The effects of interleukin-17A on T helper 2 responses in allergic airways disease

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DEDICATIONS

I dedicate this thesis to my family.

To my parents, Tosh & Yukie for their loving and unending support;

my big brother Edward; my twin sister, Stephanie, and her family (Doug, Axton & Nozomi) and

to my amazing boyfriend of many years, Simon.

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ABSTRACT

IL-17A (IL-17), a cytokine associated with severe asthma in humans, both positively and negatively regulates Th2 responses in murine models of allergic airways disease. In addition to Th17 cells, γδ T cells have also been described as a source of IL-17 in some murine models of asthma. While Th17 cells appear to act as positive regulators, some evidence suggests that IL-17-γδ T cells act as negative regulators of Th2-induced allergic airway responses. In order to better understand the effect of IL-17 on Th2-induced immune responses, we addressed the role of IL-17 sourced from $CD4^+$ T cells and γδ T cells, and as a recombinant protein, using different murine models of allergic airways disease.

We first determined the primary source of IL-17 and the role of the adjuvants, alum and complete Freund's adjuvant (CFA) on IL-17 and Th2 inflammatory responses using an intraperitoneal ovalbumin (OVA) sensitization protocol. Following airway challenge, OVA/CFA sensitized mice had significantly greater serum levels of OVA-specific IgE and airway inflammation compared to OVA/sal and OVA/alum sensitized mice, but were comparatively airway hyporesponsive. Additionally, alum and CFA skewed the frequency of IL-17-producing bronchoalveolar lavage fluid (BALF) cells from Th17 toward IL-17-γδ T cells. Increasing the frequency of IL-17-γδ T cells in OVA/CFA sensitized mice, using a γδ TCR stimulatory antibody, inhibited airway hyperresponsiveness (AHR) and eosinophilia. Together these data provide evidence that IL-17-γδ T cells negatively regulate allergic airway responses.

We next examined how recombinant IL-17 modulated airway inflammatory responses induced by IL-13, considered the major effector cytokine of Th2 cells. Two different doses of recombinant IL-17 were co-administered intranasally with IL-13. The lower dose of IL-17

enhanced IL-13-induced airway eosinophilic and lymphocytic inflammation, whereas the higher dose of IL-17 attenuated IL-13-induced AHR and eosinophilic inflammation. The adoptive transfer of γδ T cells, stimulated to express IL-17, similarly attenuated IL-13-induced eosinophilic and lymphocytic inflammation. Thus, the dose of IL-17 influences its role as a positive or negative regulator, while IL-17-γδ T cells act as a negative regulator of IL-13-induced airway responses.

Finally, we used a T cell adoptive transfer model of allergic airways disease to examine how overexpressing IL-17, specifically by $CD4^+$ T cells, modulated airway inflammatory responses. Populations of OVA-stimulated, gene-modified T cells overexpressing IL-17 were generated by transduction with recombinant retroviruses encoding IL-17. Following adoptive transfer and airway OVA challenge, our data demonstrated that BALF levels of IL-17 positively correlated with AHR. Moderate increases in BALF IL-17 were sufficient to induce airway neutrophilia, while higher BALF levels of IL-17 were associated with AHR, inflammation and enhanced Th2 responses. Thus, antigen-induced IL-17 production by $CD4^+$ T cells positively regulates Th2-induced allergic airway responses.

RÉSUMÉ

L'IL-17A (IL-17), une cytokine associée à l'asthme sévère chez les êtres humains, régule à la fois positivement et négativement les réponses des cellules Th2 chez les modèles murins de la maladie respiratoire allergique. En plus des cellules Th17, les cellules T γδ ont aussi été décrites comme étant une source d'IL-17 chez certains modèles murins de l'asthme. Alors que les cellules Th17 semblent agir en tant que régulateurs positifs, certaines données suggèrent que les cellules T IL-17-γδ agissent comme régulateurs négatifs aux réponses allergiques des voies respiratoires induites par les cellules Th2. Afin de mieux comprendre l'effet de l'IL-17 sur les réponses immunitaires induites par les cellules Th2, nous avons abordé le rôle de l'IL-17 provenant des cellules T $CD4^+$ et cellules T γδ, et en tant que protéine recombinante, en utilisant divers modèles murins de la maladie allergique des voies respiratoires.

Premièrement, nous avons déterminé la source primaire de l'IL-17 et le rôle des adjuvants, l'alum et l'adjuvant complet de Freund (ACF) sur l'IL-17 et les réponses inflammatoires des cellules Th2 en utilisant un protocole de sensibilisation intra-péritonéale avec de l'ovalbumine (OVA). Suite à l'épreuve sur les voies respiratoires, l'inflammation respiratoire et les niveaux sériques d'IgE spécifique à l'OVA étaient significativement plus élevés chez les les souris sensibilisées à l'OVA/ACF en comparaison à celles sensibilisées à l'OVA/salin ou à l'OVA/alum, même si la réponse comparative des voies respiratoires était hyporéactive. De plus, l'alum et l'ACF ont influé la fréquence de la production d'IL-17 par les cellules de fluide de lavage broncho-alvéolaire (FLBA) des cellules Th17 vers les cellules T IL-17-γδ. L'augmentation de la fréquence des cellules T IL-17-γδ chez les souris sensibilisées à

l'OVA/ACF en utilisant un anticorps stimulant TCR γδ a inhibé l'hyperréactivité des voies respiratoires (HVR) et l'éosinophilie. L'ensemble de ces données fournissent la preuve que les cellules T IL-17-γδ régulent négativement les réponses allergiques des voies respiratoires.

Nous avons ensuite examiné comment l'IL-17 recombinante modulait la réponse inflammatoire des voies respiratoires induite par l'IL-13, qui est considérée comme étant une cytokine effectrice principale des cellules Th2. Deux doses différentes d'IL-17 recombinante ont été co-administrées par voie intranasale avec l'IL-13. La plus faible dose d'IL-17 a renforcé l'inflammation éosinophilique et lymphocytaire des voies respiratoires induite par l'IL-13 alors que la dose la plus élevée d'IL-17 a atténué l'HVR induite par l'IL-13 et l'inflammation éosinophilique. Le transfert adoptif des cellules T γδ, stimulées pour exprimer l'IL-17, a atténué l'inflammation éosinophilique et lymphocytaire induite par l'IL-13 de façon similaire. Ainsi, la dose d'IL-17 influence son rôle en tant que régulateur positif ou négatif tandis que les cellules T IL-17-γδ agissent en tant que régulateurs négatifs des réponses des voies respiratoires induites par IL-13.

Finalement, des populations de cellules T stimulées par de l'OVA, génétiquement modifiées et surexprimant IL-17 ont été générées par transduction avec des rétrovirus recombinants codant pour l'IL-17. Suite au transfert adoptif et à l'épreuve de l'OVA sur les voies respiratoires, nos données ont démontré que les niveaux d'IL-17 des cellules FLBA corrélaient positivement avec l'HVR. Des augmentations modérées d'IL-17 dans les cellules FLBA ont été suffisantes pour induire la neutrophilie dans les voies respiratoires alors que des niveaux plus élevés d'IL-17 dans les FLBA ont été associés à l'HVR, l'inflammation et les réponses renforcées par les cellules Th2. Ainsi, la production d'IL-17 induite par antigènes by les

cellules T $CD4^+$ régulent positivement les réponses allergiques des voies respiratoires induites par les cellules Th2.

PREFACE & CONTRIBUTION OF AUTHORS

The following is a manuscript-based thesis containing 5 chapters. Chapter 1 is the introduction, drawing on scientific articles to summarize historical and current knowledge on subjects that are related to the thesis. Chapters 2-4 are original scholarships and make distinct contributions to knowledge. The authors, Emily Nakada (E.N.), Jichuan Shan (J.S.), Margaret Kinyanjui (M.W.), Salman Qureshi (S.Q.) and Elizabeth Fixman (E.F.) are authors that have contributed to these chapters. Chapter 5 discusses the original scholarships in the context of our current understanding of the literature.

Chapter 2 contains the manuscript, "Adjuvant-dependent regulation of interleukin-17 expressing γδ T cells and inhibition of Th2 responses in allergic airways disease." E.N. assisted in the design of the study, drafted the manuscript and carried out all experiments, assays, acquisitions and analysis, with the following exceptions: J.S. carried out airway hyperresponsiveness measurements and analysis and performed the IL-13 immunoassay; MK. carried out some real time-PCR and assisted in the analysis of some flow cytometry results and statistics; and E.F. conceived of and assisted in the design of the study, coordinated and helped draft the manuscript.

Chapter 3 contains the manuscript, "Dose-dependent effects of IL-17 on IL-13-induced airway inflammatory responses and airway hyperresponsiveness." M.W. assisted in the design of the study, drafted the manuscript and carried out some of the experiments, assays, acquisitions and analysis. J.S. treated mice with recombinant proteins, carried out all airway hyperresponsiveness measurements and analysis, performed BALF total and differential cell counts. E.N. performed some real time PCR, isolated and adoptively transferred γδ T cells,

prepared BALF cells, performed and analyzed flow cytometry for the $TLR4^{-/-}$ and adoptive transfer experiments and prepared figures for these outcomes; S.Q. provided $TLR4^{-/-}$ mice and edited the manuscript; and E.F. conceived of and assisted in the design of the study, coordinated and helped draft the manuscript.

Chapter 4 contains the manuscript, "$CD4^{+}$ T cell specific IL-17 expression: dose-dependent effects of IL-17 in allergic airways disease." E.N. assisted in the design of the study, drafted the manuscript and carried out all experiments, acquisitions and analysis, with the following exceptions: J.S. carried out all airway hyperresponsiveness measurements and analysis; M.W. transfected the Phoenix-Ecotropic retroviral packaging cell line with the IL-17pAP2 vector; E.F. conceived of and assisted in the design of the study, coordinated and helped draft the manuscript.

ABBREVIATIONS

Ag	Antigen
AHR	Airway Hyperresponsiveness
Alum	Aluminum-based adjuvant (i.e. aluminum hydroxide, imject alum)
ASF	Alternative Splicing Factor
ASM	Airway Smooth Muscle
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
CCL17	TARC (Thymus and Activation Regulated Chemokine)
C/EBP	CCAAT-enhancer-binding protein
CFA	Complete Freund's Adjuvant
DEX	Dexamethasone, a glucocorticoid class of steroids
EAE	Experimental Autoimmune Encephalomyelitis
EGFP	Enhanced Green Fluorescent Protein
Env	Envelope, proteins in the lipid layer determining retroviral tropism
ERK	Extracellular-signal-Related-Kinase
F_{ab}	Antigen-binding fragment, variable region of an antibody
FeNO	Fraction of Exhaled Nitric Oxide
FEV_1	Forced Expiratory Volume in 1 second
FOXP3	Fork-head box P3
Gag	Group antigens, proteins making up the retroviral core
GATA3	GATA-binding protein 3
GSK3β	Glycogen synthase kinase 3 beta
HDM	House Dust Mite
ICS	Inhaled Corticosteroids

IFA	Incomplete Freund's Adjuvant
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
IN	Intranasal
iNK	Invariate Natural Killer
IP	Intraperitoneal
IRES	Internal Ribosomal Entry Site
IV	Intravenous
JNK	c-Jun N-terminal Kinase
LN	Lymph Node
LPS	Lipopolysaccharide, a component of Gram(-) bacteria, TLR4 agonist
MAPK	Mitogen-Activated Protein Kinase
miRNA	MicroRNA
Mtb	*Mycobacterium tuberculosis*
NALP	NACHT, LRR and PYD domains-containing protein
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Nucleotide-binding Oligomerization Domain
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
Phoenix-Eco	Phoenix-Ecotropic
PMA	Phorbol 12-Myristate 13-Acetate
Pol	DNA polymerase, containing reverse transcriptase, integrase

RNAi	Ribonucleic Acid interference
RORγt	Retinoic acid-related Orphan Receptor γt
RSV	Respiratory Synctial Virus
SC	Subcutaneous
SCID	Severe Combined Immunodeficiency
siRNA	Small interfering Ribonucleic Acid
STAT6	Signal Transducer and Activator of Transcription 6
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-beta
Th	T helper
TLR4	Toll-Like Receptor 4
TNF	Tumor Necrosis Factor
TRAF	Tumor necrosis factor Receptor-Associated Factor
TSLP	Thymic Stromal Lymphopoitin
WT	Wild type

CHAPTER 1: INTRODUCTION

1 Severe asthma

Asthma is a chronic respiratory disease that, although rarely fatal, affects 1-18% of the population of nations worldwide (1). This equates to 300 million people and 15 million disability-adjusted life years (the 25th leading cause) and places a significant burden on an economy from direct and indirect medical costs (2). Asthma is characterized by variable airflow limitation, wheezing, chest tightness and shortness of breath (1, 3) and is typically associated with a Th2 response, elevated serum immunoglobulin (Ig)E, mucus hypersecretion, airway hyperresponsiveness (AHR), inflammation and remodeling (4-8). Even so, asthma is a heterogenous disease influenced by both genetic and environmental factors (9). The ranges in severity (mild, moderate or severe), inflammatory profile (eosinophilic and non-eosinophilic), persistence (intermittent or persistent), immune response (Th2 and/or IL-17), trigger (i.e. aspirin, allergen, cold), age of onset (child or adult), etc… associated with asthma heterogeneity (10) have been used to subdivide asthma into subcategories.

Although 10% of asthmatics are estimated to fall into the severe asthma subcategory, these individuals place a disproportionately greater burden on the health care system (3). Severe asthmatics typically experience frequent and serious exacerbations and greatly reduced lung function and they require aggressive treatment to control asthma symptoms compared to their milder counterparts (9). Until recently, the term ‘severe’ was applied to patients with severe asthma symptoms, irrespective of whether they received or were responsive to treatment (11).

The European Respiratory Society and American Thoracic Society Task Force recently reviewed and updated the definition of severe asthma in 2013, which has since been published in the European Respiratory Journal (10). Severe asthma is now defined as a disease that requires treatment with high dose inhaled corticosteroids (ICS) combined with a secondary controller (and/or systemic corticosteroids) to prevent 'uncontrolled' asthma or which remains 'uncontrolled' despite this therapy. The updated term was designed to more precisely define this unique group of difficult-to-manage asthmatics.

Severe asthmatics, defined according to the previous (12) and updated definitions (9, 13), can still be further divided into smaller groups according to inflammatory phenotypes. Two populations emerge with distinct architectural, physiological and functional characteristics when severe asthmatics, based on the earlier definition, are subdivided according to eosinophilic versus non-eosinophilic inflammatory profiles (12). The eosinophilic severe asthma group has increased $CD3^+$, $CD4^+$, $CD8^+$, $CD86^+$ and TGF-β^+ cells, more mast cells and a thicker basement membrane. Regardless of the increases, non-eosinophilic severe asthmatics have a significantly lower FEV_1 (forced expiratory volume in 1 second) than individuals from the eosinophilic group. Severe asthmatics have also been subdivided based on airway colonizing microorganism(s) and airway neutrophils (14). Compared to other severe asthmatics, those with *Moraxella catarrhalis* or a member of the *Haemophilus* or *Streptococcus* bacterial genera as the dominant airway colonizing microorganism have more airway neutrophils, longer asthma duration and a lower FEV_1, post bronchodilator treatment. However, classifications based on phenotype can be problematic, with studies showing a lack of stability in inflammatory profiles in asthmatics after a period of 12 months or more (15). For example, in one study, asthmatics, many of whom were 'severe,' were assigned to clusters according to their phenotype based either on physiological

measurements or by sputum cellularity (16). After a period of one year, 42.3% of asthmatics that had been assigned to one cluster based on sputum cellularity as a biomarker changed from their initial cluster, while only 23.6% of asthmatics classified according to physiological measurements, including lung function, changed clusters. Therefore, physiological measurements, such as FEV_1 appear more stable than biomarkers, such as sputum inflammation, and should be combined with responsiveness to high dose ICSs to classify severe asthma.

Severe asthma studies are presently moving away from classifications based on phenotype, in favour of the endotype (13). Whereas a phenotype is defined as the combined characteristics resulting from the interaction of the genes of an organism with the environment, an endotype is a subtype of a disease, the end result of a pathophysiological mechanism or response to treatment. Thus, an endotype may consist of several phenotypes, while a specific phenotype can contribute to numerous endotypes. Therefore, with the updated definition, severe asthma has transitioned from a term characterized according to phenotype to the endotype. Six asthma endotypes were initially proposed based on the seven parameters: clinical characteristics, biomarkers, lung physiology, genetics, histopathology, epidemiology, and treatment response (17). Of the six asthma endotypes, at least four are applicable to severe asthma. These are: i) severe allergic asthma; ii) late onset, persistent eosinophilic asthma; iii) aspirin exacerbated airway disease and iv) the allergic bronchopulmonary mycoses endotype (9, 13). Wenzel et al. went on to characterize five endotypes specific to severe asthma: i) severe early onset allergic; ii) adult onset, persistently eosinophilic; iii) allergic bronchopulmonary mycoses; iv) obese female; and v) neutrophilic (9). Considering the diversity of endotypes and heterogeneity of severe asthma, immune responses in asthmatics are also likely to differ with each individual.

2 Th2 responses

$CD4^+$ T helper (Th)2 cells are a subset of adaptive immune cells that express the inflammatory cytokines, interleukin (IL)-4, IL-5 and IL-13 (18). Abundant data suggest that an aberrant Th2 response contributes to the pathophysiology of asthma. Atopic asthmatics have significantly greater numbers of Th2 cells in their bronchoalveolar lavage fluid (BALF) and in lung biopsies compared to normal controls (4). Even greater numbers of these cells are recruited following airway allergen challenge (5). The increase in BALF $CD4^+$ T cells coincides with their decrease in peripheral blood, providing evidence that $CD4^+$ T cells migrate to the airway lumen from the periphery (19). Moreover, the $CD4^+$ T cells that leave the peripheral blood and are recruited to the airways following allergen exposure, are of the Th2 subset of T helper cells (20).

Not only are Th2 cells significantly increased in the airways and lungs of asthmatics, but their number and frequency correlate with classic measurements used to assess asthma severity (7). The frequency of activated Th2 cells induced by allergen challenge positively correlates with the frequency of BALF eosinophils (5). The frequency of activated BALF $CD4^+$ T cells, in unchallenged asthmatics, is also positively correlated with the frequency of eosinophils, as well as asthma symptom scores, and negatively correlated with the percent predicted FEV_1 (a measure of asthma severity) and the concentration of methacholine causing a 20% fall in FEV_1 from baseline (a measure of AHR) (21). $CD4^+$ T cells may also be involved in airway remodeling as airway smooth muscle (ASM) cell proliferation and infiltration by $CD4^+$ T cells coincide with asthma severity and these cells are in direct contact (8). Though compelling, these human studies, nevertheless, show indirect evidence of Th2 involvement in human asthma. Further support comes from an interesting study by Tournoy et al. who used human-mouse chimeras to

directly assess the role of human T cells in a murine model of asthma (22). Severe combined immunodeficiency (SCID) mice were reconstituted with human peripheral blood mononuclear cells (PBMC) from healthy, non-asthmatics donors. Following sensitization and airway challenge, murine recipients developed AHR, which was attenuated with human IL-4/IL-13 and IL-5 neutralizing antibodies.

Murine models of allergic airways disease confirm Th2 cell involvement in asthma pathogenesis. Models in which activated, antigen-specific $CD4^+$ T cells skewed toward the Th2 phenotype are transferred into naïve recipients, followed by airway challenge, show augmented allergic airways responses including AHR, eosinophilia and mucus production (23-25). Furthermore, depletion of $CD4^+$ T cells prior to antigen challenge of sensitized mice, prevents AHR and recruitment of eosinophils (26, 27). Th2 cells also mediate airway remodeling with reduced subepithelial fibrosis and a trend toward reduced epithelial thickening in response to depletion of $CD4^+$ T cells in sensitized and challenged mice (27). Furthermore, in rats, adoptively transferred $CD4^+$ T cells induce increases in ASM mass, likely by contact dependent mechanisms (28). The following sections explore the roles of Th2 associated cytokines IL-4, IL-5, IL-9 & IL-13 on allergic airway responses.

2.1 IL-4

IL-4 is a Th2 cytokine that promotes differentiation and proliferation of Th2 cells from naïve, undifferentiated T helper (Th0) cells (29). IL-4 binds 2 receptors: (1) the type I IL-4 receptor (IL-4R), which consists of the IL-4Rα and common γ chain subunits and (2) the type II IL-4R (IL-13R) comprised of the IL-4Rα and IL-13Rα1 subunits. Both IL-4 receptors regulate

Th2-related gene expression (30). IL-4 is differentially expressed in asthmatics: IL-4 levels are increased in the serum and BALF of asthmatics compared to normal controls (31, 32). Moreover, atopic asthmatics that receive recombinant human IL-4 exhibit airway hyperresponsive to methacholine and have increased eosinophils in the sputum, compared to recipients of control protein (33). IL-4 and IL-4R polymorphisms have been linked to severe asthma including the IL-4*-587T allele, which appears more frequently in the near fatal and fatal asthmatic group compared to the mild-to-moderate asthmatic and control groups (34, 35). Finally, clinical trials are underway using IL-4/IL-13 antagonists, solubilized recombinant IL-4Rα as well as IL-4Rα neutralizing antibodies to target the IL-4/IL-13 signaling pathways in order to determine their potential to treat asthma (36-39). The IL-4Rα-binding monoclonal antibody, dupilumab, was shown to be effective in significantly reducing asthma exacerbations, improving lung function and reducing biomarkers associated with a Th2 inflammatory response, after slow tapering off of treatment with ICSs (39). The solubilized human IL-4R, pitrakinra, was also able to reduce exacerbations and improve symptoms of asthma in patients with eosinophilic airway inflammation (38).

The contribution of IL-4 to the pathogenesis of asthma has been confirmed in animal models. IL-4 is significantly increased in the airways, lungs and BALF of allergen-exposed mice (40-42). In addition to differentiating Th0 cells into Th2 cells, IL-4 also enhances survival of activated T lymphocytes by upregulating expression of cell survival proteins such as Bcl-2 (43). Compared to wild-type mice, ovalbumin (OVA) sensitized and challenged IL-4-deficient mice have significantly fewer airway eosinophils and are airway hyporesponsive (44). Although IL-4 plays an important role in inducing several of the pathological features of allergic airways disease, it is unclear whether it induces these responses through IL-13. IL-4

activates signal transducer and activator of transcription (STAT)6, a transcription factor that regulates expression of several Th2 related genes including IL-4 and IL-13 (42). Perkins et al. addressed this by instilling recombinant IL-4 into the airways of IL-13 deficient (IL-$13^{-/-}$) mice (45). IL-$13^{-/-}$ mice attained similar levels of airway influx of eosinophils, AHR and goblet cell hyperplasia in response to IL-4 as the wild-type mice. However, van Panjuys and colleagues used Th2 ear infection models to demonstrate that both IL-4 and STAT6 are dispensable for Th2 cell differentiation, suggesting a separate, compensatory or overlapping pathway in differentiating Th2 cells (46). Although STAT6 and IL-4 were similarly dispensable in differentiating Th2 cells in a keyhole limpet hemocyanin model of allergic airways disease, mice deficient in either protein have significantly reduced levels of serum IgE and airway eosinophils. Overall, murine studies of allergic airways disease indicate that IL-4 plays an important role in the differentiation and proliferation of antigen-specific Th2 cells, is required at sensitization and may be sufficient, at airway challenge, to induce allergic airways disease (46-48).

