The temporal requirement of IL-4Rα signalling in allergic asthma and the role of IL-4Rα-responsive Regulatory T cells in restraining allergic airway inflammation.

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DECLARATION

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Jermaine Khumalo

November 2019
DEDICATION

This thesis is dedicated to my family; my father Qedindaba Khumalo, my beloved mother Nontokozo Khumalo, my sisters Kwandokuhle Khumalo, Simakuhle Khumalo and grandmother Adelaide Khumalo

For your unfailing support!
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Abstract

Allergic asthma is a chronic inflammatory airway disease driven predominantly by a T_{H2} immune response to environmental allergens. The asthma pathology is predominantly elicited by IL-4 and IL-13 signalling via IL-4Rα-signalling which is essential for driving T_{H2}-type immunity to allergens. Interestingly, the failure by regulatory T cells to maintain tolerance during allergic asthma, suggested to be driven by T_{H2} inflammatory signals, still remains elusive and anti-T_{H2} therapies with the potential to effectively reduce airway obstruction and inflammation in allergic asthma, have had limited success. Therefore, we aimed to investigate the function of IL-4/IL-13 responsive regulatory T cells in a T_{H2} rich environment and the temporal requirement of IL-4Rα-signalling in asymptomatic and acute airway disease.

Objective 1: We investigated potential therapeutic effects of selective inhibition of this pathway in mice with established allergic airway disease and systemically sensitised mice to prevent the onset of the disease. We used Rosa^{creERT2}IL-4Rα^{lox} mice, a novel, tamoxifen inducible IL-4Rα knockdown model to investigate the role of IL-4/IL-13 signalling during the effector phase of ovalbumin induced allergic airway disease (AAD) and for the onset of the disease. The deletion of the IL-4Rα had a therapeutic effect on established AAD and prevented the development of ovalbumin induced airway hyperreactivity, goblet cell metaplasia and eosinophilia in allergen-sensitised mice. We concluded that the abrogation of IL-4Rα signalling after allergic sensitisation would have significant therapeutic benefit for T_{H2} type allergic asthma.

Objective 2: The canonical IL-4Rα-signalling, was investigated on its role on Foxp3+ Tregs in allergic asthma with aims to re-establish tolerance during allergic asthma. We used transgenic Foxp3^{cre} IL-4Rα^{lox} mice IL-4Rα^{lox} mice to investigate the role of IL-4/IL-13 signalling during the induction or maintenance of tolerance in house dust mite-induced ADD. The depletion of IL-4Rα on Foxp3+ Tregs exacerbated airway hyperreactivity and airway inflammation in allergen-
sensitised mice. Interestingly, a reduced induction of Foxp3+ Tregs in peripheral tissue and an accompanying increased IL-33 induced ILC2 driven inflammation in the lung responsible for the exacerbation of TH2 acute disease. Conclusively, the IL-4Rα responsive Foxp3+ T regulatory cells are key in maintaining tolerance in type 2 innate immune driven allergic asthma, therefore the TH2 environment has both an innate immune specific regulative role in local lung tissue and induction of Foxp3+ Tregs in peripheral tissue during AAD.

A combined targeting of the pathogenic TH2 environment in anti-TH2 therapy and the augmentation of regulatory T cell function in the local lung tissue is necessary to inhibit both adaptive and innate drivers of TH2 inflammation in allergic disease.
Chapter 1

Introduction

1. Allergy and Asthma overview

1.1. The epidemiology of allergy and asthma

Asthma is a heterogeneous chronic inflammatory disorder associated with variable reversible airflow obstruction and bronchial hyperresponsiveness\(^1\). It commonly presents with recurrent episodes of wheezing, breathlessness, coughing, chest tightness and progressive decrease in lung function, classified as exacerbations\(^2\). These are recognised as progressively worsening acute or subacute episodes\(^3\). The intensity of exacerbations can vary from mild to very severe and even life threatening, however not necessarily correlating with disease severity\(^2,4,5\). The decrease in the expiratory airflow can be assessed by measurement of lung function using a spirometry or Peak expiratory flow (PEF)\(^2\).

Asthma is a complex disease syndrome with various presenting characteristic phenotypes namely; early onset allergic asthma, late onset eosinophilic asthma, neutrophilic asthma, obesity related and exercise induced asthma\(^6\). Among the many phenotypes, allergic asthma is mainly associated with substantial burden of disease. There is an increase in asthma and allergic disease worldwide, and this poses a global health burden across the social demographic spectrum especially in children, with a high mortality and morbidity\(^2,7\). A reported estimate of 339 million people are asthmatic worldwide and this has increased from a global burden of disease of 288 million in 2006\(^8\). This rather concerning increase in prevalence is mainly in the low and middle income countries (LMICs)\(^9\) and account for 80% of asthma-related deaths worldwide across all age groups\(^10\).

This is a significant chronic respiratory disease recognised as the most common non-communicable disease in children worldwide with a limited epidemiology. Childhood asthma prevalence is estimated at 14%, however varying widely between countries. The highest
prevalence of (>20%) is documented to be in Latin America and English-speaking countries of Australasia, Europe, North America and even South Africa. In Africa, 10-20% prevalence was observed while a lower prevalence of <5% is seen in Asia-Pacific, Eastern Mediterranean, and Northern and Eastern Europe. The difficulty in clinically distinguishing asthma from other overlapping respiratory conditions in older age groups limits standardised data survey. Strategies to reduce the burden by improved diagnosis, treatment and access to healthcare are therefore needed.

1.2. Environmental and genetic determinants

There are varied patterns of inflammation owing to common comorbidities, different treatment responses, severity and natural history predispositions thus indicating phenotypic differences that are able to influence treatment responses. Paramount to the complexity of allergic diseases are influences by genes and environmental interactions which significantly shape the development of asthma. Certain genetic loci and candidate genes have been associated with asthma phenotype like serum IgE levels, atopy and bronchial hyperreactivity. A few described positional candidate genes for instance, the Gly16 and the Glu27 polymorphism of the β2-adrenegic receptor appear to be overrepresented in severe asthma cases or are associated with less airway hyperresponsiveness (AHR), respectively. Polymorphisms of TNF-α, glutathione-S-transferase gene, NOS1 variants, and the IL-4 receptor α subunit chain also prove to be disease modifiers strongly associated with asthma susceptibility and its severity, bronchial hyperreactivity or treatment responsiveness.

Allergen exposure has been arguably misrecognised as the primary cause of asthma. This flawed classification assumes that the increasing worldwide incidence is directly proportional to the increase in aeroallergen exposure. However, epidemiological evidence from population studies is equivocal and does not indicate that allergen exposure is associated with the prevalence of asthma at the population level or a major risk factor for the primary causation of asthma in children. Non-allergen sources are recognised plausible causes of asthma attacks in the absence of an allergic response. Moreover, with an observed increased prevalence in
developed countries, the environmental cues are important drivers of aetiology and pathogenesis of allergic asthma\(^\text{12}\). These include smoking behaviour\(^\text{27}\), air pollution\(^\text{28}\), viral exposure\(^\text{29}\), occupational exposure\(^\text{30}\), even immunisation status\(^\text{31}\). Additionally, demographic factors, such as age, race and socioeconomic status appear to be risk factors for the development and progression of asthma\(^\text{7}\). Various proinflammatory mediators, released by both inflammatory and airway structural cells contribute to shaping the different asthma phenotypes and observed clinical heterogeneity\(^\text{32,33}\).

### 1.3. The natural evolution of asthma

Atopy is a major predisposing risk factor of developing asthma\(^\text{34}\). Besides atopy, the asthma phenotype is thought to begin earlier on in life, even before birth. Lavage studies of 3 year old children reveal increased mast cells and eosinophils associated with recurrent wheezing illness\(^\text{35}\). Virus induced wheezing becomes evident with persisting neutrophils in children by 5 years of age. At birth the peripheral blood mononuclear cell responses to specific stimulation in children who develop atopic disease is deficient in its capacity to generate interferon-\(\gamma\) (IFN-\(\gamma\)), thereby causing upregulation of cytokines associated with a Th2 response and the allergic phenotype\(^\text{36,37}\). This could be speculated to be a result of impaired regulation of IFN-\(\gamma\) production and its expression\(^\text{38}\). This “Th2” skewed immune system is suggested to be a result of fetal exposure of low amounts of allergen as early as 22-24 weeks of pregnancy from atopic mothers\(^\text{39}\). Other influencing factors include early exposure to microbes, cesarean delivery, breastfeeding, use of probiotics to name a few\(^\text{40}\). The Amnion and the placenta’s immune environment produce IL-4 and IL-10 cytokines further contributing to the Th2 fetal programming\(^\text{41}\). The first 3 years of life is particularly critical as the environmental events may influence the development of the asthma phenotype. Lung is incompletely developed at birth and completes its post-natal development in the first 3 years of life\(^\text{42,43}\). This post-natal development is largely shaped by encountered external environment and means that the lung remains vulnerable to pathogen colonization during these first 3 years of life\(^\text{44}\). A protective anti-Th2 response has been associated with possible microbial infections (measles/respiratory viruses/bacteria) thus providing evidence of the hygiene hypothesis which recognises early microbial interaction to be a limiting risk factor to
allergic disease\textsuperscript{45–47}. The immunological mechanism involved in the protective response to early frequent infancy infections includes an increased IL-12, IL-18 and IFN-\(\gamma\) common regulators of the prevailing T\(_H\)2 response thus reducing the susceptibility to sensitisation\textsuperscript{48}. Microbiome research has revealed that early infant asymptomatic nasopharyngeal carriage of Streptococcus, Haemophilus, and Moraxella species (typical airway bacteria) has been associated with increased risk of atopy, wheezing and later asthma symptoms\textsuperscript{49,50}. This association was specifically at 1 month and by the 2-5 year of life, the risk rates were maintained. Crosstalk between mucosal surfaces of the gastrointestinal tract (GIT) and airway pathways suggests the possible involvement of the gut microbiome in risk of allergy development\textsuperscript{51}. Five GIT bacterial genera (\textit{Veillonella}, \textit{Lachnospira}, \textit{Rothia}, \textit{Faecalibacterium}, and \textit{Bifidobacterium}) were associated with a lower incidence of atopy and wheeze in infants and therefore presumed to be associated with protection from asthma development. However, the underlying mechanism are still unknown, but mere association shows detrimental in risk of allergy at early infancy. However, at the age of 5 years atopy usually diminishes with the acquisition of antigen-specific and non-specific tolerance leading to the consolidation of a T\(_H\)1 rather than a T\(_H\)2 response\textsuperscript{52}. Interventions to reduce the risk of developing asthma early on in life include; prolonging breast-feeding, delaying weaning in infants, and avoidance of food allergens until 12 months of age in those at increased risk for asthma\textsuperscript{53,54}.

In established asthma the situation is more complex as evidenced by mixed T\(_H\) profiles superimposed upon a T\(_H\)2 response or non- T\(_H\)2 disease profiles in chronic and severe disease cases\textsuperscript{55,56}. Co-morbidities such as viral or bacterial induced exacerbations are seen to augment the eosinophil mediated inflammation causing a chronic inflammatory response as the developed immune response prolongs inflammatory cell survival\textsuperscript{34,38}. Increased chronicity leads to airway remodelling. Figure 1 summarises the above described natural maturation of asthma in humans. However, asthma is not naturally observed in mice, but with systematic sensitisation to purified allergens, similar symptoms during the disease progression are observed although not a perfect model, hence the differences should be considered if assessing results for translation to humans\textsuperscript{57,58}. 
The evolution of asthma (adopted from Holgate, 1997)

There is the confounding lack of a universally agreed definition, a differential diagnostic test as infectious or chronic conditions such as COPD have similar symptoms and often symptoms go into remission over time with absence of exposure to the offending allergen only to reappear upon re-exposure. Patient-related behaviours such as forfeiting treatment and delayed or underreporting of symptoms only when patients experience an asthmatic attack are various challenges that diagnosis and management of disease face. Unlike the experimental mouse model which recognise defined $T_H$ profiles during allergic disease, this is not similarly the case in humans as there is great heterogeneity in the phenotypes of asthmatic patients. However, improvements in the murine models has seen the introduction of mixed $T_H$ profiles induced in experiment allergic asthma to mimic the observed phenotypes in human patients.

1.4. Allergic Asthma

It is a detrimental abnormal immune-mediated response mainly directed against innocuous environmental substances (allergens), including non-infectious components of certain infectious organisms. These responses in allergic asthma are characterised by the involvement of allergen specific IgE and type 2 helper ($T_H2$) cells, even in other allergic disorders namely; anaphylaxis, allergic rhinitis and some food allergies. A predisposition to an excessive allergen specific IgE response is defined as atopy, evidence of allergic sensitisation to allergens commonly detected
by a positive skin prick test (SPT) or the presence of specific serum IgE. There is a strong association between atopic individuals and allergic disease, however not all atopic patients develop allergic disease clinical symptoms and not all diseased individuals are atopic to a range of allergens.

1.4.1. The development of allergic inflammation
1.4.1.1. Sensitisation to allergens in the airway

Sensitisation to an allergen involves ability to elicit a T<sub>H2</sub>-cell response that promotes immunoglobulin class-switch recombination in B cells driving IgE production. This initial response is key in developing allergic inflammation. The likelihood of developing clinically significant sensitisation is determined by various factors such as the host genotype, allergen type, route of allergen exposure, characteristic antigen presenting cell (APC) (myeloid and/or plasmacytoid), amount of allergen and co-exposure with environmental adjuvants (e.g. chitin, endotoxins). Determinants that influence the permeability of the epithelium barrier not only increase the likelihood of sensitisation to allergens encountered in the airway but also favour development of a subsequent T<sub>H2</sub>-cell response. The developed systemic response results in allergic disease. Some protease allergen including the major house-dust-mite allergen, Der p1, directly reduce epithelial barrier function thus increasing allergen exposure to APCs and also inducing the production of epithelial cell-derived cytokines to drive airway inflammation.

1.4.1.2. Atopy

It is difficult to ascertain when sensitisation takes place in an individual as it can be manifested in various routes and barrier surfaces, depending on a sensitising allergen. Sensitisation through inhalation involves APCs, mainly dendritic cells sampling allergen in the airway lumen across a disrupted epithelium. Some allergens cleave epithelia-cell tight junctions with their protease activity thus gaining access to the APCs. The allergen primed dendritic cells become activated and migrate to secondary lymphoid tissue draining the lung (mediastinal lymph nodes) to present processed allergen to naïve T cells. With help from ‘early interleukin 4’ produced by T<sub>fH</sub> helper cells, naïve T cells differentiate into T<sub>H2</sub> cells producing T<sub>H2</sub> pro-inflammatory cytokines namely;
IL-4 and IL-13. Some argue that basophils, DCs or ILC2 are earlier sources of IL-4. However, the established rich Th2 environment and ligation of co-stimulatory molecules between B cells and APCs in the draining lymph nodes, activate B cells to undergo immunoglobulin class-switch recombination, and expand into allergen-specific IgE antibody producing plasma cells and memory B cells. The secreted IgE diffuses locally and enters the vascular system for systemic distribution. The trafficked allergen-specific IgE antibodies bind to high affinity receptor (FcεRI) on tissue-resident mast cells, basophils and eosinophils thus sensitising them for response upon re-exposure to the allergen. This high IgE predisposition upon allergen exposure is sensitisation and there are usually no accompanying visible disease symptoms in both humans and mice. This maturation of the immune system commonly resembles immune responses to parasitic infections, however in this case it recognises innocuous substances.

1.4.1.3. Acute allergic inflammation

Upon successful allergen sensitisation, re-exposure to the same allergen leads to allergic airway inflammation. Single re-exposure to the allergen leads to acute inflammation. This is described into temporal phases namely; Early phase and late phase inflammation. Initially, an early phase reaction involves IgE-mediated type 1 immediate hypersensitivity shortly after allergen exposure, localised (urticarial) or systemic (anaphylaxis). In sensitised individuals, the allergen specific IgE bound to FcεRI on mast cells and basophils crosslinks with the allergen thus resulting in subsequent degranulation and release of histamine, leukotrienes, chemokines, and neutral proteases (chymase and tryptase). These released preformed and lipid derived mediators lead to vasodilation, increase vascular permeability with oedema, and bronchoconstriction, key in promoting the local recruitment and activation of leukocytes to the site of inflammation. The initial early phase contributes to the development of late phase reactions after 2-6hrs, peaking at 6-9hrs after allergen challenge. Chemokines secreted by degranulation of mast cells, basophils and eosinophils; CXC- chemokine ligand 8 (CXCL8) and CC-chemokine ligands (i.e. CCL2, CCL3, CCL11) locally recruit activated allergen specific Th2 cells, eosinophils, basophils and other leukocytes to the lung. The activated Th2 cells secrete cytokines namely; IL-4, IL-13, IL-5 and IL-
9 which elicit mucus hypersecretion, bronchoconstriction and eosinophil recruitment\textsuperscript{62}. This is commonly described as the effector phase of allergic airway inflammatory reaction.

1.4.1.4. Chronic allergic inflammation

Repeated allergen exposure induces persistent airway inflammation, typically characterised by the presence of increased numbers of innate and adaptive immune cells mainly at the site of inflammation, and substantial changes in the extracellular matrix and alterations in the number, phenotype and function of structural cells, classified as airway remodelling\textsuperscript{13,62}. Repeated epithelial injury from the allergen maintains secretion of alarmins; IL-33, thymic stromal lymphopoietin (TSLP), IL-25 which exacerbate the innate immune response to stimulate IL-13 and IL-5 secretion by innate lymphoid type II cells (ILC2)\textsuperscript{73}. This persistent Th2 cytokine onslaught from both adaptive allergen specific Th2 cells and innate immune cells effect airway remodelling. This includes goblet hyperplasia, metaplasia and increases in epithelial mucin. Subepithelial fibrosis increases from continued deposition of collagen I, III and V in lamina reticularis of the basement membrane zone of the submucosa\textsuperscript{62,74}. Bronchiole smooth muscle (BSM) thickening is characterised by increased deposition of extracellular matrix (ECM) proteins with significant cellular hypertrophy and hyperplasia\textsuperscript{75}. The accumulated pathological changes in the airway, predispose individuals to longer term chronic airway inflammation.
Figure 1: The development of the type 2 airway inflammatory response in allergic asthma. Initial activation is from the allergen stimuli. Antigen presenting cells harvest and process allergen, become activated and present to naïve T cells to differentiate to pathogenic Th2 inflammatory cells secreting cytokines and also induce antigen specific antibody producing B cells responsible for the asthma pathology.

