0001) and IL-10 (p<0.0001) was also demonstrated. Furthermore, iTR35 cells, IL-10+ and FoxP3+ T regs were decreased in SAR compared to NA (p=0.0003; 0.0005; p=0.001). SLIT resulted in recovery of iTR35 (p=0.016) and IL-10+ Treg (p=0.016) cells. FoxP3+ Tregs did not increased in SLIT group. TSLP to allergen-primed DCs resulted in a 97-fold increase (p=0.003) in naive T cell proliferation when compared to allergen-only primed DCs. IL-25- (p=0.426) and IL-33- (p=0.502) primed DCs did not augment this allergen-stimulated proliferative response. In the same system, IL-35 suppressed naive T cell proliferative responses when allergen+TSLP (p=0.0001) were cultured with DCs. In addition, results have shown an increase in Th2 cytokines when TSLP primed DCs are added to naive Tcells and their suppression when treated with IL-35.

Conclusion: Our findings suggest that iTR35 cells suppress grass pollen-driven Th2 responses and are induced following grass SLIT. IL-35 not only inhibits grass-pollen and TSLP-primed DC activation of naive T cells as it also suppressed Th2 cytokines induced by TSLP. The mechanism of this suppression suggests that IL-35 is a potential target for seasonal allergic rhinitis immunotherapy.

Key words: IL-35, Seasonal Allergic Rhinitis, Th2 Allergic Response, Epithelial derived cytokines, TSLP, IL-25, IL-33, Sublingual Immunotherapy.

Resumo

Introdução: A Rinite alérgica afecta um quarto da população em países desenvolvidos. A imunoterapia sublingual involve a imunoregulação de respostas Th2 e a indução de células T reguladoras, produtoras de IL-10 (Tr1). As células T reguladoras induzidas pela IL-35 (iTR35) são um novo subtipo de células T reguladoras com funcionalidade modeladora. Neste projecto, conjecturamos que a IL-35 suprime respostas do tipo Th2 induzidas pelo alergenio em causa (phlp) e induz o aumento de células iTR35 após imunoterapia sublingual. Em acréscimo, também conjecturamos que a IL-35 tem um papel supressor num modelo de resposta inflamatória Th2 induzido por citocinas proinflamatórias derivadas de células epiteliais.

Métodos: Linfócitos T efectores (CD4+CD25-) foram coletados de pacientes alérgicos a polén de gramíneas (n=12), por isolamento magnético de céluals mononucleares de sangue periférico. Células apresentadoras de antigénios foram irradiadas e cultivadas com as células T efectoras na presença de 5ug/mL de *Phleum pratense* e de 10ng/mL de IL-35: Fc. A proliferação celular foi medida pela incorporação de 3H-timidina e os níveis de citocinas produzidos por multiplex ELISA. Em seguida, a porpoção de FoxP3, IL-10 e iTR35 foi avaliada em pacientes não atópicos (NA, n=12), alérgicos (SAR, n=12) e pacientes submetidos a imunoterapia sublingual (SLIT, n=7). Finalmente, células dendriticas obtidas de pacientes alérgicos a gramíneas (n=14) foram co-estimulados na presença de TSLP, IL-25, IL-33 ou as três proteínas em simultâneo, e de 5ug/mL de *Phleum pratense* durante 24h. Estas células dendríticas foram posteriormente cultivadas com células T naive durante 6 dias a um rácio de 1:10. Ensaios de proliferação foram conseguidos por incorporação de 3H-timidina e análise de citocinas por multiplex ELISA.

Results: A IL-35 inibe significativamente a proliferação de células T efectoras, induzida pelo *Phleum pratense* (n=12; p<0.0009). Esta supressão foi associada à redução de IL-4 (p=0.0001), IL-5 (p=0.0001), IL-9 (p=0.0001), IL-13 (p=0.0009) e ao aumento de IFN-γ (p<0.0001) e IL-10 (p<0.0001). Em seguida, as células T reguladores que expressão IL-35, IL-10 e FoxP3+ foram avaliadas e observou-se um decréscimo das mesmas em SAR comparadamente a NA (p=0.0003; p=0.0005; p=0.001). Pacientes submetidos a imunoterapia sublingual demonstram um aumento em células reguladoras que expressão IL-35 (p=0.016) e IL-10 (p=0.016), não mostrando variação em T reguladores que expressão FoxP3+. As células dendriticas expostas a TSLP induziram um aumento de 97% (p=0.003) na proliferação de células T naive. No entanto, células dendríticas estimuladas com a IL-25 (p=0.426) e a IL-33 (p=0.502) não tiveram qualquer efeito na proliferação das células T naive. O papel modelador da IL-35 foi testado nas mesmas condições, mostrando supressão da proliferação de células T naive induzida por células dendríticas expostas a TSLP (p=0.0001). Por fim, a proliferação de células T naive foi associado ao aumento de citocinas envolvidas em respostas do tipo Th2, e a supressão das mesmas conseguida pela presença da IL-35.

Conclusão: Os nossos resultado sugerem um papel importante da iTR35 na supressão de respostas Th2 induzidas por phlp. Também se observou a indução destas células em pacientes que receberam tratatment sublingual. Adicionalmente, a IL-35 não só suprime proliferação de células T naive induzidas pelo efeito da TSLP nas células dendríticas, como também reduz citocinas caracteristicas the uma resposta alérgica inflamatória Th2. A IL-35 demonstra ser um potencial alvo para terapias futuras.

Palavras-chave: IL-35, Rinite alérgica, Resposta alérgica do tipo Th2, Citocinas derivadas

de células epiteliais, TSLP, IL-25, IL-33, Imunoterapia sublingual.

Index

Abbreviation List

APC	Antigen Presenting Cell	**pDCs**	Plamacytoid DCs
BFA	Brefeldin A	**PHA**	Phytohaemagglutinin
CD	Cluster of differentiation	**PMA**	Phorbol 12-Myristate 13-Acetate
DC	Dendritic cell	**PT**	Portugal
EPR	Early Phase response	**RAST**	Radioallergosorbent test
Foxp3	Forkhead box P3	**RPMI**	Roswell Park Memorial Institute
iTregs	Induced T regulatory cells		(RPMI) medium
IL-	Interleukin	**rpms**	Revolutions per minute
IgE	Immunoglobulin E	**SAR**	Seasonal Allergic Rhinitis
LFR	Late phase response	**SIT**	Specific Immunotherapy
Mϕ	Macrophage	**SLIT**	Sublingual Immunotherapy
MHC	Major Histocompatibility Complex	**TCM**	Tissue culture medium
mDC	Myeloid DCs	**TG**	Timothy Grass
nTcell	Naive T cell	**Th1**	T helper 1
N/A	Not applicable	**Th2**	T helper 2
NA	Non Atopic	**Tregs**	T regulatory cells
PBMCs	Peripheral Blood Mononuclear Cells	**TSLP**	Thymic Stromal Lymphopoietin
Phlp	Phleum pratense	**UK**	United Kingdom

Symbols

*	The definition can be found in the "Glossary" section of the attachments (Page 62)
#	Product catalogue number

Figure List

Table List

Portugal, Tão Diferente de seu Ser Primeiro

Os reinos e os impérios poderosos,
Que em grandeza no mundo mais cresceram,
Ou por valor de esforço floresceram,
Ou por varões nas letras espantosos.

Teve Grécia Temístocles; famosos,
Os Cipiões a Roma engrandeceram;
Doze Pares a França glória deram;
Cides a Espanha, e Laras belicosos.

Ao nosso Portugal, que agora vemos
Tão diferente de seu ser primeiro,
Os vossos deram honra e liberdade.

E em vós, grão sucessor e novo herdeiro
Do Bragança estado, há mil extremos
Iguais ao sangue e mores que a idade.

Luís Vaz de Camões in "Sonetos"

Chapter I: Introduction

1.1) Hypersensitivity Diseases

Individuals are said to be "sensitised" when they build up an immune response against a commonly harmless antigen. Therefore, the excessive reactions towards the specific antigen result in manifestations of hypersensitivity.

An exquisite immune system is capable of maintaining balance while eradicating infecting organisms and preventing serious cell tissue injury to the host. In contrast, a hyper-reactive individual shows signs of serious injuries to its own tissue, indicating inadequate control or inappropriate tissue targeting by their immune system.

There are four sub-types of hypersensitivity diseases named Immediate (type I) hypersensitivity, Antibody-mediated (type II) hypersensitivity, Immune complex-mediated (Type III) hypersensitivity and T-cell-mediated (type IV) hypersensitivity. Types I, II and III hypersensitivity disorders are characterised by antibody mediated injuries while type IV is defined as a hypersensitivity disorder of cell mediated injury[1–4].

It is important to emphasise that cell injury is caused by the same basic mechanisms that eradicate infectious pathogens, in which we find involved the Innate, Adaptive and Humoral immune responses. The uncontrolled balance of this cascade and the persistence of the stimuli at the site of inflammation create positive immune feedbacks, becoming difficult to terminate this pathologic cycle of immune reactions.

1.2) Immediate (Type I) Hypersensitivity – "Allergy"

Table 1.1 Causative agents of allergic diseases. The Table shows the different types of allergens, the way of dispersal and the primary site of exposure.

Immediate Hypersensitivity, most commonly known as 'Allergy', is a disorder that shows a world prevalence of 20%-30%, thus, provoking an economical and physiological burden on the individual and on society. There are various factors that aggravate allergic diseases and these include Atopy*, age (most prevalent in children), gender (most common in males),

Category			Primary site of exposure	Dispersal
Inhaled Allergens				
Outdoors	Pollens	Grass (Timothy Grass) Tree (Birch and Olive) Weed (Ragweed)	Nose, Eyes	Windborne
	Fungal Spores	Aspergillus	Nose, Eyes	
	Lower plants	Mosses Ferns	Nose, Eyes	
Indoor	Arachnids	Dust mite Storage mite	Nose, Lungs	Transient after disturbance
	Insects	Cockroach	Nose, Lungs	
	Mammals	Dogs Cats	Nose, Lungs	Airborne for many hours
Non-Inhaled Allergens				
Food	Peanut, tree nut, soy, Egg, wheat		Oral/or Skin	N/A
Bits/Stings	Hymenoptera	Bees Wasps	Skin/Circulation	
	Ticks	*Ixodes holocyclus*	Skin	

reduced microbial exposure in developed countries (hygiene hypothesis*), smoking, time of exposure to the antigen (higher exposure) and poor intrauterine nutrition[1,3].

Patients may suffer from occasionally mild (e.g. seasonal allergic rhinitis), life-long debilitating allergic reactions (asthma), or even, react with severe or fatal anaphylactic shock* (anaphilaxis*). These reactions depend on the allergen exposure (Table 1). Antigens such as airborne pollens (inhaled) and food allergens (ingested), trigger local allergic reactions on the upper and lower respiratory tracts, mouth or upper gastrointestinal tract[3]. Symptomatic hosts generally express neuronal mediated symptoms such as nasal itch, sneeze and rhinorrhoea and vascular mediated symptoms, which include nasal obstruction[3]. In the lower airways, the mediators triggered by an allergen lead to breathlessness, wheeze, cough and chest tightness[3]. Chronic symptoms may lead to the development of sinusitis, serious otitis media, conjunctivitis and lose of the senses of taste and smell. In contrast, antigens that enter the systemic (parenteral) circulation, via the intravenous administration of an antibiotic or an insect sting, can cause anaphylaxis, which is the most dramatic reaction to an immediate hypersensitivity reaction[1,3]. It is triggered seconds to minutes after exposure to the allergen and it is life-threatening as it can kill. In addition, progression of the condition can be incredibly fast and involves airway constriction, skin and intestinal irritation, altered heart rhythms and in most severe cases, complete airway obstruction, shock and death. It is important to note that there are also inhaled (for e.g. latex particles) and ingested (for e.g. peanuts) allergens that are equally capable of inducing a systemic allergic reaction[1,3].

1.3) Respiratory allergy: Allergic Rhinitis

Allergic rhinitis (AR) affects ¼ of the population in developing countries. AR can be subdivided into seasonal (also known as hay fever) and perennial (sometimes misdiagnosed as having a permanent cold), in which the former is expressed in a specific season, while the latter expresses itself all year round as the causative agent is present independently of the time of year. Therefore, it is understandable that the pollen seasons vary dependently on the country or region of exposure (Table 2)[5,6].