2.2 IL-5

Levels of IL-5 are significantly increased in the BALF of patients suffering from lung diseases associated with an aberrant airway eosinophilic response, including asthma (31). IL-5 is expressed at higher levels in bronchial biopsies of asthmatics compared to non-asthmatic controls and positively correlates with eosinophil counts (49). In early clinical trials, asthmatics treated with monoclonal antibodies against IL-5 had reduced serum and sputum eosinophils, but showed little if any improvements in physiological responses (50, 51). However, more recent

studies show a significant improvement in asthma symptoms, including exacerbations, in patients with refractory eosinophilic asthma (patients with greater than 3% eosinophils in the BALF despite ICS treatment) (52). Similar to the human data, sensitized and challenged mice have significantly increased BALF IL-5 expression compared to controls (53). Murine recipients of IL-5 expressing Th2 cells show an increase in airway eosinophils that is attenuated with a neutralizing α-IL-5 antibody (54). Furthermore, IL-5 is dispensable for mucus production (25), but its role in regulating AHR in experimental models of allergic airways disease remains unclear (53, 55).

2.3 IL-9

IL-9 was originally considered to be a Th2 cytokine (56, 57). However, it has more recently been described as the major effector cytokine for a novel subset of T helper cells called Th9 (58-60). $CD4^+$ T cells are the major source of IL-9, a glycoprotein made up of 144 amino acids that binds the IL-9 receptor, consisting of the IL-9Rα and common γ-chain subunit (57, 58). Naïve $CD4^+$ T cells differentiate into Th9 cells through the combined effects of TGF-β and IL-4, while Th2 cells are similarly skewed toward Th9 in the presence of TGF-β (57, 59).

Whether IL-9 is considered to be a Th2 or Th9 cytokine, BALF IL-9^+CD3^+ T cells and IL-9 mRNA levels from bronchial biopsies are increased in atopic asthmatics compared to atopic non-asthmatic and healthy controls (61). IL-9 mRNA levels also positively correlate with AHR and negatively correlate with FEV_1. Transgenic mice that are induced to or constitutively express IL-9 in the lungs have increased AHR, BALF eosinophils and lymphocytes, airway epithelial mast cells, collagen deposition, epithelial cell hypertrophy, mucus production and Th2 cytokine

expression (56, 62). In contrast, IL-9 deficient mice sensitized and challenged with OVA have similar airway pathologies as wild type mice including airway inflammatory profiles, mucus production, total serum IgE and BALF IL-4, IL-5, IL-13 and eotaxin levels (63). Altogether, these studies suggest that IL-9 has a positive regulatory effect on allergic airways responses that, in its absence, is adequately compensated for by alternative pathways.

2.4 IL-13

IL-13 is considered to be the main Th2 effector cytokine in the pathogenesis of asthma. IL-13 mRNA and/or protein expression are significantly increased in the bronchial mucosa, sputum, mononuclear BALF cells and ASM bundles of asthmatics compared to non-asthmatic controls and their levels correlate with asthma severity (64-68). Furthermore, IL-13 transcript and protein are increased in the BALF of asthmatics, following allergen challenge, compared to saline challenged allergic patients (68). Specific polymorphisms in the IL-13 gene have also been identified as risk factors for asthma (69). In clinical trials that use a monoclonal αIL-13 antibody, asthmatics have shown significant improvements in lung function compared to those who received a placebo, indicating its importance as a mediator of human asthma (70, 71). IL-13 stimulation of cells from a human airway epithelial cell line upregulates mRNA expression of three biomarkers called periostin, chloride channel accessory 1 (CLCA1) and Serpinβ2 (72). Based on gene expression microarrays, these three biomarkers are also upregulated in human airway epithelial cells from asthmatics compared to controls, and are attenuated in response to treatment with corticosteroids (72). Furthermore, serum periostin levels are significantly higher in asthmatics with a higher frequency and density of eosinophils in their BALF and tissue,

respectively, while periostin, CLCA1 and Serpinβ2 mRNA levels from airway epithelial cells are higher in asthmatics with a Th2-high airway inflammatory profile versus asthmatics with low numbers of Th2 airway inflammatory cells (73, 74). Overall, the data indicate that serum and/or airway epithelial cell expression of periostin, CLCA1 and Serpinβ2 are IL-13-induced biomarkers that can be used to identify eosinophilic, Th2-high and corticosteroid responsive asthmatics.

IL-13 is induced in the BALF and lungs of allergen exposed mice (75-77). It is sufficient to induce AHR, eosinophilia, lung IL-17, mucus and total serum IgE production in naïve, non-airway challenged mice (78-80). In addition to decreases in AHR and mucus secretion (78), eosinophil counts are significantly attenuated in OVA immunized and challenged mice, following treatment with a soluble IL-13Rα2 inhibitor (80). Stimulation of the type II IL-4R (IL-13R) by either IL-4 or IL-13 activates the signal transducer and activator of transcription (STAT)6 pathway, which positively regulates expression of Th2 related genes including positive feedback regulation of IL-4 and IL-13 (48). While it was initially difficult to determine whether IL-13-induced responses were truly independent of IL-4, this was shown to be the case by Grunig et al. who demonstrated that IL-4 deficient mice treated with recombinant IL-13 retained mucus induction (80). Furthermore, by transferring wild type and IL-13 deficient Th2 cells into pre-airway challenged naïve mice, Mattes et al. showed that IL-13 from Th2 cells plays an important role in regulating AHR, eosinophilia and mucus production (81). The same study showed that with the exception of mucus, the remaining features are IL-4Rα-independent, but STAT6-dependent, suggesting a novel IL-13 pathway that does not require IL-4 signaling. Overall, IL-13 is considered to be the primary Th2 effector cytokine, sufficient at inducing all of the classical features associated with asthma, even in naïve mice (48).

2.5 Eosinophils

Considering the strong association that asthma has with eosinophils, it is important to define the specific role they play in the pathogenesis of asthma. Fattouh et al. have examined the role of eosinophils in a house dust mite (HDM) model of allergic airways disease using two strains of eosinophil deficient mice and an eosinophil neutralizing antibody (82). The neutralizing antibody eliminated more than 80-85% of BALF and lung eosinophils, but had no effect on airway remodeling or the recruitment of other inflammatory cells including Th2 cells, compared to appropriate controls. The two knockout strains, which had no eosinophils (including any residual cells that would not have been neutralized with the antibody) had fewer BALF mononuclear and lung Th2 cells, but similar levels of AHR and remodeling (smooth muscle, goblet cells and collagen deposition) compared to respective controls. In addition, serum HDM-IgE levels, IL-5 and IL-13 produced by HDM cultured splenocytes were similar to control values. In a different study, it was determined that the induction of allergic airway responses in OVA and HDM sensitized and challenged, eosinophil conditional knockout mice does not depend on the presence of eosinophils at the time of sensitization (83). However, the ablation of eosinophils immediately preceding and for the duration of the airway challenges results in fewer total BALF cells, a neutrophilic airway inflammatory profile and corticosteroid insensitivity, but similar levels of BALF IL-13, goblet cell metaplasia and AHR to that of wild type controls. Thus, the absence of airway eosinophils at sensitization does not appear to significantly affect allergic airway responses, whereas ablation of eosinophils during the challenge phase induces a neutrophilic airway inflammatory profile associated with an insensitivity to corticosteroid treatment.

2.6 Th2 responsiveness to corticosteroids

For the majority of asthmatics, symptoms of disease are well controlled by conventional therapies, primarily ICS treatment (84, 85). Corticosteroids enter the cell and bind cytosolic glucocorticoid receptors that are then released from complexes with chaperone proteins before translocating to the nucleus (84). The activated receptors bind the promoter regions of anti-inflammatory genes to positively regulate their expression and/or bind pro-inflammatory transcription factors, such as nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) and STAT proteins, leading to the recruitment of corepressor proteins that inhibit the transcription of pro-inflammatory genes (84, 86). Corticosteroid insensitivity in some asthmatics may be due to reduced glucocorticoid receptor affinity for the ligand, altered receptor binding of DNA, reduced corepressor protein expression and recruitment and/or increased expression of pro-inflammatory transcription factors (13, 84, 85). Th2 inflammatory responses in asthmatics are typically attenuated by ICS treatment (67, 87). The improved FEV_1 in ICS responsive asthmatics coincides with a decrease in IL-13 $mRNA^+$ and an increase in IL-12 $mRNA^+$ cells from bronchial biopsies (67). Asthmatics treated with the corticosteroid, budesonide, also show reduced AHR and sputum eosinophilia (88-90). Th2 inflammatory responses in murine models of allergic airways disease are similarly attenuated by corticosteroids (91-93). Intranasal delivery of budesonide to OVA sensitized and challenged mice reduces bone marrow, peripheral blood and BALF eosinophils, serum and BALF IL-5 and AHR (92). Furthermore, antigen sensitized and challenged mice treated with corticosteroids have decreased goblet cell hyperplasia (94), which in one study was shown to rebound within 24 hours of corticosteroid cessation to a level higher than untreated, antigen sensitized and challenged mice (93). The rebound in goblet cell hyperplasia coincided with a comparable increase in IL-13 protein in the BALF. These data

indicate that corticosteroids can attenuate many of the pathological features associated with allergic airways disease, in particular those relating to Th2 responses, including airway eosinophilia, Th2 cytokine expression, goblet cell hyperplasia and AHR. Nevertheless, not all asthmatics are responsive to (high dose) ICSs (85, 87). More recent human and murine data suggest that corticosteroid insensitivity is closely linked with increases in airway neutrophils and IL-17 (89, 95). Severe asthma and the role of IL-17 in murine models of asthma are discussed in section 3.1.1.

3 Interleukin-17 responses

3.1 IL-17A

IL-17A is one of six members of the IL-17 family of cytokines and is the major cytokine conducting the effector phase of the Th17 response (96). It shares the highest homology, 50%, with IL-17F, another cytokine from the IL-17 family with a similar, but more moderate level of pathological activity to that of IL-17A (96, 97). Both cytokines bind the IL-17 receptor (IL-17R) consisting of the IL-17RA and IL-17RC subunits (98). Stimulation of the IL-17R involves the adaptor molecule, Act1, which activates downstream pathways including NFκB and MAPK that regulate expression of anti-microbial peptides, cytokines and chemokines (98)**.** IL-17E, otherwise known as IL-25, shares 25-35% of its homology with members of the IL-17 family, is a Th2 cytokine and contributes to the pathogenesis of asthma by promoting expression of other Th2 cytokines such as IL-4, IL-5 and IL-13 (97, 98). Much less is known of the three remaining IL-17 family of cytokines: IL-17B, IL-17C and IL-17D, which share 23-39% homology with IL-17A, but there is some evidence of their participation in regulating inflammatory responses (97, 98). IL-17A, hereafter referred to as IL-17, is a pro-inflammatory cytokine involved in host defense against extracellular bacterial and fungal infections, including *Staphylococcus aureus* and *Candida albicans* (99). However, an aberrant IL-17 response can lead to the development of an inflammatory disease such as the autoimmune diseases rheumatoid arthritis, multiple sclerosis and psoriasis (100-102), or an inflammatory airway disease like asthma (103, 104).

Prior to the discovery of IL-23, Th1 cells were thought to be the dominant T helper subset involved in the pathogenesis of autoimmune diseases (105). While IL-12 differentiates

naïve T cells into Th1 cells, IL-23 promotes pathological activity from differentiated Th17 cells, at least in part, through the induction of IL-17 (**Fig. 1.1**) (106). IL-12 and IL-23 share the IL-12p40 subunit, which dimerizes with the IL-12p35 or IL-23p19 subunit to form the active IL-12 and IL-23 cytokines, respectively (107). When clinical signs of disease in animals with experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, were attenuated with a neutralizing antibody against the IL-12p40 subunit, the data was initially interpreted to be due to the neutralization of active IL-12 (106, 108). Follow-up studies did not appear to support this conclusion (107, 109). While IL-12p35 deficient mice were prone to EAE development, mice deficient in IL-12p40 were resistant, suggesting that a molecule consisting of the IL-12p40 subunit, besides IL-12, was critical in EAE development (107). The Th17 related cytokine, IL-23, was subsequently identified as the molecule responsible (109, 110) and redirected focus to the Th17 subset of T helper cells.

IL-17 is increased in the lungs, sputum, BALF and sera of asthmatics (103, 111-114) and is linked to corticosteroid insensitivity (85). Vazquez-Tello et al. stimulated human peripheral blood mononuclear cells (PBMC) with IL-17/IL-17F and IL-23 and showed upregulation of glucocorticoid receptor β, an isoform and dominant-negative regulator of glucocorticoid receptor α,that leads to steroid insensitivity (84, 85). Combined with the recently updated definition of severe asthma (10), it follows that IL-17 expression correlates with asthma severity (111). Specific polymorphisms in the IL-17 gene are also associated with asthma (115-117) and an increase in the level of serum IL-17 is an independent risk factor for the disease (113, 118). IL-17 is specifically associated with AHR (111), mucin gene expression (119) and airway neutrophilia (114, 120, 121). Neutrophils are, in themselves, associated with severe asthma, whereas eosinophils are more closely associated with the mild to moderate phenotype (12, 122).

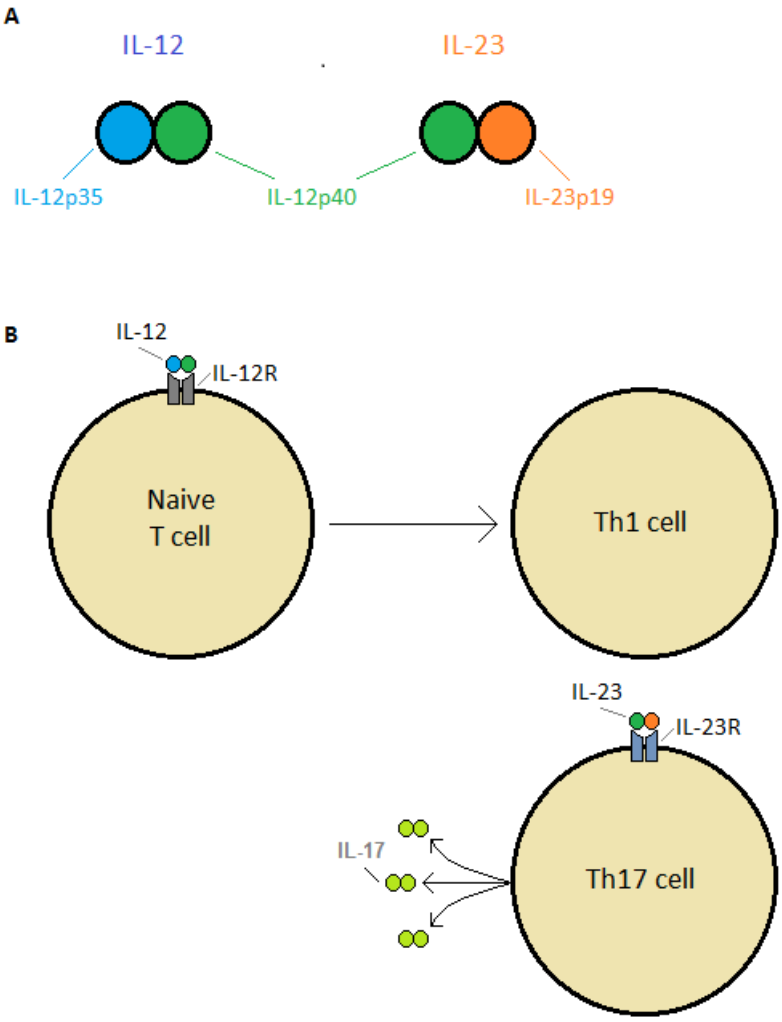
Despite the abundance of evidence corroborating the role of IL-17 as an important mediator of (severe) asthma (112, 113, 118), a clinical trial using a monoclonal human IL-17RA antibody that blocks the biological activities of IL-17, IL-17F and the Th2 cytokine, IL-17E, failed to improve asthma symptoms or lung function (123). However, conclusions specific to the role of IL-17 in human asthma are complicated by the drug's ability to block multiple signaling pathways besides those specific to IL-17. Moreover, the study did show some promising results in the treatment of a high-reversibility asthma subgroup.

Human asthma studies reporting an increase in IL-17 do not always state the cellular source of the cytokine (111, 124) and often suggest Th17 cells without co-localization experiments to confirm this assumption (112, 120). Where cellular sources of IL-17 have been identified, Th17 cells and IL-17-expressing populations of $CD8^+$ T cells, γδ T cells, neutrophils and eosinophils are all confirmed producers (103, 125-128). The source may be important since animal models of asthma indicate that IL-17 plays a dual role as both a positive and negative regulator of allergic airways disease (95, 129). It also remains unclear whether the increase in IL-17^+ cells in human asthma contributes directly to pathogenesis and/or severity or is in response to disease, recruited in order to attenuate and resolve asthma.

Experimental models of asthma indicate that IL-17 plays a dual role in allergic airways disease and that the timing and cell source may be factors that influence this activity. Temporally, when IL-17 signaling is absent at sensitization, following airway challenge, IL-17 knockout mice have lower levels of antigen-specific IgG_1 and IgE, reduced AHR and inflammation than wild type animals (130). Similarly, IL-17 signaling, which was also absent at sensitization in IL-17RA deficient mice are less airway hyperresponsive and inflamed than wild type mice (129). However, IL-17E (IL-25) signaling was also inhibited in these mice

Figure 1.1: Interleukin-12 & Interleukin-23 have an IL-12p40 subunit

IL-12 is a cytokine consisting of an IL-12p35 and IL-12p40 subunits. IL-23 also contains an IL-12p40 subunit, which is heterodimerized to the IL-23p19 subunit (A). IL-12 binds the IL-12 receptor on naïve T cells to differentiate them into Th1 cells, whereas IL-23 binds the IL-23 receptor on already differentiated Th17 cells to induce IL-17 expression.



since the IL-17RA subunit that combines with the IL-17RC subunit to form the functional IL-17 receptor, also heterodimerizes with the IL-17RB subunit to formulate the IL-25 receptor, thereby confounding interpretation of these results. In contrast, Newcombs et al. using a different strain of IL-17 deficient mice in an OVA/respiratory syncytial virus (RSV) model of allergic airways disease found that in the absence of IL-17 at sensitization, allergic airways responses are not only established, they are enhanced in comparison to wild type mice (131). In addition to sensitization, these and other studies show delivery of IL-17 at the effector phase of allergic airways disease can have both positive and negative regulatory effects (95, 129, 131, 132). These contradictory data indicate that the timing of IL-17 expression may not be the major factor determining whether IL-17 acts as a positive or negative regulator of asthma.

There is accumulating evidence that the cellular source producing IL-17 influences the outcome of IL-17 in allergic airway responses. Th17 cells are traditionally viewed as the major source of IL-17 in animal models (133). However, there is some evidence supporting macrophages (134), natural killer T cells (135, 136) and increasingly more data supporting γδ T cells as the primary source of IL-17 in allergic airways disease (129, 137). Here, I discuss the role of IL-17 in animal models, specifically originating from Th17 cells and γδ T cells, as well as in the form of recombinant protein.

3.1.1 Th17 cells

Th17 cells are a subset of T helper cells producing IL-17, IL-17F and IL-21 (138). They were first identified as a unique and separate subset from Th1 and Th2 cells by Infante-Duarte et al. using cells from OVA transgenic mice and confirmed this population of IL-17-expressing T

helper cells in the synovial fluid of arthritic patients, which was increased in comparison to normal controls (139). This was followed by studies confirming an IL-23 requirement to promote pathogenic IL-17 activity, while being dispensable for the differentiation, of Th17 cells (100, 140, 141). In some murine models of allergic airways disease, Th17 cells have been established as the major cellular source of IL-17 and are specifically associated with positively regulating inflammatory responses (133, 142). Adoptive transfer of Th17 cells into naïve or SCID mice induces airway neutrophilia, but not AHR or eosinophilia (95, 143). Zhao et al. used CD4-CreXgp130$^{flox/flox}$ mice to further examine the role of Th17 cells in an OVA model of allergic airways disease (137). These mice have $CD4^+$ T cells that are missing glycoprotein130, a transmembrane protein that complexes with the IL-6 receptor to mediate downstream IL-6 signaling, which is required for Th17 cell differentiation. Following OVA challenge, these Th17 deficient mice have significantly fewer BALF neutrophils and less airway remodeling, as assessed by reductions in collagen deposition and α-smooth muscle actin staining compared to wild type controls, despite having higher numbers of BALF Th2 cells and IL-13.

Immune responses induced in the airways by Th17 cells appear to not only be insensitive to, but may be aggravated by, steroid treatment. Under Th17 polarizing conditions, the addition of the steroid, dexamethasone (DEX), enhances Th17 cell differentiation (137). Whereas Th2 cytokine expression, goblet cell hyperplasia, AHR and recruitment of lymphocytes and eosinophils induced by the transfer of Th2 cells are attenuated by DEX, Th17 cytokine expression, goblet cell hyperplasia and airway recruitment of neutrophils induced by the transfer of Th17 cells are unaffected (95, 144). In fact, airway recruitment of neutrophils may be enhanced with DEX treatment in recipients of Th17 cells (95). Martin et al. observed that *in vitro* antigen-induced IL-17 production from lung cells harvested from recipients of adoptively

transferred Th17 cells was unaffected by DEX treatment. However, IL-17 production was sensitive to DEX treatment when lung cells were harvested from mice treated with NO_2/OVA (which induced endogenous Th17 cells). These data suggest that the lack of responsiveness to DEX treatment induced by *in vitro* differentiated Th17 cells may not accurately reflect the responsiveness to steroid treatment by endogenous Th17 cells (144). In partial support, Zhao et al. showed that collagen deposition and the recruitment of endogenous Th17 cells and eosinophils generated by mucosal sensitization of mice with OVA are significantly decreased with DEX, following chronic OVA challenge of the airways (137), whereas *in vitro* differentiation of Th17 cells, cultured under Th17-skewing conditions, is significantly enhanced by DEX. Jacobsen et al. conducted a study examining the role of eosinophils on DEX sensitivity using conditional eosinophil knockout mice (83). OVA (or HDM) sensitized and challenged conditional eosinophil deficient mice had similar AHR and recruitment of Th2 cells as wild type mice, while presenting with fewer BALF eosinophils and total cells, as well as more neutrophils. While wild type mice were responsive to DEX, eosinophil deficient mice were insensitive, that is until the eosinophils were restored in these mice, in response to an additional airway antigen challenge.

Th17 cells and regulatory T (T_{reg}) cells share a close relationship, in terms of development and the reciprocal effects they impose on one another. TGF-β is central to T_{reg} cell development (145) and combined with IL-6, differentiates murine Th17 cells (146, 147). Fork-head box p3 (Foxp3) and retinoic acid-related orphan receptor (ROR)γt are the master transcription factors regulating T_{reg} and Th17 cell differentiation, respectively (148, 149). TGF-β differentiates Th17 cells from a separate population of $CD4^+$ T cells than the Foxp3 expressing population of T_{reg} cells (146), via IL-6 suppression of TGF-β-induced Foxp3 expression (141).

Under Th17-skewing conditions, T cells are also reported to have an imbalance of these transcription factors such that insufficient Foxp3 is available to physically interact with and thus inhibit RORγt binding of the IL-17 promoter (149). Additional studies show a reciprocal decrease in frequencies of T_{reg} cells in groups of asthmatics coinciding with an increase in frequencies of Th17 cells (150, 151). Finally, Zhao et al. were able to break tolerance to OVA by co-administering OVA intranasally with IL-17. This results in increased airway eosinophils and T cells, in addition to reduced T_{reg} cell proliferation (137).

3.1.2 IL-17-γδ T cells

The γδ T cell is an innate cell involved in immune regulation including host protection against microbial infection and maintenance of normal responses in non-infectious disease (152). These cells are highly conserved and make up anywhere from 0.5-5.0% of all $CD3^+$ T cells in adult humans and rodents alike (152). They are also preferentially localized to mucosal tissue, including the lungs, where they quickly respond to infection (153, 154). They play an important role in viral and bacterial infections including influenza, *Staphylococcus aureus*, *Escherichia coli* and *Mycobacterium tuberculosis* (155). Mice that are γδ T cell deficient and intranasally instilled with *Nocardia asteroides*, an intracellular Gram(+) bacteria, quickly succumb to infection, whereas wild type mice survive infection (156). Airway recruitment of polymorphonuclear cells and macrophages is severely compromised in these mice.