1.4. Tolerance in allergic disease

1.4.1. Immunological tolerance

Tolerance to allergens involves a complex regulatory system involving multiple cell types and molecules (Figure 2). The immune interactions that make up this regulatory response are dependent on various constituents such as resident location of the antigen encounter, the type
and dose of antigen, the presence of adjuvants and immune status of the host. Mucosal surfaces are optimized for interacting with the environment thus rendering them vulnerable to invading pathogens. A protective response is therefore necessary to ensure a maintained state of unresponsiveness “immunological tolerance” against harmless environmental substances (food, commensal microbiota and even inhaled particles, such as plant pollen). Failure in maintaining tolerance results in immune pathology, such as allergic disease. Central to the maintenance of the immunological balance are effector CD4+ T helper which keep potentially pathogenic microbes at bay and various types of regulatory cells being described which predominantly suppress unwanted inflammatory response.

Allergic asthma is a highly specific, IgE-mediated hypersensitivity with allergen-specific IgE as the central mediator of the allergic response. Allergen-specific T\textsubscript{H}2 cells help orchestrate the formation of IgE-secreting plasma hence the loss of tolerance requires the uptake of the allergen by antigen presenting cells (APCs), activation and formation of allergen specific T\textsubscript{H}2 cells as the initial event underlying allergy development. Allergy in even highly atopic patients mainly develops against a limited number of “allergenic proteins” over several years of exposure, while tolerance is maintained against the majority of proteins constituting inhaled particles. Therefore, tolerance is a highly efficient process in the event of antigen-specific loss of T-cell tolerance and evasion is more a rare event even in susceptible donors.

The basic mechanisms mediating tolerance still remain elusive. What confounds this field is the technical problem of antigen-specific T cells being rare and therefore their direct identification and characterization is challenging leading to the assessment of their functional role in tolerance to be derived from mainly experimental animal models, although they do often not recapitulate critical aspects of natural allergen exposure in humans. In humans, the results are highly variable and largely influenced by the applied analysis technique. Furthermore, focus in both human and animal models is much on the few IgE-binding allergenic proteins, although additional proteins may be recognized by T\textsubscript{H}2 cells, independently of IgE. Importantly, the non-allergenic proteins (represent actually the vast majority of inhaled proteins) derived from inhaled particles
are largely ignored, although they may in fact be important targets for regulatory cells in the humans\textsuperscript{79,85}.

1.5. Mechanisms of allergic tolerance

Various non mutually exclusive mechanisms have been described to induce tolerance to exogenous, non-self-antigens. However, these mechanisms are still intrinsically at risk to the remaining or newly generated naive T cells, which are still susceptible to develop into allergenic Th2 cells\textsuperscript{80}. The induction of T helper cells with an immunosuppressive function is the most efficient way to generate a state of tolerance, by suppression of Th2 effector functions. However numerous types of suppressor/regulatory cells have been described, which significantly differ with regard to their origin, inducing conditions, long-term survival, lineage stability, and effector functions\textsuperscript{80}.

1.5.1. T regulatory cells

T regulatory cells (Tregs) are essential for maintaining local tissue homeostasis, mucosal tolerance, and are generated as a separate T-cell lineage in the thymus (tTregs) with a lineage-defined transcription factor, Foxp3\textsuperscript{86,87}. They may be also generated in the periphery from naive conventional T cells (pTreg)\textsuperscript{88,89}. The best characterized subsets comprise forkhead box P3 (FoxP3)\textsuperscript{*} CD25\textsuperscript{*} Treg cells and various T-cell subsets producing the immunosuppressive cytokine IL-10 and (Tumor Growth Factor) TGF-β\textsuperscript{90,91}. They have a broad TCR repertoire and show a distinct, stably imprinted epigenetic landscape for essential Treg genes\textsuperscript{90}. In humans, it is not clear whether natural Treg cells can become IL-10-expressing Treg cells. In animal models, Foxp3+ Treg cells can differentiate outside the thymus upon antigen recognition, and these cells can control allergic inflammation\textsuperscript{92–95,96}. Differentiation between these forms of tolerance and even relative contribution of tTregs and pTregs to airway tolerance is technically challenging, especially in humans due to a lack of appropriate markers to distinguish subsets contributing to the antigenspecific T-cell compartment\textsuperscript{76,81,82}. Besides, Human Treg cells are highly heterogeneous and difficult to identify. Generally, Treg cells suppress a variety of effector T-cell subtypes and antibody driven responses involved in the pathogenesis of allergic disease\textsuperscript{76,97}. In animal models,
Treg cells also have been shown to suppress dendritic cells, mast cells, eosinophils and basophils\textsuperscript{98-100}, however it is not clear if this is a similar case in humans. Instead, it is suggested to be mediated indirectly through suppression of T\(_H\)2 cytokines\textsuperscript{76}. Evidence revealing the specific antigen induction of effector T cells and Treg responses, and regulation of the effector T cell/Treg ratio regulated on the antigen-specific level in health and disease is still unravelling\textsuperscript{101}. Recently Tregs have been shown to directly inhibit activation of Type-2 innate lymphoid cell (IC2s), a pivotal source of T\(_H\)2 cytokines in allergic disease, via IL-10 secretion and ICOS-dependant contact\textsuperscript{102,103}.

Although the most widely studied regulatory T cells are those with FOXP3, there are also populations of Tregs that do not express FOXP3. These include three main kinds of T cells: Tr1 cells, a population activated in the periphery after antigenic stimulation in the presence of IL-10 and which express the surface markers LAG-3 (lymphocyte-activation gene 3) and CD49b in the face of absent FOXP3 and CD25 expression; Th3 cells, which are also differentiated in the periphery and these Tregs mediate the cell suppression by secreting the cytokine TGF-\(\beta\); and finally, CD8\(^+\) Tregs, described as antigen-specific memory T cells with Treg properties, which may regulate immune responsiveness by production of IL-10, TGF-\(\beta\), and IFN-\(\gamma\) though the exact mechanisms underlying this suppression are still largely unknown\textsuperscript{80,104}. 
Figure 2: Regulatory T cells mediate maintenance of immune immune homeostasis in various modes of action. They are able to suppress the inflammatory dendritic cells (DCs), inhibit the activation of effector T cells (T_{H1}, T_{H2}, T_{H22}, and T_{H17}) and type 2 innate lymphoid cells (ILC-2), block the secretion of inflammatory antibodies by antigen specific B cells, and inhibit the activation of basophils, mast cells, and eosinophils.

1.5.2. IL-10 secreting T (Tr1) cells

Interleukin (IL)-10-producing T cells originate from naive conventional T cells and thus belong to the group of peripherally induced suppressive T cells. However, it is still not clear if Tr1 cells represent a separate and stable T-cell lineage similar to Foxp3$^+$ Tregs or effector T-cell lineages$^{105,106}$. The secreted IL-10 is a major immunosuppressive cytokine especially relevant for immune homeostasis at mucosal surfaces and can be produced by basically any T-cell subset, as well as FoxP3$^+$ Tregs$^{107}$. This ability to produce IL-10 is not stably imprinted but seemingly is regulated on demand by external signals such as tissue-derived signals for Foxp3$^+$ Tregs, strong TCR signals and persistance of antigen$^{108–111}$. This rather reflects a dynamic, self-limiting state of
inflammatory response and Treg differentiation. Although IL-10 has been described in many experimental systems to downregulate Th2 responses and allergies, the association between genetic variants of the IL-10 or IL-10 receptor genes and allergic diseases or asthma are not as evident as for Foxp3. Indeed, patients with IL-10 or IL-10 receptor deficiency suffer from severe IBD but not overt allergies suggesting that IL-10 is not critical in the induction and maintenance of global tolerance against airborne antigens in humans. Of all the molecules implicated in T-cell suppression associated with allergen tolerance, IL-10 remains the most robust.

1.5.3. IL-10 secreting B (B10) cells

Mouse B cells are capable of secreting IL-10, and this subset of cells are termed B10 cells, increasingly displayed in models of diabetes and autoimmune disease. These IL-10 producing B cells maintain tolerance by suppressing allergic inflammation via induction of pulmonary Treg cells. In humans, regulatory B cells have also been implicated in the development of tolerance to food allergens. Additionally, IL-10 has been shown to selectively enhance protective immunoglobulin G type 4 (IgG4) production by human B cells, and IL-10-producing Treg cells can induce IgG4 through a variety of molecules including IL-10 and TGF-β. Expanding analysis of the IL-10-expressing cell compartment could provide new insights into IL-10 pathways that underlie allergen tolerance.

1.5.4. TGF-β-expressing T and B cells

Other regulatory cell types that have been implicated in allergen tolerance include TGF-β-expressing T and B cells. Several studies suggest an association between food allergies and a deficiency in these regulatory cells. A selective decrease in the number of TGF-β-producing T cells, but not other cytokine-expressing T cells, was reported in duodenal biopsies from children with food allergies. In other work, decreased allergen-responsive B cells expressing TGF-β (Br3) were observed in milk allergic children. The actions of TGF-β are multifold. In murine systems, TGF-β suppresses Th2 differentiation from naïve T cells, inhibits T-cell proliferation, and induces Foxp3+ Treg cells.
Protective antibodies have been observed upon successful immunotherapy thus giving rise to IgG and IgG4 antibodies as tolerance inducers. IgG4 antibodies bind Fc-γ receptors with low affinity, do not fix complement and inhibit IgG1-mediated complement activation. They have been described to decrease IgE receptor-facilitated allergen binding to B cells and also diminish activation of mast cells and basophils with this functional response correlating with clinical response. Long-term clinical tolerance may be maintained, at least in part, by the persistence of a subset of allergenspecific IgG antibodies with potent inhibitory activity.

Glycosylated (protein structures with added carbohydrate structures) antibodies have been shown to have a role in tolerance induction. The glycosylation patterns associated on the antibodies determine the pro versus anti-inflammatory functions of IgG antibodies. These antibodies bind to the C-type lectin receptors present on myeloid cells inhibiting Th1-driven inflammatory processes through enhanced induction of the inhibitory Fc receptor, FcγRIIB, on macrophages. Mice immunized with OVA under tolerogenic conditions have been shown to produce sialylated antigen-specific IgG antibodies shown to inhibit DC maturation in vitro and protect against allergic inflammation OVA-induced. In humans, sialylated allergen-specific IgG antibodies have been identified in the serum of patients receiving immunotherapy (IT) for birch pollen allergy thus suggesting antibody sialylation to be an alternate regulatory pathway in allergenic tolerance.
Figure 3: Regulatory networks and the allergic response. Multiple pathways operate to suppress Th2-driven allergic inflammation in allergen tolerance. The coloured lines denote the many afferent and efferent arms of the same pathway. Circles denote inhibitory pathways.

1.6. Challenge of diagnosing asthma

Evidence is increasing that highlights asthma to be a heterogeneous disorder distinctly represented by various inflammatory T helper cell profiles and underlying molecular mechanisms. The clinical phenotypes are usually structured according to treatment response (corticosteroid responsiveness), degree/occurrence of exacerbations, the predominant inflammatory cell profile in sputum (eosinophilic or neutrophilic) and age in childhood asthma. Structuring based on the clinical phenotypes has hindered treatment as overdiagnosis and treatment failures mire the diagnostic process. Thus ongoing improvements of clinical diagnosis with the employment of objective measures of variable airflow obstruction to support clinical diagnosis are still called for.
The most common molecular mechanism of asthma is type 2 (T\textsubscript{H}2) inflammation. Clinical studies of T\textsubscript{H}2 cytokine antagonist therapy thus far have revealed that allergic asthma disease symptoms can be caused by different pathogenic sources\textsuperscript{55,137,138}. The various aetiologies further confound diagnosis and treatment. Besides the T\textsubscript{H}2 pathway, other pathways have been investigated as potential drivers of asthma. In a study of asthmatics with varying severity of asthma, the gene expression of endobronchial tissue revealed three major distinct clusters, namely; T\textsubscript{H}2 high, T\textsubscript{H}17, and T\textsubscript{H}2/ T\textsubscript{H}17 low \textsuperscript{139}. In an in vivo preclinical model, the observed endotypes are a result of therapeutic targeting of either T\textsubscript{H}2 or a T\textsubscript{H}17 cytokines and thus resulting in amplification of the opposing pathway, therefore blocking T\textsubscript{H}2 cytokines by targeted therapy of corticosteroids induces T\textsubscript{H}17 inflammation, and \textit{vis versa}. This suggests combined targeting of a T\textsubscript{H}2 and T\textsubscript{H}17 inflammatory signatures to achieve additional efficacy over single target inhibitions. Newer diagnostic definitions such as “asthma endotypes” are also attributing the underlying molecular mechanism to improve diagnosis and therapy.

Asthma treatment has been hampered by diagnosis with treatment guidelines mostly recommending empirical approaches on the basis of clinical measures of disease severity instead of basing it on the underlying mechanism of pathogenesis\textsuperscript{55}. There is a need for understanding the mechanisms that drive the various subtypes of asthma. This would reveal potential predictive biomarkers and more effective drug targets.

1.7. Early life control of the development of allergic disease

The observed increase in incidence of allergic disease in urbanised areas worldwide has led to the reinforcement of the hygiene hypothesis, as the influence of the environmental factors are recognised to be key in the development of AD. This states that the observed increased prevalence is attributable to a reduced microbial burden during childhood, as a consequence of the Westernized lifestyle (the ‘hygiene hypothesis’)\textsuperscript{140–142}. In particular, early-life sensitisation to allergens reduces the susceptibility to the development of AD later on in life\textsuperscript{143,144}. Exposure to microbial agents during the early life can then prevent development of allergic disease. Clinical evidence has shown an association between microbial composition during early life and AD in particular the lung microbiome and development of asthma\textsuperscript{49,145}. Asthmatic patients generally
have a higher burden particularly of the Proteobacteria phylum and much more diversity in the microbiome compared to healthy patients\textsuperscript{146,147}. Perturbations in airway colonization of the oropharynx, nasopharynx and even the intestinal microbiota have been associated with increased risk of wheezing and asthma development by 5 years of age \textsuperscript{49,148,149}. However, these changes could be secondary as similar microbial dysbiosis is seen during Inflammatory bowel disease (IBD). Corticosteroid treatment has been shown to also alter the composition of the airway microbiome \textsuperscript{150}. Together, the studies highlight the association of microbiome colonisation and diversity during early life in different mucosal tissues has influence in the later development of allergy.

The environment a subject is exposed to determines the range of antigens encountered by the immune system. Commonly, exposure to farms during early childhood provides a protective effect against allergy asthma by altering the composition of the microbiota \textsuperscript{145}. Mechanistically, the farm dust and microbial products (endotoxins) induce this protection through modifying communication between epithelial cells and dendritic cells in a ubiquitin-modifying enzyme A20 dependent manner, which normally negatively regulating these responses \textsuperscript{151,152}. Simultaneously, maternal farm exposure is associated with increased regulatory T cell function in cord blood of pregnant mothers and protection from asthma in their offspring \textsuperscript{153}. Consequently, farm lifestyle children benefit from the microbial diversity hence recent attempts seek to identify potential candidate microorganisms that might be protective to development of asthma \textsuperscript{154,155}.

Interestingly, in mice the regulation of Treg cells by the microbiota during early life plays an important role in the development of allergic diseases in response to environmental exposure. Exposure to house dust mite antigens earlier on in life in mice induces Helios-negative Treg cell subsets which protect them from developing allergic airway inflammation. This protection is dependent on microbial exposure and correlates with an increase in the bacterial load and diversity in the lung and even the skin \textsuperscript{156,157}. Neonatal exposure also shows key in the development of tolerance to allergic disease. Antibiotic dysbiosis of the microbiome alters the associated Treg cell activation and increases the susceptibility to airway hyperresponsiveness in both humans and mice \textsuperscript{158,159}. Together, these reports show that early life exposure to specific
environments and microbiota alters Treg cell numbers and function leading to effects that persist into adult life and affect the predisposition to allergy (Fig 4).

Figure 4: Role of early-life microbiota in the regulation of Treg cells and development of allergic diseases in human subjects and mice. High microbial exposure during early life is associated with asthma protection and increased Treg cell function in fetal cord blood. In mice house dust mite (HDM) exposure during early life but not thereafter protects from asthma caused by the emergence of induced (Helios-negative) Treg cells. Treg cells have the capacity to accumulate in the skin of mice in response to colonization with a skin commensal bacterial strain specifically during early life and are required to establish tolerance to commensal-derived antigens in the adults.

1.8. Mouse models of allergic asthma

Numerous studies using experimental mouse models of allergic airway inflammation have delineated pathways which play a role in asthma pathobiology\textsuperscript{160–163}. The allergen challenge models aim to reproduce most of clinical features of allergic asthma in humans with either acute or chronic allergen exposure\textsuperscript{164}. Due to phenotypic heterogeneity, chronic disease is
characterised by either a predominance of eosinophilia or neutrophilia or both inflammatory profiles. Eosinophilic inflammation, which is the most common, is mostly associated with a T\(\text{H}_2\) cell mediated asthma phenotype ranging from mild to moderate and severe uncontrolled disease, while neutrophilic inflammation is predominately seen in severe forms of asthma cases mostly associated with presence of T\(\text{H}_17\) cells and its associated signature cytokine in both BALF and serum\(^{165}\). Both immunophenotypes include either allergic or non-allergic induced immune responses\(^{166}\). Most models have targeted the classical T\(\text{H}_2\) asthma phenotype characterised mainly by increased antigen-specific IgE, eosinophilia dominated airway inflammation and increased T\(\text{H}_2\) cytokines\(^{12,167}\).