Seasonal / Hay Fever		High allergen season PT	High allergen season UK
Grass	Timothy Grass	March – June	mid May – July
	Mugwort	May-July	June-July
	Plane	March-April	April-May
	Plantain	April-May	May-june
	Nettle	November-March	June-August
Tree	Birch	March - May	late March - mid May
	Olive	May-June	Data not available
	Chestnut	June-July	Data not available
	Pine	March-May	May-June
	Willow	April-June	March-April
Weed	Ragweed	Data not available	end June – September
Perennial			
House Dust Mite Cat Dog Occupational antigens		N/A	

Table 1.2. Seasonal and Perennial Allergic rhinitis. Highest seasonal allergen counts in Portugal and in the United Kingdom. The table makes a distinction between Seasonal and Perennial allergic rhinitis. In what it concerns to seasonal allergic rhinitis, the highest seasonal counts are emphasised for the most common allergens in Portugal and in the UK[5,6].

1.4) Immunological Mechanisms of Allergic Rhinitis

The clinical symptoms expressed by individuals suffering from AR can be explained by a sequence of immune events, triggered upon the entry of an allergen to the lining of the nasal mucosa. The two main characteristics of an allergic response, is the presence of Th2 (Type 2 helper) cells and allergen specific-Immunoglobulin E (IgE) antibodies.

It is believed that in AR patients, the detection of the allergen at the site of inflammation is achieved by innate immunity cells called Dendritic cells (DCs), more specifically $CD11c^+$ myeloid Dendritic cells (mDCs). These cells are antigen presenting cells (APCs), which capture and cleave the allergen into small peptides, while they mature and migrate to lymph nodes in order to present the processed antigenic peptide to naive T cells (nTcells), also known as Th0 cells. At this stage, the cross-talk between innate and adaptive immune responses is achieved. For full activation of nTcells, it is necessary the involvement of surface receptors such as the Major Histocompatibility Complex two (MHC-II) and B7-2 on DCs and the T cell receptor (TCR) and CD28 on nTcells[7]. With the presentation of the allergen to Th0 cells, these suffer a molecular changes, which allows them to produce IL-4 and therefore called IL-4 competent CD4+ T cells, becoming Th2 cells[8]. These adaptive immune cells activate T follicular helper (T_{FH}) cells, which also produce IL-4, the agent able to trigger B cell to undergo heavy-chain class switching to allergen specific IgE[9,10].

Th2 migrate to the site of inflammation, where they secrete the well known IL-4, IL-13 and IL-5 cytokines, in which the two former cytokines recruit mast cells and basophils and the latter cytokine guide the local Eosinophils to infiltrate the site of inflammation[7,11–13].

In addition, at the site of inflammation, the allergen specific IgE is known to activate Masts

cells, Basophils and Eosinophils as they, commonly, express FcεRI, which is the high-affinity receptor for the Fc portion of the ε heavy chain of IgE. Binding of IgE to the FcεRI on Masts cells, trigger degranulation of its granule stores. Mast cells granules contain chemotactic factors that recruit neutrophils and Eosinophils[1].

The secretion of histamines from Masts cells cause vasodilation, increased vascular permeability, smooth muscle contraction, and increased mucus secretion. In addition, adenosine is also secreted and it is the causative agent of bronchoconstrictions and suppression of platelet aggregation. In addition, neutral proteases such as tryptase are also released and are capable of cleaving complement components, which induce chemotactic and inflammatory mediators that may end up provoking cell damage. Furthermore, newly synthesised lipid mediators such as Prostaglandins (PGD) and Leukotrienes (LTC) are stored in Mast cells and are involved in mucus secretion (PGD_2) and, finally, several thousand times more active than histamines, LTC_4 and LTD_4 that cause vasodilation and bronchospasms. Similarly, when IgE activates Eosinophils, they also secrete LTC4 and promote inflammation by releasing platelet activating factors. Additionally, Eosinophils produce eosinophil cationic protein, which is a toxic agent known to cause cell damage. The role of IgE activated Basophils is still unclear in the allergy field and requires further analysis[1,2].

Clinically speaking, these events of inflammatory reactions cause sensitised individuals to produce an “early” phase response (EPR) responsible for acute AR and a “late” phase response (LPR) characteristic of chronic AR. The EPR occurs within 5-10 minutes and may last up to 60 minutes (Fig 1.1). This immediate phase is characterised by vasodilation, vascular leakage and smooth muscle spasm often triggered the effects of specific IgE. The LPR may be triggered 2-8 hours after the EPR and it can last for several days (Fig 1.1). This late phase reaction is expressed by cell damage to the mucosal epithelia and the most

abundant cells are neutrophils, eosinophils and T lymphocytes (in particular Th2 lymphocytes).

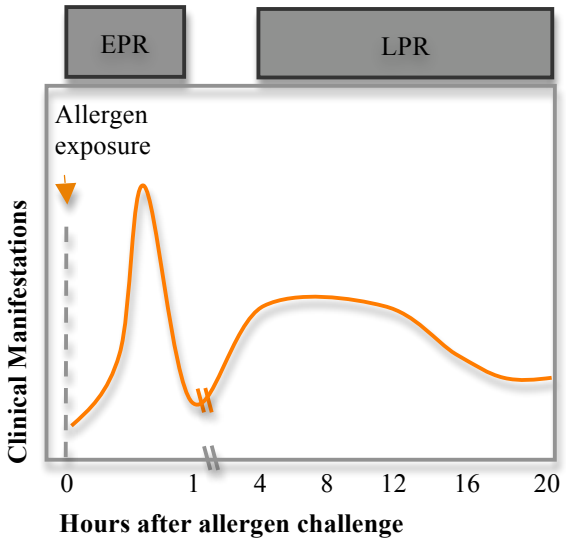

Figure 1.1 Immediate Hypersensitivity. Kinetics of the early-phase response (EPR) and late-phase response (LPR) after allergen challenge to a sensitised individual[1].

It is important to note that the cell events at the inflammatory site do not translate the changes occurring in circulation. Therefore, clinicians measure the inflammatory mediators in blood and at sites of allergen exposure. Diagnostic assays include a skin prick test and a RAST test. A Positive skin prick test to causative aeroallergens selects allergic rhinitis from non-allergic rhinitis and the RAST test consists in detecting the amount of specific IgE in blood serum. Normally, the RAST test is only requested in case the skin prick test shows unclear results. In addition, performing a histopathological exam may be also necessary as it shows oversecretion of nasal fluid containing basophils and eosinophils. Furthermore, in nasal secretions, it is also possible to measure mast cell mediators and detectable IgE, IgG and IgA[1,3,14].

1.5) Potential inflammatory mediators in SAR

In order to understand which molecules are involved in the enhancement of an allergen driven Th2 response, allergists collected cells from nasal mucosa of AR patients.

Previous results reported high expression of epithelial-derived cytokines named thymic stromal lymphopoietin (TSLP) [15], IL-25 (also known as IL-17E) [16] and IL-33[17]. Since these discoveries, research in both mice and human samples; have directed their focus towards the understanding of these non-haematopoietic-cell-derived cytokines in allergic immunity (Fig 1.2).

TSLP is an IL-7-like cytokine and its heterodimeric receptor is composed by IL-7Rα and TSLP receptor (TSLPR). Studies have identified higher affinity upon ligand binding when both receptor subunits are activated simultaneously. Reports to date have shown that TSLP is mainly secreted by epithelial cells and it is detected by mDCs by its [IL-7Rα-TSLPR] receptor. TSLP primed DCs result in the upregulation of OX40L, which is the ligand for OX40, expressed on DCs, resulting in prolonged DC survival. Such interaction is critical for the development of a Th2 driven response[11,12,18]. Primed mDCs then migrate from the site of inflammation to lymph nodes, where they present the antigen to nT cells through the major histocompatibility complex class II (MHC-II), inducing nT cell differentiation into Th2 cells[11–13,18].

IL-25 belongs to the IL-17 family and activates the IL-25 receptor (IL-25R) expressed on Basophils, Mast cells and CD25R Natural helper cells. Studies associated to asthma demonstrated that CD25R Natural helper cells secrete TNF-α, IL-1β, IL-6 and IL-8 in which IL-8 is the key cytokine for Eosinophil recruitment; and therefore IL-25 is necessary to maintain eosinophilia at the site of inflammation[10]. In addition, it has been also shown the

importance of Il-25 in activating both masts cells and basophils, which are crucial for Th2 mediated responses. Interestingly, Il-25 was found to enhance TSLP-primed DC effect for differentiation of Th2 cells in asthmatic patients[19]. In studies related to helminth infections and allergic diseases, it was shown that IL-25 triggers IL-4, IL-5 and IL-13[20–22]. The few asthma related research suggest that IL-25 may be good target for IT. However, there are no human studies relating the effect of IL-25.

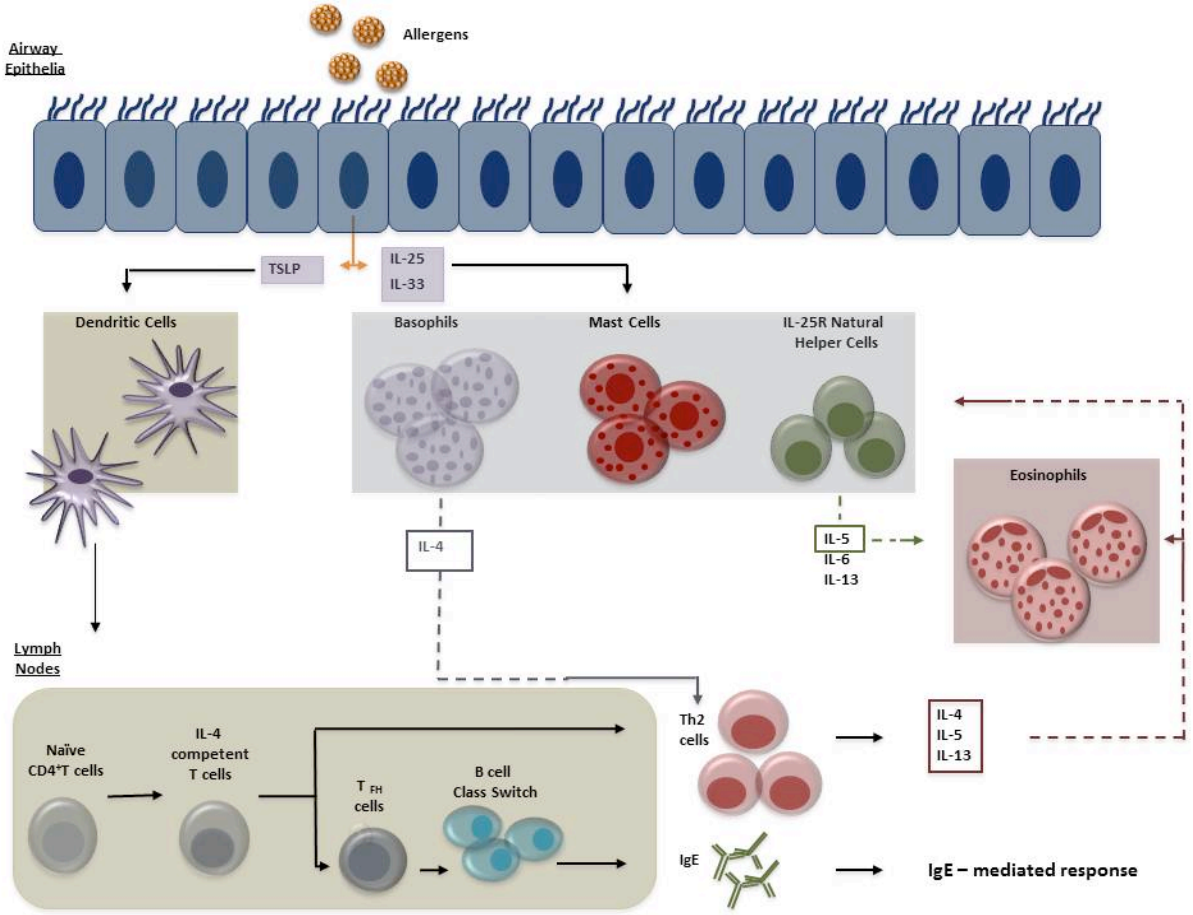

Figure 1.2. Mechanisms of seasonal allergic rhinitis. Figure adapted from [7].

IL-33 belongs to the IL-1 family and a correlation between IL-33 and the enhancement of Th2 driven inflammatory immune response was found in asthma research. IL-33 increases

IgE mediated response since it seems to activate both Mast cells and Basophils through its receptor, the ST2 receptor. IL-33 is only able to activate nTcells directly and not indirectly by priming mDCs[10,23]. Research on AR showed that IL-33 increases the production of IL-8, resulting in eosinophil recruitment[24].