The activation of γδ T cells in infection models provides evidence that evolutionarily conserved molecules derived from microbes act as the primary ligands for the γδ TCR. In reality, the specific ligand(s) that bind the γδ TCR are unclear. The adoptive transfer of $4x10^4$ γδ T cells

from OVA-tolerized mice reduces IgE production in mice that are OVA airway challenged, but not in mice challenged with Derp1, an antigenic component of HDM (152, 157). These data provide evidence of γδ TCR specificity for a unique ligand (OVA-specific); suppressive (rather than inflammatory) activity of γδ T cells; as well as alternative ligands (as opposed to pathogen-derived) for the γδ TCR. It is now generally accepted that molecules derived from the host (rather than pathogens), that are quickly upregulated in response to stress, act as the primary γδ TCR ligands (155, 158). This is further supported by data from a lung injury model induced by acute airway ozone exposure in which γδ T cell deficient mice show increased epithelial necrosis (152, 156). The authors speculate that endogenous stress molecules, released by the injured epithelium, bind to and activate the γδ TCR. Finally, as was previously mentioned, γδ T cells can have both pro-inflammatory and protective activity, depending on the circumstances of their activation (152, 156, 157). The assorted number of γδ T cell subsets, their inherent activity, their preference for specific tissues/organs and the conditions of their induction likely play an important role in determining the overall γδ T cell response (152, 159). Subsets of γδ T cells are typically categorized according to the γ and/or δ subunits that make up the TCR, but are also often categorized according to cytokine expression, predominantly IFN-γ and IL-17, which may overlap with specific γ and/or δ subunits (160-162). Although the γ and δ nomenclature system created by Garman et al. (163) is in common use, I have used the Heilig & Tonegawa system (161), similar to other labs that commonly publish using murine models of asthma (132, 152).

We and others have shown that IL-17-γδ T cells are the primary source of IL-17 in some models of allergic airways disease (79, 132, 164). It has been reported that 64% of lung IL-17-γδ T cells are of the Vγ4 subset and that the population of Vγ4 cells increases in frequency from birth so that at sexual maturity, the pulmonary γδ T cell population is primarily made up of the

Vγ4 subset (165). In addition, the frequency of IL-17 expressing Vγ4 cells in the lymph nodes can be increased by sensitizing mice with an IP injection of complete Freund's adjuvant (CFA) (166). In contrast to Th17 cells, IL-17-γδ T cells are closely associated with the negative regulation and resolution of allergic airway responses (132, 167). Adoptive transfer of IL-17 sufficient γδ T cells, $V\gamma4^+$ γδ T cells or recombinant IL-17 into sensitized and airway challenged mice attenuates AHR, inflammation and Th2 responses that are not otherwise attenuated by the transfer of IL-17 deficient γδ T cells or Th17 cells (132), indicating the importance of IL-17 producing γδ T cells, rather than the γδ T cell itself, in resolving airway inflammatory responses. The precise mechanism(s) by which IL-17 produced by Th17 cells vs. γδ T cells appears to exhibit opposing activity in allergic airways disease is/are poorly understood. However, spatially, γδ T cells are located in close proximity to the bronchial epithelium (159), and this could account for some of the differential effects of IL-17 in airway diseases.

3.1.3 Recombinant IL-17

Recombinant proteins are commonly used to study the role of specific mediators in experimental models of disease. Several systems of producing large quantities of highly purified recombinant proteins are available (168). The most popular system is *E. coli*, a Gram(-) strain of bacteria that produces large quantities of proteins in a short period of time and at a low cost compared to other systems (168, 169). However, due to its highly reduced cytoplasmic environment, it is a poor choice for producing proteins that require post-translational modifications to maintain biological activity. Yeast, insect and mammalian cell-derived recombinant protein expression systems each have their benefits and limitations (168). One of

the shared benefits of these organisms is their ability to perform post-translational modifications including phosphorylation of amino acid residues and protein glycosylation. Chapters from this thesis, specifically chapters 2 and 3, use murine recombinant IL-13 and/or IL-17 proteins derived from *E. coli*.

Recombinant IL-17 is commonly used to study IL-17 induced responses in murine models of asthma (129, 132, 142, 170, 171). While IL-17 sourced from Th17 and IL-17-γδ T cells consistently regulate airway responses in a positive and negative manner, respectively, results based on experiments performed with recombinant IL-17 are inconsistent. Recombinant IL-17 has been shown to act as a negative regulator of asthma when given during the effector/challenge phase and can improve resolution of allergic airway responses post-airway challenge (129, 132, 170). Conversely, recombinant IL-17 can also act as a positive regulator. ASM cells within the tracheal rings of mice and humans, stimulated for 12 hours with recombinant IL-17 have increased contractile force and airway luminal narrowing in response to methacholine challenge (171). Given during the challenge phase of an acute OVA allergy model, recombinant IL-17 has also been shown to enhance AHR and neutrophilia (142). The precise reasons for these differences are unclear, but may be related to details concerning their delivery, including the timing and dose, the number of instillations and the route of delivery. Several of these factors are addressed within chapters 2-4 of the thesis, as well as in the discussion.

3.1.4 Alternative cellular sources of IL-17 in the lungs

In addition to Th17 and γδ T cells, several additional cellular sources of IL-17, from both the adaptive and innate arms of immunity, have been implicated in airway inflammatory responses in asthma models (132, 134, 172, 173).

3.1.4.1 Alveolar Macrophages

Song et al. report that macrophages; not T cells, B cells, mast cells or NK cells, are the major cellular source of IL-17 in an OVA sensitized and airway challenged, murine model of allergic airways disease (134). In this study, $CD11b^{+}F4/80^{+}$ alveolar macrophages, but not interstitial macrophages were determined to be the major source of IL-17 in the lungs. Furthermore, they provided evidence that the increase in IL-17-expressing alveolar macrophages was due to mediators released from OVA-IgE activated mast cells – as opposed to recruitment of new cells. Finally, depleting alveolar macrophages with a chemical (clodronate) or treating mice with a neutralizing IL-17 antibody, reduced both AHR and inflammation, implicating alveolar macrophage derived IL-17 in these responses (134). In contrast to these data BALF macrophages from a LPS-induced murine model of asthma, in which neutrophils are the predominant inflammatory cells in the airways, did not express detectable levels of IL-17mRNA (174).

3.1.4.2 Natural Killer T cells

Natural killer (NK) T cells, which play an important role in tissue surveillance and which have also been implicated in asthma pathogenesis (despite constituting fewer than 1% of murine lymphocytes) may also be an important source of IL-17 in the inflamed allergic lung (172, 175,

176). NKT cells have combined features of both the classical T cell and natural killer (NK) cell and were initially characterized by their co-expression of the αβ-TCR and NK receptor, NK1.1. They are now generally classified as CD1d-restricted T cells with reactivity to α-galactosylceramide (αGalCer) (176). NKT cells are divided into two subgroups, according to their TCR, which are the type I or invariant natural killer (iNK) T cells that express an invariant TCR - Vα14-Jα18 in mice (or Vα24 in humans) and type II NKT cells, which have variable TCRs (176, 177). They develop in the thymus, are reactive to self-ligands and antigens presented by CD1d molecules and unlike conventional thymocytes, are generally mature and quick to migrate to sites of inflammation (176). These cells are most notable for their ability to rapidly produce large quantities of inflammatory cytokines, such as IL-4, IFN-γ and IL-17, to regulate and amplify immune responses (178, 179).

iNKT cells are present in the lungs of human asthmatic and OVA sensitized and challenged mice (173, 180, 181). Jα281$^{-/-}$ and CD1d$^{-/-}$ mice, both deficient in iNKT cells, sensitized and challenged with OVA have fewer airway eosinophils, AHR and serum OVA-specific IgE, which is restored by the transfer of wild type iNKT cells (181). iNKT cell deficient mice co-sensitized with OVA and HDM have significantly attenuated immune responses including decreases in total cells and eosinophils in the airways, serum OVA-specific IgE and IgG_1 antibodies, and reduced IL-4, IL-5, IL-13, IL-10, IL-17 and IFN-γ production from bronchial lymph node cells re-stimulated with OVA in comparison to wild type controls (136). Coquet et al. identified the CD4$^-$ subset of iNKT cells as IL-17 expressing (182). Compared to polydTA/αGalCer treated control mice, intratracheal co-administration of poly(I:C)/αGalCer induces IL-17 expression from CD4$^-$ iNKT cells, which leads to increases in (i) neutrophils and total cell numbers in the airways, (ii) BALF levels of IL-17; and (iii) AHR, features that are

inhibited by a neutralizing IL-17 antibody (175). Furthermore, ozone, a major component of air pollution, induces airways disease in mice, characterized by AHR and increases in NKT cells, macrophages, lymphocytes and neutrophils, that are reduced in IL-17 and NKT deficient mice (135).

3.1.4.3 Neutrophils

Neutrophils, $CD8^+$ T cells, B cells and lymphoid-tissue inducer-like (LTi) cells are additional cellular sources of IL-17 in the airways and lungs; however, the limited data suggests that they are normally minor sources of the cytokine and minimally affect the asthmatic response (127, 128, 172, 174, 183-185). Neutrophils from the peripheral blood of atopic asthmatics, as well as healthy controls, reportedly express IL-17, with the greatest number of neutrophils and highest frequency of $IL\text{-}17^+$ neutrophils quantified from the peripheral blood of atopic asthmatics, or more specifically from asthmatics with an allergy to fungi (128). Murine neutrophils from the BALF of intranasal (IN) LPS challenged mice, express IL-17 mRNA, although $CD4^+$ and $CD8^+$ T cells remain the major cellular sources of BALF IL-17 in this model (174). Nevertheless, in a murine model of experimental hypersensitivity pneumonitis, IL-17 was not only implicated in the development of fibrosis in the lungs; but neutrophils, monocytes and mast cells were identified as the primary sources of IL-17 in this model (186).

3.1.4.4 $CD8^+$ T cells

$CD8^+$ T cells from the peripheral blood of allergic asthmatics, as well as healthy control subjects, express IL-17 (127, 184). IL-17-expressing $CD8^+$ T cells are also known as Tc17 cells

and the ratio of the frequencies of IFN-γ-expressing $CD8^+$ T cells to Tc17 cells (IFN-γ^+:IL-17^+) in the peripheral blood is significantly lower in asthmatics compared to controls (127). In addition, a separate study that also examined PBMCs from allergic asthmatics showed significantly greater frequencies of Tc17 cells within the $CD3^+$ T cell population, compared to healthy controls (184). However, Tc17 cells may play a more important role for a sub-population of asthmatics who are allergic to fungi since the frequency of these cells from the peripheral blood of asthmatics with an allergy to fungus is much higher than asthmatics with an allergy to pollen or an indoor allergen, such as HDM, cat, dog or cockroach (128). Nevertheless, the frequencies of Th17 cells are higher than the frequencies of Tc17 cells in all three of the PBMC studies (127, 128, 184). Tc17 cells are similarly induced in murine models of asthma, including upregulated expression of IL-17mRNA from $CD8^+$ BALF cells of LPS challenged mice, and the increased frequency of Tc17 (and Th17) cells within the $CD3^+$ T cell populations from the spleen and airways of OVA/alum sensitized and challenged mice (174, 184). Adoptive transfer of Tc17 cells appear to be protective in mice given an otherwise lethal dose of influenza and is accompanied by an influx of neutrophils, which indicates a potential role for these cells in recruiting neutrophils to the airways of severe asthmatics (187).

3.1.4.5 B cells

B cells are best known for producing antibodies, but have recently been shown to produce an array of different cytokines including IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF-α and TGF-β (188). Human IL-17 mRNA was first detected in B cells using a human myeloma B cell line and their potential as a source of IL-17 was recently confirmed using purified $CD19^+$ B

lymphocytes from human tonsils (185, 189). In the latter study, both IL-17 and IL-17F mRNA and protein are detected and induced by stimulating B cells with IL-6, IL-23 and TGF-β, known mediators involved in differentiating and regulating Th17 cells and their production of IL-17 (185). B cells from mice infected with the pathogen, *trypanosome cruzi*, produce IL-17, and these IL-17 producing B cells, which outnumbered Th17 cells, were required to control infection independently of RORγt, the master transcription factor regulating differentiation of Th17 cells (190). IL-17 is also induced from human B cells sourced from human tonsils, stimulated with *T. cruzi* (190). However, the contribution and relative importance of IL-17-expressing B cells in asthma remains unknown.

3.1.4.6 Lymphoid Tissue Inducer Cells (LTi)

Finally, LTi cells are a source of IL-17 and are particularly important in a murine model of obesity-induced asthma (183). LTi cells are similar to lymphoid cells, but do not express antigen receptors (i.e. TCR) (191). LTi cells are considered to be group 3 innate lymphoid cells (ILCs), producing IL-22 and IL-17. Group 1 ILCs include NK cells and producers of IFN-γ, while group 2 ILCs include cells that produce Th2 related cytokines (191). Group 3 ILCs help regulate adaptive immune responses through $CD4^+$ T cells and are important in maintaining homeostasis of the gastrointestinal tract (192). Although group 2 ILCs are increased in the peripheral blood of allergic asthmatics and produce greater levels of IL-5 and IL-13, compared to healthy human control subjects (193), Kim et al. were the first to show group 3 ILC contributions to the pathogenesis of asthma, specifically in relation to obesity (183). In this study, mice lacking adaptive immune cells ($Rag2^{-/-}$) were fed a high-fat diet for 12 weeks, resulting in obesity

accompanied by AHR that was dependent upon IL-17 and NLRP3 (NOD-like receptor family, pyrin domain containing 3) signaling. Mice lacking IL-17 or NLRP3 that were fed a high-fat diet were still obese, but did not display AHR compared to their wild type counterparts. Furthermore, adoptive transfer of group 3 ILCs induced AHR in $Rag2^{-/-}$deficient mice following treatment with IL-1β, while in their absence, IL-1β failed to induce AHR or inflammation (183). These data provide evidence that IL-1β treatment stimulates IL-17 production from group 3 ILCs that then induce AHR in these mice. This study also found an association between IL-17-expressing ILCs in the BALF of asthmatics and asthma severity: the frequency/number of group 3 ILCs was highest in patients with severe asthma compared to both mild asthmatics and control subjects (183). Altogether, this study suggests a possible role for group 3 ILCs in asthma, particularly severe asthma associated with obesity.

4 Effects of adjuvant on IL-17 responses

Adjuvants are intended to be used to enhance the host immune response to antigen without, itself, inducing a specific immune response (194). The ideal adjuvant induces a strong humoral and/or adaptive immune response without also causing significant discomfort or damage to the host, is practical and inexpensive to produce (75, 194). They are commonly used in vaccines to prevent disease, but are also useful in producing polyclonal antibodies for research purposes and inducing animal models of disease, including autoimmune diseases like EAE and allergic diseases like asthma (75, 195, 196). The mechanism by which adjuvants were originally thought to enhance immune responses to antigen is called the 'depot effect,' which is a concept whereby antigen is emulsified with or adsorbed to the surface of an adjuvant through electrostatic and other forces that then stabilize and protect the antigen from degradation and allow for its slow release, leading to prolonged and continuous antigen exposure to host immune cells (194). More recent studies indicate that the depot effect provides only a partial mechanism of action for adjuvants as they have been shown to stimulate innate immunity (197-199).

Adjuvants very often cause mild to moderate discomfort and pain and may be injurious to the host. Complete Freund's adjuvant (CFA) is associated with a more rapid production of high titer antibodies than many, if not all, other novel and traditional adjuvants, but is also known to cause lesions at the site of injection (194, 195). In addition, CFA reportedly causes inflammation at the injection site, skin ulcerations and necrosis, fistulous tracts, self-induced trauma and muscle atrophy (200, 201). Moreover, the severity of CFA-induced lesions coincides with the quantity of mycobacterium and volume of CFA injected (200). For these reasons, CFA is no longer approved for use in human vaccines, but is still available for high titer antibody

production for research (194). Aluminum-based adjuvants (alum), Ribi adjuvants and TiterMax® also reportedly induce side effects in sensitized mice. Mice IP injected with alum may form granulomas and have inflammation lasting 8 weeks at the site of injection, show less movement and are less aware of their surroundings, while recipients of Ribi and TiterMax® may have inflammation at the site of injection, severe lesions, ulcers, abscesses and necrotizing granulomas (75, 194, 201).

Adjuvants are commonly used in animal models of allergic airways disease to enhance antigen-specific immune responses and induce pathological features that model human asthma. Airway inflammation, AHR, remodeling, antibody production and specific immune cell responses are all inducible features of asthma that are influenced by the type of adjuvant used at sensitization (75, 130, 202). The choice of adjuvant is largely dictated by the route and site of injection and the asthmatic response being studied (75, 142). Alum and CFA-dependent effects are discussed in detail below, as well as immune responses induced by antigen in the absence of adjuvant.

4.1 Sans adjuvant

Following airway challenge, the classic pathological features of allergic airways disease are inducible in mice sensitized to antigen without adding traditional adjuvants, such as alum and CFA. Several labs have published using an OVA only sensitization protocol consisting of 7 IP injections on alternating days, followed by 3 OVA airway challenges that consistently result in increased airway recruitment of total cells and/or eosinophils, AHR and antigen-specific antibody production (202-205). The multiple injections appear to be necessary, in order to

prolong antigen presentation to the host. In particular, 50 daily injections of antigen in saline induce antibody titers similar to that of a single injection of antigen in a water-in-oil emulsion (194). While antibody titers remain elevated in recipients of the single injection containing adjuvant, levels of antibody decrease significantly, following cessation of the 50 injections of OVA alone. This phenomenon can be explained through the concept of "cell selection" in which low-dose antigen maintained by its slow release from its emulsion with adjuvant leads to stimulation of high-affinity B cell receptors, whereas high-dose antigen delivered by daily adjuvant-free injections of antigen stimulate low affinity receptors on B cells (206).

There are considerable benefits to using an adjuvant-free sensitization protocol, especially when studying cell or mediator-dependent effects that are otherwise induced or significantly enhanced by the adjuvant itself. For example, mast cell, TNF and IL-17 deficient mice sensitized to OVA without adjuvant have less total and/or eosinophilic airway inflammation and are airway hyporesponsive compared to wild-type controls, thus implicating mast cells, TNF and IL-17 as positive regulators of allergic airways disease (130, 203, 204). However, the same knockout mice lose these differences when they are sensitized to OVA in the presence of alum, resulting in a different conclusion. Nevertheless, studies requiring a robust IL-17 response could benefit from, rather than be hindered by, the use of an adjuvant and are discussed in greater detail in sections 5.2 and 5.3.

The route/site of sensitization is another important factor influencing the choice of adjuvant, induction of IL-17 and allergic airway responses. The majority of these studies have specifically compared site-dependent effects to the standard IP OVA/alum sensitization protocol. These data suggest the airways as an equally or more efficient site for priming mice to produce IL-17 and induce AHR and neutrophilia (137, 142). The SC (subcutaneous) route of OVA

sensitization is reported to be better than or comparable to the IP route at inducing AHR, inflammation and Th2 cytokines (75). However, both sets of comparisons are based on protocols that use different adjuvants, making it difficult to separate adjuvant- from site-dependent effects (75, 137, 142). One study that successfully compares site-dependent effects using a single adjuvant, lipopolysaccharide (LPS), indicates that OVA/LPS sensitization via the airways induces greater influx of eosinophils and neutrophils than an IP OVA/LPS sensitization protocol (142). Adjuvant-dependent effects of LPS are also interpretable from the same data set and show greater recruitment of airway eosinophils and neutrophils in mice sensitized through the airways with OVA/LPS compared to mice sensitized with OVA alone, following airway challenge (142).

The airways are in many ways the ideal sensitization site to study allergic airways disease because of the clinical relevance they have as the primary sites of sensitization in asthmatics (207) and their sensitization and challenge induces classical features of allergic airways disease (142). Models of allergic airways disease that utilize the airways as a route of sensitization typically involve sensitization protocols that do not use conventional adjuvants and/or use allergens to which human asthmatics are commonly allergic, including species of *aspergillus*, cockroach antigens, ragweed and HDM (207). It is predicted that as many as 13% of asthmatics are allergic to species of the fungus, *aspergillus* (208). Intranasal delivery of *aspergillus fumigatus*, in a murine model of allergic airways disease leads to increases in total and antigen-specific IgE, AHR and eosinophilic inflammation (209). There is limited data on the role of IL-17 in *aspergillu*s-induced models of allergic airways disease, but one study reports that the intratracheal delivery of *aspergillus versicolor* induces strong Th17 responses, airway neutrophilia and mild AHR (210). Neutralization of IL-17 significantly increases AHR and influx of eosinophils, thus implicating IL-17 as a negative regulator of inflammation in this

model. In a cockroach model of allergic airways disease, intratracheal inhalation of cockroach fecal matter (frass) induced AHR and increased total serum IgE, as well as IL-5 and IL-13 levels in murine lungs (211). Furthermore, cultured lung cells from cockroach frass sensitized mice produce more IL-17 than lung cells from sham-sensitized mice. Intranasal delivered, ragweed models of allergic airways disease induce AHR, eosinophilic inflammation and antigen-specific IgG and IgE production and expression of Th2 cytokines and GATA-3 (40, 212). The effect of a conventional adjuvant-free ragweed model of allergic airways disease on IL-17 responses does not appear to have been studied, but an IP ragweed/alum sensitization protocol has been shown to increase BALF IL-17 expression following IN ragweed challenge (213). 50-85% of asthmatics are reported to be allergic to HDM, a real world mixture of components including the derp1 and derf1 allergens, fecal matter and LPS (214). Asthmatics and patients with rhinitis that are allergic to HDM have frequencies of peripheral blood Th17 cells, serum and sputum levels of IL-17 at baseline that are higher than controls, which are further increased following airway challenge with HDM (215).

Airway sensitization protocols are limited in their practicality to study adjuvant-dependent effects in allergic airways disease. As previously mentioned, traditional adjuvants like alum and CFA form lesions and granulomas at the site of delivery and deposits that could form a physical barrier to the exchange of oxygen, making the airways an unsuitable site in which to compare the effects of these adjuvants (75, 194, 201). Furthermore, OVA sensitization via the airways induces tolerance, necessitating the use of non-traditional adjuvants like TNF-α or low dose LPS during initial antigen exposure to break tolerance (137, 142, 216). The classical features of allergic airways disease can be induced in adjuvant-free IP and SC sensitized animals, following airway challenge (75, 202). As previously mentioned, several protocols use multiple

injections to prolong antigen presentation to host immune cells to enhance allergic airway responses to antigen in the absence of adjuvant (130, 194, 202). However, as few as 3 SC adjuvant-free injections induce increases in airway eosinophils and lymphocytes, AHR, BALF IL-5 and IL-13 (75). Furthermore, comparisons of two sensitization protocols that are free of conventional adjuvants show higher serum levels of OVA-specific IgE, but lower OVA-specific IgG_1 in SC OVA sensitized mice compared to IP OVA sensitized mice (75).

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4.2 Aluminum-based adjuvants

Aluminum-based adjuvants are commonly and herein referred to as 'alum,' which actually refers to the chemical compound, hydrated potassium aluminum sulphate (217). Alternative and more commonly used 'alums' include aluminum hydroxide; aluminum phosphate; alhydrogel®, a wet gel version of aluminum hydroxide; and imject alum, a mix of aluminum hydroxycarbonate and magnesium hydroxide. The mechanism of action of alum remains controversial, with studies both opposing and supporting the concept of the depot effect, whereby presentation of antigen is prolonged by adjuvant, thus maximizing the host immune response to antigen (194, 197, 217). Aluminum hydroxide has a large surface area, a crystalline structure and a positive charge at neutral pH; aluminum phosphate has a negative charge and amorphous structure; and while the magnesium hydroxide component of imject alum is crystalline in structure, the aluminum hydroxycarbonate component is amorphous (217). These characteristics determine how well the antigen adsorbs to and is released by alum, thereby influencing its ability to enhance the immune response to antigen.