Typical induction of this asthmatic reaction involves repeated systemic sensitisation of mice to ovalbumin (OVA) precipitated with aluminium hydroxide followed by repetitive intranasal, intratracheal or aerosol OVA challenge to induce a localised allergic response\(^{168}\). Due to weak sensitising properties of OVA, adjuvants (aluminium hydroxide, Lipopolysacharides (LPS) or endotoxins) are used to induce a more robust T\(\text{H}_2\) allergic response signal\(^{63,160,164}\). Alternatively, systemic immunisation can be bypassed by use of protease-containing allergens which sensitises lung tissue locally. These protease-derived allergens namely house dust mite antigen (HDM), Aspergillus conidia, cockroach antigens, are more “clinically relevant natural aeroallergens” and can efficiently sensitise mice through intranasal instillations without the need for adjuvants\(^{161,169,170}\). These aeroallergens are able to effect necessary epithelia injury with their protease activity and also activate innate immune cells thus fulfilling the adjuvant and antigen role\(^{171,172}\). Unlike the OVA repeated intra-peritoneal systemic immunisation, sensitisation with these protease-based aero-allergens elicits similar sensitisation in human subjects\(^{63}\). OVA sensitisation in combination with Complete Freud’s Adjuvant (CFA), a T\(\text{H}_1/\text{T}_17\) skewing adjuvant or administration with a high LPS dose (10\(\mu\)g) provides a mouse model of neutrophilic steroid resistant asthma\(^{60,173}\). The neutrophilic model is crucial as there is increasing evidence of a cooperative effect of T\(\text{H}_1/\text{T}_17\) cells with T\(\text{H}_2\) to mediate severe airway inflammation in humans and animals\(^{174–176}\). In summary, the sensitisation method and allergen choice can offer different insights into pathogenesis when modelling allergic inflammation\(^{161,168}\). However with different sensitisation and challenge models used, the basic model is consistent\(^{177}\).
Mouse models of acute and chronic allergic inflammation have been pivotal in replicating some of the hallmark features of asthma while unravelling immunopathogenesis and possible therapeutic considerations for human asthma. Knockout and transgenic mouse models have certainly added value to the understanding of specific mediators and cytokine influence in disease pathogenesis, although, varied allergic immune responses have been reported in different mouse strains. Over the years, numerous therapeutic targets for human asthma have been developed, namely; leukotriene antagonists and leukotriene receptor antagonists, mast cell depletion, IgE antagonist, anti-IL-5 monoclonal antibodies, phosphodiesterase 4 antagonists, and corticosteroids. However, all these therapeutic strategies have seen limited success, suggesting a complex nature of asthma pathogenesis.

1.9. Immunological mechanism of allergic asthma
1.9.1. Th2 based mediators

Allergic asthma is an atopic disease with symptoms of reversible airflow obstruction typically driven by a Th2 immune inflammation response with mild to severe clinical symptoms. The overall Th2 immune response involves the influence of Th2 cytokines (IL-4, IL-5 and IL-13), and the roles they play in asthma pathogenesis are understood in detail (Figure 1). The IL-4 is mainly responsible for the differentiation of naïve T cells to effector Th2 cells and B cell class switching to express allergen specific IgG1 and IgE which bind to mast cells and driving tissue inflammation. Inhibition of IL-4 and its receptor, interleukin 4 receptor alpha (IL-4Rα) in mice was shown to prevent commitment of naïve T cells to Th2 pathogenic cell and clearly reduced serum IgE production. Production of IL-5 cytokine enables eosinophil proliferation, differentiation, maturation, and recruitment to tissue cell sites and acts as a costimulatory for eosinophil activation. Mice deficient of IL-5 have shown impaired eosinophil recruitment. IL-13 is a predominant driver of asthma pathology, directly acting on epithelial and smooth muscle cells to induce AHR, mucus cell metaplasia and airway inflammation. IL-13 is able to utilise the IL-4Rα subunit for signalling, commonly shared with IL-4 and neutralisation of this receptor prevents allergen sensitisation, but not established allergy. However, there is redundancy in function between IL-4 and IL-13 with both cytokines able to induce Th2 airway inflammation.
Innate derived cytokines; IL-25, IL33 and TLSP are involved in key upstream events in airway epithelium, which shape the downstream adaptive Th2 cell response. The innate cytokines have non-redundant roles in the initiation and maintenance of Th2 response in the lung. A potential role for IL-25 is in allergen-induced trafficking and local differentiation of eotaxin to the airways thus promoting tissue eosinophilia during initiation of allergic airway disease. IL-25 deficient OVA-sensitised mice have impaired airway eosinophil infiltration and lung homing of newly produced eosinophils. IL-33 is a crucial activator of ILC2s which are potent producers of IL-13 and IL-5 in lung tissue during allergic inflammation, however experimental asthma does persist in the absence of IL-33 receptor signalling. TSLP in mice has been found to induce proliferation and differentiation of mast cells in a STAT6-dependent manner from bone marrow progenitors and amplify differentiation of alternatively activated macrophages hence contributing to airway inflammation. TSLP- or TSLP receptor-deficient mice have reduced populations of mature mast cells and drastic reduction in allergic airway inflammation with decreased BALF eosinophilia and mucus production.

1.9.2. Non-Th2 based mediators

Allergic asthma is a chronic syndrome predominantly Th2 driven, however non-Th2 disease has been observed with differing inflammatory cells and cytokine milieu. IL-17 has been shown to act on epithelial, endothelial and hematopoietic cells and can also induce the expression of pro-inflammatory cytokines which exacerbate Th2 responses and promote severe airway inflammation associated with steroid insensitivity in both human disease and experimental mouse models. IL-17 induces its effector functions by directly recruiting neutrophils or by promoting other chemokines known to activate neutrophil excavation to the site of inflammation. Its pro-inflammatory role was similarly shown in serum and sputum from patients after bronchial challenge with significant increases in the Th17 cell response IL-17 levels in late phase acute response. Case-control studies similarly confirm the higher IL-17 profile in asthmatics compared to healthy controls. Its role in goblet cell hyperplasia was shown upon depletion of the IL-17R resulting in decreased mucus secreting cells, however inflammation persisted.
IL-9 promotes mast cell functional development and has been shown to increase mast cell numbers into the lungs, contributing greatly to chronic airway inflammation\textsuperscript{200}. Blocking IL-9 signalling is protective from mast cell responses and reduce cytokine secretion especially IL-17 in chronic allergic models \textsuperscript{200,201}. Asthmatic patient studies have also shown the increased expression of IL-9 and its receptor in the airways thus enhancing mucus production, eosinophilia and AHR\textsuperscript{202}. IL-9 has also been described to enhance IL-4 mediated IgE and IgG production by B cells\textsuperscript{203}. IL-22 is thought to be produced by T\textsubscript{H}17, T\textsubscript{H}22 cells and ILCs, and is increased in sera of asthmatic patients and BAL fluid of allergic asthma mouse models\textsuperscript{204}. A dual role is proposed with its role in inhibiting proinflammatory chemokines in epithelial cells induced by IFN-\gamma. However, its enhancement of smooth muscle proliferation suggests a pathogenic role\textsuperscript{205}. Ablation if IL-22 exacerbates AHR and airway inflammation elicited by intraperitoneally sensitised mice while cutaneous sensitisation elicits a protective reduction in inflammation thus IL-22 functionality depends on route of immunisation\textsuperscript{206}.

1.9.3. IL-4/IL-13 Axis

Common in the signalling cascade and pivotal in the induction of the T\textsubscript{H}2 inflammatory response is IL-4R\textalpha, a subunit present on both type 1 and type 2 IL-4-receptor complexes (Figure 5) responsible for IL-4 only and IL-4/IL-13 signalling respectively on hematopoietic cells and/or non-hematopoietic airway cells thus effecting the various symptoms observed (AHR, airway inflammation, airway eosinophilia, mucus secretion etc.)\textsuperscript{207}. Type 1 and type 2 receptors are two of many cytokine receptors expressed on hematopoietic cells and or non-hematopoietic cells respectively. Both receptor complexes activate cytoplasmic signaling cascade molecules namely; the signal transducer and activator of transcription (STAT6), and the type 1 IL-4 receptor complex may also activate further signaling molecules, the insulin receptor substrate (IRS)-2, a molecular adaptor between diverse receptor kinases, which leads to downstream gene expression of T\textsubscript{H}2 effector that effect the symptoms of asthma (Figure 5)\textsuperscript{208}.

Figure 5: Schematic of IL-4/IL-13 signalling receptor molecules. Both type 1 and type II receptor complexes signal through the Jak/STAT cascade, with IL-4Rα associating with Jak1, γC with Jak3, and IL-13Rα1 with Tyk2. STAT molecules dimerise and nuclear translocation occurs, leading to activation of gene transcription driving pathology.

IL-13 also signals through the type 2 receptor (IL-13Rα1) and shares 25% homology with IL-4 thus the overlap in biological function. However, IL-13 has a distinct functional response on the type 2 receptor. IL-13 binds to IL-13Rα1 which complexes with the IL-4Rα subunit, forming the type II receptor. Signaling through the type I receptor leads to activation of downstream signaling adaptor molecules STAT6 and IRS-2, whereas signaling through the type II receptor predominantly activates STAT6. IL-4Rα is essential in the differentiation of Th2 responses during allergic sensitisation (priming) phase as well as effector phase, where it regulates airway inflammation and remodelling. With this receptor common in both type 1 and type 2 receptor
complexes, IL-4Rα is a valuable target asthma therapeutic research. Knockout mice deficient of IL-4Rα chain have shown reduced allergen induced airway inflammation and AHR. Our laboratory and others have elucidated the role for IL-4Rα in experimental mouse models asthma (Figure 6) and it’s a viable genetic risk marker for type-2 inflammatory endotypes and asthma exacerbations.

**Figure 6:** The role of IL-4Rα signalling in the development of T\textsubscript{H}2 inflammatory symptoms in allergic asthma. Various hematopoietic and non-hematopoietic airway cells utilise IL-4/IL-13 signalling to effect the various symptoms observed (AHR, airway inflammation, airway eosinophilia, mucus secretion). The arrows represent efferent effect of the respective cytokines and mediatory molecules. VCAM-1; vascular cell adhesion molecule 1, IgG1; Immunoglobulin G, IgE; Immunoglobulin E, Gata3; T\textsubscript{H}2 transcription factor, STAT6; signal transducer and activator of transcription 6, ECM; extracellular matrix, MHCII; major histocompatibility complex 2 and TCR; T\textsubscript{H}; t helper cell and Treg; t regulatory cell.

The above figure 6 illustrates the various roles of IL-4/IL-13 signalling in development of allergic disease with affected major inflammatory cells highlighted in blue. Arrow 1; The IL-4Rα has been shown to be vital for the in vivo differentiation of naïve T cells into pathogenic allergen specific Th2 cells\(^\text{185,212}\) and deficiency of IL-4Rα on CD4\(^+\) T cells has led of increased Th17 cells\(^\text{215}\). Arrow 2; IL-4/IL-13 signalling mediates differentiation and isotype class-switching of B cells to induce IgE synthesis\(^\text{216}\). Arrows 3; There is a downstream effect of IL-4Rα signalling eliciting IL-5-dependent eosinophil and IL-9-dependent mast cell recruitment and activation\(^\text{208}\). Arrow 4; Plasma cell secreted IgE migrate to the local tissue and bind to IgE receptors on mast cells, basophils, and eosinophils, later crosslinking with allergen then inducing the release of inflammatory mediators (histamine, leukotrienes, prostaglandins and more cytokines). Arrows 5; Secreted mediators also induce smooth muscle contraction and vasodilatation\(^\text{217}\). However, IL-13 signalling on smooth muscles and epithelium cells is responsible for ASM hyperplasia, and AHR and involves promotion of smooth muscle migration, proliferation and contractility in vivo\(^\text{218,219}\). Arrows 6; Secreted Th2 cytokines, IL-13 and IL-4 act directly on airway epithelial cells inducing mucus secretion, goblet cell hyperplasia and\(^\text{220,221}\).

Arrow 7; IL-4, together with IL-13 also contribute to inflammatory cell recruitment by inducing the upregulation of vascular cell adhesion molecules-1 on the vascular endothelium, which facilitates transmigration of T cells, eosinophils, monocytes, and basophils into airway tissues\(^\text{222}\). Arrow 8; IL-4Rα signalling is a key regulator of tissue fibrosis in asthma with a regulatory role on the extracellular matrix deposition during chronic airway remodelling\(^\text{208}\). This involves mediating the production of epithelial TGFβ, a profibrotic cytokine known to transform fibroblasts into myofibroblasts. This initiates the secretion of matrix proteins and enzymes (metalloproteases) involved in subepithelial fibrosis\(^\text{223,224}\). Arrow 9; Adoptively transferred IL-4Rα\(^+\) macrophages are shown to sufficiently enhance Th2-driven, allergic inflammation. The IL-4Rα signalling stimulates the alternative activation of the macrophages to M2 polarised alveolar macrophages and contribute to Th2 driven allergic inflammatory asthma exacerbations in the lung by secreting cytokines and other pro-inflammatory mediators\(^\text{225,226}\). Although, IL-4Rα signalling has been
described in many cell types, it is still unclear what the function of the receptor is in other cell types e.g. regulatory T cells.

1.10. Treatment options thus far

Currently two key asthma treatments are given; Bronchodilators (β2-adrenergic receptor agonists); they function to reverse airway narrowing by relaxing airway smooth muscle, and corticosteroids; anti-inflammatory drugs that are particularly effective in supporting type 2 immune responses treating the underlying airway inflammation11. Many patients with asthma symptoms are controlled with low doses of ICS or leukotriene receptor agonist55. This first line treatment option is for patients with frequent or persistent asthma symptoms and preferable inhalation is more effective than the oral route with limited side effects. Recently ICS and long-acting β2-agonist (LABA) combination to control persistent or difficult to treat asthma has been included into the Essential Medicines List by the WHO for asthma patient disease management even though combinatory therapy has been implemented for decades 11. However, steroid-resistant, severe asthma and mixed Th2/Th17 asthma phenotypes do persist hence new treatment strategies are necessary.

With increasing knowledge on the mediators of allergic inflammation and the specific roles in pathogenesis of the disease, multiple potential therapeutic targets for asthma have been tested. The first approved immunomodulatory treatment in 2002 was Omalizumab (Xolair; Genentech/Novartis), a non anaphylactogenic monoclonal antibody which functions to deplete IgE thus disarming mast cells and basophils, blocking overall IgE effects. This was shown in early clinical studies with acceptable safety and efficacy as additional therapy to ICS treatment227,228. The main effects involve decreasing asthma exacerbation rates and as combinatory therapy it is shown to reduce the maintenance dose or administered oral corticosteroids. However, there are modest effects on the forced expiration volume (FEV) (a hallmark symptom for measuring airflow and lung functionality) and asthma symptoms229,230.
With more than half of asthma patients exhibiting persistent eosinophilia, IL-5 inhibitors were a potential therapeutic target. Mepolizumab (Nucala; GlaxoSmithKline) an anti-IL-5 humanized monoclonal antibody which binds to IL-5 preventing IL-5Rα binding was recently (2015) approved for clinical use as treatment in severe eosinophilic asthma patients, leading to reduced exacerbation rates, improved FEV and asthma symptoms score\textsuperscript{231,232}. This improved asthma control was also accompanied by a decreased daily dose of administered corticosteroids\textsuperscript{233}.

IL-13 is the main pathology driver in allergic asthma, it binds to two receptors; the heterodimeric combination of IL-13Rα1 and IL-4Rα, and the monomeric IL-13Rα2 (Figure 5). Moreover, IL-4 binds to heterodimeric combination of IL-13Rα1 and IL-4Rα, and the heterodimeric γC chain and IL-4Rα (Figure 5). For both IL-4 and IL-13 mediators, separate monoclonal antibody therapeutics were tested in phase II clinical trials. Lebrikizumab (Genentech/Roche) interferes with IL-13 binding to IL-4Rα to prevent formation of IL-13/IL-13Rα1 signalling complexes, GSK679586 (GlaxoSmithKline) and Tralokinumab (Medimmune/AstraZeneca) block binding of IL-13 to IL-13Rα1 and to IL-13Rα2. These IL-13 inhibiting immunotherapies in steroid-naive asthma patients did not improve lung function, asthma control or asthma exacerbations\textsuperscript{234–236}. Subgroup analysis based on asthma biomarkers of Tralokinumab trial studies showed reduced exacerbations with greatest effects seen in patients with high peristonin levels in serum. As for IL-4 targeted therapy, the IL-4Rα was the opted immunotherapy target which blocks allergic T\textsubscript{H}2 inflammation through inhibition of the IL-4Rα subunit (Figure 5). Pitrakinra (Aerovant), a recombinant IL-4 mutein and competitive antagonist of IL-4/IL-13 pathways, was unsuccessful in alleviating asthma symptoms with only patients of a specific genotype benefiting\textsuperscript{237}. Dupilumab (Regeneron Pharmaceuticals) treatment, an anti-IL-4Rα monoclonal antibody, on the other hand resulted in improved lung function and asthma control\textsuperscript{238,239}. A current phase III trial is ongoing (ClinicalTrials.gov identifier: NCT02414854), although the drug has been approved for treatment of atopic dermatitis\textsuperscript{240}. Inhibition of T\textsubscript{H}2 regulators (IL-25, IL-33 and TSLP) has not had much inhibitors reach clinical testing, but AMG 157 (Amgen) an anti-TSLP human monoclonal IgG2λ antibody which disrupts interaction of TSLP with its receptor has shown favourable effect\textsuperscript{55}. There was decreased sputum eosinophils, FEV1, and exhaled nitric oxide (FeNO), critical asthma biomarkers in Amgen treated
individuals with mild asthma\textsuperscript{241}. With just a single study, and currently on-going phase 2 studies (ClinicalTrials.gov identifier NCT02698501 and NCT02237196) these findings indicate a vital role of TSLP in allergen-induced airway response and initiation of inflammation. Other target mediators include anti-IL-2, anti-TNF-\(\alpha\), anti-IL-9 and anti-OXO4L although much work still is required to render the biologics efficacious and safe for asthma treatment in the future\textsuperscript{242–245}.

The great challenge of asthma management with the observed tailored immunotherapy is the heterogeneity of the disease syndrome, hence more research is still required. The above mentioned therapeutic interventions have modestly alleviated the asthma burden with a predominantly limited success despite standard treatment care. There is a requirement for genotypic and phenotypic classification of disease to aid in clinical treatment for more personalised therapy and provide more insight with ongoing research.
1.11. Thesis Aims and Objectives

Current asthma treatment with anti-inflammatory drugs merely reduces the symptoms of allergic disease and not prevent progression of the T\(_h\)2 immune response. The emergence of non-T\(_h\)2 or mixed T\(_h\) cell immune response profiles also confounds diagnosis and treatment of allergic asthma. However, with a predominant T\(_h\)2 immune profile amongst asthmatic patients, we sort to investigate the role of **IL-4R\(\alpha\)** signalling in allergic disease.