In summary, these epithelial-derived cytokines have shown to, synergistically, maintain a Th2 response. However, in AR patients, the way they work independently from each other to achieve such end, has not been tested and therefore remains unclear.

1.6) Potential Immunotherapy targets in SAR

The main aim of research is to increase knowledge on the immune mechanisms underlying AR, in order to crack the puzzle of inflammatory modulation for a better diagnosis and reduced symptom score. Immunotherapy targets the site of inflammation in order to regulate and/or redirect the course of response.

Studies have indicated the involvement of specific subsets of T cells with the role of regulating allergen specific Th2 mediated allergic immune responses (Fig 1.3). These cells are called regulatory $CD4^+$T cells (T_{reg}) and they can be subdivided into the naturally occurring T_{reg} (nT_{reg}) cells found in the thymus, which are characterised by $CD4^+CD25^+FOXP3^+$ T_{reg} cells and the induced $CD4^+$ $CD25^+$ $FOXP3^-$ T_{regs} (iT_{regs}), found in the periphery, which can be IL-10 producing Tr1 or IFN-γ producing Th1 cells[25]. Interestingly, there are cells that contain both Tr1 and Th1 phenotype. In other words, these Tr1/Th1 cells are activated by IL-10 and IL-12 derived APCs and secret both IL-10 and IFN-γ. This may indicate that they are suffering a phenotype change from a Th1 to a Tr1 cell or the way around. These cells, however, are able to both inhibit Th2 cells and impair B cell IgE class switch, redirecting it towards IgG1, IgG4 or IgA Abs[26]. It has been shown in a couple of

studies the increase of IL-10 and IFN-γ after specific immunotherapy (SIT) [27,28]. However, a study involving sublingual immunotherapy (SLIT), in which patients obtained increased IL-10 and IFN-γ, did not translate its effect in a clinical point of view, since the patients' symptoms were not alleviated despite the immunological changes[29].

The question remaining is if either these cells can regulate inflammation by suppressing Th2 responses or change their course from a Th2 to a Th1 phenotype.

Therefore, in this project, we focus on a novel target named IL-35[30], found by Collison et al. in 2007. IL-35 belongs to the IL-12 family, which include the pro-inflammatory cytokines IL-12 and IL-23, and the anti-inflammatory cytokines IL-27 and the Il-35. Its structure consists of the EBV-induced gene 3 (EBI3) subunit of IL-27 and the p35 subunit of IL-12[31]. IL-35 demonstrated to inhibit T cell proliferation.

In addition, Collison's work also demonstrated that IL-35 has the effect of suppressing T cell proliferation, resulting in reduced inflammation[32]. One year earlier, mouse studies were showing that IL-35 reduces IL-17, an inflammatory cytokine, thus inhibiting proliferation of Th17 by inducing $CD4^{+}CD25^{+}Foxp3^{+}$ T_{reg} [33]. Untill this point, it was suspected the anti-inflammmatory role of IL-35, however its mechanism was still unclear. It wasn't until later, in 2010, that Collison was able to show IL-35 inducible FoxP3- Tregs[34]. Soon after, in 2011, transwell plate experiments revealed that iTr35 cells implement contact free suppression through IL-35 and partially IL-10[3536].

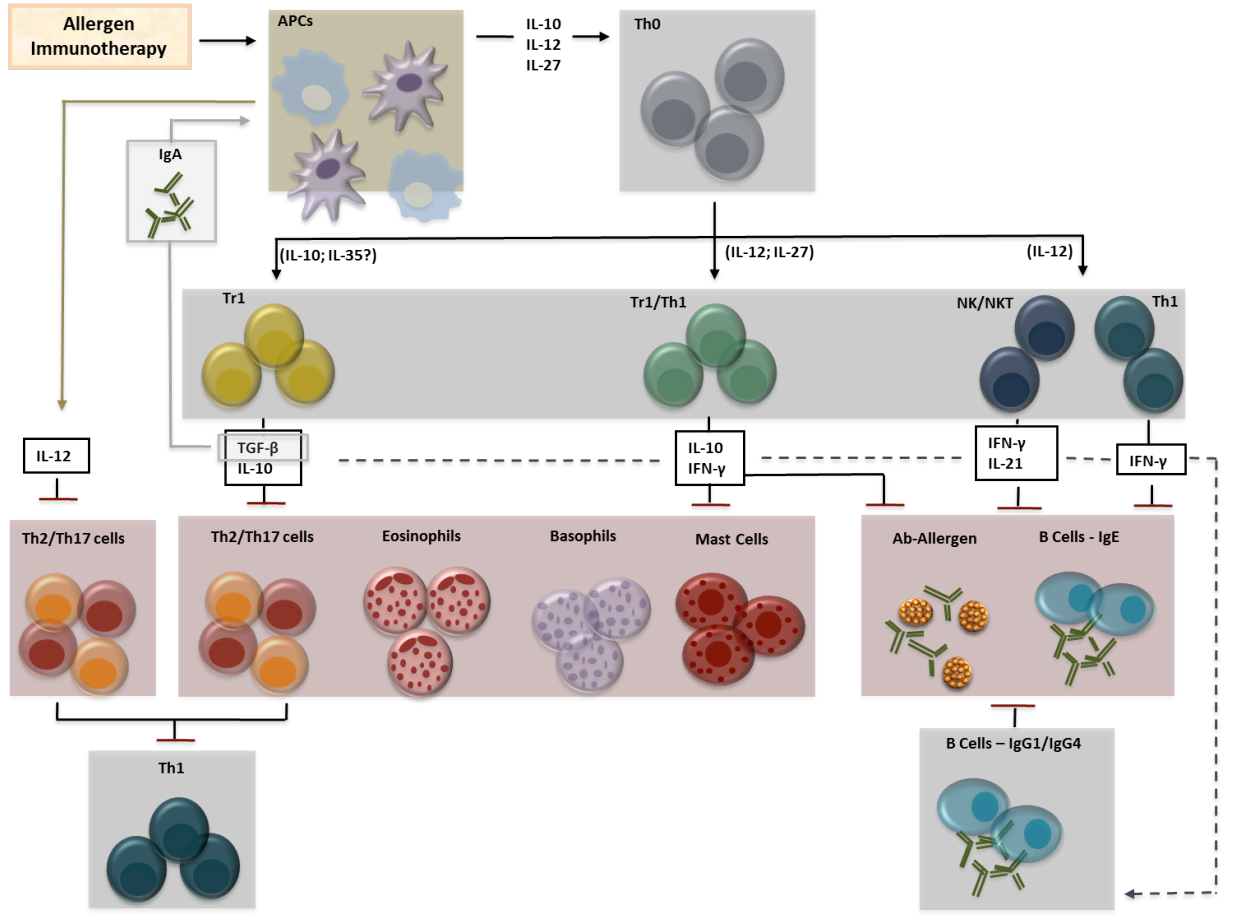

Figure 1.3 Mechanisms of allergen specific immunotherapy. Figure adapted from [37].

Hypotheses

- Grass pollen-SLIT treated patients have higher proportions of Foxp3+, inducible IL-10 (TR1) and IL-35+Tregs (iT_R35) compared to untreated allergics.
- IL-35 suppresses grass pollen-specific effector Th2 responses following ex-vivo grass pollen stimulation.
- Epithelial derived mediators/cytokines such as thymic stromal lymphopoetin, interleukin -25 and 33 primed DCs when stimulated with grass pollen allergen drives naïve T to diferenciate into allergen-specific Th2 cells that produce IL-5 and IL-13.
- IL-35 supresses TSLP, IL-25 and IL-33 responses on naïve T cells.
- IL-35 supresses Th2 polarisation from Th0 cells.

This project will attempt to accomplish the underlying aims:

- To assess the proportion of Foxp3, IL-10+ and IL-35+ Tregs in SAR, SLIT and NA.
- To develop an *in vitro* model of allergen-driven Th2 response induced by TSLP, IL-25 and IL-33 primed DCs.
- To analyse the behaviour of IL-35 on allergen-driven Th2 responses.
- To optimise a T cell polarisation protocol towards Th1 and Th2 cells, to run in the lab, daily for future IL-35 studies.

Chapter II: Methodology

2.1 Ethical Statement

The clinical practice implemented in this project was in accordance with the guidelines of the Ethics Committee of the Royal Brompton and Harefield Hospital NHS trust. All tissue and blood samples were collected with prior written approval from all patients involved. Samples were collected according to good clinical practice.

2.2 Skin prick test and ImmunoCap

ImmunoCap was used to verify the allergic sensitisation of patients quantifying specific and total and specific IgE in blood serum (Phadia). The protocol was followed as instructed by the manufacture.

2.3 PBMC isolation

Venous blood was collected into Lithium-heparin tubes and kept sterile. Blood was then centrifuged for 10 minutes at 20°C at 1500rpms. Plasma was removed and stored for future serology work. Following serum extraction and storage, RPMI-1640 without glutamine (Gibco, Invitrogen, UK) was added to equal the volume of serum removed. 35ml of the diluted sample was layered carefully over 15ml of Ficol (VWR International Ltd., UK) within a 50ml tube. After a 25 minute centrifugation at 2200 rpms at 20°C (without centrifuge brake), the layer of peripheral blood mononuclear cells (PBMCs) which is isolated and kept in tissue culture medium.

2.4 Thawing PBMCs from Liquid Nitrogen

PBMCs initially stored in cryovials were removed from liquid nitrogen and 70% thawed with agitation in a 37°C waterbath. Cells were then transferred from cryovials to 10mL tubes, immersed in RPMI-1640 and centrifuged at 1500RPM for 10min. Any supernatant was then

discarded and samples resuspended in 5mL RPMI-1640. A 20µL cell suspension in trypan blue at a 1:5 dilution was then loaded into a haemocytometer chamber and a live cell number calculated.

2.5 Cell staining of FoxP3+Treg, IL10+CD4+Treg and IL-35+CD4+Treg

Cells were stained with the surface staining protocol indicated below (2.10) for APC CD4+ (clone: RPA-T4, BD Bioscences, USA) and APC/Cy7 CD25+ (clone: M-A251, BD Bioscience). Intracellular and nuclear stainings were performed accordingly to the protocol below (2.11) of PerCP IL12p35 (clone: 27537, R&D), AlexaFluor488 EBI3(clone 607201 , R&D) and V450 FOXP3 (clone: 236A/E7, BD Bioscience) and Pe/Cy7 IL-10 (JE53-9D7, Biolegend).

2.6 $CD4^+CD25^-$ T effector cells antigen-specific stimulation and IL-35 neutralisation

The separated $CD4^+CD25^-$ T_{eff} cells were incubated in 1:1 ratio with irradiated Ag-presenting cells (APC) in concentration $300x10^6$ cells/well. In each well 3ug/ml PhlP (Alk abello, Denmark) was added along with 10 ng/ml IL-35 (Enzo life Science,UK). When determining the best concentration of Phl P to be used experiments were carried out with 0,1, 3, 5 and 10 ug/ml. The plates were cultured for 9 days at 37^0C in 5% CO_2 and pulsed for the last 18 hours with [^{3}H] - thymidine. Proliferation was measured using the described above method.

2.7 T cell polarisation using epithelial derived cytokines and IL-35 suppression

PBMCs were isolated from 200 ml of whole blood from allergic rhinitis patients. DCs and naive T cells (nT cells) were purified from isolated PBMCs, using Pan-DC Pre-enrichment Kit and naïve CD4+ T enrichment kits respectively (Stem Cell technologies, UK). DCs and nT cells were counted, using a haemocytometer and dead cell excluded, Trypan blue (Sigma

Aldrich Company Ltd., UK). $5x10^3$ cells of Dcs were added in 50µl of TCM per well. 15ng/ml TSLP (eBioscience,UK), 10ng/ml IL-25 (R&D,UK) and 10ng/ml IL-33 (R&D) were added as described for 24h, where used timothy grass pollen, Phleum Partenses allergen (Phlp) was added at 5µg/ml (ALK-Abelló, Denmark). At 24h primed, DCs were co-cultured with naive T cells, at $5x10^4$ cells per well. In some experiments, IL-35 was added IL-35 (Enzo life Science) at 10ng/ml. Phytohaemagglutanin (PHA) (10µg/ml) was added as positive control for T cell proliferation. At day six of co-culture, some wells were pulsed with 1µl of 3H-Thymidine to determine proliferation. Following a further 16h, cells were harvested onto filtermats (Harvester 96Tomtec) and beta counts detected by 1450 Micro Beta Trilux. Non-pulsed cell supernatants were stored at -20°C for cytokine analysis by Multiplex ELISA.