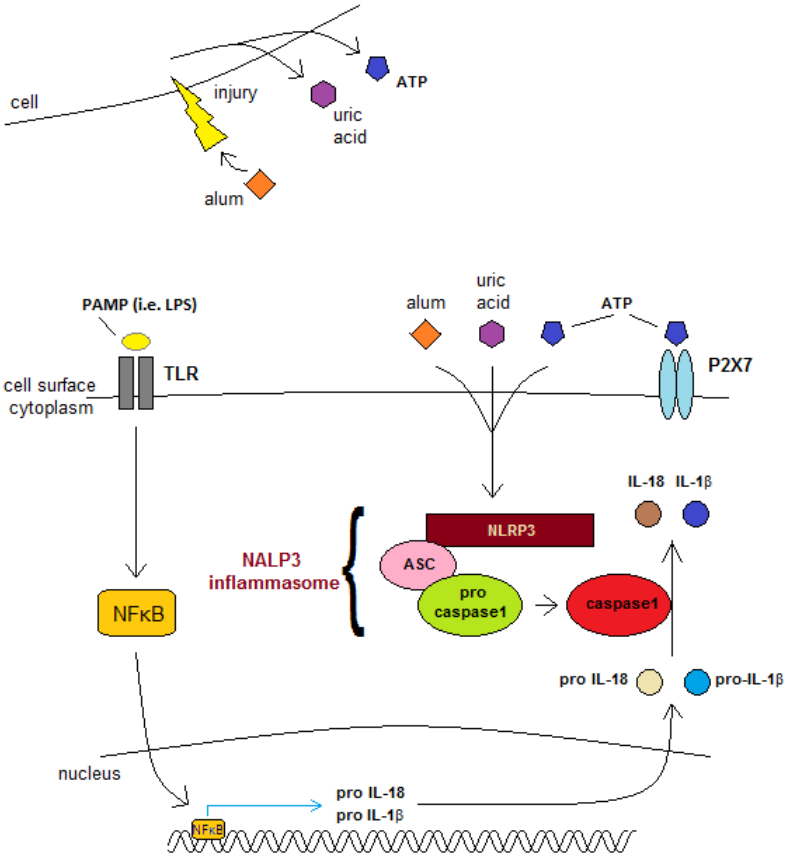
Alum has repeatedly been shown to signal through the NALP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome, which consists of NLRP3, the adaptor molecule ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain) and pro-caspase-1 (**Fig 1.2**) (199, 218, 219). Alum does not directly stimulate TLR4 (toll-like receptor 4), but requires activation of this pathway (by LPS) to produce the pro forms of IL-1β and IL-18, which along with IL-33 are cleaved into their active forms by the capase-1 component of the NALP3 inflammasome (220). ATP (adenosine triphosphate) and the crystalline form of uric acid are endogenous ligands of NLRP3 (199, 217, 218). Alum is hypothesized to release endogenous ligands of NLRP3, in response to injury sustained at the site of injection, which then activate the inflammasome; however, a study by Eisenbarth et al. provides evidence that alum itself is also a NLRP3 ligand (199). Using uricase to degrade uric acid and P2X7R (an ATP purinoreceptor) deficient mice to inhibit uric acid and ATP signaling, respectively, the authors were able maintain production of mature IL-1β and IL-18 at similar levels to controls. Alum, delivered through an IP injection, appears to signal through the NALP3 inflammasome to enhance serum levels of OVA-specific IgG in sensitized mice and total BALF inflammatory cells in OVA sensitized & challenged mice, as well as increasing production of IL-5 from OVA re-stimulated draining lymph nodes from OVA sensitized mice (199, 221). Nevertheless, while the NALP3 inflammasome is required to produce mature IL-1β and IL-18, there are studies indicating that NALP3 is dispensable for alum to boost adaptive immune responses, such as enhancing IgG production; IP OVA induced $CD4^+$ and $CD8^+$ T cell recruitment to the mediastinal lymph nodes and spleen; and eosinophil, neutrophil, monocyte and dendritic cell recruitment to the peritoneal cavity (218, 222). These studies indicate that the immune boosting effects of alum on adaptive immune responses are accomplished through a

NALP3 inflammasome-independent pathway. In addition, mast cells, macrophages and eosinophils are also inessential to the alum-enhanced priming of T cells, production of antigen-specific antibodies and biasing of T cells toward the Th2 phenotype (222). Altogether, the data indicate that alum may not directly signal through TLR4, but its activation produces the pro forms of cytokines that are cleaved into their active form by the caspase-1 component of the NALP3 inflammasome. Furthermore, alum appears to signal through the NALP3 inflammasome to produce active IL-1β, IL-18 and IL-33 cytokines, but signaling through the NALP3 inflammasome may not be responsible for the adjuvanticity of alum.

The most common protocols utilized in studies of allergic airways disease typically involve 1 to 3 IP injections of OVA in alum, followed by one to several OVA airway challenges (75, 199). These models consistently induce airway eosinophilia, AHR, antibody production and Th2 cytokine production (75, 130, 132, 142). Surprisingly, studies comparing the effects of different sensitization protocols on allergic airway responses often combine two or more variables, such as the type of adjuvant or antigen concentration with different routes, numbers or timing of exposure. This is because the primary objective of these studies is to find a protocol that more strongly induces specific features of allergic airways disease (i.e. Th2, IL-17, airway eosinophilia, AHR, etc…), than the standard IP OVA/alum sensitization protocol (75, 142). The few experiments that do report on alum as a single variable generally show alum enhancement of Th2 inflammatory responses. For example, following OVA challenge, IP OVA/alum sensitized mice have fewer airway neutrophils, and more eosinophils; frequencies of IL-4, IL-17 and CCR6 (an extracelluar Th17 cell marker) expressing BALF and/or lung T cells; IL-13, IL-17 and IL-23 BALF levels; median fluorescence intensity (MFI) values for RORγt, the master transcription factor regulating Th17 cell differentiation; and serum OVA-specific IgG_1 levels, compared to IP

Figure 1.2: Alum signaling through the NALP3 inflammasome

Alum may directly and/or indirectly signal through the NALP3 inflammasome, which consists of NLRP3, ASC and pro-caspase1. The endogenous molecules, uric acid and ATP, directly bind NLRP3, whereas alum may directly bind to or indirectly activate NLRP3 by causing cell injury, resulting in the release of endogenous ligands (i.e. uric acid and ATP) that bind NLRP3. Upon activation of the NALP3 inflammasome, inactive pro-caspase1 is catalyzed into active capase1, which cleaves pro forms of the cytokines, IL-1β, IL-18 and IL-33 into their active forms. Alum does not signal directly through TLRs, however activation of TLR4 by LPS activates the NFκB pathway, which regulates expression of the pro forms of IL-1β and IL-18 that are cleaved into their active forms by the NALP3 inflammasome.



OVA sensitized mice (75, 137, 142). As previously mentioned, alum may also mask the role of certain cell types and mediators including IL-17, TNFα and mast cells, which could affect the conclusions of a study (130, 204, 222, 223).

Data relevant to alum-dependent effects on allergic airway responses is not entirely limited to models of allergic airway disease. Alum also increases recruitment of total cells, neutrophils, eosinophils and monocytes to and enhances levels of IL-6 and IL-1β in the peritoneal lavage fluid of IP OVA sensitized mice (221). Furthermore, in the absence of antigen, an IP injection of alum recruits significantly greater numbers of IL-4^+ eosinophils, neutrophils, dendritic cells, NK and NKT cells, but fewer IL-4^+ mast cells and macrophages to the peritoneal cavity compared to mice injected with PBS alone (222). These IP alum mice also have greater levels of IL-5, IL-1β, IL-6, keratinocyte chemoattractant (CXCL1), eotaxin, monocyte chemoattractant protein-1 (MCP-1), but not IL-17 or IFN-γ protein in their peritoneal lavage fluid. Brewer et al. report that subcutaneous injections of alum, with OVA, enhance plasma levels of OVA-IgG_1, which is partially dependent on IL-4 and independent of IL-6 and TNF-receptor 1; induce OVA-IgE through an IL-4-dependent pathway; and induce Th2 responses in the absence of IL-4 and IL-13 signaling (224-226).

4.3 Complete and incomplete Freund's adjuvant

Incomplete Freund's adjuvant (IFA) consists of a light mineral oil and a surfactant agent, whereas complete Freund's adjuvant (CFA) also has heat killed *Mycobacterium tuberculosis* (*Mtb*) as an additional component (194). Typically, the antigen is added to an aqueous solution before mixing vigorously with an equal volume of IFA or CFA to create an emulsion (194, 201).

CFA was first introduced by Freund in the 1930's and was quickly recognized for its ability to enhance the host immune response to antigen, largely based on its ability to stimulate production of high titer, high affinity antibodies (194, 227-229). However, because of the pain, discomfort and injury associated with CFA, its use is highly regulated or restricted (200, 228). As a result, IFA often replaces CFA during secondary injection of antigen intended to boost the immune response to antigen (201). Similar immunological responses are achieved with IFA as with CFA as a booster injection, following a primary injection of antigen with CFA, but with significantly fewer adverse effects (200, 201).

As with many other adjuvants, the mechanism of action of CFA is controversial (199, 230-232). Firstly, CFA is thought to act as a depot (194). CFA consists of microbial components that are recognized ligands for several toll-like receptors (TLR) including TLR2, 4 and 9, which would suggest a possible immuno-stimulatory mechanism (196, 230). All TLRs, with the exceptions of TLR3 and 4, rely on the MyD88 pathway of signaling, while TLR3 signals through the TIR domain-containing adapter inducing interferon beta (TRIF) pathway and TLR4 can signal through both (233). Mice deficient in MyD88 produce less serum IL-17 and have a lower frequency of IL-17$^+$ splenic CD3$^+$ T cells 10 days after sensitization with myelin oligodendrocyte glycoprotein/CFA; and myeloid dendritic cells from these mice express less IL-6 and IL-23 mRNA following *in vitro* stimulation with heat killed *Mtb* (196, 230, 231). In contrast, IL-6 and IL-23 mRNA expression, as well as IL-17 responses are significantly higher in TLR4 deficient mice, indicating that CFA both positively and negatively regulates IL-17 responses through different TLRs (231). However, production of different classes of antibodies generally remains unaffected in MyD88 and TRIF double knockout mice, injected with CFA (or IFA or alum), indicating that CFA boosts antibody production through a TLR-independent mechanism (232,

233). Muramyldipeptide (MDP), another component of CFA, is recognized by nucleotide-binding oligomerization domain (NOD)2, which could account for the TLR-independent effects of CFA (232). In addition, NALP3 inflammasome involvement in CFA/IFA-dependent responses is controversial. Eisenbarth et al. reported that CFA and IFA stimulated, LPS primed, macrophages do not produce mature IL-1β, suggesting that the adjuvanticity of CFA and IFA relies on mechanism(s) aside from NALP3 (199). In contrast, the heat-killed *Mtb* component of CFA alone has been shown to induce mature IL-1β and IL-18 production from dendritic cells, which is significantly attenuated by a caspase-1 inhibitor and in caspase-1 and NLRP3 deficient dendritic cells (198), the result of which is reduced IL-17 production from co-cultured $CD4^+$ T cells.

CFA has historically been considered a Th1 adjuvant because of its ability to induce Th1 responses, including IFN-γ production (194, 234). Nevertheless, CFA is combined with myelin-associated proteins to induce experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, which is strongly associated with Th17/IL-17 responses (196, 234). Moreover, two recent studies provide evidence that CFA is also a potent inducer of Th17 immunity. Smith et al., examined EAE development in mice sensitized to myelin proteins with either CFA (essentially IFA + *Mtb*) or IFA combined with *Citrobacter rodentium* (*C. rodentium*), which typically induces a robust IL-23-dependent Th17 response in an infection model (234). Their data demonstrate that *C. rodentium*/IFA induced far fewer Th1 and Th17 cells in the central nervous system than mice exposed to CFA (234). *C. rodentium*/IFA exposed mice also had less severe, slow progressing, late onset EAE. Altogether their data indicate that in spite of its strong association with Th1 responses, CFA could also be considered a Th17 skewing adjuvant. An additional study compares mice sensitized with a myelin protein in the presence of

CpG/IFA, CpG being a known Th1 skewing agent, to mice sensitized to myelin protein in CFA (235). Similar frequencies of Th1, but significantly more Th17 cells differentiate in mice from the CFA group compared to the CpG/IFA group. Together these studies provide evidence that CFA is more of a mixed Th1/Th17 adjuvant than solely Th1.

The very limited data specifically examining CFA-dependent effects on allergic airway responses as a single variable indicate that, following OVA challenge, mice IP OVA sensitized with CFA have similar numbers of eosinophils, neutrophils, Th1, Th2 and Th17 cells recruited to the airways as mice IP OVA sensitized with alum (137). Production of a wide spectrum of the plasma immunoglobulins IgG_1, IgG_{2a} and IgE are significantly higher in SC OVA/CFA sensitized mice compared to mice SC OVA sensitized without conventional adjuvant (224, 225). Thus, despite the limited data, there is some evidence that CFA may enhance asthma- and Th17-related responses.

5 Manipulation of protein expression in animal models of asthma

Animal models of human disease, including models of allergic airways disease, are often criticized for not providing data that translates well in clinical trials (236). It has been suggested that this may have more to do with a lack of understanding of the pathophysiology of the disease, including complex interactions between mediators and overlapping pathways, rather than the animals themselves and is furthermore, an issue not limited to diseases of the airways (236). Notwithstanding these concerns, animal models of allergic airways disease have provided invaluable data that has broadened our understanding of asthma and its therapies (207, 237, 238). Murine models are particularly useful for studying the immunological and inflammatory aspects of allergic airways disease, especially with the abundance of peptides, proteins and monoclonal antibodies available. Moreover, advances in animal engineering and technologies, including transgenic and knockout mice, have been particularly useful and are herein discussed. In view of the positive and negative regulatory effects that IL-17 has on Th2-inflammatory responses and the influence that variables, such as the cellular source of IL-17, have on downstream IL-17 signaling events, several different approaches will likely be required to fully understand the complex nature of the IL-17 response as it applies to allergic airways disease.

5.1 Viral vector-based genetic engineering of target cells for adoptive transfer

Considering their natural ability to infect cells, viral vectors are an important means of delivering and expressing exogenous genetic information in transfected cells and can be divided into two groups according to how the viral sequence is incorporated by the host: non-integrative

and integrative techniques (239). Non-integrative viral vector-based techniques involve transfecting cells with DNA or RNA-based viral vectors coding gene(s) that do not integrate directly into the host genome, resulting in the transient expression of the gene(s)-of-interest. Recombinant adenoviruses are an example of a non-integrating viral vector, consisting of double-stranded DNA (240). Integrative viral vector-based techniques typically involve gamma retroviral vectors that incorporate exogenous genetic material directly into the host genome resulting in stable expression of the genes (239). Retroviral particles are enveloped viruses that contain two copies of the retroviral RNA genome, reverse transcriptase and integrase enzymes within the capsid that are required for integration of the retroviral genome into that of the host. On the retroviral envelope are cell surface glycoproteins recognized by specific receptors expressed on target cells (239, 241) that allow the viral envelope to fuse with the cell membrane, internalizing the virus where the capsid degrades. Viral RNA is released and reverse transcribed into cDNA that is subsequently integrated into the host genome. Retroviruses readily transduce proliferating cells, gaining access to the host genome while the nuclear membrane is disassembled during cell division (239, 242). The obvious limitation is that gamma retroviruses transduce only actively dividing cells, a limitation that can also be an advantage. For example, retroviruses are being tested as an *in vivo* means of targeting cancerous cells as they divide, without harming the quiescent, healthy cells surrounding them, by delivering a suicide gene, the product of which induces cell death (243). We and others have also taken advantage of their specificity for dividing cells in order to target antigen-specific $CD4^+$ T cells in animal models of human disease (28, 244, 245).

Lentiviruses are a subtype of retroviruses that are able to transduce cells that are not actively dividing as they contain a central termination signal that specifically assists in the import

of the pre-integration complex past the intact nuclear membrane (246). Thus, lentiviruses allow for the stable expression of gene(s)-of-interest in target cells that are terminally differentiated.

In Chapter 4, we utilized the pAP2 retrovirus, a retrovirus that has been used previously by the Fixman lab (242, 244), to target OVA-specific $CD4^+$ T cells from OVA sensitized mice for transduction during *in vitro* re-stimulation with OVA. The pAP2 retroviral vector was designed by Galipeau et al. and contains a multi-cloning site for insertion of the gene-of-interest upstream of an internal ribosomal entry site (IRES) and the gene encoding enhanced green fluorescence protein (EGFP) (243). In transduced cells, the retroviral RNA is produced as a bicistronic message and the IRES allows efficient translation of EGFP protein, which is used to identify transduced cells.

The pAP2 plasmid was transfected into a retroviral packaging cell line called Phoenix-ecotropic (Phoenix-Eco), derived from human embryonic kidney, 293 T cells, and which contain the necessary genes to produce, package and release fully functional retroviral particles that target and transduce dividing, murine and rat cells (244, 247). Phoenix cells produce group antigens (gag), proteins that make up the retrovirus capsid; DNA polymerase (pol), a combination of reverse transcriptase, RNase H and integrase; and envelope (env), a protein present in the lipid layer of the membrane and which determines the tropism of the retrovirus. Gag-pol are encoded on a separate construct from env and the pAP2 plasmid and are under the control of different promoters to minimize the risk of a recombination event that would create wild type retroviruses (247). Altogether, the protein products of the gag, pol, env genes and the pAP2-derived retrovirus genome are packaged into intact virions that are released by Phoenix-eco cells.

5.2 Recombinant proteins

Recombinant proteins have many applications in drug discovery and research. Recombinant cytokines are commonly used in murine models of allergic airways disease to better understand their role in disease pathogenesis. The most commonly used expression system is *Escherichia coli* (*E. coli*) because of the relative ease with which the system can be manipulated, the low cost and the short time required for protein production (169, 248). It is also the expression system used to generate the recombinant IL-13 and IL-17 used in experiments conducted in Chapter 3. Mammalian expression systems have some notable benefits compared to *E. coli* and insect cell expression systems, including post-translational modifications and folding of proteins to generate bioactive proteins (248). However, IL-13 and IL-17 do not require post-translational modifications that would necessitate the added time and expense associated with the mammalian system (248).

The commercial availability of a growing number of an already long list of recombinant proteins provides opportunities to study the role of single or multiple mediators in models of disease. In asthma studies, recombinant proteins are most often delivered directly to the airways (45, 249). Recombinant Th2 cytokines generally augment features of asthma. Examples include data demonstrating that intratracheal delivery of IL-4 or IL-13 to naïve mice induces AHR, eosinophilia and goblet cell hyperplasia (45). However, as discussed in Chapter 1, section 3 (Interleukin-17 responses), while IL-17 that originates from Th17 and γδ T cells respectively augments and attenuates airway responses in murine models of asthma, there is conflicting data in terms of the effect of recombinant IL-17 protein (129, 142). Studies specifically using recombinant IL-17 to examine the role of IL-17 on allergic airway responses are discussed in detail in section 3.1.3 of Chapter 1 of the thesis and is appropriately titled, ‘recombinant IL-17.’

Slight variations in sensitization and challenge protocols, the strain of mouse and protein delivery could contribute to the contradictory effects of recombinant IL-17 on airway responses. In terms of their delivery, recombinant proteins are typically given at very high quantities, on multiple occasions and delivered through the airway lumen, whereas the expression of endogenous proteins are typically induced by a trigger, such as exposure to allergen and continuously produced by structural cells and/or inflammatory cells recruited to specific areas of the airways and lungs. Thus, recombinant proteins have been useful in determining the role of single, as well as multiple mediators in murine models of asthma, but may have their limitations. In the case of IL-17, these limitations are more evident because of the contrasting data generated with recombinant IL-17.

5.3 Antibodies

Antibodies, otherwise known as immunoglobulins, are glycoproteins produced by plasma cells that recognize foreign antigens, signaling additional immune cells to mount a coordinated immune response (250). The specificity of antibodies for a particular antigen makes them useful for research, as well as for targeted therapy (251). Antibodies can be divided into two groups, polyclonal and monoclonal, based on the number of antibody-producing cells that are involved in their production. Polyclonal antibodies are produced from different cell lineages recognizing multiple epitopes for a single antigen and are widely used in research and diagnostics for their ability to recognize and amplify signals, as well as the relative low cost of production (250, 251). Monoclonal antibodies come from a single cell lineage, recognizing a single epitope on an

antigen, and are often used *in vivo* to neutralize or stimulate targeted mediators and/or cells (250).

Today, there are several asthma-related monoclonal antibodies available to treat severe asthma patients who, by definition, have asthma symptoms that are difficult to control, following a course of treatment that includes high dose ICSs combined with a secondary controller (10). Omalizumab is a commercially available antibody targeting IgE that significantly reduces the number of yearly exacerbations and improves clinical and several quality of life outcomes of asthma (252, 253). Mepolizumab and Reslizumab are monoclonal antibodies targeting IL-5 that are well tolerated and improve lung function and asthma questionnaire scores, most notably in asthmatics with the highest airway eosinophils (254, 255). Benralizumab is an anti-IL-5Rα antibody that decreases blood eosinophil counts and the severity and rate of asthma exacerbations, when administered SC or IV (256, 257). Dupilumab is a monoclonal antibody inhibiting the biological effects of both IL-4 and IL-13 by binding the IL-4Rα subunit (39, 258). Wenzel et al. gave SC dupilumab to highly eosinophilic, persistent, severe asthmatics and reduced the frequency of exacerbations and improved several lung function measurements, but induced more side effects (39). Finally, AMG-157 is a novel antibody targeting thymic stromal lymphopoietin (TSLP), a Th2 related epithelial cell-derived cytokine, was recently shown to improve FEV_1 and to reduce both blood and sputum eosinophils (259).

Monoclonal antibodies are also important in animal research, neutralizing and stimulating target mediators and/or cells in order to determine their role in physiological and pathological responses (252, 260). Antibodies targeting protein mediators neutralize by preventing the mediator from binding and activating their receptors (261). However, when the target of an antibody is a receptor, the antibody can be stimulatory (agonistic) or inhibitory/neutralizing

(competitive antagonistic or allosteric antagonistic) (262, 263). Allosteric antagonists bind different sites from the active site, while competitive antagonists bind the active site directly, preventing activation of the target receptor (263). Agonistic and antagonistic antibodies can have identical constant regions, while the variable regions of their light and heavy chains may share less than 50% sequence homology, have opposing effects, and still bind the same receptor (264). Mijares et al. also show evidence that the same sequence for a single antigen-binding fragment (F_{ab}) can be influenced into acting as an agonist or antagonist (262). These authors previously showed that a monoclonal antibody for the β_2-adrenoceptor called Mab6H8 acts as an agonist, whereas an identical F_{ab} only fragment of Mab6H8 has 5-10x lower affinity for the β_2-adrenoceptor, has no agonistic effect, but competes with the β_2-agonist Clenbuterol for the receptor (262). The authors speculate that the difference in the affinity of the F_{ab} for a receptor determines agonist/antagonist activity. Another study examines the binding sites of several monoclonal antibodies for the α I domain of the integrin $\alpha_L\beta_2$ and discusses specific characteristics of their binding sites that could determine agonostic/antagonistic activity of an antibody (263). Overall, these studies indicate that there are several characteristics of monoclonal antibodies and their receptor binding sites that could influence their activity.