**AIM 1:** Temporal role of IL-4R\(\alpha\) signalling in ovalbumin induced allergic asthma using the \(\textit{Rosa}\text{\textsuperscript{creERT2}IL-4R\(\alpha\)-}\text{\textsuperscript{-lox}}\) mice

Animal models of allergic asthma have defined periods for development of disease. Our aim was then to define the role of IL-4R\(\alpha\) signalling prior to onset of disease and during active disease. The hypothesis was that, disruption of the IL-4R\(\alpha\) would prevent both onset of disease and active disease. These temporal points represent asymptomatic cases and allergic cases respectively. The specific objectives include:

- Oral gavage of tamoxifen before and after sensitization with Ovalbumin for the prophylactic and therapeutic models respectively.
- An invasive measurement of respiratory mechanics and lung function
- Assessment of cytokine and antibody responses in vivo and ex vivo
- Investigation of inflammatory cellularity in the local lung and peripheral draining lymphoid tissue

**AIM 2:** The role of IL-4R\(\alpha\) signalling on the regulatory T helper cells in the pathogenesis of HDM-induced allergic asthma using \(\textit{FoxP3}\text{\textsuperscript{Cre}IL-4R\(\alpha\)-}\text{\textsuperscript{-lox}}\) mice.

The absence of tolerance during active disease is still elusive. We hypothesized that the T\(_h\)2-rich environment during allergic disease is modulating the functionality of regulatory T cells and therefore the IL-4/IL-13 T\(_h\)2 cytokines impair tolerance. Our aim was to define the effect of **IL-**
4Rα signalling on the functionality of T regulatory cells (T regs) during allergic disease. The hypothesis was that with disruption of IL-4Rα signalling on T regs, the functionality of the Tregs would be altered. We then aimed to investigate the T reg cell specific contributions of IL-4Rα signalling in the development of Ovalbumin and House dust mite-induced allergic airway disease (AAD) in mice. The specific objectives include:

- Sensitization with House dust mite (HDM) allergen.
- An invasive measurement of respiratory mechanics and lung function.
- Assessment of cytokine and antibody responses in vivo and ex vivo.
- Characterisation of inflammatory cells in the local lung and peripheral draining lymphoid tissue, specifically the regulatory T cells.
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Chapter 2:
2. The temporal requirement of IL-4Rα signalling in allergic asthma

Abstract

Background: Allergic asthma is a chronic inflammatory airway disease driven predominantly by a Th2 response to environmental allergens. IL-4Rα signalling is key for driving Th2-type immunity to allergens. Anti-Th2 therapies have the potential to effectively reduce airway obstruction and inflammation in allergic asthma.

Objective: We investigated potential therapeutic effects of selective inhibition of this pathway in mice with established allergic airway disease. We further investigated if IL-4Rα disruption in systemically sensitised mice can prevent the onset of the disease.

Methods: We used Rosa^{creERT2} IL-4Rα^{−/lox} mice, a tamoxifen inducible IL-4Rα knockdown model to investigate the role of IL-4/IL-13 signalling prior to the onset of the disease and during the effector phase in the ovalbumin-induced allergic airway disease.

Results: Deletion of IL-4Rα demonstrated a therapeutic effect, on established AAD and prevented development of OVA induced airway hyperreactivity, goblet cell metaplasia and eosinophilia in allergen-sensitised mice. Interestingly, IL-4Rα knockdown after allergic sensitisation did not induce Th17, neutrophilic inflammatory response as observed in global IL-4Rα-deficient mice after intranasal allergen challenge.

Conclusion: Abrogation of IL-4Rα signalling after allergic sensitisation would have significant therapeutic benefit for Th2-type allergic asthma.
2.6. Introduction

Airway hyperresponsiveness (AHR), pulmonary inflammation, eosinophilia and mucus hyperplasia are hallmarks of allergic asthma. Indeed, chronic disease is driven predominantly by Th2 immune responses with pathology largely caused by IL-4 and IL-13 signalling, which share a common pleotropic c receptor, IL-4Rα subunit. The IL-4/IL-13 axis has been a target for allergic asthma treatment, however, anti-Th2 based therapies for asthma have seen limited success in clinical trials. The therapy conundrum is further compounded by phenotypic heterogeneity amongst asthma cases, limited treatment options for steroid resistant cases and possible non-Th2 inflammatory mechanisms involved in asthma pathogenesis. However, targeting Th2-type inflammation still remains a promising therapeutic approach for a large proportion of carefully stratified asthma patients.

Key in the IL-4/IL-13 axis is the IL-4Rα signalling which has been identified as a potential target for asthma therapies. Deficiency of IL-4Rα in allergen sensitised mice show reduced allergen induced symptoms such as AHR, eosinophilia and mucus hyperplasia in vivo. We and others have also illustrated various cell-specific role of IL-4Rα signalling during the development of allergic disease pathology. However, limited successful anti-Th2 based therapies for asthma calls for better understanding of the mechanisms involved in successful therapy. Hence, investigated in vivo requirements of IL-4Rα signalling after allergic sensitisation prior to the onset of disease as well as after Th2 pulmonary allergic airway lung inflammation. We hypothesised that temporal significance of IL-4Rα signalling might highlight its prophylactic and therapeutic relevance, especially since most patients are diagnosed only after an asthmatic attack episode of established disease. Resurgence of side effects such as Th17 responses have been a concern for anti-Th2 targeted therapies, we further hypothesised that our approach will counter these Th17 responses.

We developed a tamoxifen inducible, conditional IL-4Rα knockdown murine model (Rosa
creERT2/IL-4Rα/lox) and sought to investigate the temporal role of the receptor signalling during the effector phase (therapeutic) and priming/sensitisation phase (prophylactic) of allergic asthma. Rosa
creERT2,
IL-4Rα^flox^, IL-4Rα^lox^ and IL-4Rα^-/^- mice were sensitised with OVA/alum complex intraperitoneally and challenged intranasally with OVA to induce murine allergic airway disease. Temporal deletion of IL-4Rα chain after the sensitisation phase prevented disease development, whereas temporal deletion during the effector phase reduced most diseases signs such as, AHR, eosinophilia as well as goblet cell metaplasia. Unexpectedly, temporal deletion of IL-4Rα signalling in both prophylactic and therapeutic models reduced T_h17-type and neutrophilic airway inflammation, suggesting an unprecedented effect in non-T_h2 type allergic airway inflammatory responses. We thus conclude that abrogation of IL-4Rα signalling after allergic sensitisation would have significant therapeutic benefit for T_h2 type asthma without inducing potentially detrimental T_h17 responses.
2.7. Methods

2.7.1. Mouse strains

We generated an inducible IL-4Rα deletion mouse, Rosa\textsuperscript{creERT2}IL-4Rα\textsubscript{lox}/lox\textsuperscript{237} on a BALB/c background by intercrossing transgenic Rosa\textsuperscript{creERT2}IL-4Rα\textsubscript{lox}/lox C57BL/6 mice\textsuperscript{238} IL-4Rα\textsubscript{lox}/lox BALB/c mice (Supplementary Fig 1). IL-4Rα\textsubscript{lox}\textsuperscript{239} and IL-4Rα\textsubscript{lox}\textsuperscript{-240} mice on a BALB/c background were used as control animals. Eight- to twelve-week old mice were used for the experiments and housed in independently ventilated cages under specific pathogen-free conditions in the University of Cape Town Animal Facility. Animal procedures were approved by the university of Cape Town Animal Ethics committee (Reference number, 015/009).

2.7.2. Models of allergic airway disease

2.7.2.1. Prophylactic model: Post-sensitisation IL-4Rα knockdown in ovalbumin-induced allergic airway disease

Mice were sensitised intraperitoneally with (50µg in 200µl) of ovalbumin (OVA) adsorbed to 0.65% alum (Sigma-Aldrich, Aston Manor, South Africa) on days 0, 7, 14\textsuperscript{241}. The tamoxifen and oil treatment was done by oral gavage of 100µl of 2.5mg/day tamoxifen (TAM) solubilised in vegetable oil (OIL) or 100µl of vegetable oil respectively, on days 15, 16, 17, 18. On days 23, 24, 25, mice were intranasally challenged with 100µg of OVA/alum under anaesthesia with ketamine (ANaket-V; Centaur Labs, Johannesburg, South Africa) and xylazine (Rompun; Bayer, Isando, South Africa) as previously described\textsuperscript{241}. AHR was measured on day 26. After the procedure, mice were euthanised with halothane and tissue samples were collected for analysis.

2.7.2.2. Therapeutic model: Post-effector phase IL-4Rα knockout in ovalbumin-induced allergic airway disease.

Mice were sensitised intraperitoneally with (50µg in 200µl) of OVA/alum on days 0, 7, 14. On days 23, 24, 25, mice were intranasally challenged with 100µg of OVA/alum under anaesthesia. Tamoxifen and OIL treatment was done on days 26, 27, 28, 29. Mice were intranasally challenged again on days 34, 35, 36 and AHR was measured on day 37. After procedure, mice were euthanised and tissue samples were collected for analysis.
2.7.3. Lung function measurements

Airway resistance and elastance of the whole respiratory system (airways, lung chest wall) after intranasal challenge was determined by forced oscillation measurements as described previously \(^{241}\) with the Flexivent system (SCIREQ, Montreal, Canada) using the single compartment ("snapshot") perturbation. Measurements were carried out on mice with increasing doses of acetyl-β-methylcholine (methacholine, Sigma-Aldrich, Aston Manor, South Africa) treatment. Differences in the dose-response curves were analysed by repeated measures ANOVA with Bonferroni post-test. Only mice with acceptable measurements for all doses (coefficient of determination >0.90) were included in the analysis.

2.7.4. Analysis of cell populations by flow cytometry

Bronchoalveolar lavage (BAL) cells were obtained as previously described\(^{242}\). Single-cell suspensions were prepared from lymph nodes in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco, Paisley, United Kingdom) by passing them through 40μm filter. To obtain single cell suspensions from lung tissues, a left lobe lung was digested for 1 hour at 37°C in RPMI (Gibco, Paisley, United Kingdom) containing 13mg/mL DNase I (Roche, Randburg, South Africa) and 50U/mL collagenase IV (Gibco, Waltham, Massachusetts) and passed through 70μm filter. Single cells were then blocked with 24G2 for 30min at 4°C, followed by surface staining with fluorophore conjugated antibodies for 30min at 4°C in the dark.

Antibodies used in these experiments included, phycoerythrobilin (PE)-conjugated anti-Siglec-F (clone, E50-2440), FITC-conjugated anti-GR-1 (clone, RB6-8C5), Allophycocyanin (APC)-conjugated anti-CD11c (clone, HL3), V450 conjugated anti-CD11b (clone, M1/70), PE-conjugated anti-CD124 (IL-4Rα, M-1). AlexaFlouro 700-conjugated anti-CD3ε (clone, 145-2C11), V500- anti-CD4 (clone, RM4-5), APC-Cy7-conjugated anti-CD19 (clone, 1D3) and anti-CD8 (clone, 53-6.7) were purchased from BD Pharmagen, AlexaFlouro 700- conjugated anti-MHC II (clone, M5/114) and Live/dead Fixable Yellow stain (Qdot605 dead cell exclusion dye) were purchased from eBiosciences. Cells were acquired using Fortessa (BD Biosciences, Erembodegem, Belgium), and data were analysed with Flowjo version 10 software (TreeStar, Ashland, Ore)
2.7.5. Histology and immunohistochemistry

Lungs were fixed in 4% formaldehyde/PBS and imbedded in paraffin. Tissue sections were stained with periodic acid-Schiff for mucus secretion, haematoxylin and eosin (H&E) staining for inflammation. Image analysis was performed on NIS Elements (Nikon Instrument, Tokyo, Japan). Mucus quantification was carried out using the automated NIS Elements software by defining regions of Interest (ROIs) which are the individual bronchioles on cut lung sections to be analysed for mucus staining and using threshold quantification of the mucus stain in the specific ROIs NIS Elements (Nikon Instrument, Tokyo, Japan). Area of staining is defined as total area of mucus secretion per area of bronchiole epithelial lining. Lung sections from individual mice were assessed, and data from 2 experiments were pooled (n=4-6 mice per experiment).

2.7.6. Antibody and cytokine ELISAs

Antibody ELISAs were carried out as previously described using 5µg/ml of OVA to coat for specific IgE and IgGs. For in vitro cytokine production analysis, single cell suspensions were prepared from mediastinal lymph nodes of OVA-treated and littermate control mice. Cells (2x10^5 cells, in 200µL) were incubated for 5 days in IMDM/10% FCS (Delta Bioproducts, Kempton Park, South Africa) in 96-well plates. Cells were either stimulated with OVA (50 µg/mL) or anti-CD3 (10 µg/mL) and supernatants were collected after incubation period. Concentrations of IL-2, IL-4, IL-5, IL-12, IFN-γ (BD Biosciences), IL-13 (R&D Systems, Minneapolis, Minn), IL-17 and IL-23 (BioLegend) were measured using ELISA assays according to the manufacturer’s protocol.

Statistical analysis

P values were calculated in GraphPad Prism 6 (GraphPad Software, Inc, San Diego, California) by using nonparametric Mann-Whitney student t-test or Two-way ANOVA with Bonferroni’s post-test for multiple comparisons, results were presented as SE of the mean. Differences were considered significant if p was <0.05.
2.8. Results

2.8.1. Characterisation of IL-4Rα expression in Rosa\textsuperscript{creERT2}\textsuperscript{IL-4Rα/lox} mice

The Rosa\textsuperscript{creERT2}\textsuperscript{IL-4Rα/lox} mouse strain has been previously characterised on a C57BL/6 background strain by our laboratory and shown to have an impaired expression of IL-4Rα expression on lung and lymph node tissue upon tamoxifen treatment\textsuperscript{243}. Here we characterised IL-4Rα expression in Rosa\textsuperscript{creERT2}\textsuperscript{IL-4Rα/lox} mouse strain on the BALB/c background under ovalbumin induced allergic asthma. We induced deletion of IL-4Rα in two models, prophylactic (where mice were fed tamoxifen orally after sensitisation before acute challenge with OVA) and therapeutically, where mice were fed tamoxifen orally after sensitisation and acute challenge with OVA) (Fig 7, A). There was efficient knockdown of IL-4Rα in both lung and lymph node tissue after tamoxifen treatment compared to vehicle treated mice (Fig 7, B and C). We further evaluated cell type-specific deletion of IL-4Rα and showed significantly reduced expression in inflammatory cells such as CD4\textsuperscript{+} T cells, B cells and dendritic cells, but not macrophages in lung tissue of TAM-treated mice compared to the OIL-treated littermate control (Fig 7, D and E). The expression levels of IL-4Rα after tamoxifen knockdown were similar to those observed in IL-4Rα global knockout mice (Fig 7, B-E). Hence, we conclude that IL-4Rα was efficiently knocked down upon treatment with tamoxifen.
Figure 7: Prophylactic and therapeutic inducible deletion of IL-4Rα in OVA induced allergic airway inflammation. A, Schematic diagram of IL-4Rα deletion using the tamoxifen inducible mouse model (ROSA^cre^IL-4Rα^-/lox^ mice) in a prophylactic (top panel) and therapeutic model (bottom panel). Mice were fed tamoxifen (2.5 mg) orally for 4 days either after sensitisation (prophylactic) or after first challenge (therapeutic). B, IL-4Rα expression in different organs including BALF, lung and mediastinal lymph nodes in the prophylactic model. C, IL-4Rα expression in different organs including BALF, lung and mediastinal lymph nodes in the therapeutic model. D, IL-4Rα expression in different lung cell types including B cells (CD19^+^MHCII^+^), T cells (CD3^+^MHCII^-^CD11c^-^), CD4^+^ T cells (CD3^+^CD4^+^MHCII^-^CD11c^-^), dendritic cells (CD11c^high^SiglecF^low^MHCII^hi^) and alveolar macrophages (CD11c^hi^SiglecF^hi^MHCII^low^) in a prophylactic model. E, IL-4Rα expression in different lung cell types including B cells (CD19^+^MHCII^+^), T cells (CD3^+^MHCII^-^CD11c^-^), CD4^+^ T cells (CD3^+^CD4^+^MHCII^-^CD11c^-^), dendritic cells (CD11c^high^SiglecF^low^MHCII^hi^) and alveolar macrophages (CD11c^hi^SiglecF^hi^MHCII^low^) in a therapeutic model. Data shown mean ±SDs from 1
2.8.2. Knockdown of the IL-4Rα signalling prophylactically prevented the development of AHR and airway inflammation

Mice deficient of IL-4Rα are protected from OVA-induced allergic airway disease\textsuperscript{161}. Here, we explored the temporal requirement of this signalling pathway post-sensitisation with OVA/alum. We assessed AHR after OVA challenge and found TAM-Rosa\textsuperscript{creERT2}IL-4Rα\textsuperscript{-/-} mice to have significantly reduced airway resistance and elastance compared to the OIL-Rosa\textsuperscript{creERT2} IL-4Rα\textsuperscript{-/-} littermate control (Fig 8, A and B). We also observed significantly increased resistance and elastance in OVA challenged and OIL-Rosa\textsuperscript{creERT2} IL-4Rα\textsuperscript{-/-} when compared to PBS challenged control mice. This demonstrated that deletion of IL-4Rα after sensitisation prevented development of AHR following allergen challenge.
Figure 8: Prophylactic deletion of the IL-4Rα restores normal lung function and reduces allergic airway inflammation. Mice (ROSAcreIL-4Rαlox/lox) were sensitised with OVA/Alum on days 0, 7 and 14 and fed TAM or OIL on days 15 to 18 and challenged with OVA on days 23-25. Analysis was done on day 26. A, Airway resistance. B, Airway elastance were measured with increasing doses of methacholine. C, Total lung cells and bronchoalveolar lavage fluid cells. D, Total number of neutrophils (CD11c<sub>low</sub>CD11b<sub>high</sub>Ly6G<sub>high</sub>) and eosinophils (CD11c<sub>low</sub>CD11b<sub>high</sub>Ly6G<sub>low</sub>SiglecF<sub>hi</sub>) in the BALF were stained and analysed by Flow cytometry. E, Histology analyses of lung sections (magnification X200), stained with H&E (bottom) and PAS (top). F, Automated quantification of the area (µm²) of mucus staining per analysed bronchiole epithelial lining was carried out using NIS elements imaging software. Shown is mean ±SDs from one representative of 3 independent experiments (n= 5-8). Significant differences are described as: *p<.05, **p<.01 ***p<.001.
We measured total numbers of infiltrating cells, eosinophils and neutrophils in the BAL fluid and lung tissue and found total cells and eosinophils to be reduced in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice (Fig 8, C and D). We observed increased neutrophil infiltration in IL-4Rα<sup>-/-</sup> mice, which was absent in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>-lox</sup> mice (Fig 8, D). Furthermore, we observed reduced mucus hypersecretion (Fig 8, E and F) and lung inflammation (Fig 8, E) in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>-lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>-lox</sup> mice. Taken together, this data demonstrates that IL-4Rα-dependent signalling post sensitisation is important in regulating airway inflammation and that IL-4Rα-dependent signalling prior to sensitisation may contribute to airway neutrophilia.