2.8 TSLP receptor expression on DCs

DCs were purified from isolated PBMCs and stained for flow cytometric analysis at 0 and 24h following stimulation with phlp and TSLP. PE $TSLPR^+$ (clone:147036, R&D) and APC^+ IL-7Rα (clone:40131, R&D) were used to check TSLP receptor heterodimer, APC-Cy7 $CD1c^+$ (clone: L161, eBioscience) and V450 $CD11c^+$ (clone:B-ly6, BD, UK) for myeloid DCs, PerCpCy5.5 $CD123^+$ (clone:6H6, Biolegend,UK) and FITC $CD303^+$ (clone:AC144, Miltenyi Biotec,UK) for plasmacytoid DCs.

2.9 T cell polarisation towards Th1 or Th2 cells

After PBMC and nT cell isolation, nT cells were counted and equally divided into three, 1µ/ml αCD3 (R&D) and 2µ/ml αCD28 (R&D) pre-coated, 24well plates (Previously incubated for 4h in the incubator at 37°C and 5% CO_2). Non-polarised nT cells were stimulated with 2ng/ml IL-2 (clone: R&D) alone. Th1 polarised cells were polarised with

2ng/ml IL-2 (clone: R&D), 2.5ng/ml rhIL-12 (R&D,UK) and 5µ/ml αhIL-4 (clone:MP4-25D2, BD). Th2-polarised cells were stimulated with 2ng/ml rhIL-2 (R&D), 12.5ng/ml rhIL-4 (R&D), 5µ/ml αhIL-10 (clone: JES3-19F1, BD) and 5µ/ml αhIFN-γ (clone:B27, BD). This experiment was carried out in three atopic patients (Fig 2.1).

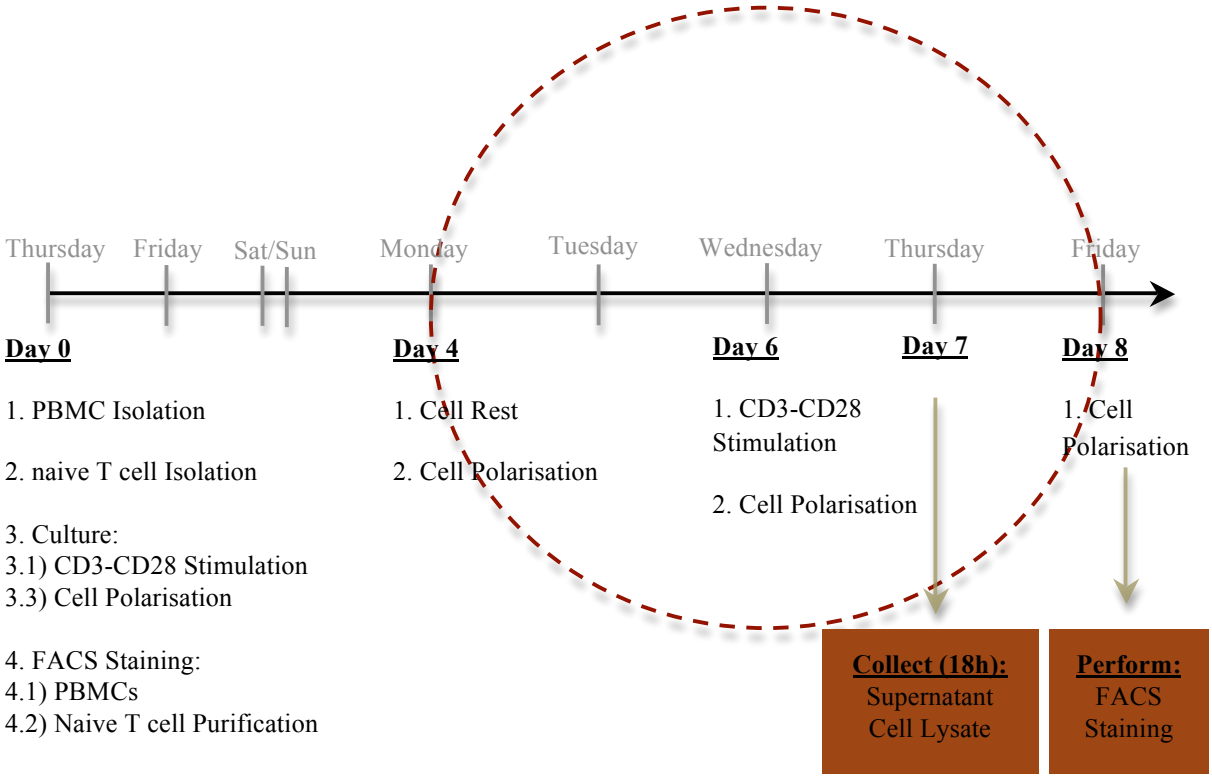

Figure 2.1 T cell Polarisation Protocol

Weekly, prior to re-stimulation cells were stained for flow cytometry with the following fluorochrome-coupled antibodies:

To check purification of naïve T cells from isolated PBMCs on day zero, the staining antibodies used were: PE-Cy7 $CD4^+$ (clone: SK3, BD), APC-Cy7 $CD25^+$ (clone:BC96, Biolegend), PE $CD45RO^+$ (clone: BD) and FITC $CD45RA^+$ (clone: BD). Purification was achieved at 99.8% nTcell isolated from PBMCs. (Fig 2.2)

Weekly staining involved Pacific Blue $CD4^+$ (clone: RPA-T4, BD), APC-Cy7 $CD25^+$ (clone:BC96 Biolegend), APC IL-13^+ (clone: BD), PE GATA-3^+ (clone: eBioscience), IFN-γ^+, and PE-Cy7 T-bet^+ (clone: eBioscience) fluorescence antibodies.

Cell lysates were stored to analyse gene expression by q-PCR as described below and cell supernatants stored for cytokine analysis by multiplex ELISA (Fig 2.1).

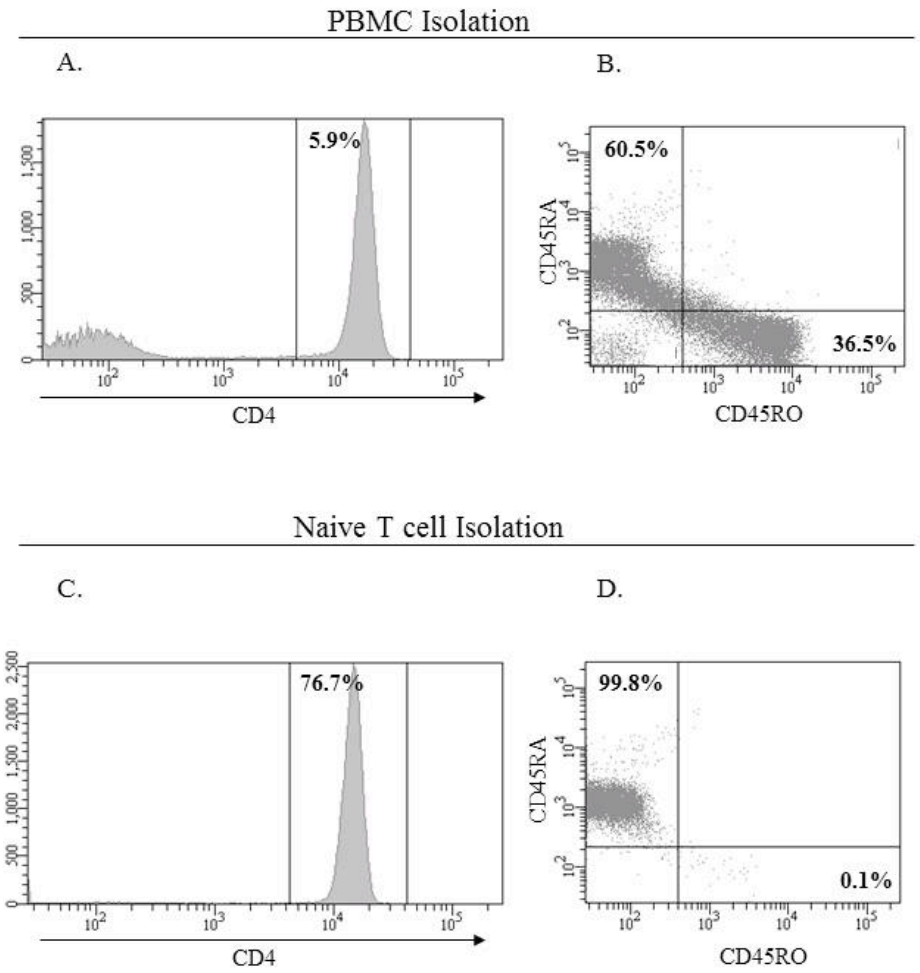

Figure 2.2. Naive T cell purification check. PBMCs were isolated from whole blood and naive T cells were further purified. PBMCs and naive T cells were collected and stained for cell surface receptors: CD4 cell marker indicating T cells, CD45RO effector T cells and CD45RA naive T cells. A and B) FACS data of PBMC staining showed the presence of 5.9% of T cells, 36.5% of effector T cells and 60.5% of naive T cells. C and D) After isolating naive T cells from PBMCs, Cell staining results demonstrated that 76.7% of the cells in culture are T cells and 99.8% of those cells express the CD45RA surface marker. Results suggest that the isolation of naive T cells were performed successfully and further experiments with naive T cells can be carried on.

2.10) Surface staining

Followed the addition of 10µl of FC Blocker for 15 minutes, surface staining was added accordingly for 40 minutes. Cells were then washed with BD Cell staining buffer and run through flow cytometry.

2.11) Intracellular and nuclear staining by Flow cytometry

For cytokine intracellular staining, cells were stimulated with PMA (Sigma-Aldrich), Ionomycin (Sigma-Aldrich) for one hour and BFA (BD #347688) added for the remaining 4h. The protocol used for the intracellular and nuclear staining was the BD transcription factor Buffer set staining (BD # 562574). Very briefly, 500x10^6 per sample were stimulated as indicated above and 10µl of FC Blocker added for 15 minutes. Surface staining was then added for 50 minutes at 4°C. Cells were washed twice with BD cell staining buffer. 1 ml of 1x Fix/Perm solution (BD) was added to the cells prior to a 50 minute incubation at 4°C. Cells were submitted to three washes with the BD 1xPerm/wash solution and incubated for 50 minutes with the corresponding intracellular staining. After a two time wash, cells were run through flow cytometry.

2.12) Gene expression assay by qPCR

Cells were collected and lysed with RLT Buffer (Qiagen) containing 1% β-Metacaptoethenol (Sigma-Aldrich, UK), 16h after polyclonal stimulatiom with αCD3 (R&D) and αCD28 (R&D) antibodies and kept at -20°C. Cell lysates were added to the QIA Cube (Qiagen), which uses the RNeasy Mini Isolation Kit (Qiagen) and QIA shredder (Qiagen), for RNA extraction from cell lysates. Nanodrop was performed in order to check the concentration and purity of RNA isolated. Once confirmed, it was added per mix 4µl of 5xTaqman RT Buffer (Thermo Scientific), 2µl dNTPs Thermo Scientific), 1µl Random Hexomers (Thermo

Scientific), 1µl Reverse Transcriptase (Thermo Scientific), 0.5µl RNase inhibitor and the 11.5µl RNA sample, which are then run in Sense Quest Labcycler for cDNA production. The following program wasused: 25°C for 10 minutes, 47°C for 1h and 70°C for10miutes. cDNA is analysed by Nanodrop and diluted 1:10 in molecular biology water, in epMotion machine together with the corresponding primers (1.6µl) mixed with Syber Green (5µl) (AB Applied Biosystems). All the primers used for this experiment were ordered from Sigma-Aldrich. The housekeeping gene more appropriate for the experiment was EF2A and the primers of interest IL-4, IL-5, GATA 3 IFN-µ and T-Bet (Table 2.1).