Several monoclonal antibodies that are commercially available for research are characterized and sold as neutralizing antibodies, even while there is evidence that they are stimulatory (265, 266). The GL3 and UC7-13D5 clones of the γδ TCR (one of which is used in Chapter 2 of this thesis) have been used primarily as neutralizing antibodies, despite the fact that receptor binding by either antibody induces internalization of the target receptor, leaving the cell intact but "invisible" to detection by a second γδ TCR antibody (265, 266). A δ-TCR reporter mouse expressing GFP and activation markers CD69 and CD44 was used to show the existence

of GL3 and UC7-13D5 antibody-stimulated, receptor-internalized, "invisible" γδ T cells and to demonstrate that these "invisible" cells were actually activated, rather than inhibited or neutralized. The same study also provided evidence that the epitopes recognized by these GL3 and UC7-13D5 antibodies partially overlap and thus, these antibodies compete for the same epitope on the γδ TCR. This is important because a second receptor antibody is often used to confirm neutralization of the target cell by the neutralizing antibody, which if they are in competition for the same epitope, would diminish/reduce detection of the target cell, rendering the cell 'invisible'. The important points made in these studies are likely applicable to stimulatory and neutralizing antibodies for other receptors and showcase the importance of verifying antibody activity before drawing any conclusions.

5.4 Gene-modified animals

Gene-modified mice, widely used in research to study gene/protein function are also applied in Chapter 3 of the thesis. Transgenic animals have exogenous genetic material that has been introduced and integrated into their genome (240). The first transgenic mice were created in 1974, when mouse blastocysts were microinjected with simian virus 40 viral DNA and transferred into pseudo-pregnant surrogate mothers (267). It was not until 1981 that the first transgenic mice capable of passing on foreign material to offspring were created (268-270). Today, there are broad applications for, and a growing number of techniques used, to generate transgenic animals (240). The technology is being applied in agriculture to improve animal breeding and the quality of animal products; in medicine, to reduce the rate of rejection in xenotransplantation of organs, create and improve pharmaceutical products and provide gene

therapy for genetic diseases; and in science, to study specific genes and induce disease in animals for research (240).

Transgenic mice are commonly used in allergy models and have helped clarify the role of Th17 cells and IL-17 in allergic airways disease. For example, antigen-induced AHR, inflammation, BALF levels of IL-17 and frequencies of lung Th17 cells were increased in IL-23 transgenic mice, overexpressing IL-23 in lung epithelial cells (143). Similarly, IL-17 transgenic mice, overexpressing IL-17 in lung epithelial cells, have greater inflammation, collagen deposition, mucus production, alveolar wall thickening and chemokine expression than control mice (271). As previously mentioned, murine models used to study allergic airways disease commonly use OVA for sensitizations and challenges (75), making OVA TCR transgenic mice particularly useful as they express the αβ T cell receptor (TCR) specific for the OVA323-339 epitope on nearly all $CD4^+$ T cells and have been created in both the BALB/c and C57Bl/6 strains of mice called DO11.10 and OTII, respectively (272-274). $CD4^+$ T cells from OVA TCR transgenic mice, cultured under Th2 and Th17 skewing conditions and adoptively transferred into naïve or SCID mice, have been used to address the role of antigen-specific Th2 and Th17 cells in allergic airways disease, (25, 81, 95, 143). The results of these studies are discussed in Chapter 1, sections 2 (Th2 response) and 3.1.1 (Th17 cells), as well as in Chapter 5, section 4 (IL-17 effects on Th2 responses), but overall show that the adoptive transfer of OVA-specific Th2 cells into naïve recipients induces AHR and inflammation, characterized by eosinophils and Th2 cytokine expression in the BALF and/or lungs, whereas the adoptive transfer of OVA-specific Th17 cells recruits neutrophils to the airways, is resistant to attenuation by corticosteroids, but also enhances Th2 responses, following OVA airway challenge (23, 25, 81, 95, 143). Similar to DO11.10 and OTII mice, alternative TCR transgenic mice are being used to

study allergic airways disease, including a *derp1* transgenic mouse expressing a TCR specific for an epitope on the *Dermatophagoides pteronyssinus* species of HDM, which is a more physiologically appropriate allergen to which 50-80% of asthmatics are allergic (77, 275, 276). Derp1 transgenic mice challenged via the airways with a derp1 peptide, without prior priming, have increased lymphocytes and eosinophils in their airways, goblet cell hyperplasia and mucus production (275). Although the study did not look at IL-17 responses, these transgenic mice may be useful as HDM is a natural allergen; IL-17 responses are augmented in HDM allergic human asthmatics (215), post HDM airway challenge; and because very little is yet known about the effects of HDM on IL-17 responses.

Specific-gene targeting technology not only applies to gain-of-function (knockin), but loss-of-function (knockout) animals as well (240, 277). The vast majority of the estimated 3000+ knockout strains of mice have been created by means of this technology where by homologous recombination, the targeted gene of an embryonic stem cell is disrupted (277). Some of the benefits of this technology are that the role of a single protein can be examined under many different physiological and pathological conditions and the disrupted gene is transmitted through the germline to offspring; however, with traditional knockout technology, the loss-of-function of certain genes is embryonic or early postnatal lethal, or impacts development of the animal in such a way as to allow normally recessive pathways to compensate for the loss-of-gene function (240, 277).

The development of conditional knockout technology improves upon some of the problematic issues associated with traditional knockout animals. By using conditional knockout animals, the loss-of-gene function can be temporally and spatially controlled, allowing researchers to better address the role of a gene at a specific stage of development when certain

diseases emerge (i.e. adult cancers), or in specific types of cells or tissues where certain diseases emerge (i.e. lung epithelial cells in asthma) (277).

Conventional and conditional knockout mice are especially useful in elucidating the role of single mediators and cell types in allergic airways disease, including Th17 related cytokines (25, 226). An IL-17R knockout study reported the requirement of IL-17 at sensitization to induce allergic airways disease; however, IL-17F also signals through the same receptor, while the IL-25R shares the IL-17RA subunit of the receptor in common with IL-17R (129). In another study, IL-17 deficient mice produce significantly less OVA-specific IgE and IgG_1; are more airway hyperresponsive; have higher total airway inflammatory cells and BALF levels of IL-4 and IL-5, compared to wild type mice (130). A comparison study looked at the roles of IL-17 and IL-17F using IL-17- and IL-17F-deficient mice in a murine model of asthma and showed IL-17F, but not IL-17 involvement in airway neutrophilia and attenuation of Th2 responses; while IL-17, but not IL-17F appeared to be involved in enhancing Th2 responses (278). Conditional eosinophil knockout mice were also effective in addressing the functional role of eosinophils on allergic airways responses (83). The study showed AHR to be unaffected by the presence of eosinophils, although these mice were corticosteroid insensitive. In these ways, gene-modified animals have contributed a great deal to our understanding of the roles of specific immune mediators and cell types in murine models of disease.

6 Summary

Asthma, although rarely fatal, affects 300 million people worldwide and is typically associated with a Th2 immune response, characterized by airway hyperresponsiveness, eosinophilia and remodeling and is normally treated with corticosteroids. While this may describe most mild and moderate asthmatics, severe asthmatics are associated with a Th17 immune response, airway hyperresponsiveness, neutrophilia and poor or uncontrolled asthma symptoms despite treatment with high dose inhaled corticosteroid combined with a secondary controller.

Data from murine models of allergic airways disease indicate that IL-17 may regulate allergic airway responses through modulation of Th2 responses. However, this is complicated by data indicating that IL-17 can act as both a positive and negative regulator of Th2 responses. Additional evidence suggesting that this role may be influenced by the cellular source of IL-17 led us to formulate 2 main questions: (1) What is the primary cellular source(s) of IL-17 in murine models of asthma and (2) Does the cellular source of IL-17 differentially affect Th2 immune responses in allergic airways disease. The first objective was addressed using both allergen and non-allergen models. We also determined whether the primary cellular source could be altered by the conditions of sensitization in mice sensitized IP with OVA in the presence or absence of the adjuvants, alum and CFA. The second objective was addressed by looking at the effects of IL-17 delivered as a recombinant protein or produced/delivered by γδ T cells and $CD4^+$ T cells. We hypothesized that that the primary source of IL-17 in our models would be $CD4^+$ T cells, followed by γδ T cells and that OVA/CFA>OVA/alum>OVA/sal sensitized and challenged mice would have higher frequencies and numbers of $IL\text{-}17^+$ cells, AHR and inflammation. We

also hypothesized that recombinant IL-17 would enhance IL-13-induced AHR and eosinophilia and that IL-17 from γδ T cells would inhibit, while IL-17 from $CD4^+$ T cells would enhance AHR and inflammation in the mouse.

Elucidating the IL-17 pathway and factors influencing the IL-17 response may allow us to identify and target mediators that activate the negative regulatory pathway or inhibit the positive regulatory pathway of IL-17, which could better control asthma symptoms in severe asthmatics.

The following chapters examine the effects of IL-17 on Th2 responses using different murine models of allergic airways disease and discuss factors that influence and pathways involved in regulating the IL-17-response.

7 References

1. GINA. 2014. Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma, Vancouver, USA. 1-134.
2. Masoli, M., D. Fabian, S. Holt, R. Beasley, and P. Global Initiative for Asthma. 2004. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 59: 469-478.
3. Moore, W. C., E. R. Bleecker, D. Curran-Everett, S. C. Erzurum, B. T. Ameredes, et al. 2007. Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. *J. Allergy Clin. Immunol.* 119: 405-413.
4. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, et al. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326: 298-304.
5. Robinson, D., Q. Hamid, A. Bentley, S. Ying, A. B. Kay, and S. R. Durham. 1993. Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J. Allergy Clin. Immunol.* 92: 313-324.
6. Van der Pouw Kraan, T. C., J. S. Van der Zee, L. C. Boeije, E. R. De Groot, S. O. Stapel, and L. A. Aarden. 1998. The role of IL-13 in IgE synthesis by allergic asthma patients. *Clin. Exp. Immunol.* 111: 129-135.
7. Kay, A. B. 1997. T cells as orchestrators of the asthmatic response. *Ciba Found. Symp.* 206: 56-67; discussion 67-70, 106-110.

8. Ramos-Barbon, D., R. Fraga-Iriso, N. S. Brienza, C. Montero-Martinez, H. Verea-Hernando, et al. 2010. T Cells localize with proliferating smooth muscle alpha-actin+ cell compartments in asthma. *Am. J. Respir. Crit. Care Med.* 182: 317-324.

9. Wenzel, S. 2012. Severe asthma: from characteristics to phenotypes to endotypes. *Clin. Exp. Allergy* 42: 650-658.

10. Chung, K. F., S. E. Wenzel, J. L. Brozek, A. Bush, M. Castro, et al. 2014. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur. Respir. J.* 43: 343-373.

11. Bousquet, J., E. Mantzouranis, A. A. Cruz, N. Ait-Khaled, C. E. Baena-Cagnani, et al. 2010. Uniform definition of asthma severity, control, and exacerbations: document presented for the World Health Organization Consultation on Severe Asthma. *J. Allergy Clin. Immunol.* 126: 926-938.

12. Wenzel, S. E., L. B. Schwartz, E. L. Langmack, J. L. Halliday, J. B. Trudeau, et al. 1999. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am. J. Respir. Crit. Care Med.* 160: 1001-1008.

13. Campo, P., F. Rodriguez, S. Sanchez-Garcia, P. Barranco, S. Quirce, et al. 2013. Phenotypes and endotypes of uncontrolled severe asthma: new treatments. *J. Investig. Allergol. Clin. Immunol.* 23: 76-88; quiz 71 p follow 88.

14. Green, B. J., S. Wiriyachaiporn, C. Grainge, G. B. Rogers, V. Kehagia, et al. 2014. Potentially pathogenic airway bacteria and neutrophilic inflammation in treatment resistant severe asthma. *PLoS One* 9: e100645.

15. Newby, C., L. G. Heaney, A. Menzies-Gow, R. M. Niven, A. Mansur, et al. 2014. Statistical cluster analysis of the british thoracic society severe refractory asthma registry: clinical outcomes and phenotype stability. *PLoS One* 9: e102987.

16. Kupczyk, M., B. Dahlen, P. J. Sterk, E. Nizankowska-Mogilnicka, A. Papi, et al. 2014. Stability of phenotypes defined by physiological variables and biomarkers in adults with asthma. *Allergy*.

17. Lotvall, J., C. A. Akdis, L. B. Bacharier, L. Bjermer, T. B. Casale, et al. 2011. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. *J. Allergy Clin. Immunol.* 127: 355-360.

18. Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255-281.

19. Gerblich, A. A., H. Salik, and M. R. Schuyler. 1991. Dynamic T-cell changes in peripheral blood and bronchoalveolar lavage after antigen bronchoprovocation in asthmatics. *Am. Rev. Respir. Dis.* 143: 533-537.

20. Crimi, E., D. Gaffi, E. Frittoli, B. Borgonovo, and S. E. Burastero. 1997. Depletion of circulating allergen-specific TH2 T lymphocytes after allergen exposure in asthma. *J. Allergy Clin. Immunol.* 99: 788-797.

21. Robinson, D. S., A. M. Bentley, A. Hartnell, A. B. Kay, and S. R. Durham. 1993. Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: relation to asthma symptoms, lung function, and bronchial responsiveness. *Thorax* 48: 26-32.

22. Tournoy, K. G., J. C. Kips, and R. A. Pauwels. 2001. The allergen-induced airway hyperresponsiveness in a human-mouse chimera model of asthma is T cell and IL-4 and IL-5 dependent. *J. Immunol.* 166: 6982-6991.

23. Cohn, L., J. S. Tepper, and K. Bottomly. 1998. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J. Immunol.* 161: 3813-3816.

24. Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186: 1737-1747.

25. Cohn, L., R. J. Homer, H. MacLeod, M. Mohrs, F. Brombacher, and K. Bottomly. 1999. Th2-induced airway mucus production is dependent on IL-4Ralpha, but not on eosinophils. *J. Immunol.* 162: 6178-6183.

26. Gavett, S. H., X. Chen, F. Finkelman, and M. Wills-Karp. 1994. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.* 10: 587-593.

27. Nabe, T., T. Morishita, K. Matsuya, A. Ikedo, M. Fujii, et al. 2011. Complete dependence on CD4+ cells in late asthmatic response, but limited contribution of the cells to airway remodeling in sensitized mice. *J. Pharmacol. Sci.* 116: 373-383.

28. Ramos-Barbon, D., J. F. Presley, Q. A. Hamid, E. D. Fixman, and J. G. Martin. 2005. Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma. *J. Clin. Invest.* 115: 1580-1589.

29. Levine, S. J., and S. E. Wenzel. 2010. Narrative review: the role of Th2 immune pathway modulation in the treatment of severe asthma and its phenotypes. *Ann. Intern. Med.* 152: 232-237.

30. Chatila, T. A. 2004. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends Mol. Med.* 10: 493-499.

31. Walker, C., W. Bauer, R. K. Braun, G. Menz, P. Braun, et al. 1994. Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am. J. Respir. Crit. Care Med.* 150: 1038-1048.

32. Daher, S., L. M. Santos, D. Sole, M. G. De Lima, C. K. Naspitz, and C. C. Musatti. 1995. Interleukin-4 and soluble CD23 serum levels in asthmatic atopic children. *J. Investig. Allergol. Clin. Immunol.* 5: 251-254.

33. Shi, H. Z., J. M. Deng, H. Xu, Z. X. Nong, C. Q. Xiao, et al. 1998. Effect of inhaled interleukin-4 on airway hyperreactivity in asthmatics. *Am. J. Respir. Crit. Care Med.* 157: 1818-1821.

34. Sandford, A. J., T. Chagani, S. Zhu, T. D. Weir, T. R. Bai, et al. 2000. Polymorphisms in the IL4, IL4RA, and FCERIB genes and asthma severity. *J. Allergy Clin. Immunol.* 106: 135-140.

35. Al-Muhsen, S., A. Vazquez-Tello, A. Alzaabi, M. S. Al-Hajjaj, H. H. Al-Jahdali, and R. Halwani. 2014. IL-4 receptor alpha single-nucleotide polymorphisms rs1805010 and rs1801275 are associated with increased risk of asthma in a Saudi Arabian population. *Ann. Thorac. Med.* 9: 81-86.

36. Antoniu, S. A. 2010. Pitrakinra, a dual IL-4/IL-13 antagonist for the potential treatment of asthma and eczema. *Curr Opin Investig Drugs* 11: 1286-1294.

37. Borish, L. C., H. S. Nelson, M. J. Lanz, L. Claussen, J. B. Whitmore, et al. 1999. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. *Am. J. Respir. Crit. Care Med.* 160: 1816-1823.

38. Hambly, N., and P. Nair. 2014. Monoclonal antibodies for the treatment of refractory asthma. *Curr. Opin. Pulm. Med.* 20: 87-94.

39. Wenzel, S., L. Ford, D. Pearlman, S. Spector, L. Sher, et al. 2013. Dupilumab in persistent asthma with elevated eosinophil levels. *N. Engl. J. Med.* 368: 2455-2466.

40. Justice, J. P., M. T. Borchers, J. J. Lee, W. H. Rowan, Y. Shibata, and M. R. Van Scott. 2002. Ragweed-induced expression of GATA-3, IL-4, and IL-5 by eosinophils in the lungs of allergic C57BL/6J mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 282: L302-309.

41. Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, et al. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* 183: 109-117.

42. McCusker, C. T., Y. Wang, J. Shan, M. W. Kinyanjui, A. Villeneuve, et al. 2007. Inhibition of experimental allergic airways disease by local application of a cell-penetrating dominant-negative STAT-6 peptide. *J. Immunol.* 179: 2556-2564.

43. Vella, A., T. K. Teague, J. Ihle, J. Kappler, and P. Marrack. 1997. Interleukin 4 (IL-4) or IL-7 prevents the death of resting T cells: stat6 is probably not required for the effect of IL-4. *J. Exp. Med.* 186: 325-330.

44. Brusselle, G., J. Kips, G. Joos, H. Bluethmann, and R. Pauwels. 1995. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell Mol. Biol.* 12: 254-259.

45. Perkins, C., M. Wills-Karp, and F. D. Finkelman. 2006. IL-4 induces IL-13-independent allergic airway inflammation. *J. Allergy Clin. Immunol.* 118: 410-419.

46. van Panhuys, N., S. C. Tang, M. Prout, M. Camberis, D. Scarlett, et al. 2008. In vivo studies fail to reveal a role for IL-4 or STAT6 signaling in Th2 lymphocyte differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 105: 12423-12428.

47. Paul, W. E. 2010. What determines Th2 differentiation, in vitro and in vivo? *Immunol. Cell Biol.* 88: 236-239.

48. Wills-Karp, M. 2004. Interleukin-13 in asthma pathogenesis. *Immunol. Rev.* 202: 175-190.

49. Hamid, Q., M. Azzawi, S. Ying, R. Moqbel, A. J. Wardlaw, et al. 1991. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J. Clin. Invest.* 87: 1541-1546.

50. Leckie, M. J., A. ten Brinke, J. Khan, Z. Diamant, B. J. O'Connor, et al. 2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356: 2144-2148.

51. Kips, J. C., B. J. O'Connor, S. J. Langley, A. Woodcock, H. A. Kerstjens, et al. 2003. Effect of SCH55700, a humanized anti-human interleukin-5 antibody, in severe persistent asthma: a pilot study. *Am. J. Respir. Crit. Care Med.* 167: 1655-1659.

52. Busse, W. W., J. Ring, J. Huss-Marp, and J. E. Kahn. 2010. A review of treatment with mepolizumab, an anti-IL-5 mAb, in hypereosinophilic syndromes and asthma. *J. Allergy Clin. Immunol.* 125: 803-813.

53. Nagai, H., S. Yamaguchi, and H. Tanaka. 1996. The role of interleukin-5 (IL-5) in allergic airway hyperresponsiveness in mice. *Ann. N. Y. Acad. Sci.* 796: 91-96.

54. Kaminuma, O., A. Mori, K. Ogawa, A. Nakata, H. Kikkawa, et al. 1997. Successful transfer of late phase eosinophil infiltration in the lung by infusion of helper T cell clones. *Am. J. Respir. Cell Mol. Biol.* 16: 448-454.

55. Shardonofsky, F. R., J. Venzor, 3rd, R. Barrios, K. P. Leong, and D. P. Huston. 1999. Therapeutic efficacy of an anti-IL-5 monoclonal antibody delivered into the respiratory tract in a murine model of asthma. *J. Allergy Clin. Immunol.* 104: 215-221.

56. Temann, U. A., G. P. Geba, J. A. Rankin, and R. A. Flavell. 1998. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J. Exp. Med.* 188: 1307-1320.

57. Goswami, R., and M. H. Kaplan. 2011. A brief history of IL-9. *J. Immunol.* 186: 3283-3288.

58. Stassen, M., E. Schmitt, and T. Bopp. 2012. From interleukin-9 to T helper 9 cells. *Ann. N. Y. Acad. Sci.* 1247: 56-68.

59. Dardalhon, V., A. Awasthi, H. Kwon, G. Galileos, W. Gao, et al. 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat. Immunol.* 9: 1347-1355.

60. Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, et al. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* 9: 1341-1346.

61. Shimbara, A., P. Christodoulopoulos, A. Soussi-Gounni, R. Olivenstein, Y. Nakamura, et al. 2000. IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J. Allergy Clin. Immunol.* 105: 108-115.

62. Temann, U. A., P. Ray, and R. A. Flavell. 2002. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J. Clin. Invest.* 109: 29-39.

63. McMillan, S. J., B. Bishop, M. J. Townsend, A. N. McKenzie, and C. M. Lloyd. 2002. The absence of interleukin 9 does not affect the development of allergen-induced pulmonary inflammation nor airway hyperreactivity. *J. Exp. Med.* 195: 51-57.

64. Saha, S. K., M. A. Berry, D. Parker, S. Siddiqui, A. Morgan, et al. 2008. Increased sputum and bronchial biopsy IL-13 expression in severe asthma. *J. Allergy Clin. Immunol.* 121: 685-691.

65. Berry, M. A., D. Parker, N. Neale, L. Woodman, A. Morgan, et al. 2004. Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. *J. Allergy Clin. Immunol.* 114: 1106-1109.

66. Humbert, M., S. R. Durham, P. Kimmitt, N. Powell, B. Assoufi, et al. 1997. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J. Allergy Clin. Immunol.* 99: 657-665.

67. Naseer, T., E. M. Minshall, D. Y. Leung, S. Laberge, P. Ernst, et al. 1997. Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. *Am. J. Respir. Crit. Care Med.* 155: 845-851.

68. Huang, S. K., H. Q. Xiao, J. Kleine-Tebbe, G. Paciotti, D. G. Marsh, et al. 1995. IL-13 expression at the sites of allergen challenge in patients with asthma. *J. Immunol.* 155: 2688-2694.

69. Cui, L., J. Jia, C. F. Ma, S. Y. Li, Y. P. Wang, et al. 2012. IL-13 polymorphisms contribute to the risk of asthma: a meta-analysis. *Clin. Biochem.* 45: 285-288.

70. Corren, J., R. F. Lemanske, N. A. Hanania, P. E. Korenblat, M. V. Parsey, et al. 2011. Lebrikizumab treatment in adults with asthma. *N. Engl. J. Med.* 365: 1088-1098.

71. Scheerens, H., J. R. Arron, Y. Zheng, W. S. Putnam, R. W. Erickson, et al. 2014. The effects of lebrikizumab in patients with mild asthma following whole lung allergen challenge. *Clin. Exp. Allergy* 44: 38-46.

72. Woodruff, P. G., H. A. Boushey, G. M. Dolganov, C. S. Barker, Y. H. Yang, et al. 2007. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc. Natl. Acad. Sci. U. S. A.* 104: 15858-15863.

73. Bhakta, N. R., O. D. Solberg, C. P. Nguyen, C. N. Nguyen, J. R. Arron, et al. 2013. A qPCR-based metric of Th2 airway inflammation in asthma. *Clin Transl Allergy* 3: 24.