2.8.3. IL-4Rα signalling is necessary for inducing a T<sub>H</sub>2 allergic airway immune response in a prophylactic model

We measured T<sub>H</sub>2 cytokines after stimulation of lymph nodes with anti-CD3 (Fig 9, A) or OVA antigen (Fig 9, B) in prophylactic model. Temporal deletion of IL-4Rα in a prophylactic model significantly reduced IL-4, IL-5 and IL-13 production in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> (Fig 9, A and B). This was consistent with reduced pathology showing that even temporal deletion of IL-4Rα is necessary for the induction of a T<sub>H</sub>2 allergic airway responses. We analysed phosphorylation of downstream transcription factor, STAT6 by flow cytometry in lung, lymph node and lung CD4<sup>+</sup> T cells (Fig 9, C and D). There was a significant reduction in phosphorylated STAT6 expression in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>-lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>-lox</sup> mice.

We then assessed the effect of temporal deletion of the IL-4Rα on the humoral response by measuring serum titres of OVA-specific IgG<sub>1</sub>, IgG<sub>2a</sub>, IgE (Fig 9, E) and total IgE (Fig 9, F). There was significantly reduced OVA-specific IgG<sub>1</sub>, IgE and total IgE in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>-lox</sup> mice, indicating a reduction of type-2 associated antibody secretion by B cells. These results correlate with a reduction in AHR, mucus secretion and allergic lung inflammation, when IL-4Rα was t prior to challenge with OVA. Taken together, these results
demonstrate that temporal deletion of IL-4Rα has a profound effect on induction of T<sub>H2</sub> type allergic airway responses in sensitised mice and in subsequent disease development.

![Image of a bar graph showing cytokine levels](image)

**Figure 9:** Prophylactic deletion of the IL-4Rα impairs the induction of a T<sub>H2</sub> cell mediated cytokine and humoral response in allergic airway inflammation. T<sub>H2</sub> associated cytokine production in prophylactic OVA induced allergic asthma (as described in Fig 1, A) were measured in ex vivo restimulated MLN by ELISA. A, anti-CD3 stimulated and B, OVA stimulated). C and D, Histogram showing Impairment of pSTAT6 expression in OVA-challenged mediastinal lymph nodes, lung tissue and lung CD4<sup>+</sup> T cells of ROSA<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice after induced gene deletion was evaluated by flow cytometry and represented as MFI expression histograms of pSTAT6 in the prophylactic model. Data representative mean ±SDs from one of 2 experiments (n=5-7). MFI,
Median fluorescence intensity. E, Antibody production in prophylactic depletion model; OVA-specific IgG1, IgG2a and IgE. F, Total IgE. Data shows mean ±SDs from 1 representative experiment of 3 independent experiments carried out (n= 5). Significant differences are described as: *p < .05, **p < .01, ***p < .001.

2.8.4. Deletion of IL-4Rα signalling after established allergic airway disease reduces AHR and airway inflammation

We assessed temporal requirement of IL-4Rα signalling in a therapeutic model as described in Fig 1A. Firstly, we assessed lung function and observe a significant reduction in the airway resistance in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>−/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>−/lox</sup> mice (Fig 10, A and B). We also observed significantly increased resistance and elastance in OVA challenged OIL treated Rosa<sup>creERT2</sup>IL-4Rα<sup>−/lox</sup> when compared to PBS challenged control mice. This demonstrated a protective therapeutic effect of IL-4Rα temporal deletion in an established allergic airway disease.
Figure 10: Therapeutic deletion of the IL-4Rα reduces airway hyperresponsiveness and allergic airway inflammation. Mice (ROSA<sup>cre</sup>IL-4Rα<sup>-/lox</sup>) were sensitised with OVA/Alum on days 0, 7 and 14 challenged with OVA on days 23-25, fed TAM or OIL on days 27 to 30 by oral gavage and then challenged with OVA on days 34-36. Analysis was done on day 37. A, Airway resistance B, Airway elastance measured with increasing doses of methacholine. C, Total lung cells and bronchoalveolar lavage fluid cells. D, Total number of neutrophils (CD11<sup>c</sup>lowCD11b<sup>high</sup>Ly6G<sup>high</sup>) and eosinophils (CD11<sup>c</sup>lowCD11b<sup>high</sup>Ly6G<sup>low</sup>SiglecF<sup>hi</sup>) in the BALF were stained and analysed by Flow cytometry. E, Histology analyses of lung sections (magnification X200), stained with H&E (bottom) and PAS (top). F, Automated quantification of the area (μm<sup>2</sup>) of mucus staining per analysed bronchiole epithelial lining was carried out using NIS elements imaging software. Shown
is means ±SDs from one representative experiment of 3 independent experiments (n= 5-8). Significant differences are described as *p < .05, **p < .01, ***p < .001.

We then measured total infiltrating cells in BAL fluid and lung tissue and found them to be reduced in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice (Fig 10, C). This correlated with a reduction in airway eosinophilia in BAL of TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice (Fig 10, D). Global IL-4Rα deficient mice had significantly higher neutrophilic infiltration in the BALF when compared to F when compared to TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice, which correlated with increased total cell counts in this group (Fig 10, C and D). When analysing lung pathology by histology, we observed reduced mucus hypersecretion (Fig 10, E and F) and lung inflammation (Fig 10, E) in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice. This data together with AHR suggested that IL-4Rα signalling is crucial in maintaining allergic airway inflammation in established disease.

2.8.5. T<sub>H</sub>2 immune response and antibody production is maintained after temporal deletion of IL-4Rα signalling in established allergic airway disease

We have shown that temporal deletion of IL-4Rα during the effector stage reduced AHR and lung inflammation. We then measured T<sub>H</sub>2 cytokines in mediastinal LN stimulated with OVA antigen (Fig 11, A) or anti-CD3 (Fig 11, B). Interestingly, T<sub>H</sub>2 cytokine (IL-4 and IL-5) levels were similar between TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice in both OVA (Fig 11, B) and anti-CD3 stimulated (Fig 11, A) mLN. We then assessed humoral responses by measuring levels of OVA-specific serum antibodies and total IgE. We observed no reduction in OVA-specific IgG1, IgE (Fig 11, C), although, a reduction in total IgE was observed (Fig 11, D) when comparing TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice. However, there was no reciprocal increase in IgG2a, a type 1 associated antibody, therefore suggesting the reduced IgE was independent of a type 1 antagonistic effect. Taken together, this data suggested that the reduced signs of allergic airway inflammation and AHR during temporal deletion of IL-4Rα were unlikely to be due to a reduced type 2 cytokines.
Figure 11: Therapeutic deletion of the IL-4Rα has a maintained T<sub>H</sub>2 cell mediated cytokine response even with reduced airway inflammation. T<sub>H</sub>2 associated cytokine production in therapeutic OVA induced allergic asthma were measured in ex vivo restimulated MLN by using ELISA. A, anti-CD3 stimulated and B, OVA stimulated). C, Antibody production in prophylactic depletion model; C, OVA-specific IgG<sub>1</sub>, IgG<sub>2a</sub> and IgE, D, Total IgE. Data shows mean ±SDs from 1 representative experiment of 3 independent experiments carried out (n= 5). Significant differences are described as: *p < .05, **p < .01, ***p < .001.

Functionally distinct dendritic cells (DCs) are central in not only inducing, but also suppressing T<sub>H</sub>2 or T<sub>H</sub>17 immune response through secretion of T<sub>H</sub> cell polarising cytokines<sup>244–248</sup>. We investigated DC compartment in the lung draining lymph nodes (Fig 12, A and B). We observed a reduction in migratory CD11b<sup>+</sup> DCs in both prophylactic (Fig 12, C) and therapeutic (Fig 12, D) IL-4Rα temporarily deleted TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>-/-lox</sup> mice compared to OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>-/-lox</sup> mice. These migratory DCs are implicated in trafficking of antigens to the lymphoid tissue during sensitisation and induction of a T<sub>H</sub>2 cell mediated response<sup>248</sup>. These results suggested a possible
necessity of IL-4Rα signalling in trafficking of CD11b+ DCs for establishing T_{H}2 cell mediated inflammation in allergic airway disease.

Figure 12: The dendritic cell compartment was altered with temporal depletion of the IL-4Rα gene. A, A representative gating strategy for migratory DCs (CD11c^+ MHCII^hi) and residential DCs

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(CD11c*MHCII<sup>int</sup>) in mediastinal lymph nodes stained by flow cytometry. **B**, gating strategy of the CD103<sup>+</sup> migratory DCs and CD11b<sup>+</sup> migratory DCs. **C**, Plotted mean ± SDs proportions of migrated cell populations following OVA airway exposure in a prophylactic model. **D**, Plotted mean ± SDs proportions of migrated cell populations following OVA airway exposure in a therapeutic model. Significant differences among groups are represented as (*p < .05, **p < .01, ***p < .001).

### 2.8.6. T<sub>H</sub>17 induced neutrophilia is dependent on IL-4Rα deletion prior to sensitisation

We observed airway neutrophilia in IL-4Rα<sup>-/-</sup> mice, but not in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice in both prophylactic and therapeutic deletion of IL-4Rα signalling (Fig 8, D and 10, D). We had shown previously that increased production of IL-17 after disruption of IL-4Rα expression on CD4<sup>+</sup> T cells was responsible for increased IL-17 production and airway neutrophilia<sup>191</sup>. IL-17 levels were measured on supernatants of ex vivo anti-CD3 (Fig 13, A and C) and OVA re-stimulated mLN (Fig 13, B and D) in both prophylactic (Fig 13, A and B) and therapeutic models (Fig 13, C and D). IL-17 found to be increased in IL-4Rα<sup>-/-</sup> mice, but not in TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice (Fig 13, A-D).

Additionally, IFN-γ was increased in IL-4Rα<sup>-/-</sup> mice compared to the other groups (Fig 13, A-D), further confirming that anti-T<sub>H</sub>2 effect observed in the TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> was IFN-γ - independent. We further investigated a possible DC compartment responsible for the consequential T<sub>H</sub>17 response (Fig 12, A and B). There was increased migratory CD103<sup>+</sup> DCs in mLN of IL-4Rα<sup>-/-</sup> mice compared to TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice and OIL-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice in prophylactic (Fig 12, C) and therapeutic (Fig 12, D) models. The CD103<sup>+</sup> DCs have been shown to control T<sub>H</sub>17 polarisation by secreting polarising cytokines IL-23 and IL-2<sup>248,249</sup>. We also found increased levels of IL-23 and IL-2 in OVA restimulated mLN in IL-4Rα<sup>-/-</sup> mice compared to TAM-Rosa<sup>creERT2</sup>IL-4Rα<sup>/lox</sup> mice in a prophylactic model (Fig 12, B). The results highlight a potential involvement of migratory CD103<sup>+</sup> DCs together with IL-23 in maintaining expansion of T<sub>H</sub>17 cells observed in IL-4Rα deletion prior to sensitisation. Our approach of temporal IL-4Rα deletion supports a critical role in the establishment and maintenance of disease and further highlight its therapeutic potential in many asthma disease endotypes.
Figure 13: Prophylactic and therapeutic deletion of IL-4Rα reduces a Th17-associated cytokine response. Th1 and Th17 associated cytokine production levels were measured in ex vivo restimulated MLN by using ELISA in both models. A, Prophylactic model anti-CD3 stimulated and B, OVA stimulated cytokine production. C, Therapeutic anti-CD3 stimulated and D, OVA stimulated cytokine. Data shows mean ±SDs from 1 representative experiment of 3 independent experiments. Significant differences are shown as: *p < .05, **p < .01, ***p < .001.
Supplementary Figure 1. Mouse breeding schematic of Rosa^{Cre−/−} IL-4Rα^{−/lox} BALB/c mice. Rosa^{Cre−/−}IL-4Rα^{−/lox} C57BL/6 mice were intercrossed with IL-4Rα^{lox/lox} BALB/c mice to generate Rosa^{Cre−/−} IL-4Rα^{−/lox} BALB/c mice.
2.9. Discussion

The critical importance of IL-4/IL-13 axis as a driver of T\textsubscript{H}2 immunity in allergic asthma has been established with IL-4R\textalpha being central mediator of disease pathology\textsuperscript{124,161}. We and others have previously demonstrated cell-specific function IL-4R\textalpha signalling in the development of allergic disease\textsuperscript{161,188,191,195,197,228,230,242}. However, temporal role of IL-4R\textalpha signalling in vivo, during sensitisation and effector phases of allergic disease is not completely clear. In this study, we developed an inducible IL-4R\textalpha deletion model, allowing us to conditionally delete receptor signalling. In this approach we could investigate the role of IL-4/IL-13 signalling during sensitisation and the effector phase of OVA-induced AAD in systemically sensitised animals. Our findings show that temporal deletion of IL-4R\textalpha signalling post-sensitisation prevents development of ovalbumin-induced airway hyperreactivity, eosinophilia and goblet cell hyperplasia. Therefore, our data supports a hypothesis that abrogation of IL-4R\textalpha signalling would have significant therapeutic benefit for T\textsubscript{H}2 type allergic asthma. Our study contributes to already existing literature on the central role of IL-4R\textalpha subunit in allergic asthma and guides currently ongoing phase III clinical trials targeting IL-4R\textalpha in various asthma endotypes.

Of key importance in establishing a T\textsubscript{H}2 driven airway pathology is a presence of canonical IL-4R\textalpha signalling, which when absent in global IL-4R\textalpha- deficient mice leads to the amelioration of IL-4/IL-13 dependent allergic airway disease\textsuperscript{185,250,251}. We observed a reduction in AHR, eosinophilia, mucus hypersecretion and pathology with temporal deletion of IL-4R\textalpha after sensitisation phase or during effector phase. This indicates that anti-IL-4R\textalpha therapies may be suitable for therapeutic treatment of acute allergic disease patients and even as prophylactic intervention in asymptomatic, but sensitised patients, who represent 50%-60% of asthma patients\textsuperscript{252,253} and are highly at risk of developing asthma or eczema\textsuperscript{254,255}.

We show that diminished T\textsubscript{H}2 cell response in our study is not caused by an increased IFN-\gamma response (Type 1 immune response) as previously suggested\textsuperscript{256}. The reduction in T\textsubscript{H}2 responses in our study correlated with reduced STAT6 phosphorylation\textsuperscript{251}. Of significant note is a potential requirement for IL-4R\textalpha signalling in migration of CD11b\textsuperscript{+} DCs to secondary lymphoid tissues for
antigen sensitisation and priming of a T_{H2} cell mediated immune response. This would be consistent with previous studies showing ILC2-derived IL-13 being important in priming migration of DC to mediastinal lymph nodes through CCL21 chemoattractant^{257}. It is also possible that IL-4Rα in DC is crucial for recruitment of memory T_{H2} cells to the lung through production of chemokines such as CCL17^{41,248}. IL-4 and IL13 have been shown to regulate chemokine production by DCs or macrophages^{258,259} and monocyte-derived DCs have been implicated in being predominant chemokine producers, which are known to regulate migration of T_{H2} cells^{248}. This modulation of DC compartment suggests an additional diverse mechanism involved in augmenting allergic airway inflammation with temporal deletion of IL-4Rα signalling post allergen sensitisation.

Deletion of IL-4Rα subunit after established T_{H2} disease shows a reduction in ovalbumin induced AHR and airway inflammation despite an unresolved T_{H2} cytokine production. This is suggestive of a maintained T_{H2} memory cell recall from systemic OVA sensitisation which is IL-4/IL-13-independent^{158,260–262}. This is in contrast to similar temporal deletion of IL-4Rα signalling prior to secondary infection with *Nippostrongylus brasiliensis* showing a recall role in driving of type 2 immune responses and clearance of the parasites^{243}. In this helminth infection model, IL-4Rα signalling is vital in not only initiating, but also maintaining a functional T_{H2} driven pathology even with maintained T_{H2} type cytokine production. However, we show evidence that IL-4Rα signalling is not necessary for restimulation of a new T_{H2} cell response in allergic airway disease. This is consistent with previous studies where blocking IL-4/IL-13 axis after an established T_{H2} response with an antagonist did not affect ongoing cytokine production by IL-4 secreting CD4+ T cells^{158}. Recently, it was shown using single cell RNA sequencing that basal stem cells from nasal epithelial polyps have a strong IL-4/IL-13 memory signature that persists even after dupilumab treatment^{263}. This stem cell memory may be what drives disease persistence especially in established T_{H2} airway responses.

The successful utility of targeting IL-4Rα signalling has led to clinical trials for dupilumab, a human monoclonal antibody for anti-T_{H2} therapy in allergic asthma patients and atopic dermatitis
Concern arises on unprecedented $T_{h1}/T_{h17}$ polarising inflammation with anti-$T_{h2}$ interventions in humans and mice. Our mouse model resolves a $T_{h2}$ response without inducing any potential $T_{h1}/T_{h17}$ responses even with established disease, thus emphasising on a potential benefit of anti-IL-4Rα therapies as a viable $T_{h2}$ treatment option. Conversely, Stat6-deficient or IL-4Rα-deficient mice have shown increased IgG2α and IgG2b secretion even with resolution of $T_{h2}$ inflammation. Recently, Rorγt-deficient T cells were shown to suppress $T_{h2}$ and $T_{h17}$ differentiation via upregulated BCL6 expression in the airways. $Bcl6$, a transcriptional repressor which antagonises both Rorγt and Gata3, is high in IL-4 committed T follicular helper cells, a precursor of $T_{h2}$ cells upon secondary antigen challenge. The anti-$T_{h2}$ outcome observed with Rorγt deficiency in T cells could be a result of impaired Tfh developmental plasticity to develop into effector T cells. On the other hand, temporal deletion of IL-4Rα on T cells might disrupt Tfh cell responses during sensitisation phase, preventing their IL-4 commitment into effector $T_{h2}$ cells.