	Forward primer 5'-3'	**Reverse primer 5'-3'**
EF2A	CTG AAC ATC CAG GCC AAT	GCCGTGTGGCAATCCAAT
IL-4	AAA CGG CTC GAC ACG AAC CT	ACTCTGCTTGGCTTCCTTCACA
IL5	AGC TGC CTA CGT GTA TGC CA	GCAGTGCCAAGGTCTCTTTCA
GATA-3	GCG GGC TCT ATC ACA AAA TGA	GCTCTCCTGGCTGCAGACAGC
IFN-γ	TCT GGA AAC GAT GAA ATA TAC AAG TT	GTA ACA GCC AAG AGA ACC CAA AA
T-Bet	GAT GCG CCA GGA AGT TTC AT	GCA CAA TCA TCT GGG TCA CAT T

Table 2.1: Forward and reverse primer sequences for Th1 and Th2 related targets.

2.1) Cytokine Analysis by Multiplex ELISA

Milliplex MAP Kits (Luminex Millipore, Germany) were used to preform multiplex ELISA. Both Th1/Th2 and Th17 human magnetic bead panel kits were used to quantify cytokine concentrations in supernatant (#HCYTOMAG-60K, #HTH17MAG-14K). Manufacture's protocol was followed and MAGPIX Luminex XMap Technology was used to run the analysis.

2.14) Statistical Analysis

Analysis of all data was conducted with GraphPad Prism 5.01 software (San Diego, USA). Non-parametric FACS data was processed with a wilcoxon test, with statistical significance represented as ($p<0.05$).

Chapter III: Results

3.1) Rhinitis symptom scores declines after sublingual immunotherapy

Subjects treated with sublingual immunotherapy (n=7), were asked to grade the severity of their allergic rhinitis. Untreated SAR patients (n=12) and NA patients (n=12) were recruited for the same evaluation. SLIT patients demonstrated a significant improvement in AR symptom scores of 47% (p=0.006) compared to SAR patients.

3.2) IL-10 + and IL-35 +, but not, FoxP3 + Treg is increased following SLIT

SLIT (n=7), SAR (n=12) and NA (n=12) provided whole blood from which PBMC were isolation for FoxP3+, IL-10 and IL35+ cell staining. By analysing the $CD4^+$ T cell population for Foxp3 expression, no statistically discernible difference between SAR and SLIT patients was noted (p=0.688) (Fig 3.1B), suggesting that the T_{reg} numbers remain unchanged between treated and untreated patients. Intracellular fluorescent Ab FACS staining for IL-10 revealed a noticeable increase in $IL\text{-}10^+CD4^+CD25^+Foxp3^+$ T_{reg} cells from SLIT PBMCs as opposed to those from SAR (p=0.016) patients (Fig 3.7C). In contrast, NA patients show the highest percentage of IL-10 producing Tregs (p=0.001). Intracellular fluorescent Ab FACS staining for the subunits of IL-35 (Ebi3 & IL-12P35) demonstrated a clear increase in induced $IL\text{-}35^+$ $CD4^+CD25^+FoxP3^+$ T_{reg} population of PBMCs for SLIT-treated compared to SAR (p=0.016) patients (Fig 3.1D). SAR has significantly decreased proportions of IL-35+ Tregs on compared to NA (p=0.0005).

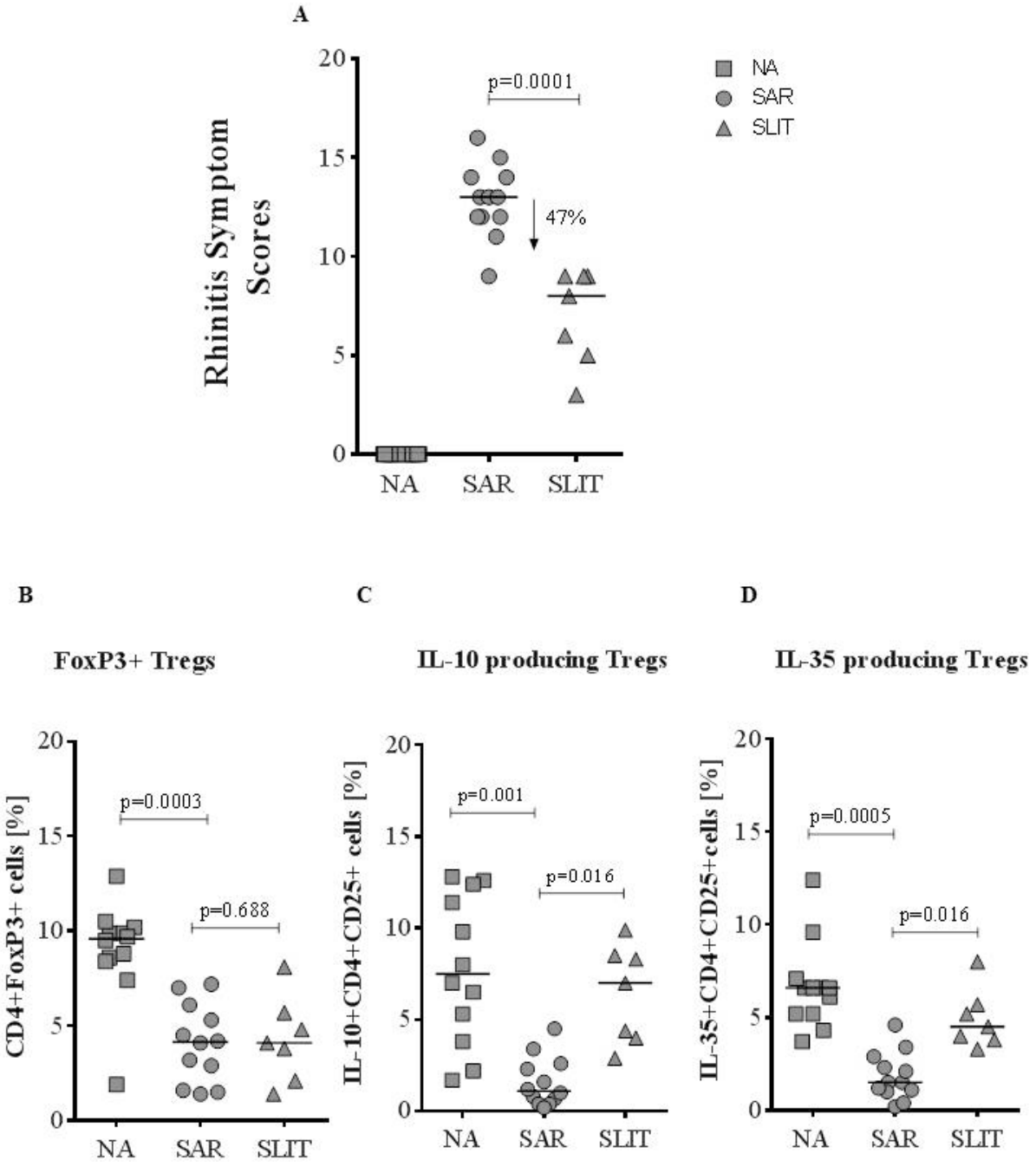

Figure 3.1. SA symptom score and PBMC surface and intracellular staining of different subtypes of Tregs in SAR and SLIT patients. NA, SAR and SLIT patients A) provided their RA symptom score. B) FoxP3 + Tregs, C) IL-10 producing Tregs and D) IL-35 producing Tregs of these same patients were measured by intracellular staining. SLIT patients recover IL-10 and Il-35 producing Tregs (p=0.016 of both), however FoxP3+ is maintained, compared to SAR patients (p=0.688).

3.3) IL-35 suppresses allergen driven Teff cell proliferation and induces Th2 differentiation

Teff cells isolated from PBMCs of SAR (n=12) patients, were treated with or without IL-35. Proliferation assay results showed a statistically significant suppression of Teff cell proliferation upon addition of IL-35 ($p<0.0001$) (Fig. 2A). In addition, IL-35 suppressing effect was tested by adding an IL-35 neutralising antibody, which successfully recovered the inhibitory effect of the anti-inflammatory cytokine ($p=0.0009$) (Fig. 2B).

Cytokine levels were measured, in order to depict the role of IL-35 in Th2 responses. Th2 suffered a statistically significant decrease (IL-4 $p=0.0001$, IL-5 $p=0.0001$, IL-9 $p=0.0001$ and IL-13$p=0.0009$) (Fig 2C), while inducing IFN-γ ($p<0.0001$) (Fig 2D) and IL-10 ($p<0.0001$) (Fig 2E), suggesting not only that IL-35 suppresses Th2 responses but also deviated the response to what it seems to be a T regulatory response.

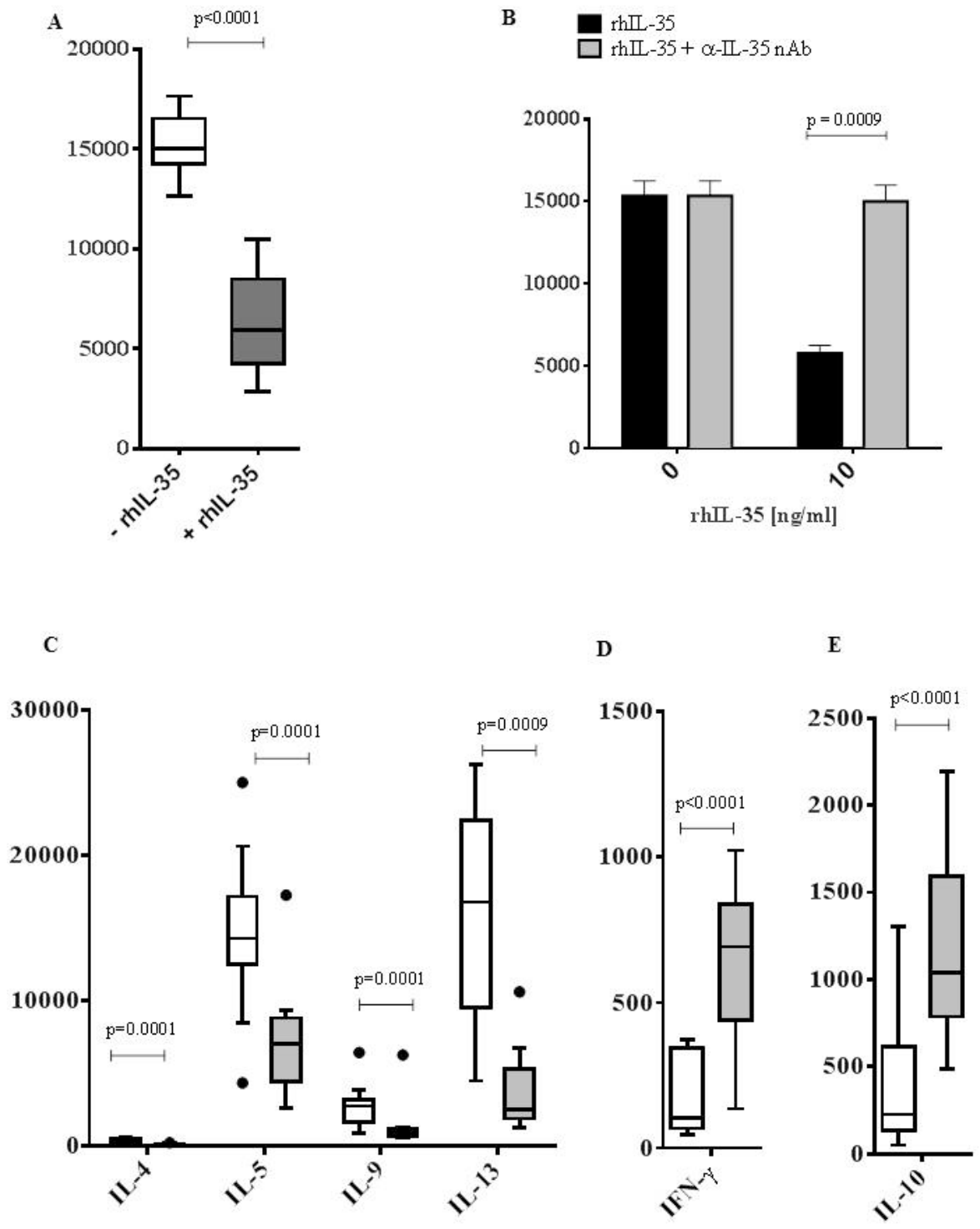

Figure 3.2. Teff cells treated with or without IL-35 were submitted to a proliferation assay as to cytokine analysis. A) Teff cells were treated with and without IL-35 and the B) role of IL-35 was tested by adding an IL-35 neutralising Ab. C-E) Cytokine behaviour to IL-35 was also analysed. Data are shown as mean+/-SEM. IL-35 suppressed Teff cell proliferation (p,0.0001) and Th2 cytokine production: IL-4, IL-5 and IL-9 (p=0.0001) and IL-13 (p=0.0009). IFN-γ (p<0.0001) and IL-10 (p<0.0001) were increased in the presence of IL-35.