74. Jia, G., R. W. Erickson, D. F. Choy, S. Mosesova, L. C. Wu, et al. 2012. Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. *J. Allergy Clin. Immunol.* 130: 647-654 e610.

75. Conrad, M. L., A. O. Yildirim, S. S. Sonar, A. Kilic, S. Sudowe, et al. 2009. Comparison of adjuvant and adjuvant-free murine experimental asthma models. *Clin. Exp. Allergy* 39: 1246-1254.

76. Chiba, Y., S. Nakazawa, M. Todoroki, K. Shinozaki, H. Sakai, and M. Misawa. 2009. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. *Am. J. Respir. Cell Mol. Biol.* 40: 159-167.

77. Ro, E. J., P. H. Cha, H. Y. Kim, Y. H. Cho, J. W. Park, et al. 2013. House dust mite allergen Der f 2 induces interleukin-13 expression by activating the PI3K/Akt pathway. *Immunol. Res.* 56: 181-188.

78. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, et al. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258-2261.

79. Kinyanjui, M. W., J. Shan, E. M. Nakada, S. T. Qureshi, and E. D. Fixman. 2013. Dose-dependent effects of IL-17 on IL-13-induced airway inflammatory responses and airway hyperresponsiveness. *J. Immunol.* 190: 3859-3868.

80. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, et al. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261-2263.

81. Mattes, J., M. Yang, A. Siqueira, K. Clark, J. MacKenzie, et al. 2001. IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. *J. Immunol.* 167: 1683-1692.

82. Fattouh, R., A. Al-Garawi, M. Fattouh, K. Arias, T. D. Walker, et al. 2011. Eosinophils are dispensable for allergic remodeling and immunity in a model of house dust mite-induced airway disease. *Am. J. Respir. Crit. Care Med.* 183: 179-188.

83. Jacobsen, E. A., W. E. Lesuer, L. Willetts, K. R. Zellner, K. Mazzolini, et al. 2014. Eosinophil activities modulate the immune/inflammatory character of allergic respiratory responses in mice. *Allergy* 69: 315-327.

84. Adcock, I. M., and P. J. Barnes. 2008. Molecular mechanisms of corticosteroid resistance. *Chest* 134: 394-401.

85. Vazquez-Tello, A., R. Halwani, Q. Hamid, and S. Al-Muhsen. 2013. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J. Clin. Immunol.* 33: 466-478.

86. Ito, K., K. F. Chung, and I. M. Adcock. 2006. Update on glucocorticoid action and resistance. *J. Allergy Clin. Immunol.* 117: 522-543.

87. Chung, K. F. 2012. Inflammatory biomarkers in severe asthma. *Curr. Opin. Pulm. Med.* 18: 35-41.

88. Leigh, R., D. Vethanayagam, M. Yoshida, R. M. Watson, T. Rerecich, et al. 2002. Effects of montelukast and budesonide on airway responses and airway inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 166: 1212-1217.

89. Gauvreau, G. M., M. D. Inman, M. Kelly, R. M. Watson, S. C. Dorman, and P. M. O'Byrne. 2002. Increased levels of airway neutrophils reduce the inhibitory effects of inhaled glucocorticosteroids on allergen-induced airway eosinophils. *Can. Respir. J.* 9: 26-32.

90. Gauvreau, G. M., I. Sulakvelidze, R. M. Watson, M. D. Inman, T. J. Rerecich, and P. M. O'Byrne. 2000. Effects of once daily dosing with inhaled budesonide on airway hyperresponsiveness and airway inflammation following repeated low-dose allergen challenge in atopic asthmatics. *Clin. Exp. Allergy* 30: 1235-1243.

91. Ano, S., Y. Morishima, Y. Ishii, K. Yoh, Y. Yageta, et al. 2013. Transcription factors GATA-3 and RORgammat are important for determining the phenotype of allergic airway inflammation in a murine model of asthma. *J. Immunol.* 190: 1056-1065.

92. Shen, H., P. M. O'Byrne, R. Ellis, J. Wattie, C. Tang, and M. D. Inman. 2002. The effects of intranasal budesonide on allergen-induced production of interleukin-5 and eotaxin, airways, blood, and bone marrow eosinophilia, and eosinophil progenitor expansion in sensitized mice. *Am. J. Respir. Crit. Care Med.* 166: 146-153.

93. Southam, D. S., R. Ellis, J. Wattie, W. Glass, and M. D. Inman. 2008. Goblet cell rebound and airway dysfunction with corticosteroid withdrawal in a mouse model of asthma. *Am. J. Respir. Crit. Care Med.* 178: 1115-1122.

94. Henderson, W. R., Jr., G. K. Chiang, Y. T. Tien, and E. Y. Chi. 2006. Reversal of allergen-induced airway remodeling by CysLT1 receptor blockade. *Am. J. Respir. Crit. Care Med.* 173: 718-728.

95. McKinley, L., J. F. Alcorn, A. Peterson, R. B. Dupont, S. Kapadia, et al. 2008. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* 181: 4089-4097.

96. Hymowitz, S. G., E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, et al. 2001. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* 20: 5332-5341.

97. Pappu, R., V. Ramirez-Carrozzi, N. Ota, W. Ouyang, and Y. Hu. 2010. The IL-17 family cytokines in immunity and disease. *J. Clin. Immunol.* 30: 185-195.

98. Gu, C., L. Wu, and X. Li. 2013. IL-17 family: cytokines, receptors and signaling. *Cytokine* 64: 477-485.

99. van de Veerdonk, F. L., M. S. Gresnigt, B. J. Kullberg, J. W. van der Meer, L. A. Joosten, and M. G. Netea. 2009. Th17 responses and host defense against microorganisms: an overview. *BMB Rep* 42: 776-787.

100. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, et al. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233-240.

101. van Beelen, A. J., M. B. Teunissen, M. L. Kapsenberg, and E. C. de Jong. 2007. Interleukin-17 in inflammatory skin disorders. *Curr. Opin. Allergy Clin. Immunol.* 7: 374-381.

102. Ouyang, W., J. K. Kolls, and Y. Zheng. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28: 454-467.

103. Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, et al. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430-438.

104. Al-Ramli, W., M. Al Samri, and Q. Hamid. 2008. Th-17 cell-related cytokines' potential role in the pathogenesis of severe asthma. *J. Asthma* 45 Suppl 1: 41-44.

105. Adorini, L. 1999. Interleukin-12, a key cytokine in Th1-mediated autoimmune diseases. *Cell. Mol. Life Sci.* 55: 1610-1625.

106. Croxford, A. L., P. Kulig, and B. Becher. 2014. IL-12-and IL-23 in health and disease. *Cytokine Growth Factor Rev.*

107. Becher, B., B. G. Durell, and R. J. Noelle. 2002. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J. Clin. Invest.* 110: 493-497.

108. Leonard, J. P., K. E. Waldburger, and S. J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med.* 181: 381-386.

109. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421: 744-748.

110. Wakashin, H., K. Hirose, I. Iwamoto, and H. Nakajima. 2009. Role of IL-23-Th17 cell axis in allergic airway inflammation. *Int. Arch. Allergy Immunol.* 149 Suppl 1: 108-112.

111. Barczyk, A., W. Pierzchala, and E. Sozanska. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 97: 726-733.

112. Al-Ramli, W., D. Prefontaine, F. Chouiali, J. G. Martin, R. Olivenstein, et al. 2009. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J. Allergy Clin. Immunol.* 123: 1185-1187.

113. Agache, I., C. Ciobanu, C. Agache, and M. Anghel. 2010. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir. Med.* 104: 1131-1137.

114. Jiang, K., H. B. Chen, Y. Wang, J. H. Lin, Y. Hu, and Y. R. Fang. 2013. [Changes in IL-17 and TGF-beta1 levels in serum and bronchoalveolar lavage fluid and their clinical significance among children with asthma]. *Zhongguo Dang Dai Er Ke Za Zhi* 15: 604-608.

115. Chen, J., Y. Deng, J. Zhao, Z. Luo, W. Peng, et al. 2010. The polymorphism of IL-17 G-152A was associated with childhood asthma and bacterial colonization of the hypopharynx in bronchiolitis. *J. Clin. Immunol.* 30: 539-545.

116. Maalmi, H., A. Beraies, R. Charad, J. Ammar, K. Hamzaoui, and A. Hamzaoui. 2014. IL-17A and IL-17F genes variants and susceptibility to childhood asthma in Tunisia. *J. Asthma* 51: 348-354.

117. Wang, J. Y., S. D. Shyur, W. H. Wang, Y. H. Liou, C. G. Lin, et al. 2009. The polymorphisms of interleukin 17A (IL17A) gene and its association with pediatric asthma in Taiwanese population. *Allergy* 64: 1056-1060.

118. Chien, J. W., C. Y. Lin, K. D. Yang, C. H. Lin, J. K. Kao, and Y. G. Tsai. 2013. Increased IL-17A secreting CD4+ T cells, serum IL-17 levels and exhaled nitric oxide are correlated with childhood asthma severity. *Clin. Exp. Allergy* 43: 1018-1026.

119. Chen, Y., P. Thai, Y. H. Zhao, Y. S. Ho, M. M. DeSouza, and R. Wu. 2003. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J. Biol. Chem.* 278: 17036-17043.

120. Bullens, D. M., E. Truyen, L. Coteur, E. Dilissen, P. W. Hellings, et al. 2006. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir. Res.* 7: 135.

121. Linden, A., and M. Adachi. 2002. Neutrophilic airway inflammation and IL-17. *Allergy* 57: 769-775.

122. Fahy, J. V., K. W. Kim, J. Liu, and H. A. Boushey. 1995. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J. Allergy Clin. Immunol.* 95: 843-852.

123. Busse, W. W., S. Holgate, E. Kerwin, Y. Chon, J. Feng, et al. 2013. Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *Am. J. Respir. Crit. Care Med.* 188: 1294-1302.

124. Sun, Y. C., Q. T. Zhou, and W. Z. Yao. 2005. Sputum interleukin-17 is increased and associated with airway neutrophilia in patients with severe asthma. *Chin. Med. J.* 118: 953-956.

125. Cosmi, L., R. De Palma, V. Santarlasci, L. Maggi, M. Capone, et al. 2008. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J. Exp. Med.* 205: 1903-1916.

126. Zhao, Y., J. Yang, Y. D. Gao, and W. Guo. 2010. Th17 immunity in patients with allergic asthma. *Int. Arch. Allergy Immunol.* 151: 297-307.

127. Zhao, Y., J. Yang, and Y. D. Gao. 2011. Altered expressions of helper T cell (Th)1, Th2, and Th17 cytokines in CD8(+) and gammadelta T cells in patients with allergic asthma. *J. Asthma* 48: 429-436.

128. Ramirez-Velazquez, C., E. C. Castillo, L. Guido-Bayardo, and V. Ortiz-Navarrete. 2013. IL-17-producing peripheral blood CD177+ neutrophils increase in allergic asthmatic subjects. *Allergy Asthma Clin. Immunol.* 9: 23.

129. Schnyder-Candrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, et al. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203: 2715-2725.

130. Nakae, S., Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, et al. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 17: 375-387.

131. Newcomb, D. C., M. G. Boswell, S. Reiss, W. Zhou, K. Goleniewska, et al. 2013. IL-17A inhibits airway reactivity induced by respiratory syncytial virus infection during allergic airway inflammation. *Thorax* 68: 717-723.

132. Murdoch, J. R., and C. M. Lloyd. 2010. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing {gamma}{delta}T cells. *Am. J. Respir. Crit. Care Med.* 182: 464-476.

133. Lajoie, S., I. P. Lewkowich, Y. Suzuki, J. R. Clark, A. A. Sproles, et al. 2010. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat. Immunol.* 11: 928-935.

134. Song, C., L. Luo, Z. Lei, B. Li, Z. Liang, et al. 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J. Immunol.* 181: 6117-6124.

135. Pichavant, M., S. Goya, E. H. Meyer, R. A. Johnston, H. Y. Kim, et al. 2008. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J. Exp. Med.* 205: 385-393.

136. Wingender, G., P. Rogers, G. Batzer, M. S. Lee, D. Bai, et al. 2011. Invariant NKT cells are required for airway inflammation induced by environmental antigens. *J. Exp. Med.* 208: 1151-1162.

137. Zhao, J., C. M. Lloyd, and A. Noble. 2013. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol.* 6: 335-346.

138. Oboki, K., T. Ohno, H. Saito, and S. Nakae. 2008. Th17 and allergy. *Allergol Int* 57: 121-134.

139. Infante-Duarte, C., H. F. Horton, M. C. Byrne, and T. Kamradt. 2000. Microbial lipopeptides induce the production of IL-17 in Th cells. *J. Immunol.* 165: 6107-6115.

140. Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278: 1910-1914.

141. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, et al. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235-238.

142. Wilson, R. H., G. S. Whitehead, H. Nakano, M. E. Free, J. K. Kolls, and D. N. Cook. 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 180: 720-730.

143. Wakashin, H., K. Hirose, Y. Maezawa, S. Kagami, A. Suto, et al. 2008. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* 178: 1023-1032.

144. Martin, R. A., J. L. Ather, R. Daggett, L. Hoyt, J. F. Alcorn, et al. 2013. The endogenous Th17 response in NO2-promoted allergic airway disease is dispensable for airway hyperresponsiveness and distinct from Th17 adoptive transfer. *PLoS One* 8: e74730.

145. Peng, Y., Y. Laouar, M. O. Li, E. A. Green, and R. A. Flavell. 2004. TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc. Natl. Acad. Sci. U. S. A.* 101: 4572-4577.

146. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, et al. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441: 231-234.

147. Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, et al. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8: 967-974.

148. Ivanov, II, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, et al. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121-1133.

149. Ichiyama, K., H. Yoshida, Y. Wakabayashi, T. Chinen, K. Saeki, et al. 2008. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *J. Biol. Chem.* 283: 17003-17008.

150. Agarwal, A., M. Singh, B. P. Chatterjee, A. Chauhan, and A. Chakraborti. 2014. Interplay of T Helper 17 Cells with CD4(+)CD25(high) FOXP3(+) Tregs in Regulation of Allergic Asthma in Pediatric Patients. *Int. J. Pediatr.* 2014: 636238.

151. Barczyk, A., W. Pierzchala, G. Caramori, R. Wiaderkiewicz, M. Kaminski, et al. 2014. Decreased percentage of CD4(+)Foxp3(+)TGF-beta(+) and increased percentage of CD4(+)IL-17(+) cells in bronchoalveolar lavage of asthmatics. *J Inflamm (Lond)* 11: 22.

152. Born, W. K., M. Lahn, K. Takeda, A. Kanehiro, R. L. O'Brien, and E. W. Gelfand. 2000. Role of gammadelta T cells in protecting normal airway function. *Respir. Res.* 1: 151-158.

153. Lahn, M. 2000. The role of gammadelta T cells in the airways. *J. Mol. Med.* 78: 409-425.

154. Augustin, A., R. T. Kubo, and G. K. Sim. 1989. Resident pulmonary lymphocytes expressing the gamma/delta T-cell receptor. *Nature* 340: 239-241.

155. Zheng, J., Y. Liu, Y. L. Lau, and W. Tu. 2013. gammadelta-T cells: an unpolished sword in human anti-infection immunity. *Cell. Mol. Immunol.* 10: 50-57.

156. King, D. P., D. M. Hyde, K. A. Jackson, D. M. Novosad, T. N. Ellis, et al. 1999. Cutting edge: protective response to pulmonary injury requires gamma delta T lymphocytes. *J. Immunol.* 162: 5033-5036.

157. McMenamin, C., C. Pimm, M. McKersey, and P. G. Holt. 1994. Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science* 265: 1869-1871.

158. Aydintug, M. K., C. L. Roark, J. L. Chain, W. K. Born, and R. L. O'Brien. 2008. Macrophages express multiple ligands for gammadelta TCRs. *Mol. Immunol.* 45: 3253-3263.

159. Wands, J. M., C. L. Roark, M. K. Aydintug, N. Jin, Y. S. Hahn, et al. 2005. Distribution and leukocyte contacts of gammadelta T cells in the lung. *J. Leukoc. Biol.* 78: 1086-1096.

160. O'Brien, R., N. Jin, Y. Huang, M. K. Aydintug, C. Roark, and W. Born. 2010. Characteristics of IL-17-producing gammadelta T cells. *Immunity* 32: 1; author reply 2-4.

161. Heilig, J. S., and S. Tonegawa. 1986. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature* 322: 836-840.

162. Shibata, K., H. Yamada, M. Nakamura, S. Hatano, Y. Katsuragi, et al. 2014. IFN-gamma-producing and IL-17-producing gammadelta T cells differentiate at distinct developmental stages in murine fetal thymus. *J. Immunol.* 192: 2210-2218.

163. Garman, R. D., P. J. Doherty, and D. H. Raulet. 1986. Diversity, rearrangement, and expression of murine T cell gamma genes. *Cell* 45: 733-742.

164. Nakada, E. M., J. Shan, M. W. Kinyanjui, and E. D. Fixman. 2014. Adjuvant-dependent regulation of interleukin-17 expressing inverted question mark inverted question mark T cells and inhibition of Th2 responses in allergic airways disease. *Respiratory research* 15: 90.

165. Sim, G. K., R. Rajaserkar, M. Dessing, and A. Augustin. 1994. Homing and in situ differentiation of resident pulmonary lymphocytes. *Int. Immunol.* 6: 1287-1295.

166. Roark, C. L., Y. Huang, N. Jin, M. K. Aydintug, T. Casper, et al. 2013. A canonical Vgamma4Vdelta4+ gammadelta T cell population with distinct stimulation requirements which promotes the Th17 response. *Immunol. Res.* 55: 217-230.

167. Hahn, Y. S., C. Taube, N. Jin, K. Takeda, J. W. Park, et al. 2003. V gamma 4+ gamma delta T cells regulate airway hyperreactivity to methacholine in ovalbumin-sensitized and challenged mice. *J. Immunol.* 171: 3170-3178.

168. Sugiki, T., T. Fujiwara, and C. Kojima. 2014. Latest approaches for efficient protein production in drug discovery. *Expert Opin Drug Discov* 9: 1189-1204.

169. Chen, R. 2012. Bacterial expression systems for recombinant protein production: E. coli and beyond. *Biotechnol Adv* 30: 1102-1107.

170. Tian, B. P., W. Hua, L. X. Xia, Y. Jin, F. Lan, et al. 2014. Exogenous Interleukin-17A Inhibits Eosinophil Differentiation and Alleviates Allergic Airway Inflammation. *Am. J. Respir. Cell Mol. Biol.*

171. Kudo, M., A. C. Melton, C. Chen, M. B. Engler, K. E. Huang, et al. 2012. IL-17A produced by alphabeta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat. Med.* 18: 547-554.

172. Cua, D. J., and C. M. Tato. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat. Rev. Immunol.* 10: 479-489.

173. Umetsu, D. T., and R. H. Dekruyff. 2010. Natural killer T cells are important in the pathogenesis of asthma: the many pathways to asthma. *J. Allergy Clin. Immunol.* 125: 975-979.

174. Ferretti, S., O. Bonneau, G. R. Dubois, C. E. Jones, and A. Trifilieff. 2003. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J. Immunol.* 170: 2106-2112.

175. Vultaggio, A., F. Nencini, S. Pratesi, G. Petroni, S. Romagnani, and E. Maggi. 2012. Poly(I:C) promotes the production of IL-17A by murine CD1d-driven invariant NKT cells in airway inflammation. *Allergy* 67: 1223-1232.

176. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23: 877-900.

177. Umetsu, D. T., and R. H. Dekruyff. 2010. The regulatory role of natural killer T cells in the airways. *Int. J. Biochem. Cell Biol.* 42: 529-534.

178. Kronenberg, M., and L. Gapin. 2002. The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol.* 2: 557-568.

179. Nembrini, C., B. J. Marsland, and M. Kopf. 2009. IL-17-producing T cells in lung immunity and inflammation. *J. Allergy Clin. Immunol.* 123: 986-994; quiz 995-986.

180. Akbari, O., J. L. Faul, E. G. Hoyte, G. J. Berry, J. Wahlstrom, et al. 2006. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N. Engl. J. Med.* 354: 1117-1129.

181. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, et al. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9: 582-588.

182. Coquet, J. M., S. Chakravarti, K. Kyparissoudis, F. W. McNab, L. A. Pitt, et al. 2008. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc. Natl. Acad. Sci. U. S. A.* 105: 11287-11292.

183. Kim, H. Y., H. J. Lee, Y. J. Chang, M. Pichavant, S. A. Shore, et al. 2014. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat. Med.* 20: 54-61.

184. Li, K., Z. Wang, Y. Cao, H. Bunjhoo, J. Zhu, et al. 2013. The study of the ratio and distribution of Th17 cells and Tc17 cells in asthmatic patients and the mouse model. *Asian Pac. J. Allergy Immunol.* 31: 125-131.

185. Vazquez-Tello, A., R. Halwani, R. Li, J. Nadigel, A. Bar-Or, et al. 2012. IL-17A and IL-17F expression in B lymphocytes. *Int. Arch. Allergy Immunol.* 157: 406-416.

186. Hasan, S. A., B. Eksteen, D. Reid, H. V. Paine, A. Alansary, et al. 2013. Role of IL-17A and neutrophils in fibrosis in experimental hypersensitivity pneumonitis. *J. Allergy Clin. Immunol.* 131: 1663-1673.

187. Hamada, H., L. Garcia-Hernandez Mde, J. B. Reome, S. K. Misra, T. M. Strutt, et al. 2009. Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. *J. Immunol.* 182: 3469-3481.

188. Bao, Y., and X. Cao. 2014. The immune potential and immunopathology of cytokine-producing B cell subsets: A comprehensive review. *J. Autoimmun.* 55C: 10-23.

189. Zhou, L., S. Peng, J. Duan, J. Zhou, L. Wang, and J. Wang. 1998. A human B cell line AF10 expressing HIL-17. *Biochem. Mol. Biol. Int.* 45: 1113-1119.

190. Bermejo, D. A., S. W. Jackson, M. Gorosito-Serran, E. V. Acosta-Rodriguez, M. C. Amezcua-Vesely, et al. 2013. Trypanosoma cruzi trans-sialidase initiates a program independent of the transcription factors RORgammat and Ahr that leads to IL-17 production by activated B cells. *Nat. Immunol.* 14: 514-522.

191. Kumar, V. 2014. Innate lymphoid cells: new paradigm in immunology of inflammation. *Immunol. Lett.* 157: 23-37.

192. Hepworth, M. R., L. A. Monticelli, T. C. Fung, C. G. Ziegler, S. Grunberg, et al. 2013. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature* 498: 113-117.

193. Bartemes, K. R., G. M. Kephart, S. J. Fox, and H. Kita. 2014. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J. Allergy Clin. Immunol.* 134: 671-678 e674.

194. Stills, H. F., Jr. 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. *ILAR J* 46: 280-293.

195. Smith, D. E., M. E. O'Brien, V. J. Palmer, and J. A. Sadowski. 1992. The selection of an adjuvant emulsion for polyclonal antibody production using a low-molecular-weight antigen in rabbits. *Lab. Anim. Sci.* 42: 599-601.

196. Libbey, J. E., and R. S. Fujinami. 2011. Experimental autoimmune encephalomyelitis as a testing paradigm for adjuvants and vaccines. *Vaccine* 29: 3356-3362.

197. Hutchison, S., R. A. Benson, V. B. Gibson, A. H. Pollock, P. Garside, and J. M. Brewer. 2012. Antigen depot is not required for alum adjuvanticity. *FASEB J.* 26: 1272-1279.

198. Lalor, S. J., L. S. Dungan, C. E. Sutton, S. A. Basdeo, J. M. Fletcher, and K. H. Mills. 2011. Caspase-1-processed cytokines IL-1beta and IL-18 promote IL-17 production by gammadelta and CD4 T cells that mediate autoimmunity. *J. Immunol.* 186: 5738-5748.