Development of $T_{h17}$ responses has been suggested as a potential detrimental side-effect of anti-allergic therapies targeting the IL-4/IL-13/IL-4Rα signalling pathway. The exact mechanism of how the $T_{h17}$ response is potentiated is elusive. In our previous work we have shown that disruption of IL-4Rα-signalling specifically on CD4+ T cells results in antigen-specific $T_{h17}$ responses after allergic sensitisation. Similar to the global IL-4Rα knockout mice, deficiency of IL-4Rα subunit on CD4+ T cells was prior to sensitisation. In this study, $T_{h17}$ responses were only observed in global IL-4Rα-deficient mice, but not in TAM-Rosa$^{creERT2}$ IL-4Rα$^{lox}$ mice, where IL-4Rα signalling was disrupted only after sensitisation. Thus, our study supports a concept that IL-4Rα signalling directly prevents differentiation of CD4+ T cells into $T_{h17}$ cells during allergen sensitisation and reveals a temporal IL-4Rα dependency in development of a $T_{h17}$ airway inflammation. Strikingly, with absence of IL-4Rα signalling prior to sensitisation, we observe a modulation of a DC compartment in lymphoid tissue. A predominant increased migratory CD103+ DC profile and antigen specific IL-23 secretion were observed and are both known to be responsible for maintenance of memory $T_{h17}$ cells. Shalaby et al, similarly revealed pathogenic $T_{h17}$ cells in airway inflammation which was mainly driven by cDC IL-23. However, their model...
relied on environmental adjuvants (HDM and LPS) driving a TLR4-dependent co-stimulation with OVA to expand or maintain chronic airway inflammation\textsuperscript{249}.

In conclusion, we provide further evidence for a therapeutic potential for blocking IL-4Rα signalling in acute disease cases as well as prophylactic possibility in cases of asymptomatic atopic patients in T\textsubscript{H}2-type allergic airway inflammatory responses.

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Chapter 3:

3. The role of IL-4Rα-responsive Regulatory T cells in allergic airway inflammation

Abstract

**Background:** Allergic asthma is caused by a pathogenic, Th2 hyper-inflammatory airway response to inert antigens. This hyper-inflammatory response to these innocuous particles during allergic asthma has been linked to failure by regulatory T cells (Tregs) to promote tolerance or their instability and plasticity towards a pathogenic effector phenotype.

**Objective:** Th2-type immunity in allergic asthma, is mainly driven by the canonical IL-4Rα-signalling. The importance of Tregs in allergic asthma is well documented, however, significance of IL-4Rα-signalling in Treg function is unknown. We investigated the *in vivo* role of IL-4Rα signalling on Foxp3⁺ Tregs in allergic asthma. We aimed to understand the requirement of IL-4Rα-signalling on Tregs in their ability to establish tolerance to common allergens and in their stability.

**Methods:** We used a cell-specific transgenic mouse, *Foxp3<sup>cre</sup>IL-4Rα<sup>-/lox</sup>* mice (where IL-4Rα chain is specifically knocked out/down in Foxp3⁺ Treg cells). We sensitised and challenged *Foxp3<sup>cre</sup>IL-4Rα<sup>-/lox</sup>* mice or littermate control mice with house dust mite (HDM) or ovalbumin (OVA). For HDM, mice were sensitised intratracheally (i.t.) at day 0 and challenged intranasally (i.n.) on days 6 to 10. Lung function and immunological assays were analysed at day 14. In the OVA model, *Foxp3<sup>cre</sup>IL-4Rα<sup>-/lox</sup>* mice or littermate control mice were sensitised intraperitoneally (i.p.) with OVA/alum on days 0, 7 and 17, challenged on days 23 to 25 with analysis done on day 26. We measured AHR, lung pathology, cellular infiltrate and cytokines by Flexivent, histology, flow cytometry and ELISA, respectively.

**Results:** The knockdown of IL-4Rα-responsive Foxp3⁺ Tregs exacerbated airway hyperreactivity and mucus hypersecretion in OVA and HDM-sensitised mice. Moreover, there was reduced induction of Foxp3⁺ Tregs in IL-4Rα-deficient Tregs, which was accompanied by increased IL-33 “alarmin” production and ILC2 in the lung. Interestingly, T cell derived Th2 cytokines were reduced in both peripheral and local tissue.
3.6. Introduction

Immunological tolerance to aeroallergens has been predominantly maintained by T regulatory cells (Tregs) in the lung \(^{272-274}\). The Treg mechanisms include the suppression of pathogenic effector T cells established by anti-inflammatory IL-10 and TGF-β cytokine secretion \(^{274}\), cell-cell contact suppression with costimulatory receptors, CTLA4\(^{275}\), ICOS\(^{77}\) and PD1\(^{273}\) or depletion of peptide-MHC II from dendritic cells \(^{276}\). The suppressive mechanism is dependent on environmental cues, immune response type and biological context\(^{272}\). However, the Tregs which are key in maintaining tolerance fail to control allergic inflammation and thus their role during active allergic disease has been poorly understood\(^{274}\).

Allergic asthma involves a predominant chronic dysregulated Th2 immune response comprised of; airway hyperresponsiveness, increased allergen specific IgE secretion, influx of effector T cells producing Th2 cytokines in the lung and goblet cell hyperplasia \(^{141}\). The failure to develop tolerance has been suggested to be a result of Th2 cytokines in the prevailing Th2 environment impairing the induction of allergen specific Treg cells by reprogramming them toward a pathogenic effector T cell phenotype\(^{277-280}\). Defects in Treg function in both human and mice are associated with autoimmune diseases due to uncontrolled lymphocytes proliferative diseases \(^{281}\). Patients with Foxp3 mutations and mice deficient of Foxp3, exhibit pathologies in mucosal tissues associated with allergic inflammation, suggesting that Tregs are key regulators of allergic inflammation \(^{282,283}\). Tregs can act in a site specific and cell intrinsic manner exerting their regulatory functions, once recruited to the site of inflammation.

There is evidence to suggesting the functional instability of Tregs in allergic disease\(^{278}\) even in food allergy\(^{284}\). This paradigm of a Th2 dependent impairment of Treg function suggests a requirement for IL-4/IL-13 and its pleotropic receptor signalling on Tregs in allergic asthma. Mechanistically, IL-4 acts via IL-4Rα signalling resulting in activation of downstream transcription factor STAT6, which is thought to disrupt differentiation of CD4\(^{+}\) T cells into Foxp3\(^{+}\) Tregs in vitro\(^{285}\). Furthermore, in humans, some IL-4Rα polymorphisms have been associated with destabilised Foxp3 transcription factor in Treg cells, thus reprogramming them into pathogenic
ex-Foxp3⁺ T H2 or ex-Foxp3⁺ T H17 cells 277,286–288. However, evidence has also shown an IL-4Rα-independent Treg conversion to ex-Foxp3 T H2 cells in HDM induced airway inflammation not inspired by T H2 cytokine signalling 287. This highlights the complexity in failure to induce or maintain tolerance by Tregs during allergic asthma and the controversy of the T H2 environment in impairing Foxp3⁺ T reg regulation induced in vivo during allergic asthma. We thus sort to investigate the functional role of IL-4/IL-13 responsive T regs in hindering tolerance during allergic asthma by knocking out IL-4Rα on Foxp3⁺ Tregs. We found that IL-4Rα signalling on Foxp3⁺ Tregs was important in recruitment of Tregs into the lung, a site of allergic inflammation. Selective deletion of IL-4Rα signalling on Foxp3⁺ T regs resulted in increased allergic airway inflammation, airway hyperresponsiveness and reduced T cell derived T H2 type cytokines. Interestingly, we found increased innate lymphoid cell type 2 cells and their secreted T H2 type cytokines, which suggested them being a major player in driving allergic asthma pathogenesis.
3.7. Methodology

3.7.1. Mouse strains

Eight- to 10-week old Foxp3CreIL-4RαfloX, IL-4RαfloX and IL-4Rα−/− mice on a BALB/c background were used for the experiments and housed in independently ventilated cages under specific pathogen-free conditions in the University of Cape Town Animal Facility. Animal procedures were approved by the university of Cape Town Animal Ethics committee (Reference number 014/019, 018/013).

3.7.2. Mouse models

3.7.2.1. House Dust-mite induced allergic airway disease

Mice were sensitised intratracheally with 1µg of HDM (Geer laboratories, Lenoir, USA) on day 0. The HDM contained D. pteronyssinus (Der p1), endotoxin content of 25500 EU/vial, protein content of 39.6 mg/vial and the Lot number was 262538. Mice were intranasally challenged with 10µg of HDM under anaesthesia on days 6, 7, 8, 9 and 10 as previously described290. AHR was measured on day 14. After the procedure, mice were euthanised and tissue samples were collected for analysis.

3.7.2.2. Ovalbumin induced allergic airway disease

Mice were sensitised intraperitoneally with (50µg in 200µl) of ovalbumin (OVA) adsorbed in 0.65% alum on days 0, 7, 14241. On days 23, 24, 25, mice were intranasally challenged with 100µg of OVA/alum under anaesthesia as previously described241. AHR was measured on day 26. After the lung functionality procedure, mice were euthanised and samples collected for analysis.
3.7.3. Airway Hyperresponsiveness

Airway resistance and elastance of the whole respiratory system (airways, lung, chest wall) after intranasal challenge was determined by forced oscillation measurements as described previously\textsuperscript{228} with the Flexivent system (SCIREQ, Montreal, Canada) by using the single compartment (“snapshot”) perturbation. Measurements were carried out on mice with increasing doses of acetyl-\(\beta\)-methacholine (Methacholine, Sigma-Aldrich, Aston Manor, South Africa) treatment. Differences in the dose-response curves were analysed by repeated-measures ANOVA with Bonferroni post-test. Only mice with acceptable measurements for all doses (coefficient for determination >0.90) were included in the analysis.

3.7.4. Flow cytometry

BALF cells were obtained as previously described\textsuperscript{291}. Single cell suspensions were prepared from lymph nodes in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco, Paisley, United Kingdom) by passing them through 100\(\mu\)m filter. To obtain single cell suspensions from lung tissues, a left lobe lung was digested for 1 hour at 37\(^\circ\)C in RPMI (Gibco, Paisley, United Kingdom) containing 13mg/mL DNase I (Roche, Randburg, South Africa) and 50U/mL collagenase IV (Gibco, Waltham, Massachusetts) and passed through 70\(\mu\)m filter. IL-4R\(\alpha\) surface expression was detected on lymph node cells, lung cells and BAL fluid cells by phycoerythrin (PE) anti-CD124 (IL-4R\(\alpha\), M-1). Cell subpopulations were identified with Alexa Fluor 400, BD Horizon V500, BD horizon V450, PerCP-Cy5.5, APC, APC-Cy7, Fluoroscein isothiocyanate, PE, PE-Cy7 or biotinylated monoclonal antibodies against anti-CD3\(\varepsilon\) (clone, 145-2C11), anti-CD4 (clone, RM4-5), anti-CD8 (clone 53-6.7), anti-CD19 (clone, 1D3), CD25 (clone PC61), CD45 (clone A20, anti-CD103 (clone, M290), anti-Ly6G (clone, RB6-8C5), anti-CD11c (clone, HL3), anti-MHCII (clone, M5/114) eBioscience, Siglec F (clone, E50-2440), anti-CD11b (clone, M1/70), EpCAM (clone, G8.8), Lineage (Cat 561317), T1ST2 (Cat FAB10041A), SCA1 (clone D7), Foxp3 (clone, FJK-16s) and intracellular cytokine stains IL-4 (clone, 11B11), IL-5 (Cat 554395), IL-13 (clone, eBio13A), IL-10 (Cat 554466), IL-17 (clone
eBio17B7), IL-33 (Cat IC3626P) and IFN-γ (XMG1.2). Biotin-labelled antibodies were detected with streptavidin conjugated PE-Texas Red. All antibodies were from BD Pharmingen (San Diego, CA). For staining (1x10⁶) were labelled and washed in PBS, 3% FCS FACS buffer. For intracellular cytokine staining, cells were restimulated with phorbal myristate acetate (50ng/mL, Sigma-Aldrich), ionomycin (250ng/mL, Sigma-Aldrich), and monensin (200mM, Sigma-Aldrich) in 10% FCS supplemented IMDM for 6h at 37°C then, surface stained before fixing in 2% PFA, permeabilised and stained for intracellular cytokines. Data was acquired on the LSR Fortessa machine (BD immunocytometry system, San Jose, CA, USA) and data were analysed using Flowjo software (Treestar, Ashland, OR, USA).

3.7.5. Histology and Immunohistochemistry

Lungs were fixed in 4% formaldehyde/PBS and embedded in paraffin. Tissue sections were stained with periodic acid-Schiff for mucus secretion, and haematoxylin and eosin (H&E) staining for inflammation. Image analysis was performed on NIS Elements (Nikon Instruments, Tokyo, Japan). Mucus quantification was carried out using the automated NIS Elements software by defining regions of Interest (ROIs) which are the individual bronchioles on cut lung sections to be analysed for mucus staining and using threshold quantification of the mucus stain in the specific ROIs NIS Elements (Nikon Instruments, Tokyo, Japan). Area of staining is defined as total area of mucus secretion per area of bronchiole epithelial lining. Lung sections from individual mice were assessed, and data from 3 experiments were pooled (n=4-6 mice per experiment).

3.7.6. Antibody and cytokine ELISAs

Antibody ELISAs were carried out as previously described²⁹¹ using 5 µg/mL HDM to coat for specific IgE and specific IgGs. For in vitro cytokine production analysis, single cell suspensions were prepared from mediastinal lymph nodes of HDM treated and littermate control mice²⁹¹. Cells (2x10⁵ cells, in 200µL) were incubated for 5 days in 10% FCS supplemented IMDM (Delta
Bioproducts, Kempton Park, South Africa) in 96-well plates. Cells were either stimulated with HDM (30µg/mL) or anti-CD3 (10µg/mL) and supernatants were collected after a 5day incubation period. BAL cells were isolated from BALF and the supernatant assessed for cytokine production after quantifying the protein concentration. Concentrations of IL-4, IL-5, IFN-γ, (BD Biosciences, IL-13, IL-33 (R&D Systems, Minneapolis, Minn), IL-17 and IL-10 (BioLegend) were measured using ELISA assays according to the manufacturer’s protocol.

Statistical Analysis

P values were calculated in GrapPad Prism 5 (GrapPad Software, Inc, San Diego, CA, USA) by using ANOVA with Bonferroni’s post-test or repeated measures ANOVA, and results era presented as mean ± (SD). Differences were considered significant if P was <.05.
3.8. Results

3.8.1. IL-4Rα signalling on Foxp3+ Tregs is required for infiltration of CD4+ CD25+ Foxp3+ Tregs in the lung and modulates expansion of CD4+ T cells in vivo.

*Foxp3*<sup>cre</sup>*IL-4Rα<sup>−/lox</sup> mice* have been previously characterised on a BALB/c background by our laboratory and shown to have an impaired expression of IL-4Rα CD4+ CD25+ Foxp3+ Tregs in both lung and lymph node tissue under steady state<sup>289</sup>. We induced allergic asthma by sensitising and challenging mice with HDM allergen (Fig 14, A). We show by flow cytometry, a significantly reduced expression of IL-4Rα subunit on CD4+ CD25+ Foxp3+ T regulatory cells in the lung and lymph node tissue in *Foxp3*<sup>cre</sup>*IL-4Rα<sup>−/lox</sup> mice* compared to *IL-4Rα<sup>−/lox</sup>* littermate control mice, confirming effective deletion of IL-4Rα (Fig 14, B-E). There was upregulation of IL-4Rα Tregs in *IL-4Rα<sup>−/lox</sup>* mice treated with HDM compared to PBS treated mice Tregs, suggesting a necessity for Treg IL-4Rα signalling during allergic airway inflammation (Fig 14, D and E). There was an impaired expansion of CD4+CD25+ Foxp3+ Tregs, but similar expression of Foxp3 on Tregs in MLN of *Foxp3*<sup>cre</sup>*IL-4Rα<sup>−/lox</sup> mice* compared to littermate control mice (Fig 14, F and H; Fig 18, gating strategy). Lung tissue Treg expansion was maintained in both *Foxp3*<sup>cre</sup>*IL-4Rα<sup>−/lox</sup> mice* and *IL-4Rα<sup>−/lox</sup>* mice (Fig 14, G). However, there was a reduction in Foxp3 expression on Treg in *Foxp3*<sup>cre</sup>*IL-4Rα<sup>−/lox</sup> mice* compared to *IL-4Rα<sup>−/lox</sup>* mice, highlighting a possible instability (Fig 14, I). These findings suggest that selective deletion of IL-4Rα signalling on Foxp3+ cells impairs Treg and stability at site of inflammation during HDM induce allergic asthma.
Figure 14: Induced impairment of IL-4Rα expression on CD4+ CD25+ FoxP3+ T regulatory cells. A, Schematic of the HDM induced airway inflammation model. B - E, Impairment of IL-4Rα expression in HDM-challenged (B and D) mediastinal lymph node and (C and E) lung tissue of
HDM-challenged FoxP3creIL-4Rαlox/lox mice evaluated by flow cytometry and represented as MFI expression histograms. F and G, Proportion and cell numbers of CD4+ CD25+ FoxP3+ T regs in (F) MLN and (G) Lung tissue. H, The expression of FoxP3 transcription factor on the CD4+ CD25+ FoxP3+ T regs in (H) MLN and (I) Lung tissue. Representative of 3 independent experiments. MFI, Median fluorescence intensity. Significant differences are described as: *P < .05, **P < .01, ***P < .001.
Figure 15: Deletion of the IL-4Rα on CD4⁺ CD25⁺ FoxP3⁺ Tregs exacerbates airway hyperresponsiveness and mucus hyperplasia in HDM airway inflammation. A, Airway resistance and, B, Airway elastance were measured. Significant differences between FoxP3<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice and IL-4Rα<sup>-/lox</sup> mice: *P<.05, **P<.01 P values: Comparison of dose-response curves to inhaled methacholine. Data representative Means ± SEMs from 2 experiments (n>8). MFI, Median fluorescence intensity. C, Total cellular infiltration in bronchoalveolar lavage fluid and, D, Total lung cells. E, Histology analyses of lung sections (magnification X200), stained with PAS. F, Automated quantification of the area (µm²) of mucus staining per analysed bronchiole epithelial lining was carried out using NIS elements imaging software. Shown is one representative experiment of 3 independent experiments (n= 5-8). Significant differences are described as: *P < .05, **P < .01, ***P < .001. G, Total number of eosinophils (CD11c<sup>low</sup>CD11b<sup>high</sup>Ly6G<sup>low</sup> SiglecF<sup>hi</sup>) in the BALF and, H, Eosinophils in Lung tissue were stained and analysed by flow cytometry. I, Consequent CD4⁺ T cell proportions in the MLN and, J, Lungs after HDM induced airway inflammation.