3.4) TSLP, but not Il-25 and IL-33 primed DCs induce T cell proliferation in an allergen driven response and Th2 differentiation

A proliferation assay (n=14) of nT cell co-cultured with TSLP primed DCs was performed and demonstrated a statistically significant induction in nT cell proliferation (p=0.003). Equally, dendritic cells primed with TSLP, IL-25 and IL-33 showed statistically significant proliferative effect on nT cells (p=0.0001). In contrast, there are no alterations in the proliferation of nTcells when exposed to DCs that had been previously exposed to IL-25 (p=0.426) or IL-33 (p=0.502) (Fig 3.3A).

In addition, Supernatants were collected (n=7) and analysed in multiplex for IL-5 and IL-13 (Fig 3.3B) as to IFN-γ (Fig 3.3C). TSLP+Phlp primed DCs clearly trigger T cells to secrete IL-5 and IL-13 production. Unexpectedly, a modest increase in IFN-γ was also seen. Cytokine results were not statistically significant.

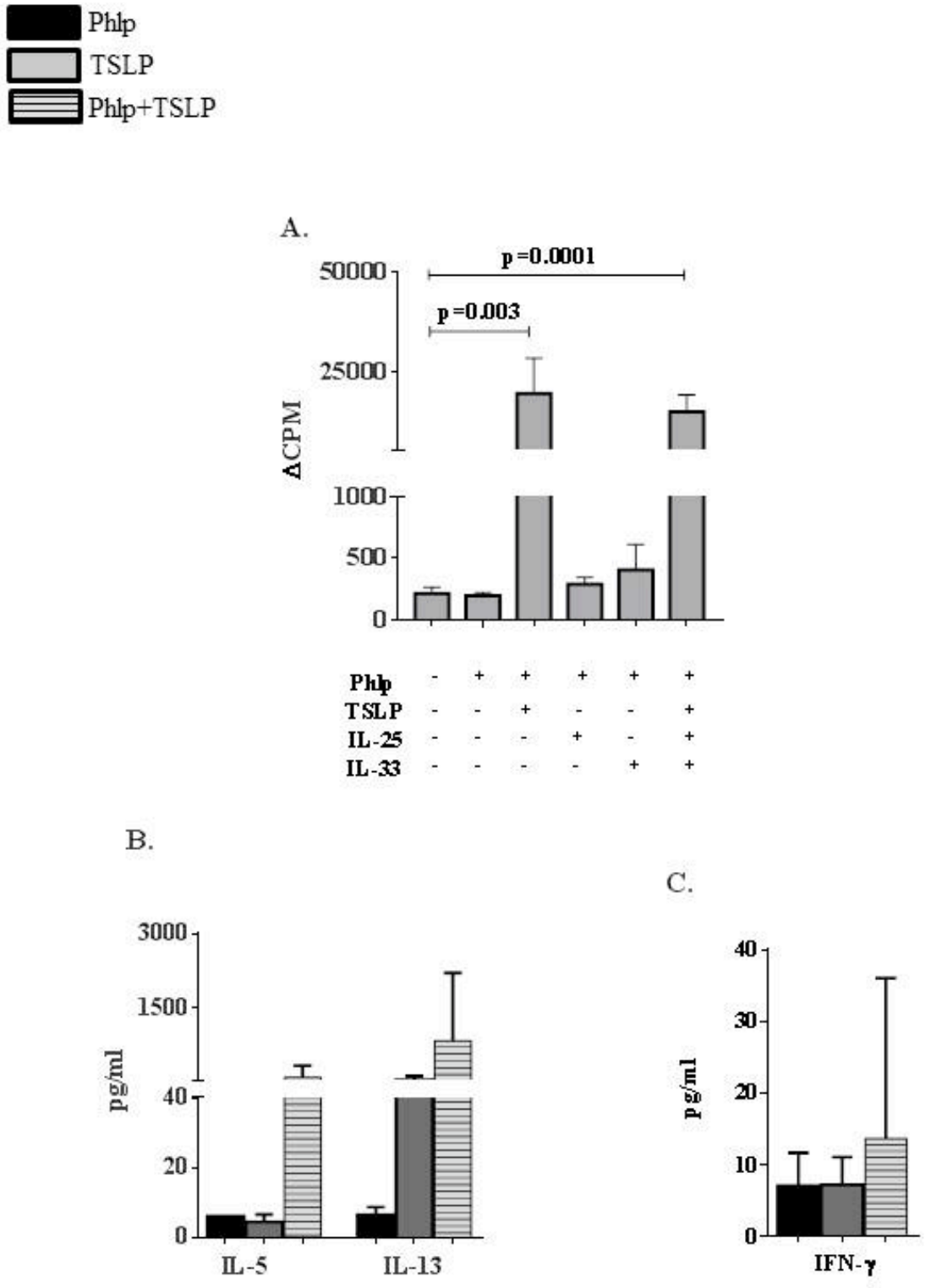

Figure 3.3. Epithelial-derived pro-allergic mediator TSLP but not IL-25 and IL-33 induces grass pollen-specific naive T cell proliferation and Th2 cytokines. A) TSLP, IL-25 and IL-33 primed DCs stimulated with/without Phlp were co-culture with naive T cells and proliferative responses were measured. B) IL-5, IL-13 and C) IFN-γ were measured from cell culture supernatants. Data are shown as mean+/-SEM. TSLP+Phlp primed DCs induce nTcell proliferation (p=0.003) as well as the three epithelial-derived cytokines+Phlp (p=0.0001).

3.5) TSLP Receptor is expressed in higher amounts in SAR compared to NA patients

Purified DCs of SAR volunteer patients (n=3) and NA volunteer patients (n=3) were isolated and stained for myeloid ($CD11c^+/CD1c^+$ and $CD11c^+/CD1c^-$) and plasmacytoid DCs ($CD123^{+/}CD303^+$). Flow cytometry plots (Fig 3.4A) show increased staining of the TSLPR fluorescence fluorochrome in SAR compared to NA patients. In addition, the bar chart (Fig 3.4B) clearly confirms the high percentage of TSLPR in all cell types in SAR patients compared to NA patients. In addition, the higher proportion of TSLPR in $CD11c^+/CD1c^-$ and $CD123^{+/}CD303^+$ cells compared to $CD11c^+/CD1c^+$ cells in DCs of SAR patients can be easily seen by analysing the bar chart (Fig 3.4B).

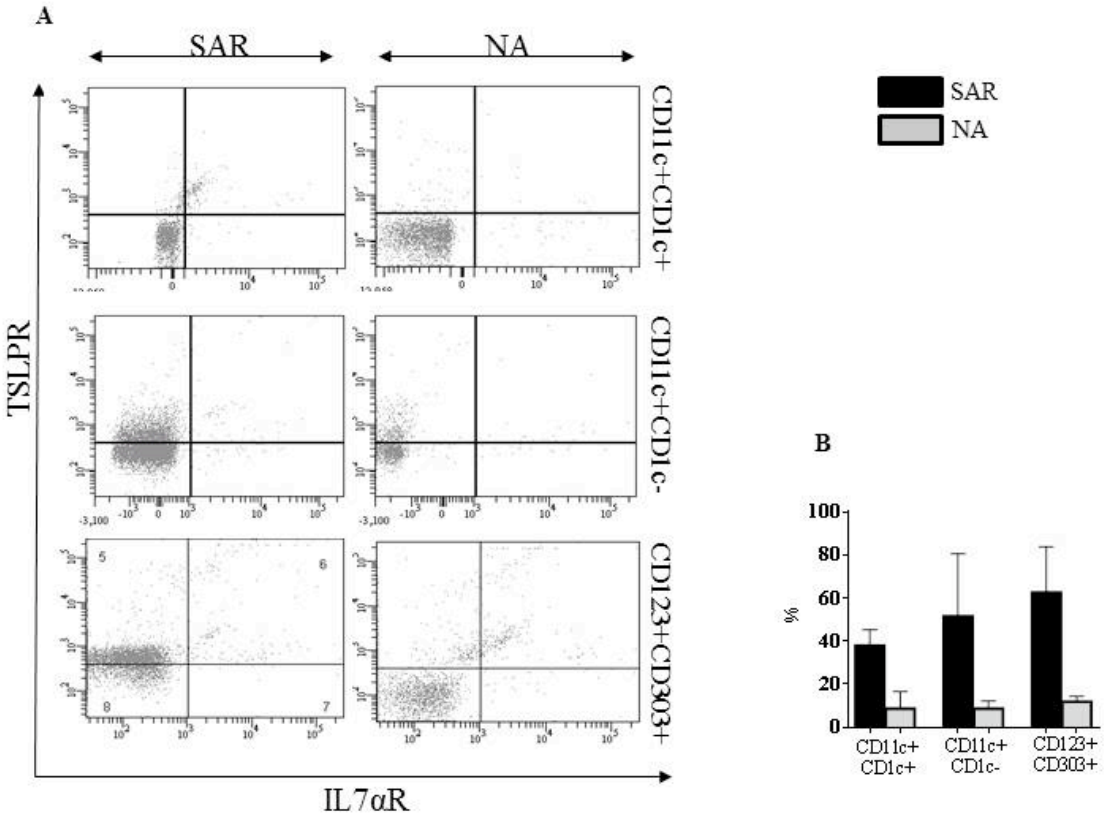

Figure 3.4. Effect of TSLP on TSLPR expression on mDCs and pDCs obtained from SAR and NA patients. Dendritic cells were primed with Phlp+TSLP TSLPR was assessed on Myeloid ($CD11c^+/CD1c^+$), (CD11c+/CD1c-) and plasmacytoid ($CD123^+/CD303^+$) DCs. A) Representative flow cytometry plots showing expression of TSLPR on CD11c+CD1c+, CD11c+CD1c- and CD123+CD303+ DCs. B) Proportion of TSLPR on CD11c+CD1c+, CD11c+CD1c- and CD123+CD303+ DCs obtained from SAR (n=3) and NA (n=3). Data are shown as mean+/-SEM.

3.6) IL-35 suppresses TSLP primed DCs driven naive T cell proliferation and induces Th2 response deviation

TSLP+Phlp primed DCs were co-cultured with n T cells with or without IL-35. Proliferation data (n=14) (Fig 3.5A) demonstrated the inhibitory effect of IL-35 on nT cell proliferation induced by TSLP primed DCs (p=0.0001).

Cytokine measurements (n=7) of Th2 related cytokines, IL-5 and IL-13, show a clear decrease in IL-35 treated nT cells (Fig 3.5B). Interestingly, IFN-γ also suffered a modest decrease in IL-35 treated cells (Fig 2.5C). However, cytokine suppression values of the three measurements are not statistically significant.