199. Eisenbarth, S. C., O. R. Colegio, W. O'Connor, F. S. Sutterwala, and R. A. Flavell. 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453: 1122-1126.

200. Broderson, J. R. 1989. A retrospective review of lesions associated with the use of Freund's adjuvant. *Lab. Anim. Sci.* 39: 400-405.

201. Leenaars, P. P., C. F. Hendriksen, A. F. Angulo, M. A. Koedam, and E. Claassen. 1994. Evaluation of several adjuvants as alternatives to the use of Freund's adjuvant in rabbits. *Vet. Immunol. Immunopathol.* 40: 225-241.

202. Blyth, D. I., M. S. Pedrick, T. J. Savage, E. M. Hessel, and D. Fattah. 1996. Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am. J. Respir. Cell Mol. Biol.* 14: 425-438.

203. Williams, C. M., and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* 192: 455-462.

204. Nakae, S., C. Lunderius, L. H. Ho, B. Schafer, M. Tsai, and S. J. Galli. 2007. TNF can contribute to multiple features of ovalbumin-induced allergic inflammation of the airways in mice. *J. Allergy Clin. Immunol.* 119: 680-686.

205. Hessel, E. M., A. Zwart, E. Oostveen, A. J. Van Oosterhout, D. I. Blyth, and F. P. Nijkamp. 1995. Repeated measurement of respiratory function and bronchoconstriction in unanesthetized mice. *J. Appl. Physiol.* 79: 1711-1716.

206. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Adv. Immunol.* 10: 1-50.

207. Chapman, D. G., J. E. Tully, J. D. Nolin, Y. M. Janssen-Heininger, and C. G. Irvin. 2014. Animal models of allergic airways disease: where are we and where to next? *J. Cell. Biochem.* 115: 2055-2064.

208. Agarwal, R., and A. Chakrabarti. 2013. Allergic bronchopulmonary aspergillosis in asthma: epidemiological, clinical and therapeutic issues. *Future Microbiol.* 8: 1463-1474.

209. Mehlhop, P. D., M. van de Rijn, A. B. Goldberg, J. P. Brewer, V. P. Kurup, et al. 1997. Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc. Natl. Acad. Sci. U. S. A.* 94: 1344-1349.

210. Mintz-Cole, R. A., A. M. Gibson, S. A. Bass, A. L. Budelsky, T. Reponen, and G. K. Hershey. 2012. Dectin-1 and IL-17A suppress murine asthma induced by Aspergillus versicolor but not Cladosporium cladosporioides due to differences in beta-glucan surface exposure. *J. Immunol.* 189: 3609-3617.

211. Page, K., J. R. Ledford, P. Zhou, K. Dienger, and M. Wills-Karp. 2010. Mucosal sensitization to German cockroach involves protease-activated receptor-2. *Respir. Res.* 11: 62.

212. Wang, Y., Y. Li, J. Shan, E. Fixman, and C. McCusker. 2011. Effective treatment of experimental ragweed-induced asthma with STAT-6-IP, a topically delivered cell-penetrating peptide. *Clin. Exp. Allergy* 41: 1622-1630.

213. Srivastava, K. D., D. Dunkin, C. Liu, N. Yang, R. L. Miller, et al. 2014. Effect of Antiasthma Simplified Herbal Medicine Intervention on neutrophil predominant airway inflammation in a ragweed sensitized murine asthma model. *Ann. Allergy. Asthma. Immunol.* 112: 339-347 e331-332.

214. Nelson, R. P., Jr., R. DiNicolo, E. Fernandez-Caldas, M. J. Seleznick, R. F. Lockey, and R. A. Good. 1996. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. *J. Allergy Clin. Immunol.* 98: 258-263.

215. Bajoriuniene, I., K. Malakauskas, S. Lavinskiene, J. Jeroch, and R. Sakalauskas. 2013. Th17 response to Dermatophagoides pteronyssinus is related to late-phase airway and systemic inflammation in allergic asthma. *Int. Immunopharmacol.* 17: 1020-1027.

216. Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 196: 1645-1651.

217. Hogenesch, H. 2012. Mechanism of immunopotentiation and safety of aluminum adjuvants. *Front. Immunol.* 3: 406.

218. Franchi, L., and G. Nunez. 2008. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. *Eur. J. Immunol.* 38: 2085-2089.

219. Li, H., S. Nookala, and F. Re. 2007. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release. *J. Immunol.* 178: 5271-5276.

220. De Gregorio, E., E. Tritto, and R. Rappuoli. 2008. Alum adjuvanticity: unraveling a century old mystery. *Eur. J. Immunol.* 38: 2068-2071.

221. Kool, M., V. Petrilli, T. De Smedt, A. Rolaz, H. Hammad, et al. 2008. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J. Immunol.* 181: 3755-3759.

222. McKee, A. S., M. W. Munks, M. K. MacLeod, C. J. Fleenor, N. Van Rooijen, et al. 2009. Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol.* 183: 4403-4414.

223. Nakae, S., H. Suto, G. J. Berry, and S. J. Galli. 2007. Mast cell-derived TNF can promote Th17 cell-dependent neutrophil recruitment in ovalbumin-challenged OTII mice. *Blood* 109: 3640-3648.

224. Brewer, J. M., M. Conacher, A. Satoskar, H. Bluethmann, and J. Alexander. 1996. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *Eur. J. Immunol.* 26: 2062-2066.

225. Brewer, J. M., M. Conacher, M. Gaffney, M. Douglas, H. Bluethmann, and J. Alexander. 1998. Neither interleukin-6 nor signalling via tumour necrosis factor receptor-1 contribute to the adjuvant activity of Alum and Freund's adjuvant. *Immunology* 93: 41-48.

226. Brewer, J. M., M. Conacher, C. A. Hunter, M. Mohrs, F. Brombacher, and J. Alexander. 1999. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J. Immunol.* 163: 6448-6454.

227. Opie, E. L., and J. Freund. 1937. An Experimental Study of Protective Inoculation with Heat Killed Tubercle Bacilli. *J. Exp. Med.* 66: 761-788.

228. Warren, H. S., F. R. Vogel, and L. A. Chedid. 1986. Current status of immunological adjuvants. *Annu. Rev. Immunol.* 4: 369-388.

229. Freund, J., and E. L. Opie. 1938. Sensitization and Antibody Formation with Increased Resistance to Tuberculous Infection Induced by Heat Killed Tubercle Bacilli. *J. Exp. Med.* 68: 273-298.

230. Marta, M., U. C. Meier, and A. Lobell. 2009. Regulation of autoimmune encephalomyelitis by toll-like receptors. *Autoimmun Rev* 8: 506-509.

231. Marta, M., A. Andersson, M. Isaksson, O. Kampe, and A. Lobell. 2008. Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* 38: 565-575.

232. Ishii, K. J., and S. Akira. 2007. Toll or toll-free adjuvant path toward the optimal vaccine development. *J. Clin. Immunol.* 27: 363-371.

233. Gavin, A. L., K. Hoebe, B. Duong, T. Ota, C. Martin, et al. 2006. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* 314: 1936-1938.

234. Smith, A. J., Y. Liu, H. Peng, R. Beers, M. K. Racke, and A. E. Lovett-Racke. 2011. Comparison of a classical Th1 bacteria versus a Th17 bacteria as adjuvant in the induction of experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 237: 33-38.

235. Tigno-Aranjuez, J. T., R. Jaini, V. K. Tuohy, P. V. Lehmann, and M. Tary-Lehmann. 2009. Encephalitogenicity of complete Freund's adjuvant relative to CpG is linked to induction of Th17 cells. *J. Immunol.* 183: 5654-5661.

236. Vasquez, Y. R., and D. Spina. 2000. What have transgenic and knockout animals taught us about respiratory disease? *Respir. Res.* 1: 82-86.

237. Zosky, G. R., and P. D. Sly. 2007. Animal models of asthma. *Clin. Exp. Allergy* 37: 973-988.

238. Allen, J. E., R. J. Bischof, H. Y. Sucie Chang, J. A. Hirota, S. J. Hirst, et al. 2009. Animal models of airway inflammation and airway smooth muscle remodelling in asthma. *Pulm. Pharmacol. Ther.* 22: 455-465.

239. Nowakowski, A., A. Andrzejewska, M. Janowski, P. Walczak, and B. Lukomska. 2013. Genetic engineering of stem cells for enhanced therapy. *Acta Neurobiol. Exp. (Wars.)* 73: 1-18.

240. Miao, X. 2013. Recent advances in the development of new transgenic animal technology. *Cell. Mol. Life Sci.* 70: 815-828.

241. Koo, B. C., M. S. Kwon, and T. Kim. 2014. 6 - Retrovirus-Mediated Gene Transfer. In *Transgenic Animal Technology (Third Edition)*. C. A. Pinkert, ed. Elsevier, London. 167-194.

242. Kinyanjui, M. W., D. Ramos-Barbon, A. Villeneuve, and E. D. Fixman. 2006. Enhanced transduction of antigen-stimulated T lymphocytes with recombinant retroviruses concentrated by centrifugal filtration. *J. Immunol. Methods* 314: 80-89.

243. Galipeau, J., H. Li, A. Paquin, F. Sicilia, G. Karpati, and J. Nalbantoglu. 1999. Vesicular stomatitis virus G pseudotyped retrovector mediates effective in vivo suicide gene delivery in experimental brain cancer. *Cancer Res.* 59: 2384-2394.

244. Kinyanjui, M. W., M. Tamaoka, and E. D. Fixman. 2007. T-helper type 2-dependent early recruitment of antigen non-specific CD4+ T cells in experimental asthma. *Clin. Exp. Allergy* 37: 1455-1466.

245. Costa, G. L., J. M. Benson, C. M. Seroogy, P. Achacoso, C. G. Fathman, and G. P. Nolan. 2000. Targeting rare populations of murine antigen-specific T lymphocytes by retroviral transduction for potential application in gene therapy for autoimmune disease. *J. Immunol.* 164: 3581-3590.

246. Morgan, R. A., and S. Kakarla. 2014. Genetic modification of T cells. *Cancer J.* 20: 145-150.

247. Swift, S., J. Lorens, P. Achacoso, and G. P. Nolan. 2001. Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. *Curr. Protoc. Immunol.* Chapter 10: Unit 10 17C.

248. Koehn, J., and I. Hunt. 2009. High-Throughput Protein Production (HTPP): a review of enabling technologies to expedite protein production. *Methods Mol. Biol.* 498: 1-18.

249. Pope, S. M., E. B. Brandt, A. Mishra, S. P. Hogan, N. Zimmermann, et al. 2001. IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J. Allergy Clin. Immunol.* 108: 594-601.

250. Wootla, B., A. Denic, and M. Rodriguez. 2014. *Polyclonal and monoclonal antibodies in clinic*.

251. Frenzel, A., M. Hust, and T. Schirrmann. 2013. Expression of recombinant antibodies. *Front. Immunol.* 4: 217.

252. Eisenberg, S. 2012. Biologic therapy. *J. Infus. Nurs.* 35: 301-313.

253. Caminati, M., G. Senna, M. Guerriero, A. R. Dama, F. Chieco-Bianchi, et al. 2015. Omalizumab for severe allergic asthma in clinical trials and real-life studies: what we know and what we should address. *Pulm. Pharmacol. Ther.*

254. Lim, H., and P. Nair. 2015. Efficacy and safety of reslizumab in patients with moderate to severe eosinophilic asthma. *Expert Rev. Respir. Med.*: 1-8.

255. Ortega, H. G., M. C. Liu, I. D. Pavord, G. G. Brusselle, J. M. FitzGerald, et al. 2014. Mepolizumab treatment in patients with severe eosinophilic asthma. *N. Engl. J. Med.* 371: 1198-1207.

256. Nowak, R. M., J. M. Parker, R. A. Silverman, B. H. Rowe, H. Smithline, et al. 2015. A randomized trial of benralizumab, an antiinterleukin 5 receptor alpha monoclonal antibody, after acute asthma. *Am. J. Emerg. Med.* 33: 14-20.

257. Castro, M., S. E. Wenzel, E. R. Bleecker, E. Pizzichini, P. Kuna, et al. 2014. Benralizumab, an anti-interleukin 5 receptor alpha monoclonal antibody, versus placebo

for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study. *The Lancet. Respiratory medicine* 2: 879-890.

258. Vatrella, A., I. Fabozzi, C. Calabrese, R. Maselli, and G. Pelaia. 2014. Dupilumab: a novel treatment for asthma. *Journal of asthma and allergy* 7: 123-130.

259. Gauvreau, G. M., P. M. O'Byrne, L. P. Boulet, Y. Wang, D. Cockcroft, et al. 2014. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *N. Engl. J. Med.* 370: 2102-2110.

260. Nelson, A. L., E. Dhimolea, and J. M. Reichert. 2010. Development trends for human monoclonal antibody therapeutics. *Nature reviews. Drug discovery* 9: 767-774.

261. Janeway, C. 2005. *Immunobiology : the immune system in health and disease*. Garland Science, New York.

262. Mijares, A., D. Lebesgue, G. Wallukat, and J. Hoebeke. 2000. From agonist to antagonist: Fab fragments of an agonist-like monoclonal anti-beta(2)-adrenoceptor antibody behave as antagonists. *Mol. Pharmacol.* 58: 373-379.

263. Lu, C., M. Shimaoka, A. Salas, and T. A. Springer. 2004. The binding sites for competitive antagonistic, allosteric antagonistic, and agonistic antibodies to the I domain of integrin LFA-1. *J. Immunol.* 173: 3972-3978.

264. Lee, U. H., K. B. Kwack, J. W. Park, and B. S. Kwon. 2002. Molecular cloning of agonistic and antagonistic monoclonal antibodies against human 4-1BB. *Eur. J Immunogenet.* 29: 449-452.

265. Koenecke, C., V. Chennupati, S. Schmitz, B. Malissen, R. Forster, and I. Prinz. 2009. In vivo application of mAb directed against the gammadelta TCR does not deplete but generates "invisible" gammadelta T cells. *Eur. J. Immunol.* 39: 372-379.

266. Kaufmann, S. H., C. Blum, and S. Yamamoto. 1993. Crosstalk between alpha/beta T cells and gamma/delta T cells in vivo: activation of alpha/beta T-cell responses after gamma/delta T-cell modulation with the monoclonal antibody GL3. *Proc. Natl. Acad. Sci. U. S. A.* 90: 9620-9624.

267. Jaenisch, R., and B. Mintz. 1974. Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proc. Natl. Acad. Sci. U. S. A.* 71: 1250-1254.

268. Palmiter, R. D., R. L. Brinster, R. E. Hammer, M. E. Trumbauer, M. G. Rosenfeld, et al. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* 300: 611-615.

269. Palmiter, R. D. 1998. Transgenic mice--the early days. *Int. J. Dev. Biol.* 42: 847-854.

270. Gordon, J. W., and F. H. Ruddle. 1981. Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science* 214: 1244-1246.

271. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, et al. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133-1141.

272. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* 250: 1720-1723.

273. Robertson, J. M., P. E. Jensen, and B. D. Evavold. 2000. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J. Immunol.* 164: 4706-4712.

274. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34-40.

275. Jarman, E. R., K. A. Tan, and J. R. Lamb. 2005. Transgenic mice expressing the T cell antigen receptor specific for an immunodominant epitope of a major allergen of house dust mite develop an asthmatic phenotype on exposure of the airways to allergen. *Clin. Exp. Allergy* 35: 960-969.

276. Gregory, L. G., and C. M. Lloyd. 2011. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* 32: 402-411.

277. Gierut, J. J., T. E. Jacks, and K. M. Haigis. 2014. Strategies to achieve conditional gene mutation in mice. *Cold Spring Harb Protoc* 2014: 339-349.

278. Yang, X. O., S. H. Chang, H. Park, R. Nurieva, B. Shah, et al. 2008. Regulation of inflammatory responses by IL-17F. *J. Exp. Med.* 205: 1063-1075.

CHAPTER 2: Adjuvant-dependent regulation of interleukin-17 expressing γδ T cells and inhibition of Th2 responses in allergic airways disease

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1 Abstract

Background: Th2 immune responses are linked primarily to mild and moderate asthma, while Th17 cells, Interleukin-17A (IL-17) and neutrophilia have been implicated in more severe forms of disease. How Th2-dependent allergic reactions are influenced by Th17 and IL-17-γδ T cells is poorly understood. In murine models, under some conditions, IL-17 promotes Th2-biased airway inflammatory responses. However, IL-17-γδ T cells have been implicated in the inhibition and resolution of allergic airway inflammation and hyperresponsiveness (AHR).

Methods: We compared airway responses in Balb/c mice sensitized to OVA with (and without) a Th2-skewing aluminum-based adjuvant and the IL-17 skewing, complete Freund's adjuvant (CFA). AHR was measured invasively by flexi-Vent, while serum OVA-IgE was quantified by an enzyme immunoassay. Airway inflammatory and cytokine profiles, and cellular sources of IL-17 were assessed from bronchoalveolar lavage and/or lungs. The role of γδ T cells in these responses was addressed in OVA/CFA sensitized mice using a γδ T cell antibody.

Results: Following OVA challenge, all mice exhibited mixed eosinophilic/neutrophilic airway inflammatory profiles and elevated serum OVA-IgE. Whereas OVA/alum sensitized mice had moderate inflammation and AHR, OVA/CFA sensitized mice had significantly greater inflammation but lacked AHR. This correlated with a shift in IL-17 production from CD4+ to γδ T cells. Additionally, OVA/CFA sensitized mice, given a γδ TCR stimulatory antibody, showed increased frequencies of IL-17-γδ T cells and diminished airway reactivity and eosinophilia.

Conclusions: Thus, the conditions of antigen sensitization influence the profile of cells that produce IL-17, the balance of which may then modulate the airway inflammatory responses, including AHR. The possibility for IL-17-γδ T cells to reduce AHR and robust eosinophilic

inflammation provides evidence that therapeutic approaches focused on stimulating and increasing airway IL-17-γδ T cells may be an effective alternative in treating steroid resistant, severe asthma.

2 Background

Asthma is a complex disease characterized by airway inflammation, hyperresponsiveness (AHR) and variable airflow obstruction (1, 2) mediated, at least in part, by an aberrant T helper (Th)2 (2-4) and/or Th17 response (5-7). Th2 cells and the associated cytokines, interleukin (IL)-4, IL-5 and IL-13, are increased in the bronchoalveolar lavage (BAL), sputum and bronchial biopsies of asthmatics and are linked to airway eosinophilia, IgE and reduced lung function (3, 4, 8, 9). Th17 cells and their prime effector cytokine, IL-17A (hereafter referred to as IL-17), have more recently been implicated in asthma pathogenesis. IL-17 expressing cells are increased in the BAL, sputum, bronchial biopsies and in peripheral blood of asthmatics (5-7) and are correlated with neutrophilic airway inflammation and resistance to corticosteroid treatment in moderate-to-severe asthmatics (10-12). Although much of the human data is approached from either the Th2 or Th17 perspective, they are unlikely to be mutually exclusive. Asthmatics have been reported to have increases in both Th2- and Th17-related cytokines (13, 14) and Cosmi et al. have reported increases in a unique subset of CD4+ T cells expressing both IL-4 and IL-17 (15). Asthmatics can also present with a combined eosinophilic/neutrophilic airway inflammatory profile (16, 17), which may reflect a mixed immune response.

Eosinophilic airway inflammation, AHR and mucus production are features of asthma that have been successfully modeled in Th2-driven experimental asthma in the mouse (18, 19). A widely used method of antigen sensitization involves intraperitoneal (IP) injection(s) of ovalbumin (OVA) adsorbed to an aluminum-based adjuvant (hereafter referred to as alum), a potent inducer of the Th2 response (20). Comparatively, animal studies of IL-17 show an important role for Th17 cells on airway neutrophilia and steroid resistance (21, 22). Intranasal

instillations, as well as epicutaneous routes of OVA sensitization, have been shown to induce a more robust IL-17 response compared to IP injections of OVA/alum (23, 24). Furthermore, complete Freund's adjuvant (CFA), a strong inducer of IL-17 typically used in experimental autoimmune encephalomyelitis models, has seen limited use as an IL-17-skewing adjuvant in murine models of allergic asthma (22, 25).

The Th2/IL-17 relationship has been assessed in different Th2-driven models of experimental asthma. A growing body of evidence suggests that IL-17, when sourced from Th17 cells, enhances Th2-induced eosinophilic inflammation and AHR (24, 26), whereas we and others have shown that IL-17-γδ T cells negatively regulate these responses (27, 28). We have now compared how two widely used adjuvants, alum and CFA, modulate OVA-induced allergic airways disease. Our primary goals were to induce a mixed Th2/IL-17 inflammatory response and to identify the cellular source(s) of IL-17. Following airway OVA challenge, OVA/alum and OVA/CFA sensitized mice exhibited antigen-induced airway eosinophilia and had similar total IL-17$^+$ and Th17 BAL fluid cell numbers. However, the influx of inflammatory cells into the lung, as well as serum OVA-IgE levels, and BAL fluid IL-17-γδ T cells were all significantly enhanced in OVA/CFA over OVA/alum sensitized mice following OVA challenge. Nevertheless, AHR was completely absent in these highly inflamed animals, which corresponded to a shift in IL-17 production from CD4$^+$ to γδ T cells. Our secondary goal was to define the role of IL-17-γδ T cells on AHR and inflammation. In mice treated with a γδ TCR stimulatory antibody, the frequency of IL-17-γδ T cells in OVA/CFA sensitized mice was increased. Consistent with a negative regulatory role for these cells, AHR and eosinophilia were both significantly decreased in these mice. Overall, our data demonstrate that the conditions of initial antigen sensitization direct production of IL-17 from different populations of cells, which in turn,

may have the ability to modulate Th2-biased airway inflammatory responses. These data also suggest that specific enhancement of IL-17-γδ T cells could inhibit allergic airways responses.

3 Methods

Animals

Wild type Balb/c mice originating from Charles River Laboratories (Montreal, QC, Canada) were bred and used at ages 6-10 weeks at the Meakins-Christie Laboratories Animal Facility. Animal studies were approved by the McGill University Animal Care Committee and performed following the Canadian Council on Animal Care guidelines.

Sensitization & airway challenge

Mice were sensitized intraperitoneally (IP) with 100µg OVA (Sigma-Aldrich, St. Louis, Minnesota, USA) in sterile PBS with either a 10% solution of Imject Alum (Thermo Scientific, Rockford, Illinois, USA), a 50% emulsion of Complete Freund's Adjuvant (Sigma-Aldrich) or without adjuvant on days 0 & 7. Each OVA group had a respective control group injected without OVA. All mice were lightly anaesthetized with isoflurane and challenged intranasally (IN) with 30µl of PBS containing 50µg of OVA on days 28, 29 & 30 and sacrificed 24 hours after the last airway challenge.

γδ TCR antibody treatment

Balb/c mice were OVA/CFA sensitized and OVA challenged according to the protocol above, with the following exceptions: mice were alternatively challenged on days 42, 43 & 44 and received a total of two 80µg IV injections of the UC7-13D5 γδ TCR antibody or Armenian hamster isotype control antibody (Biolegend, San Diego, California, US) 2 days and 6 hours before the first airway challenge. Flow cytometry analysis of BAL fluid and lung cells confirmed

an increase in the frequency of IL-17-γδ T cells in mice receiving the γδ TCR stimulatory antibody.

Analysis of airway hyperresponsiveness & airway inflammation

The AHR readouts, resistance and elastance, were taken from mice anaesthetized with a cocktail of xylaxine and sodium pentobarbital, followed by an injection of the paralyzing agent, pancuronium bromide. Measurements were determined using the flexiVent small animal ventilator (SCIREQ, Montreal, Quebec, Canada) by exposing mice to increasing concentrations of aerosolized methacholine. Following assessment of AHR, BAL was performed. Cells, recovered from two-1ml PBS lavages of the airways, were pooled and red blood cells lysed before counting total cells. A portion of the cells was centrifuged onto glass slides and stained using Diff-Quick (Fisher Scientific, Ottawa, Ontario, Canada) from which a differential cell count, based on a 300 cell count from five to ten fields, was obtained. BAL fluid from the first lavage was frozen and maintained at -80°C for later cytokine/chemokine protein analysis.