3.8.2. IL-4Rα-responsive CD4⁺ CD25⁺ Foxp3⁺ T regulatory cells are required to control airway hyperresponsiveness hyperplasia in HDM allergic airway inflammation.

STAT6 expression <i>in vivo</i> suppresses Tregs during allergic lung inflammation and is suggested to dampen their suppressive function<sup>292</sup>. We investigated the influence of IL-4Rα responsive Tregs in lung pathology during HDM allergic asthma. Sensitisation and challenge with HDM, showed significantly increased airway resistance and elastance in Foxp3<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice compared to IL-4Rα<sup>-/lox</sup> mice and PBS treated control mice (Fig 15, A and B). We measured goblet cell hyperplasia by staining for mucus secretion in lung tissue sections and observed a significant increase in mucus production in Foxp3<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice compared to IL-4Rα<sup>-/lox</sup> mice (Fig 15, E and F). Staining with H&E revealed similar cellular infiltration around peribronchial and perivascular areas of lung tissue section (Fig 15, E). Interestingly, there was a visible increase in alveolar spaces in the Foxp3<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice compared to IL-4Rα<sup>-/lox</sup> mice with H&E staining thus suggesting
increased epithelial damage or failed tissue repair. The increased airway obstruction correlated with modest increases in cellular infiltration in BALF and a significant increase in cellularity in lung tissue of Foxp3creIL-4Rα/flx mice compared to IL-4Rα/flx mice (Fig 15, C and D). Moreover, an accompanying increase in proportion of CD4+ T cells in lung tissue of Foxp3creIL-4Rα/flx mice compared to IL-4Rα/flx mice was observed suggestive of impaired T helper (Th) cell suppression by Tregs in Foxp3creIL-4Rα/flx mice (Fig 15, J). Interestingly, eosinophil numbers in BAL fluid were significantly reduced in Foxp3creIL-4Rα/flx mice compared to IL-4Rα/flx mice, which is in contrast to the observed increase in lung tissue (Fig 15, G and H). Taken together these results demonstrate that depletion of IL-4Rα on Tregs exacerbates AHR, mucus secretion and lung eosinophilia.

Figure 16: Antibody production is maintained with depletion of IL-4/IL-13 responsive Tregs. Antibody production in HDM induced allergic inflammation model, A, HDM-specific IgG1. B, HDM-specific IgG2. C, HDM-specific IgG2a. D, HDM-specific IgE and E, Total IgE. Data from 1
representative experiment of 3 independent experiments carried out (n= 5). Significant differences are described as: *P < .05, **P < .01, ***P < .001.

We then measured serum levels of allergen-specific antibodies and total IgE. We found similar levels of HDM-specific IgG$_1$ between Foxp3$^{cre}$IL-4R$\alpha^{-/-}\text{lox}$ mice compared to IL-4R$\alpha^{-/-}\text{lox}$ mice (Fig 13, A), and there was no induction of HDM-specific IgG$_{2b}$ production (Fig 16, B). However, there was a significant reduction in HDM-specific IgG$_{2a}$ in Foxp3$^{cre}$IL-4R$\alpha^{-/-}\text{lox}$ mice compared to IL-4R$\alpha^{-/-}\text{lox}$ mice (Fig 16, C). There was a modest reduction in HDM specific IgE production (Fig 16, D), while total IgE secretion was similar between Foxp3$^{cre}$IL-4R$\alpha^{-/-}\text{lox}$ mice and IL-4R$\alpha^{-/-}\text{lox}$ mice (Fig 16, E). This result showed a maintained antibody production in HDM-induced allergic asthma.
Figure 17: Impairment of HDM specific T\(_h\)2 associated cytokine production in peripheral lymphoid and local lung tissue of FoxP3\(^{Cre}\)IL-4R\(\alpha^{lox}\) mice with HDM induced airway inflammation. T\(_h\)2 associated cytokine production in HDM induced allergic asthma were measured in ex vivo restimulated MLN by using ELISA, and both MLN and lung tissue by flow
cytometry. A, Cytokines from HDM restimulated MLN cells. Data representative Means ± SEMs from 3 experiments (n>10). B, Cytokine production by CD4+ T cells from the MLN tissues cells, C, cytokine secretion by CD4+ T cells from the Lung tissue cells.

3.8.3. Altered T<sub>H</sub>2 immunity in lymphoid and local tissue of Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice upon acute HDM challenge

Crucial in inducing AHR and mucus hyperplasia are IL-13 and IL-5 cytokines, respectively<sup>162</sup>. We measured production of T<sub>H</sub>2 cytokines in both anti-CD3 and HDM restimulated MLN and observed a significant reduction in HDM-specific IL-13 and IL-5 cytokine production in Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice and IL-4Rα<sup>/lox</sup> mice and a modest increase in HDM-specific IL-4 production (Fig 17, A). Interleukin 10 and IFN-γ cytokines were increased in the MLN of Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice and IL-4Rα<sup>/lox</sup> mice (Fig 17, A). Similarly, the anti-CD3 had reduced IL-4, IL-5, IL-10 and IL-13 in the Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice compared to IL-4Rα<sup>/lox</sup> mice although not significant (Fig 17, B). This suggested that IL-4Rα deficient Tregs were crucial in limiting T<sub>H</sub>2 induction in periphery tissue. We assessed intracellular cytokine secretion by CD4<sup>+</sup> T cells in the MLN and found CD4<sup>+</sup> IFN-γ<sup>+</sup>, CD4<sup>+</sup> IL-13<sup>+</sup> and CD4<sup>+</sup> IL-17<sup>+</sup> T cells to be significantly reduced, while CD4<sup>+</sup> IL-10<sup>+</sup> and CD4<sup>+</sup> IL-4<sup>+</sup> T cells were similar between Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice and IL-4Rα<sup>/lox</sup> mice (Fig 17, C and Fig 25, gating strategy in the MLN). Total numbers in the MLN were maintained in the Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice and IL-4Rα<sup>/lox</sup> mice. We then evaluated the site of allergic inflammation, lung tissue and found a significant reduction in the proportions of CD4<sup>+</sup> IL-4<sup>+</sup>, CD4<sup>+</sup> IL-13<sup>+</sup>, CD4<sup>+</sup> IL-10<sup>+</sup> and CD4<sup>+</sup> IL-17<sup>+</sup> T cells in Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice compared to IL-4Rα<sup>/lox</sup> mice (Fig 17, D and Fig 26, gating strategy in the lung). The T cell numbers had a reduced trend in the Foxp3<sup>cre</sup>IL-4Rα<sup>/lox</sup> mice compared to IL-4Rα<sup>/lox</sup> mice, although not significant. The observed reduction in T<sub>H</sub>2 cytokines together with IL-10 in lung tissue suggests some site specific mechanisms of T<sub>H</sub>2 regulation by IL-4Rα responsive Tregs. Despite the decreased T cell derived T<sub>H</sub>2 inflammatory responses disease pathology was unaltered. Taken together, there is a reduced T cell mediated T<sub>H</sub>2 immune response in lymphoid and local lung tissue with deletion of IL-4Rα on Foxp3 regulatory T cells.
Figure 18: Exacerbated innate induced T\textsubscript{H}2 associated cytokine production in lungs of \textit{Foxp3\textsuperscript{\textasciitilde}}\textit{IL-4R\textalpha/\textlambda} mice upon acute HDM challenge. BAL cytokines were measured by ELISA and corrected to the protein concentration. Significant differences among groups are represented as (*P<.05, **P<.01, ***P<.001). A, IL-4 B, IL-5 C, IL-13 D, IL-33 E, Proportion of innate lymphoid type II cells (ILC2s), CD45\textsuperscript{-}Lineage\textsuperscript{-}SCA\textsuperscript{+}T1ST2\textsuperscript{+}, were measured by flow cytometry. T\textsubscript{H}2 associated cytokine production by ILC2s was assessed. F, IL-5 secretion. G, IL-13 secretion.

3.8.4. IL-4R\textalpha responsive CD4\textsuperscript{+} CD25\textsuperscript{+} Foxp3\textsuperscript{+} T regulatory cells modulate IL-33 production and ILC2 expansion in the lung

Since we could not detect increased CD4\textsuperscript{+} T cell derived T\textsubscript{H}2-associated cytokines in the lung of \textit{Foxp3\textsuperscript{\textasciitilde}}\textit{IL-4R\textalpha/\textlambda} mice, we then investigated non-T cell derived mediators driving the observed pathology. We measured T\textsubscript{H}2 cytokine production in BALF and observed a significant increase in total IL-5, IL-13 protein in the BAL, along with alarmin, IL-33 protein, but not IL-4 in \textit{Foxp3\textsuperscript{\textasciitilde}}\textit{IL-4R\textalpha/\textlambda} mice compared to \textit{IL-4R\textalpha/\textlambda} mice (Fig 18, A-D). This data was consistent with the increased lung pathology we observed as IL-33 is a known key amplifier of mucosal and systemic...
innate allergic responses thus suggestive of an increased IL-33-mediated T\(_{H2}\) inflammatory response. These results demonstrate a role for IL-4R\(_{\alpha}\)-responsive CD4\(^+\)CD25\(^+\) Foxp3\(^+\) T regulatory cells in controlling AHR and mucus hyperplasia during acute lung inflammation by modulating innate cytokine production.

Type 2 Innate lymphoid cells (ILC2s) are recognised as the main producers of IL-5 and IL-13 in lung tissue and are IL-33 responsive. Hence, we investigated this T cell-independent T\(_{H2}\) cytokine source and how it is modulated by IL-4R\(_{\alpha}\)-responsive CD4\(^+\)CD25\(^+\) Foxp3\(^+\) T regulatory cells. With an increase in total IL-33 protein production in BALF (Fig 18, D), a corresponding significant increase in lung ILC2 expansion was observed in Foxp3\(^{cre}\)IL-4R\(_{\alpha}\)\(^{-}\)lox mice compared to IL-4R\(_{\alpha}\)\(^{-}\)lox mice (Fig 18, E and Fig 24). Conversely, the increase in lung ILC2 cells was complemented by an increase in ILC2\(^+\)IL-5\(^+\)and ILC2\(^+\)IL-13\(^+\) IL-5\(^+\) producing cells in Foxp3\(^{cre}\)IL-4R\(_{\alpha}\)\(^{-}\)lox mice compared to IL-4R\(_{\alpha}\)\(^{-}\)lox mice (Fig 18, F and G; Fig 24). This result highlights the regulatory role of IL-4R\(_{\alpha}\)-responsive Tregs in controlling local lung tissue inflammation, particularly ILC2 derived T\(_{H2}\)-associated cytokine production during HDM induced allergic airway inflammation.

Figure 19: IL-4R\(_{\alpha}\)-responsive CD4\(^+\) CD25\(^+\) FoxP3\(^+\) T regulatory cells regulate epithelial IL-33 during allergic airway inflammation. A, Proportion of epithelial cells, CD45\(^-\)EpCam\(^+\) MHCII\(^-\), in the HDM model B, Innate cytokine production from epithelial cells measured by flow cytometry, C, Proliferative marker Ki67 expression was assessed and represented as MFI. Significant differences are described as: *P < .05, **P < .01.
Barrier surfaces such as epithelial cells are the major producers of IL-33, an alarmin, during allergic airway inflammation. \textit{Foxp3}^{cre}\textit{IL-4Rα}^{−\text{lox}} mice had increased proportion of epithelial cells compared to IL-4Rα^{−\text{lox}} littermate control (Fig 19, A). The lung epithelia significantly secreted more IL-33 in \textit{Foxp3}^{cre}\textit{IL-4Rα}^{−\text{lox}} mice compared to IL-4Rα^{−\text{lox}} mice, thus confirming its function as a mediator of ILC2s induction (Fig 19, B and Fig 25, gating strategy). However, this increase in proportion of lung epithelia was not accompanied by an increase in proliferation Ki67 marker on epithelial cells, but showed similar levels between \textit{Foxp3}^{cre}\textit{IL-4Rα}^{−\text{lox}} mice and IL-4Rα^{−\text{lox}} mice (Fig 19, C). Taken together, we conclude that IL-4Rα-responsive Tregs act in a local tissue-specific manner and modulate epithelial innate cytokine production during acute HDM induced allergic airway inflammation thereby suppressing inflammation.

3.8.5. Contradictions of OVA induced allergic asthma model in IL-4Rα deficient Foxp3\textsuperscript{+} Tregs.

We used OVA/alum to induce allergic asthma (Fig 20, A) and measured lung functionality and airway inflammation. Airway hyperresponsiveness was increased in the \textit{Foxp3}^{cre}\textit{IL-4Rα}^{−\text{lox}} mice compared to IL-4Rα^{−\text{lox}} mice (Fig 20, B and C) however, in the repeat experiment we observed no increase in the AHR (Fig 20, D and E). There was a consistent increase in BALF cellular infiltration, accompanied by increased eosinophilia, alveolar macrophages, dendritic cells and CD3\textsuperscript{+} Lymphocytes in the \textit{Foxp3}^{cre}\textit{IL-4Rα}^{−\text{lox}} mice compared to IL-4Rα^{−\text{lox}} mice (Fig 20, F-L). This result showed an inconsistency in AHR thus we resorted to HDM allergen model.
Figure 20: of the IL-4Rα on CD4+ CD25+ FoxP3+ T regs exacerbates airway inflammation in Ovalbumin induced-airway inflammation. A, Schematic of ovalbumin-induced allergic asthma mouse model. B, Airway resistance and, C, Airway elastance were measured for Experiment 1. D, Airway resistance and, E, Airway elastance were measured for Experiment 2. Significant differences between Foxp3<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice and IL-4Rα<sup>lox/lox</sup> mice: *P < .05, **P < .01. P values: comparison of dose-response curves to inhaled methacholine. F, Total cellular infiltration in bronchoalveolar lavage fluid and G, total lung cells. Cellular infiltration in BAL fluid H, eosinophils and I, neutrophils. J, CD3+ Lymphocytes K, alveolar macrophages and L, Dendritic cells.
3.9. Discussion

Regulatory T cells are known to suppress Th2 immunity through various mechanisms such as regulating T cell recruitment and homing into inflamed tissue sites, limiting DC activation and recently, and suppressing ILC2 inflammatory cytokine production\textsuperscript{272,280,292–294}. Defects in Treg function is associated with developing autoimmune diseases and depletion \textit{in vivo} exacerbates Th2 inflammation in allergy\textsuperscript{70,280,284}. Intriguingly, in allergic asthma tolerance is diminished, suggesting a possible disruption of Tregs by a dominant Th2 microenvironment\textsuperscript{295}. Hence, there are suggestions of functional and phenotypic modulation of Tregs by inflammatory TH2 cytokines such as IL-4 or IL-13 both in vitro and in vivo, however, the role of these IL-4/IL-13 responsive Tregs in allergic asthma still remain unknown\textsuperscript{280,285}. We therefore investigated the \textit{in vivo} role of IL-4/IL-13 responsive Tregs in HDM and OVA induced allergic airway inflammation. Our findings demonstrated that IL-4R\(\alpha\) signalling on Tregs is crucial not only in the induction of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Treg and modulation of T cell derived Th2 immune response, but also controls local tissue innate cytokine driven allergic airway inflammation.

A common role for Tregs is in controlling the adaptive allergen-specific Th2 immune response\textsuperscript{296}. We expected that specific deletion of IL-4R\(\alpha\) on Tregs would result in an unrestrained allergen-specific Th2 responses as previously shown in mice temporally deficient of Tregs or STAT6-deficient mice\textsuperscript{70,274,292,297,298}. However, in our model an unrestrained Th2 associated cytokine inflammatory response is not T cell derived as expected, but innate cell driven. Pillemer and colleagues showed that adoptively transferred IL-4R\(\alpha\) deficient Tregs prior to sensitisation were still able to suppress T cell specific responses during OVA induced allergic airway inflammation regardless of their lack of IL-4 responsiveness, thus similarly confirming the reduced T cell derived Th2 cytokine secretion in both the lung and lymphoid tissue of our model\textsuperscript{285}. Besides, the ability of IL-4R\(\alpha\) unresponsive Tregs to suppress T cells in the lymphoid tissue could possibly be through immunosuppressive allergen specific IL-10 and IFN-\(\gamma\) secretion hence the overall anti-Th2 effect\textsuperscript{299}. However, unstable Tregs in the periphery and local tissue are known to increase antigen-specific ex-Foxp3 IFN-\(\gamma\)
producing cells which may counter a \( \text{T}_{\text{h}}2 \) allergic inflammatory response\(^{300}\). Previously, IL-10\(^+\) Tregs were shown to suppress the development of ILC2 driven papain-induced airway inflammation through an IL-33/Mast cell axis, however, in our study this tolerance is disrupted with a decrease in \( \text{T} \) cell derived regulatory IL-10 secretion in lung tissue\(^{301}\). Therefore, the impaired \( \text{T} \) cell derived \( \text{T}_{\text{h}}2 \) response in the local tissue was independent of IL-10 secretion.

We recently demonstrated exacerbated mucus production and \( \text{T}_{\text{h}}2 \) cytokine secretion in \( \text{FoxP}3^{\text{cre}}\text{IL-4R}^{\text{a/\text{lox}}} \) mice, in two helminth infection models of \text{Schistosoma mansoni} or \text{Nippostrongyulus brasiliensis}\(^{289}\). In these helminth infection models, there was reduced trafficking of IL-4R\(^\alpha\)-deficient Tregs into the sites of inflammation and reduced suppressive function, shown by exaggerated effector type-2 cytokine production by CD4\(^+\) \( \text{T} \) cells. Interestingly, in a HDM induced allergic asthma model, Treg accumulation in lung tissue was not affected, but function of Tregs was reduced, which contributed to exacerbated allergic disease. This revealed that IL-4R\(^\alpha\)-responsive Tregs are key in controlling both adaptive and innate immune response in different disease settings and highlights the requirement for Treg-IL-4R\(^\alpha\) expression, not only in maintaining the Treg populations but also suppression of innate immune responses. Although \( \text{T} \) cells commonly contribute to the severity of asthma they were redundant in the exacerbation of airway hyperreactivity and remodelling with depletion of IL-4R\(^\alpha\) signalling on Tregs. This rather novel Treg function in regulating innate cellular responses involves restraining of lung epithelial-derived IL-33 secretion, in turn leading to reduced ILC2 expansion, known to independently drive allergic asthma through IL-4, IL-5 and IL-13 production\(^{302-304}\). Subsequently, this innate-type 2 cytokine provokes recruitment of eosinophils, mast cells basophils in the local tissue, contributing to the exacerbated disease\(^{304-306}\).