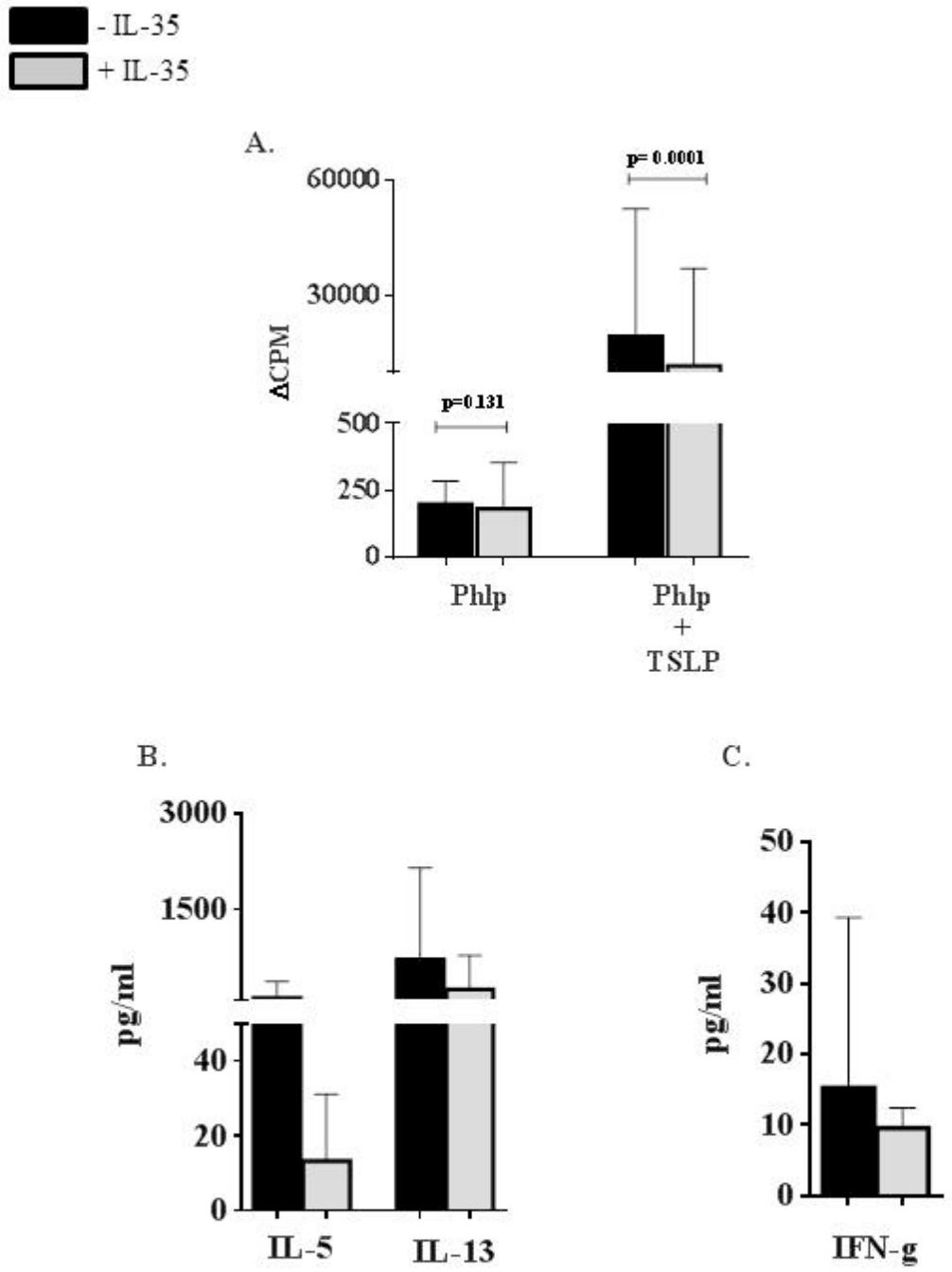

Figure 3.5. Effect of IL-35 on naive T cells exposed to Phlp and Phlp+TSLP primed DCs. After priming DCs for 24h with Phlp or Phlp+TSLP, these were cultured with naive T cells and treated with IL-35. A) proliferation assay and (B, C) cytokine analysis was measured . Data are shown as Mean +/- SEM. Suppression of nT cell proliferation induced by TSLP+Phlp primed DCs, was statistically significant after adding IL-35 (p=0.001).

3.7) Naive T cells fully polarised into Th1 cells after 2 weeks of treatment

Naive T cells received a follow up of 4 weeks, treated with Th1 polarising antibodies, in order to achieve total polarisation into Th1 cells. In addition, a line of non-polarised cells (NP) or, T helper 0 cells (Th0), cells was carried out in order to serve as a control. Intracellular staining demonstrated full polarisation of Th1 cells since these showed to express 0.3% of IL-13, 21.4% of IFN-γ, 0.5% of GATA-3 and 50.0% of T-bet. Non-polarised T cells showed the same value for IL-13 (0.3%), decreased IFN-γ (5.7%) and increased GATA-3 (7.1%) and T-bet (71.4%). Therefore, by comparison of Th1 cell with NP cells, it can be confirmed the total polarisartion of Th1 cells (Fig 3.6). At the end of week 3 Th1 cells start to dye.

3.8) Naive T cells fully polarised into Th2 cells after 3 weeks of treatment

Naive T cells also received a follow up of 4 weeks, treated with Th2 polarising antibodies, in order to achieve a total polarisation towards the Th2 phenotype. In addition, a line of Th0 cells was created to serve as a polarisation control. Th2 took longer than Th1 cells to get fully polarised, detecting a full polarisation on Wk3, by expressing 8.5% IL-13, 2.2% IFN-γ,79.1% GATA-3 and an unexpected 29.9 % T-bet. Comparing these results with Th0 cells, there is an increase of IL-13 and GATA-3 as NP cells show 0.3% and 5.4% respectively. In contrast, NP cells show higher values of GATA-3 (7.1%) and T-bet (71.4%). By comparing Th2 cells to the Th0, control cells, Th2 has found its polarisation at week 3 (Fig 3.6). Cells start dying at the end of week four.

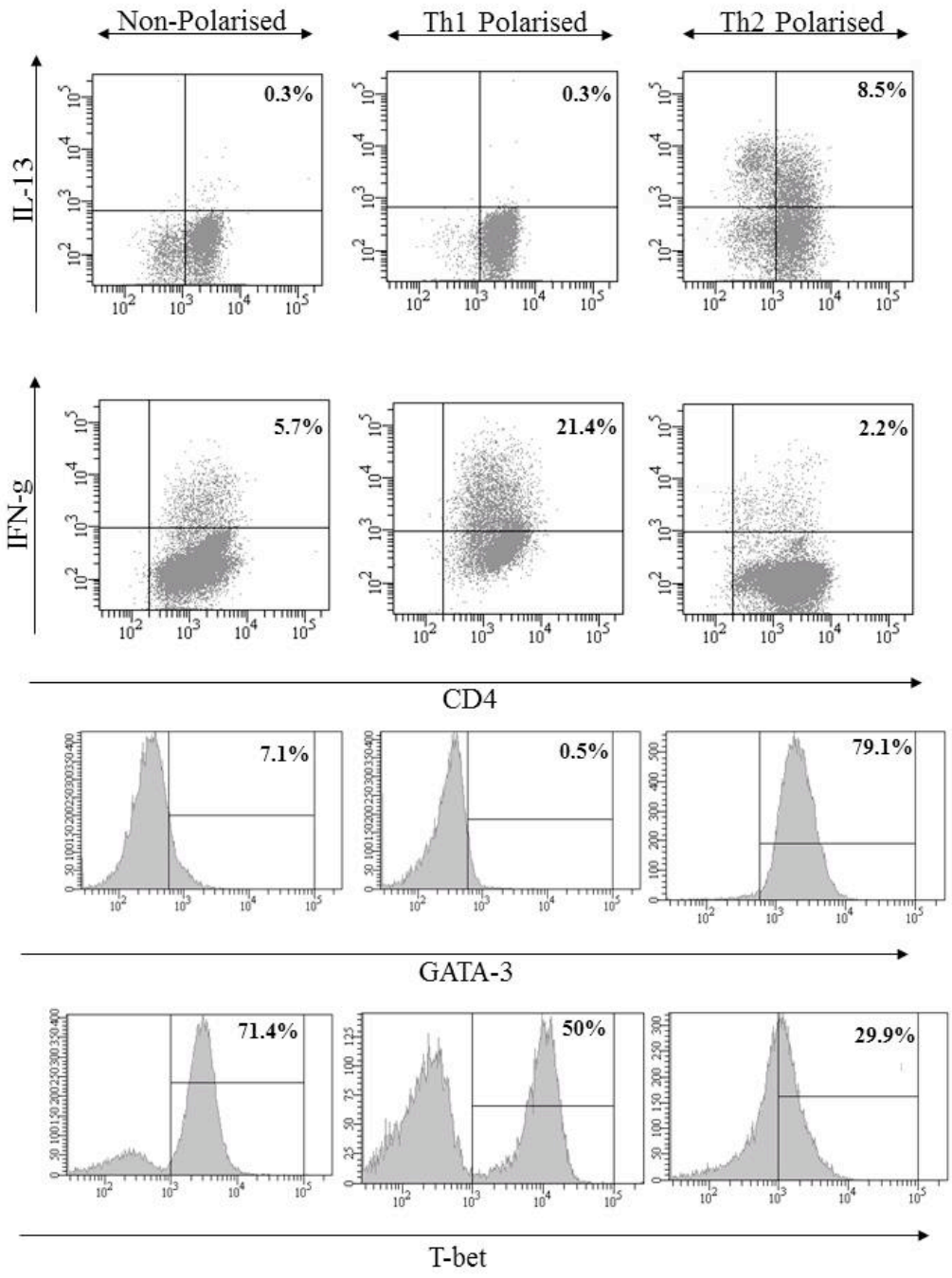

Figure 3.6. Surface, intracellular and nuclear staining of Th1 and Th2 markers, of fully polarised cells. Naive T cells received a follow up of 4 weeks, treated with polarising antibodies, in order to achieve total polarisation into Th1 or Th2 cells by the second and third week of treatment, respectively. The graph shows staining of Th1 and Th2 fully polarised T cells and of the non-polarised T cells

3.9) Polarisation of Th1 and Th2 cells were confirmed by Multiplex and q-PCR

Weekly supernatants were collected and the cytokine analysis of the supernatants in culture (Fig 3.7A) showed absence of IL-4, low amounts of IL-5 and IL-9 and high number of IL-9 cytokine in Th1 polarised cells. In contrast, Th2 T cells showed high levels of IL-4, increased IL-5 and IL-9 compared to Th0 and Th1 polarised T cells.

Together with the multiplex data, mRNA gene expression was also performed. Weekly cell lysates were collected from the three polarising cells. Gene expression (Fig 3.7B) of Th1 and Th2 polarised cells compared to Th0 T cells was carried out. Th1 polarised cells showed increased T-bet and high expression of IFN-γ compared to the control. In contrast, Th2 polarised cells exhibited an increased in the amount of IL-4 and GATA-3.

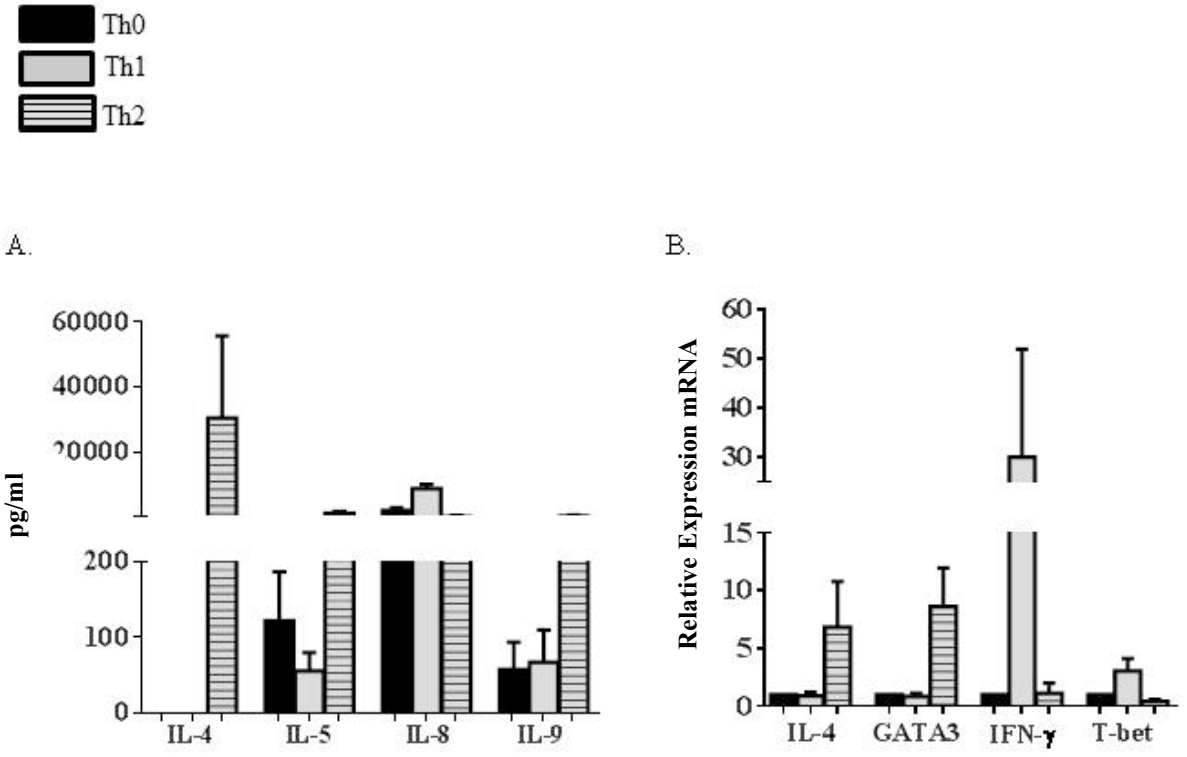

Figure 3.7. Cytokine and gene expression analysis of polarised T cells. Th1and Th2 polarised cell lysates and supernatants were collected. This graph represents data from week 2 for Th0 and Th1 cells and week 3 for Th2 cells. A) demonstrates cytokine analysis while B) shows the relative expression mRNA for Th1 and Th2 gene targets.

Chapter IV: Discussion

Due to the increased prevalence of allergic rhinitis world wild, SCIT was developed. However, reports have shown occurrences of patients with severe or rarer fatal events, due to this therapy. Therefore, the prescription of SCIT has been restricted and alternative methods, such as SLIT are being improved in order to offer a solution to patients that react badly to SCIT.

Inspired by the cases of unsuccessful SCIT treatment, this investigation has attempted to illustrate the important immunosuppressive properties of IL-35 iTregs and their characteristic cytokine IL-35 in grass pollen sublingual immunotherapy. This novel subset of T_{reg} cells, were first identified in humans in 2010[38].

Results conducted in this project identified in PBMCs of NA (n=12), SAR (n=12) and SLIT-treated (n=7) patients, a recovery in IL-$10^{+}CD4^{+}CD25+$ T_{reg} (Tr1) (Fig 3.1C) and IL-$35^{+}CD4^{+}CD25+$ T_{reg} (Fig 3.1D) in SLIT patients that received treatment for 2 years. These results were in accordance with a RA symptom score enquiry answered by these same SLIT patients, who scored an average reduction of 47% (p=0.0001) in their symptoms of allergic rhinitis compared to untreated SAR patients (Fig 3.1A). This revival in the population of Tr1 cells in SLIT–treated patients has been noted in several grass pollen SCIT studies[39], while also being alluded to in grass pollen SLIT findings, via an increase in IgG4 serum levels[40]. In contrast, on analysis of $Foxp3^{+}CD4^{+}$ T cells it was interesting to note that natural Foxp3+Tregs remained unchanged between SAR and SLIT-treated patients (Fig 3.1B). However, some aeroallergen immunotherapies have expressed an initial peak in Foxp3 following treatment, only to subside back to atopic levels after prolonged treatment.[41]

So far this data is as expected and is a reproducibility of results shown in previous studies, consequently validating the efficacy of the SLIT treatment under investigation.

Additionally, in order to discover the behaviour of IL-35 in different *in vitro* inflammatory reactions, an *in vitro* model of Th2 allergic inflammation was developed by simultaneously testing the role of epithelial-derived TSLP, IL-25 and IL-33 cytokines in SAR. Finally, Th1 and Th2 cells were polarised *in vitro,* from original nT isolated cells. This protocol was optimised over time in order to be used for further IL-35 analysis.

IL-25 and IL-33 together with TSLP are believed to be the initial power at site of allergen contact, for the induction of an allergic response. Following this understanding, the three cytokines were tested for their role on DCs. The proliferation assay concerning DCs primed with the epithelial derived cytokines (Fig 3.3A), demonstrated an impressive effect of TSLP primed DCs (in the presence of phlp) in inducing proliferation of nT cells ($p=0.003$). Interestingly, when priming DCs with TSLP, IL-25 and IL-33, their exposure to nT cells also showed a bust in nT cell proliferation ($p=0.0001$). However, the difference between the proliferation triggered by TSLP primed DCs and the three epithelial cytokines primed DCs does not show significance ($p=0.583$). Therefore, the proliferation achieved in the second experiment is believed to happen due to the presence of TSLP, but not IL-25 or IL-33, as these alone had no indirect effect on nT cells ($p=0.426$ and $p=0.502$; respectively).

In fact, while previous studies proved that TSLP acts on OX40 receptor located on the surface of DCs[13], IL-25 and IL-33 receptors have not been shown to have any effect on these innate cells. There is however, one report[42] suggesting the expression of the IL-33 receptor, ST2, on naive T cells only, therefore, explaining the failure of IL-33 primed DCs in triggering nT cell proliferation (Fig 3.3A). In addition, eventhough there is no significance between TSLP primed DCs and [TSLP+IL-25+IL-33] primed DCs, a previous experiment hypothesised the enhancement of TSLP effect in the presence of IL-25[19].

As the effect of TSLP on DCs indicated aggressiveness towards nT cells, resulting in its proliferation (Fig. 3.3A), cytokine analysis were measured in culture supernatants (Fig 3.3B and Fig 3.3C). Interestingly, TSLP primed DCs induced mainly IL-5 and IL-13 production by T cells, suggesting that these have suffered a deviation towards a Th2 like phenotype. Unexpectedly, naive T cells also showed an increase in the levels of IFN-γ, which still remains to be explained. According to mouse studies TSLP was shown to induce Th2 allergic immune responses. Thus, and most importantly, recognised as a potential target for AR immunotherapy.

As our results show a clear involvement of TSLP in Timothy grass AR, the next question was if either the expression of TSLPR varies on DCs of Timothy grass (TG) sensitised patients compared to healthy controls. There are different subtypes of DCs such as the myeloid ($CD11c^{+}$/$CD11c^{+}$) and plasmacytoid DCs ($CD123^{+/}CD303^{+}$). The role of these two subtypes is still unclear. Therefore, while staining for the TSLPR and IL-7α, plasmacytoid and myeloid DCs were also stained, in order to check the expression of these two chains in different DC cell subsets. However, the number of patients used were reduced (n=3 SAR and n=3 NA) the surface staining of TSLPR chain successfully showed an increased expression in SAR compared to NA patients (Fig 3.4). Interestingly, $CD11c^{+}CD11c^{-}$ DCs cells showed increased TSLPR compared to $CD11c^{+}$/$CD11c^{+}$ myeloid DCs. In addition, similarly to $CD11c^{+}CD11c^{-}$ DCs, increased TSLPR chain was seen in $CD123^{+/}CD303^{+}$ plasmacytoid DCs. Furthermore, it was previously published that if TSLP binds to both chains of its heterodimer receptor, TSLPR and IL-7αR, the downstream signal will be stronger than if it binds to TSLPR chain alone. Our results demonstrate much lower % of both chains than TSLPR alone (Fig 3.4A). Further experiments must be done to increase the number of patients, in order to prove this preliminary data as a true, statistically significant result.

Furthermore, it would be interesting to use immunohistochemistry to colocalise the two chains of the heterodimeric TSLP receptor. This could proof that the TSLPR and IL-7αR present are found together, as an heterodimeric receptor, rather than just spread individually throughout the surface of pDCs and mDCs.

Once the in vitro model of a Th2 response had been developed successfully, the IL-35 effect was analysed. In the presence of IL-35 (10ng/ml), T cell proliferation induced by TSLP primed DCs, showed a clear suppression by achieving a significant value of p=0.0001 (Fig 3.5 A). Following the results obtained for the suppression in proliferation, the cytokine phenotype secreted by nT cells, were looked at. Interestingly, not only the Th2 related cytokines (IL-5 and IL-13) were suppressed (Fig 3.5B) as the Th1 cytokine, IFN-γ suffered a modest reduction (Fig 3.5B), in the presence of IL-35. The question that arises is if either these changes are a result of reduced proliferation or deviation in response. Independently of the answer, both would be a result of the IL-35 treatment.

At last but not least, nT cells were polarised into Th1 and Th2 cells by a follow up of 4weeks. Weekly, intracellular staining by flow cytometry, cytokine analysis by multiplex ELISA and gene expression by q-PCR were performed in order to check for polarisation. In Fig 3.6, FACS plots represent Th1 at Week2 and Th2 cells at week 3. As obtained higher amounts of IFN-γ in Th1 than Th0 cells, shows indication of polarisation. In contrast eventhough there is more T-bet in Th0 than Th1, it doesn't rule out the opinion that these cells are fully polarised, as it can be observed a high number of dying cells in Th1 culture. Additionally, Th2 showed very cleary, increased in both IL-13 and GATA-3, leaving no doubt of it's polarising phenotype. We can now use Th1 cells at week 2 and Th2 cells at week 3, for further IL-35 testing.

Chapter VI: Future Studies

The characterisation of iTr35 cells is very recent; as a consequence little is understood with regards to its mechanisms of suppression and its clinical relevance towards allergy and immunotherapy. I believe that this project would benefit from the following assays and avenues of research concerning iTr35 cells:

- As an additional validation towards IL-35 as the primary suppressive mechanism of IL-35 in the Th2 model developed involving primed DCs and TSLP could be repeated in a transwell plate. Providing the pore size of the membrane between the wells is smaller than the width of a T cell (0.4μM in diameter would suffice), this assay could identify if the mode of iTr35 cell suppression is cell-cell mediated. So far only Collison *et al*., 2009 has implemented this assay to suggest that the suppressive capacity of iTr35 cells is only IL-35 and partially IL-10 dependent.
- Additional in vivo assays could be attempted involving transgenic mice with a conditional knockout genes coding for the subunits of IL-35. The mice could be exposed to a number of aeroallergens (grass pollen, birch pollen, dust mite) and their peripheral Th2 response quantified with and without the induction of the genes coding for EBi3 and IL-12p35. Furthermore, allergic mouse models could be treated with recombinant IL-35 intravenously and any impact on the Th2 response scrutinised.
- The impact of IL-35 on B cells is still poorly understood. B cells could be isolated from SAR patient PBMCS, stimulated with grass pollen allergen (Phl P) and cultured alongside incremental doses of IL-35. Any resulting Ig isotypes could then be identified with an enzyme-linked immunosorbent assay (ELISA), thus determining if IL-35 instigates B cell class switching. If an Ig isotype is recognised its quantity can

then be analysed in the serum of SLIT patients to gauge the impact of IL-35 on the treatment.

- As this project has demonstrated a recovery in peripheral quantity of iTr35 cells following grass pollen SLIT, it may be interesting to attempt similar findings in other allergen derived SLIT treatments, such as birch pollen and house dust mite.
- It would be useful to repeat the T cell polarisation to check the behaviour of IL-35 on these cells. Other cell type such as Th17 could also be polarised and IL-35 effect tested.
- Finally, not forgetting the pro-inflammatory effect of TSLP, I would increase the number of peitents to check the TSLPR on the surface of different DC subtypes not only in NA, SAR bur also in SLIT patients. I belive that te produced preliminary data suggests that TSLPR can potentially become an immunotherapy target.

Chapter VII: Conclusion

This project has been the first to identify a clear recovery in the quantity of iTr35 cells within the peripheral blood of SAR patients treated with SLIT. In addition, IL-35 was shown to have a suppressor role in allergen-driven Teff cells and TSLP-primed DC induced Th2 responses. Cell lines Th1 and Th2 polarised T cells were optimised for further analysis of IL-35.

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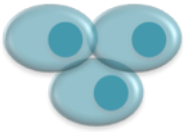
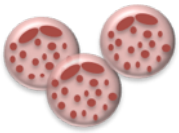
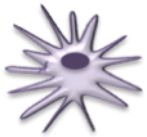
Attachments

Glossary

Anaphylactic shock	It is a sudden, life-threatening allergic reaction, defined by systemic vasodilatation and consequent sharp drop in blood pressure and bronchial spasm with shortness of breath. Therefore, tissues and organs receive inadequate blood flow and consequently decreased oxygen supply and deposits of waste products. Thus, possibly resulting in circulatory collapse and death within minutes
Atopy	Atopy refers to the inherited tendency for T cell deviation towards a Th2 immune response and the hyperproduction of IgE antibodies to common environmental allergens. 80% of the atopic individuals have a family background of allergy.
Hygiene Hypothesis	Hygiene Hypothesis argues that the lack of microbial burden, such as exposure to bacteria and parasites, as a child, increases the susceptibility to develop allergic diseases, by deviating their lymphocytes towards a Th2 rather than a Th1 cytokine profile.

Key to illustrations in the introduction

	Airway Epithelium		Naïve $CD4^+$ Tcells
	Allergen		Natural killer cells
	Antibodies		Specific allergen antibody
	Basophiles		T Follicular Helper Cells
	B cells		T helper 0 cells
	Plasma cells secreting antibodies		T helper 1
	Eosinophils		T helper 1 like T regulatory 1 cells
	Immunogenic Dendritic cells		T helper 2
	Interleukin 4 competent Tcells		T helper 2 and T helper17 cells
	IL-25R Natural Helper Cells		Type 1 regulatory Tcells
	Macrophages		Tolerogenic Dendritic cells
	Mast Cells		

Relevant Courses

- Statistical analysis course
- Radiation course

Awards

- Travel Grant from the European academy of allergy and Clinical immunology (EAACI).
- Spot Award by the Department of allergy and clinical Immunology – Imperial College London.

Publications

- Abstract submission for EAACI winter school , Austria 2013: Oral Presentation