The crucial network of this interaction between epithelial cells and ILC2 generates sustained IL-33 production which is key in the development of a persistent chronic asthma\(^{307}\). Furthermore, we speculate the epithelial derived IL-33 upregulation decreases epithelial repair leading to an increased susceptibility to allergen sensitisation\(^{308}\), which would normally be controlled by IL-
4Rα-responsive Tregs. This is evident in the lung tissue of IL-4Rα-responsive Treg having increased airspaces/alveoli airsacs, a clear sign of increased epithelial lung damage. This could also be supported by studies showing that Treg cells can promote proliferation in alveolar epithelial cells and lung repair after LPS induced acute lung injury, but this integral role is elicited through a CD103-dependent cell-cell contact manner. Although in our study, the observed epithelial cell expansion is seemingly a result of goblet cell hyperplasia instead of increased proliferation because Ki67 was not significantly upregulated. Additionally, a lung tissue repair role of Tregs has been described for utilising Treg-amphiregulin secretion, however, this is independent of its regulatory T cell suppressive function.

The sustained barrier disruption in allergic asthma is induced by protease based aeroallergens which disrupt gap junctions leading to increased epithelial IL-33 cytokine production. The epithelial derived IL-33, through a positive feedback, dysregulates the regulatory T cells and further impairs the establishment of tolerance in the lung further increasing barrier leakiness, thus increasing alarmin secretion and persistence of disease. IL-33 and associated SNPs are known correlates of disease severity in asthmatic patients thus our report further emphasises the need for characterising innate driven asthmatic patients in the vast heterogeneity of allergic disease to identify persistent asthma phenotypes. Meanwhile, the control of a predominantly ILC2 driven innate immune response in allergy is still fairly elusive with recent propositions for control reported to involve direct (ICOS)–ICOS ligand cell contact for Treg cell–mediated ILC2 suppression.

Adhesion molecules (e.g E-cadherin-KLRG1 interactions) have also shown to be potent regulators of ILC2 proliferation and cytokine secretion in response to IL-33 in allergic asthma. We previously have shown that IL-4Rα deficient Tregs have a reduced expression of GATA3 and Foxp3 in lymphoid tissue upon helminth infection accompanied with a compromised Th2 immune suppressive function. On the other hand, GATA3 deficient Tregs also have an impaired Th2 suppressive role which correlates with a reduced Foxp3 expression as GATA3 functions to control
Foxp3 expression\textsuperscript{316,317}. This intrinsic defect in Tregs has a reduced ST2 expression thus a diminished responsiveness to IL-33 which is crucial for activating the production of amphiregulin, a key mediator for tissue repair in the lung\textsuperscript{317–319}. We therefore think that failure in the IL-4Rα deficient Tregs to produce amphiregulin and regulate tissue repair would see the increased alarmin production and this regulatory role is shown to be independent of the suppressive function\textsuperscript{319}. We also speculate that IL-4Rα deficient Tregs have an altered IL-33 responsiveness which reduces the competition for IL-33 with ILC2s thus promoting ILC2 activation and innate immune driven inflammation. Further compounding the observed break in tolerance might be IL-33 feedback inhibition of lung Tregs to suppress Th2 allergen responses\textsuperscript{280,284}. Therefore, a possible regulatory interaction of IL-4 responsive Tregs and lung tissue repair exists to modulate the secretion of the innate cytokines, although still requires further investigation\textsuperscript{77,209}.

IL-4Rα deficient Tregs did not alter antibody production, which suggested that they had no function in B cell ability to class switch and produce IgG1 or IgE. This is further supported by no reduction in IL-4 production in lymph nodes, which is required for B cell class switching. Overall, the tissue specificity in Treg function during airway inflammation is well documented\textsuperscript{317,318,320} and the IL-4 responsive Tregs seemingly function differently in lymphoid and local tissue. We therefore demonstrate an important indirect role of IL-4 responsive Treg cells in suppression of IL-33/ILC2s mediated airway inflammation, and highlight the critical contribution of Treg cell–mediated modulation of epithelial cell derived innate cytokines. Thus, a reduction in Th2 responses might not only be Treg cell–mediated suppression by inhibition of CD4\textsuperscript{+} T cells and ILC2 cytokine secretion, but also by modulation of epithelial barrier leakiness. Further studies are necessary to investigate the mechanism of epithelial immunity modulation by Tregs. Additionally, the study’s relevance to anti-IL-4Rα therapy seeks to advice on possible significant effects of therapy interfering with Treg based tolerance\textsuperscript{285} as the blockade of IL-4Rα not only would ameliorate antigen specific Th2 inflammation but also unfortunately deplete IL-4 responsive Tregs which we show to be key in restraining IL-33-mediated allergic inflammation.
Using OVA allergen, an increase in airway inflammation is similarly noted in the BAL confirming the augmentation of the airway disease. In contrast, the AHR was not clearly exacerbated with deficiency of the IL-4 responsive Tregs, probable due to an experimental influence than a biological effect of gene deletion. With regards to the OVA/alum induction, it has been previously shown that OVA instillations can spontaneously induce tolerance in mice which is Treg or IL-10-producing dendritic cell-dependent \(^{40,321-323}\). Subtle dose differences and adjuvant exposure instillations can cause significant differences in the quantity and quality of mucosal effector responses induced \(^{160}\). Our model uses 3 sensitisations prior to the intranasal challenges and we suspect the observed AHR inconsistencies could be due to the OVA/alum immunisations. Thus the use of HDM allergen which is more consistent and also includes stimulation of a robust innate inflammatory response is used to describe the role of IL-4 responsive Tregs. Therefore, the AHR inconsistencies from the 2 experiments would require a repeat experiment or a separate tolerance model (includes multiple OVA/alum sensitisation instillations) to investigate a possible effect of a break in tolerance by IL-4Rα deficient Tregs.

3.10. Conclusion

We reveal an immunoregulatory role by IL-4Rα-responsive Tregs in modulating ILC2 expansion and function \textit{in vivo}, establishing an important checkpoint for controlling ILC2-mediated T\(_{H2}\) immune responses and restoring Treg induced tolerance in allergy. The T\(_{H2}\) inflammatory environment thus proves crucial in providing stimulating soluble factors which alter Treg function, however this observed altered functionality seems to be tissue specific. Here we provide evidence for the requirement of IL-4Rα signalling in Tregs in limiting IL-33 derived ILC2 driven AHR and airway inflammation in allergic asthma.
Flow cytometry Gating Strategies

Figure 21: Gating schematic for CD4⁺ CD25⁺ FoxP3⁺ T regs (Lung and MLN)
Figure 22: Gating schematic for $T_H2$ cytokine secretion in the MLN.
Figure 23: Gating schematic for $T_h2$ cytokine secretion in the Lung
Figure 24: Gating schematic for ILC2s populations and ILC2 cytokine production in lung
Figure 25: Gating schematic for Lung epithelia and IL-33 producing epithelial cells
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Allergic asthma is a chronic inflammatory airway disease driven predominantly by a $\text{T}_\text{H}2$ immune response to harmless environmental allergens. The asthma pathology is predominantly elicited by IL-4 and IL-13 cytokines via IL-4R$\alpha$-signalling which is essential for driving $\text{T}_\text{H}2$-type immunity to aeroallergens. The deletion of IL-4R$\alpha$ subunit or its IL-4/L-13 signalling in experimental mouse models has shown a significant reduction in asthma symptoms and polymorphism of the receptor subunit in humans has been associated with an increased risk in asthma. There has been limited success in the therapeutic treatment of allergic asthma due to the still elusive mechanisms on the critical drivers of allergic disease. Recently Dupilumab, a monoclonal antibody therapy targeting the IL-4R$\alpha$ was approved as therapy for atopic dermatitis however with limited success in asthma treatment. We therefore hypothesised that IL-4R$\alpha$ signalling was indeed key in driving allergic disease and successful genetic disruption would prevent onset of disease and even treat active disease. Our study clearly shows that deletion of the IL-4R$\alpha$ target prevented development of ovalbumin induced airway hyperreactivity, goblet cell metaplasia and eosinophilia in allergen-sensitised mice. Similarly, with other inhibition methods of the IL-4/IL-13 pathway, there has been efficient prevention of the development of allergic airway pathologic conditions but not with established disease. Differences could be due to how long term the inhibitory method was, in our model, with maintained genetic disruption for 16 weeks post knockout induction. However, we demonstrate that abrogation of IL-4R$\alpha$ signalling after allergic sensitisation or with established disease would have significant therapeutic benefit for $\text{T}_\text{H}2$ type allergic asthma. Furthermore, evidence for the therapeutic potential for blocking IL-4R$\alpha$ in acute disease cases even in the presence of persistent $\text{T}_\text{H}2$ cytokine secretion highlights the need for therapy focus on targeting and disabling the machinery that maintains and activates the persisting long lived memory responses on re-exposure to allergen.
The suppression of type 2 inflammation has raised concerns on the reciprocal promotion of T\textsubscript{H}17 responses which are associated with severe endotypes of asthma\textsuperscript{331,14}. Previously, we showed the reciprocal development of T\textsubscript{H}17 neutrophilic inflammation with global knockout of the IL-4R\textalpha and this was dependent on IL-4R\textalpha gene disruption specifically on T cells\textsuperscript{334}. To add to that, we suggest that the concerning reciprocal T\textsubscript{H}17 response (associated with severe cases of allergic disease) is a result of disruption of the gene prior to allergic sensitisation. An inducible conditional knockout model of the IL-4R\textalpha on T cells specifically would confirm and reveal the underlying mechanism that regulate this temporal switch. Severe asthma cases associated with T\textsubscript{H}17 neutrophilic inflammation comprise 10% of asthma cases and the IL-4R\textalpha gene has been suggested to classify steroid responsiveness\textsuperscript{335,336}. Our work could likely advice for further research assessing questions as to whether type 2 immunity directed therapy could result in a low T\textsubscript{H}2 endotypes with reciprocal T\textsubscript{H}17 inflammation. Do patients with predisposed polymorphisms to the IL-4R\textalpha or IL-4/IL13 genes prior to sensitisation develop T\textsubscript{H}17 inflammation later on in life? If yes, then the gene would be a biomarker for susceptibility to allergic disease and even severe forms of neutrophilic inflammation. Finally, is there a necessity for combined therapy targeting both the T\textsubscript{H}2 and T\textsubscript{H}17 inflammatory drivers to achieve maximum therapeutic efficacy? We address the concerns of anti-T\textsubscript{H}2 therapy in both mice and humans, leading to a concomitant increase in T\textsubscript{H}17 neutrophilic inflammatory responses associated with severe cases of asthma. The research seeks to advise current ongoing research on anti-T\textsubscript{H}2 therapy in both mice and humans.

Besides targeting the drivers of type 2 inflammation in preventing the onset of and active disease, one of the paramount aims of therapy has been to regain tolerance to aeroallergens, therefore the failure to regulate sensitisation to allergens suggest an inhibitory effect of the predominantly prevailing T\textsubscript{H}2 environment on the regulatory T cells. The failure to maintain tolerance by regulatory T cells during T\textsubscript{H}2 allergic asthma, remains incompletely defined while anti-T\textsubscript{H}2 therapies, with a potential to effectively reduce airway obstruction and inflammation in allergic asthma have had limited success in clinical trials. We therefore investigated the role of IL-4/IL-13 signalling in the regulation of allergic asthma by T regulatory cells during allergic disease.
The TH2 environment has been suggested to destabilise Foxp3+ Tregs, reprogramming them to pathogenic effector T cells which exacerbate allergic disease (1–4). We show that in the absence of intact IL-4Rα signalling on FoxP3+ T regulatory cells, there is exacerbated airway hyperresponsiveness and mucus secretion during HDM induced allergic asthma. This augmented TH2 inflammation was a result of an increase in IL-33 induced airway inflammation driven by type 2 innate lymphoid cells, instead of the expected “unrestrained” T cell driven TH2 pathology. The IL-4Rα signalling on Tregs seemingly regulates the ILC2 driven pathology in the localised lung further supporting the evidence that the inflammatory environment is crucial in altering Treg function (2, 3). However, this highlights IL-4/IL-13 signalling as a key mechanism for controlling ILC2 innate mediated immune response. Besides controlling the innate immune response, Tregs have been key regulators of barrier immunity too via induction of regulatory myeloid populations to control lung epithelial tissue repair during active disease. A rather interesting T cell specific role was seen with a reduction in particularly T cell derived TH2 cytokines in both the local and peripheral tissue. Our study shows a dual role of the TH2 environment. Firstly, IL-4/IL-13 signalling specifically inhibits T cell targeted inhibition and also controls the activation of ILC2s to HDM sensitised mice. This rather novel role of IL-4-responsive Tregs in modulating the epithelial derived innate cytokines highlights the need to further elucidate the immunological mechanisms of modulating epithelial repair and regulating IL-33-mediated airway inflammation to provide a better understanding of innate immune cell driven pathogenesis.

A composite therapeutic strategy would have to include not only combined T cell and innate immune response directed therapy response but also induce tolerance in the process. Current therapeutic attempts have been singular target strategies mostly focused on inhibiting a prevailing TH2 inflammatory response leading to emergence of type 2 independent driven diseases. Corticosteroid therapy has been shown to promote Foxp3+ Treg cell numbers while maintaining their suppressor function and a decrease in effector T cell. However, developing anti-IL-4Rα therapy has a potential to restore T cell-specific tolerance by regulatory T cells but also impair Treg-specific inhibition of key innate immune drivers which show significant in driving
allergic disease. Additionally, there is an associated intrinsic loss in transcriptional regulatory drivers, e.g. FoxP3 expression (although tissue specific) of the Tregs with disruption of the IL-4Rα gene \(^{34}\). Moreover, combined Treg therapy in addition to the corticosteroid therapy treatment would probable help ameliorate type 2 innate immune cell driven allergic asthma while promoting tolerance \(^{339,340}\). We highlight that not only is there a temporal-dependence on effective anti-T\(_{H2}\) therapy in asthma patients but, the similarly disrupted T\(_{H2}\) environment sustains intrinsic regulatory functions of T regulatory cells during allergic disease.

4.1. Limitations and setbacks of the study
Interpretation of translational relevance remains limited by the experimental animal models utilised. Although limited, much has been learned with regards to the pathobiology of allergic disease \(^{341}\). The idea of allergy or atopic disease being the consequence of type-2 inflammation is rather simple and most clinical studies demonstrate that human T cells do not obviously polarized in a similar manner \(^{342}\). The various contributing factors result in a complex and somewhat variable pathogenesis between individual patients and in the same patient at different stages of their disease \(^{341}\). Partly this is due to the inherent difference amongst humans and mice in terms of the anatomical, physiological, and immunological contributors \(^{343,344}\). Specific difficulty is that most of AD models are acute and transient following initial allergen sensitisation and challenge, while human allergic disease develop gradually and chronic models are difficult to establish in mice thus a cautious interpretation of the mouse model results is necessary \(^{345}\).

Nonetheless, although no mouse model fully mimics the full range of clinical manifestations of the human allergic diseases, many do reproduce a collection of the features that characterize its most common forms (major allergic disease determinants).

Mouse models in allergy provide unique opportunities to gain insights into mechanisms of disease pathogenesis, which otherwise are impossible to perform in complex human populations. The predominantly used inbred nature of experimental mouse models offer reduced variability and high reproducibility on the same genetic background. This inbred genetic background might mask other key pathways in the diverse phenotypes seen in humans, due to
limitation in genetic diversity. Various endotypes of the heterogeneous nature of allergic asthma in humans clearly highlight the influence of genetic variability, thus experimental allergic asthma models utilising outbred mice are encouraged. Humanised mouse models are gaining momentum to try combat these caveats of using inbred experimental mouse models. On the other hand, genetic editing is far from being used as a treatment strategy which makes interpretation of gene knockout/deletion experiments, merely a report on a gene functionality in an intact immune system (animal models) instead of advice on medical intervention options.

4.2. Future Prospective

Considering the promising results obtained from this study, further investigations are necessary. With the protective outcome of temporal deletion of IL-4Rα prior to sensitisation or during active disease, immune responses to Th2 immune response directed comorbidities should be assessed to determine if there is impaired development of effective Th2 immune responses e.g. helminth infection. Susceptibility to Th17 and Th1 inflammatory diseases would also highlight the possible cross-effect in other immune responses. Most cases of asthma are already established disease cases, therefore there is a need to further track the fate of residual memory Th2 cell responses which remains even after amelioration of disease symptoms. Possible relapse is a likely outcome if the memory cells are long-lived and thus treatment should also involve targeting antigen-specific memory cells. The regulatory arm is a vital target for restoration of tolerance to allergens. Future studies should focus on tolerance models to assess the influence of IL-4 responsive T regs in suppressing aberrant innate type 2 responses both in vitro and in vivo. To delineate the innate specific targeted suppression by IL-4 responsive T regs, innate immune responses can be assessed in RAG-deficient mice, lacking a functional adaptive response. Adoptively transferring IL-4Rα deficient T regs into RAG-deficient mice sensitised with allergen will confirm the lack of suppression of ILC2 driven allergic disease. Intrinsic alteration to the suppressive function of Tregs can be assessed by sequencing for conserved non-coding sequences (CNS) methylated genes associated with FoxP3 stability and Treg suppressive functionality. The series of experiments will
unravel a key regulatory role on epithelial repair and hopefully direct treatment towards investigation of ways to restore barrier protection against allergens.

The thesis results reveal a previously unappreciated temporal function of IL-4Rα-signalling in protection against established allergic asthma and suggests potential outcomes and concerns of IL-4Rα-targeted therapy. Furthermore, this thesis highlights an important role of IL-4Rα-signalling in T regulatory cell function, in maintaining tolerance during allergic disease. Therefore, more strategic approaches, combining Th targeted therapy and maintaining tolerance to aero allergens are needed for treatment of allergic disease.

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