Detection of OVA-specific IgE

Preceding removal of the lungs, the chest cavity was opened and blood collected by cardiac puncture. The blood was left at room temperature for 30 min to facilitate coagulation before centrifugation and separation of serum for storage at -80°C for future analysis. OVA-specific IgE was quantified using a modified protocol of the ELISA MAX mouse IgE kit (Biolegend, San Diego, California, USA). The kit protocol was followed with the following exceptions: wells used to measure OVA-specific IgE from serum samples were coated with 100µl of a 20µg/ml OVA solution overnight at 4°C in place of the coating antibody. Serum samples were incubated

with an equal volume of protein G sepharose overnight at 4°C. Following centrifugation, the supernatants were added to wells during the sample incubation step.

Preparation of lung cells

The right lung was stored in RNAlater (Ambion, Carlsbad, California, USA) at -20°C for analysis by real-time PCR. A single cell suspension of lung cells was obtained from the left lung of each mouse by first mincing the lung and then incubating the tissue in 1ml of serum-free DMEM (Life Technologies, Carlsbad, California) containing Liberase TM (100µg/ml; Roche, Indianapolis, Indiana, USA) combined with collagenase XI (250µg/ml), hyaluronidase 1a (1mg/ml) and DNase I (200µg/ml, Sigma-Aldrich) for 1h at 37°C. The reaction was stopped with a 20mM final concentration of EDTA (29). Red blood cells were lysed following enzymatic digestion.

Flow cytometric analysis

Single cell preparations of BAL and lung cells were incubated for 4h in 1ml of RPMI media containing 1% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (Life Technologies); 0.67 µl/ml GolgiStop from the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, San Diego, California, USA); 50ng/ml phorbol 12-myristate 13-acetate (PMA) and 500ng/ml ionomycin (Sigma-Aldrich). Surface and intracellular cytokine staining were performed according to the kit protocol. Cells were double or triple stained with a combination of the following antibodies: α-CD4-Pacific Blue, α-γδ TCR-Fluorescein Isothiocyanate (GL3 clone), α-IL-17-AlexaFluor 647, α-CD3-Pacific Blue (BD Biosciences) or α-Vγ4-Fluorescein Isothiocyanate (UC3-10A6 clone) (Biolegend). Stained cells were processed using an LSRII

flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, Oregon, USA).

RNA purification & real time PCR analysis

Lung tissue was homogenized and total RNA isolated following the PureLink RNA Mini Kit (Ambion) protocol. RNA was reverse-transcribed into cDNA using Oligo dT primers and SuperScript II (Life Technologies). Real-time quantitative PCR amplification was performed with Power SYBR Green (Applied Biosystems, Warrington, UK) using the Applied Biosystems PCR system. Cycle threshold values were first normalized to Gapdh gene expression before quantification by the comparative threshold cycle method to obtain the gene expression levels from lungs of OVA sensitized and challenged mouse groups, relative to the saline control group (30).

Quantitative analysis of BAL fluid mediators

BAL fluid cytokine and chemokine levels were quantified with the Q-View Imager using the 16-plex mouse cytokine screen (Quansys Biosciences, Logan, Utah, USA). IL-13 levels in the BAL fluid were quantified using the ELISA Ready-SET-Go kit (ebioscience, San Diego, California, USA).

Statistical analysis

Data are expressed as the mean +SEM. Multiple comparisons (i.e. antigen- and adjuvant-dependent effects) were analyzed by two-way ANOVA, followed by the Holm-Sidak post hoc test. Single comparisons (between the 3 OVA-sensitized groups) were analyzed by one-way

ANOVA, followed by the Holm-Sidak post hoc test. Single comparisons (between the 2 antibody treated groups) were analyzed by an unpaired, two-tailed t-test. p-values less than 0.05 were considered statistically significant. Figures and statistics were analyzed using GraphPad Prism 6 (La Jolla, California, USA).

4 Results

Enhanced airway inflammation, but lack of AHR, in mice sensitized to OVA in the presence of CFA

In order to establish a mixed model of allergic asthma in which the IL-17 response could be assessed within an in vivo Th2 environment, we intraperitoneally (IP) sensitized mice with OVA in the absence (OVA/sal group) or presence of the adjuvants, alum (OVA/alum group) or CFA (OVA/CFA group). We confirmed induction of several classic features associated with allergic airways disease and differentiated OVA-specific (§) from adjuvant-specific (*) effects **(Fig 2.1)**. OVA-IgE was selectively detected in all OVA sensitized and challenged mice and was present at significantly higher levels in OVA/CFA mice **(Fig 2.1A)**. Total cells, eosinophils, neutrophils and lymphocytes were significantly increased in the BAL fluid of OVA/CFA sensitized mice (solid bar) compared to the CFA control (striped bar). In contrast, inflammation was not significantly changed in OVA/sal mice and only eosinophils were significantly increased in OVA/alum sensitized mice **(Fig 2.1B)**. Moreover, following OVA challenge, OVA/CFA mice had significantly more macrophages, eosinophils, neutrophils and lymphocytes, resulting in 3 and 5.5 fold more total cells recovered compared to OVA/alum and OVA/sal mice, respectively. With regard to BAL fluid cell frequencies, eosinophils were increased in all OVA-sensitized mice compared to their respective controls, primarily at the expense of macrophages **(Fig 2.2)**. OVA/alum sensitized and challenged mice had greater frequencies of BALF eosinophils than OVA/sal mice, while OVA/CFA mice had greater frequencies of eosinophils, as well as lower frequencies of both macrophages and lymphocytes compared to OVA/sal. Regardless of

adjuvant, a mixed eosinophilic/neutrophilic inflammatory profile was observed in all OVA sensitized groups following OVA challenge.

To assess possible mechanisms by which inflammatory cell recruitment differed, differences in lung chemokine expression were assessed following OVA challenge. Levels of mRNA encoding the macrophage chemoattractant, CCL2 (MCP-1), the eosinophil chemoattractants CCL11 (eotaxin-1) and CCL24 (eotaxin-2), and the neutrophil chemoattractant, CXCL1, were increased in OVA/CFA sensitized mice compared to OVA/sal and/or OVA/alum sensitized mice with these differences reaching statistical significance for CCL2, CCL11, and CXCL1 **(Fig 2.1C)**. Consistent with the overall increase in BAL fluid inflammatory cell influx, mRNA levels for several chemokines were greater in OVA/CFA mice compared to OVA/sal and/or OVA/alum mice. Moreover, OVA/CFA mice were also the only group to show any significant OVA-specific increases in chemokine expression, with the exception of the neutrophil chemoattractant, CXCL5.

Following OVA challenge, airway resistance **(Fig 2.3A)** and elastance **(Fig 2.3B)** were significantly increased in OVA/alum sensitized mice compared to OVA/CFA mice, across multiple concentrations of methacholine (left panels), and to OVA/sal mice at the highest dose (right panels). Moreover, the OVA-dependent increases (§) in airway resistance and elastance that were observed in OVA/alum (and OVA/sal) sensitized mice were completely absent in OVA/CFA sensitized mice. Thus, although OVA-IgE and airway inflammation were significantly enhanced in OVA/CFA sensitized mice **(Fig 2.1A, B)**, AHR was absent. Although adjuvant-only controls showed subtle differences in respiratory resistance and elastance at the 50mg/ml methacholine dose **(Fig 2.3 A & B, right panels)**, these differences were not statistically different. BAL fluid was analyzed for Th1, Th2 and Th17 related cytokines. Levels

Figure 2.1: Serum OVA-specific IgE and airway inflammatory responses are enhanced in OVA/CFA sensitized mice

BALB/c mice were IP OVA sensitized without adjuvant (OVA/sal), or in the presence of alum (OVA/alum) or CFA (OVA/CFA). Corresponding control groups were injected with saline, alum or CFA. (A) Serum OVA-IgE levels. OVA-IgE was undetectable in control mice. Mean (+SEM) from 8-12 mice per group from a minimum of 3 independent experiments. One-way ANOVA, Holm-Sidak. (B) BAL fluid differential cell counts from OVA sensitized groups (solid bars) and their respective adjuvant controls (striped bars). Mean total cells macrophages, eosinophils, neutrophils and lymphocytes (+SEM) are shown for 11-16 total mice from at least 3 independent experiments. Two-way ANOVA, Holm-Sidak. (C) Relative expression of CCL2, CCL11, CCL24, CXCL1 and CXCL5 in the lung following OVA challenge assessed by real-time PCR. Expression is presented as fold increase relative to the saline control. Results represent the means (+SEM) from 5-6 total mice per group from a minimum of 2 independent experiments. Two-way ANOVA, Holm-Sidak. (A-C) $^{\S}p<0.05$ OVA sensitized groups vs. respective controls (antigen-dependent effects), *$p<0.05$ comparisons between OVA groups (adjuvant-dependent effects).

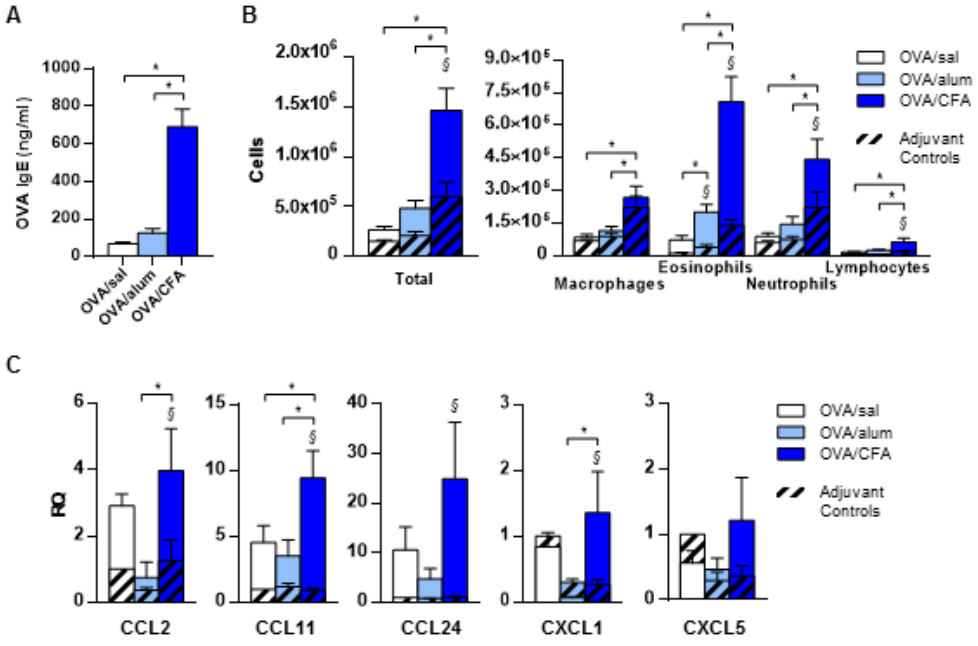


Figure 2.2: Frequencies of BAL fluid inflammatory cells

Frequencies of macrophages, eosinophils, neutrophils and lymphocytes in the BAL fluid of OVA sensitized mice and their respective adjuvant controls. Frequencies shown are from 11-16 total mice from at least 3 independent experiments. Two-way ANOVA, Holm-Sidak. $^{\S}p<0.05$ OVA sensitized groups vs. respective controls (antigen-dependent effects); $*p<0.05$ compared to the OVA/sal group, $\wedge p<0.05$ compared to the CFA control group (adjuvant-dependent effects). Additional relevant comparisons were not significantly different.

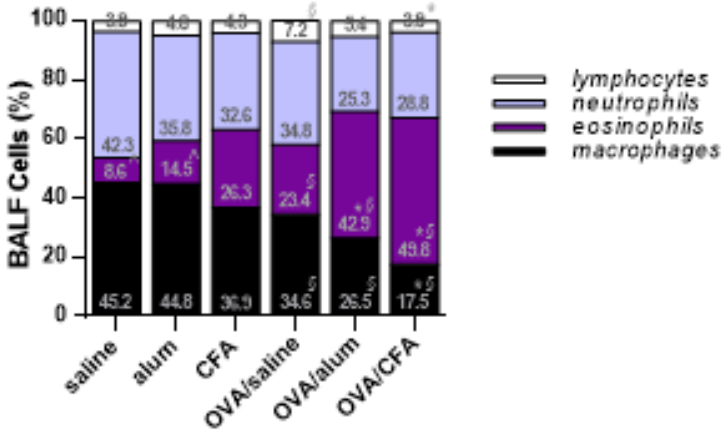
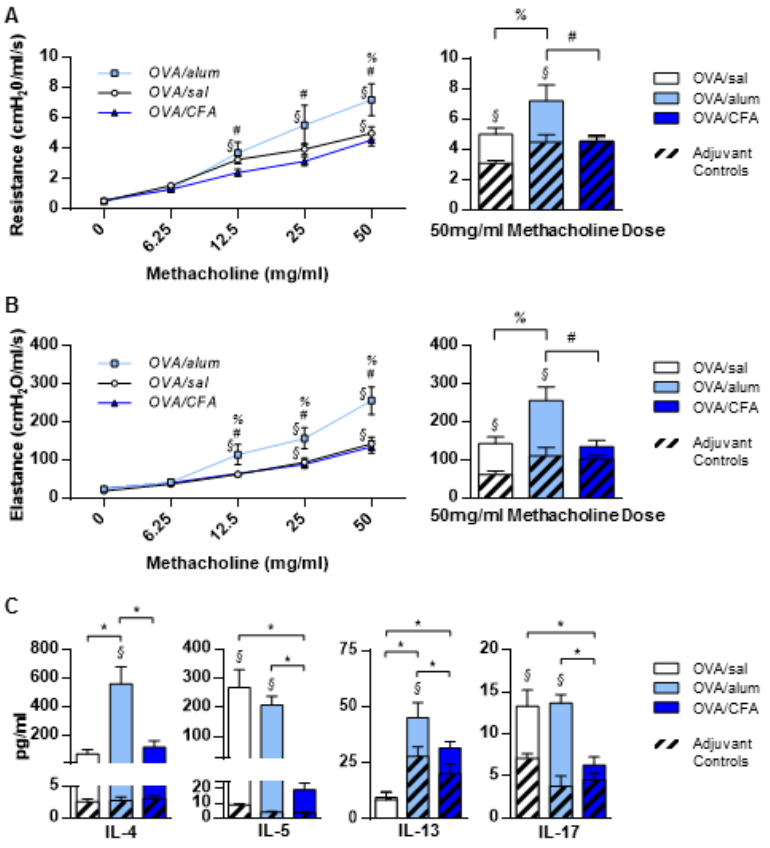


Figure 2.3: AHR is absent in OVA/CFA sensitized mice and Th1/Th2 BAL fluid cytokine levels are influenced by the adjuvant used during OVA sensitization

Total lung resistance and elastance were assessed 24h after the last airway OVA challenge. Mean (±SEM) respiratory system (A) resistance and (B) elastance to increasing concentrations of methacholine (left panels) for the 3 OVA-sensitized mouse groups, as well as at the 50mg/ml concentration (right panels) for all 6 groups. Control groups were removed from left panel figures for clarity. Data from 8-14 total mice per group from 2 independent experiments. (C) Mean BAL fluid levels (+SEM) of IL-4, IL-5, IL-13 & IL-17 were assessed from 7-12 total mice per group, from at least 2 independent experiments. (A-C) Two-way ANOVA, Holm-Sidak. $^{\S}p<0.05$ OVA sensitized groups versus respective controls (antigen-dependent effects); $^{*}p<0.05$ comparisons within the OVA sensitized groups (adjuvant-dependent effects), specifically $^{\#}p<0.05$ OVA/alum vs. OVA/CFA, $^{\%}p<0.05$ OVA/alum vs. OVA/sal.



of IFN-γ were generally low in all groups (data not shown). In OVA/sal sensitized mice, OVA challenge increased levels of IL-5 and IL-17 **(Fig 2.3C)**. OVA/alum sensitized mice had increased IL-4, IL-5, IL-13 and IL-17 levels compared to alum-sensitized mice, post airway challenge. OVA/alum mice also had significantly greater levels of these cytokines compared OVA/sal and/or OVA/CFA mice. Interestingly, OVA/CFA sensitized mice showed no differences in cytokine levels compared to CFA-sensitized mice.

When emulsified with OVA at sensitization, CFA increases IL-17-γδ T cell numbers in the BAL fluid

Following OVA challenge, IL-17 expressing cells were present in the BAL fluid of all mice, regardless of the conditions of OVA sensitization **(Fig 2.4A)**. Although there was a trend toward a reduced frequency of IL-17$^+$ cells in mice sensitized with OVA/CFA, this difference did not reach statistical significance **(Fig 2.4B)**. Nevertheless, when taking into account the overall inflammatory cell influx into the BAL fluid, OVA/CFA sensitized mice had larger numbers of IL-17$^+$ cells compared to the other OVA sensitized groups, though this difference was only significant when compared to the OVA/sal group **(Fig 2.4C)**. No antigen-dependent differences were observed in the OVA sensitized mouse groups compared to their respective controls.

Several cell types, including Th17 cells (24, 31), γδ T cells (27, 28) and alveolar macrophages (32) have been identified as sources of IL-17 in murine models of allergic airways disease. Following OVA challenge, we identified the major IL-17 producing cell(s) in both the BAL and lungs of OVA/sal, OVA/alum and OVA/CFA sensitized mice. After gating on IL-17$^+$ cells, the major sources of IL-17 were identified as CD4$^+$ (Th17) and γδ (IL-17-γδ) T cells,

though the ratio differed depending on the conditions of OVA sensitization **(Fig 2.5A)**. The ratio of the frequency of IL-17-γδ to Th17 cells in the BAL fluid established that Th17 cells were the major source of IL-17 in OVA/sal sensitized mice; that Th17 and IL-17-γδ T cells contributed almost equally to IL-17 production in OVA/alum sensitized mice; and that IL-17-γδ T cells were the main producers of IL-17 in OVA/CFA sensitized mice **(Fig 2.5B)**. Similar total numbers of Th17 cells were present in the BAL fluid of all OVA sensitized and challenged mice **(Fig 2.5C)**, but greater numbers of total IL-17-γδ T cells were recovered from OVA/CFA sensitized mice compared to OVA/alum and OVA/sal mice. Only the OVA/alum group had significantly greater numbers of Th17 cells compared to its respective control **(Fig 2.5C, left panel)**, whereas antigen-dependent differences were absent for total IL-17-γδ T cells **(Fig 2.5C, right panel)**. The ratios of total IL-17-γδ to Th17 cells for all OVA sensitized mouse groups were similar to the ratios of the frequencies of the same cells **(Fig 2.5D)**, with OVA/CFA sensitized mice having a significantly larger ratio to both OVA/sal and OVA/alum mice. The median fluorescence intensity (MFI) of IL-17 was greater in IL-17-γδ T cells compared to Th17 cells from the BAL fluid **(Fig 2.6A, B)** for all OVA sensitized groups, indicating that, while the frequencies of cell types expressing IL-17 were modified by the type of adjuvant present at sensitization, the amount of IL-17 expressed by these individual cells was not affected.

Activating IL-17-γδ T cells leads to inhibition of airway eosinophilia and AHR

To examine the impact of elevated numbers of IL-17-γδ T cells present in mice sensitized with OVA/CFA, these mice were treated with a γδ TCR antibody, UC7-13D5. Consistent with a study demonstrating that this antibody activates γδ T cells in vitro and in vivo (33), UC7-13D5 treated OVA/CFA sensitized mice exhibited increased frequencies of BAL fluid IL-17-γδ T cells

Figure 2.4: IL-17 expressing cells are present in the BAL fluid of all OVA sensitized and challenged mice

Cells were stimulated with PMA/ionomycin and stained to detect IL-17. (A) Representative flow cytometry plots are shown. (B) The frequencies of IL-17 expressing cells for the OVA groups are shown. One-way ANOVA, Holm-Sidak. (C) The total number of IL-17 expressing cells are shown for OVA groups. Two-way ANOVA, Holm-Sidak. Mean values (+SEM) from 7-11 total mice per group, from 2 independent experiments. (B-C) *p<0.05 comparisons between OVA groups.

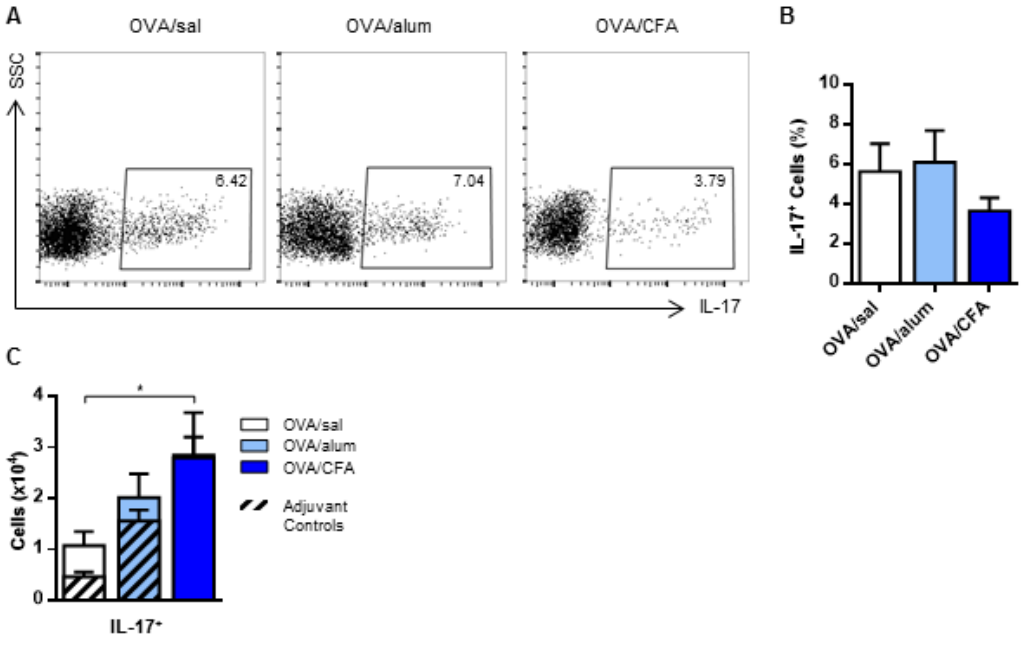


Figure 2.5: OVA/CFA sensitized mice have more γδ$^+$IL-17$^+$ T cells in the BAL fluid

BAL fluid cells were stimulated with PMA/ionomycin and triple stained with α-CD4, α-γδ TCR and α-IL-17 antibodies. (A) Representative flow cytometry plots of cells in BAL fluid (top panel) and lung (bottom panel) gated first to identify IL-17$^+$ cells and subsequently to identify frequencies of CD4 and γδ T cells. (B) The frequency distribution of IL-17$^+$ cells within the CD4 and γδ T cell populations is presented as the ratio (γδ/CD4) of these cells. One-way ANOVA, Holm-Sidak. (C) Total numbers of CD4$^+$IL-17$^+$ and γδ$^+$IL-17$^+$ populations calculated from the frequency of these cells and the total cell counts are shown. Two-way ANOVA, Holm-Sidak. (D) The distribution of total γδ$^+$IL-17$^+$ and CD4$^+$IL-17$^+$ T cells is presented as the ratio (γδ/CD4) of these cells. One-way ANOVA, Holm Sidak. (B-D) Data are from 7-11 total mice per group from at least 2 independent experiments. *p<0.05 comparisons between OVA groups (adjuvant-dependent effects), §p<0.05 OVA sensitized groups versus respective controls (antigen-dependent effects).

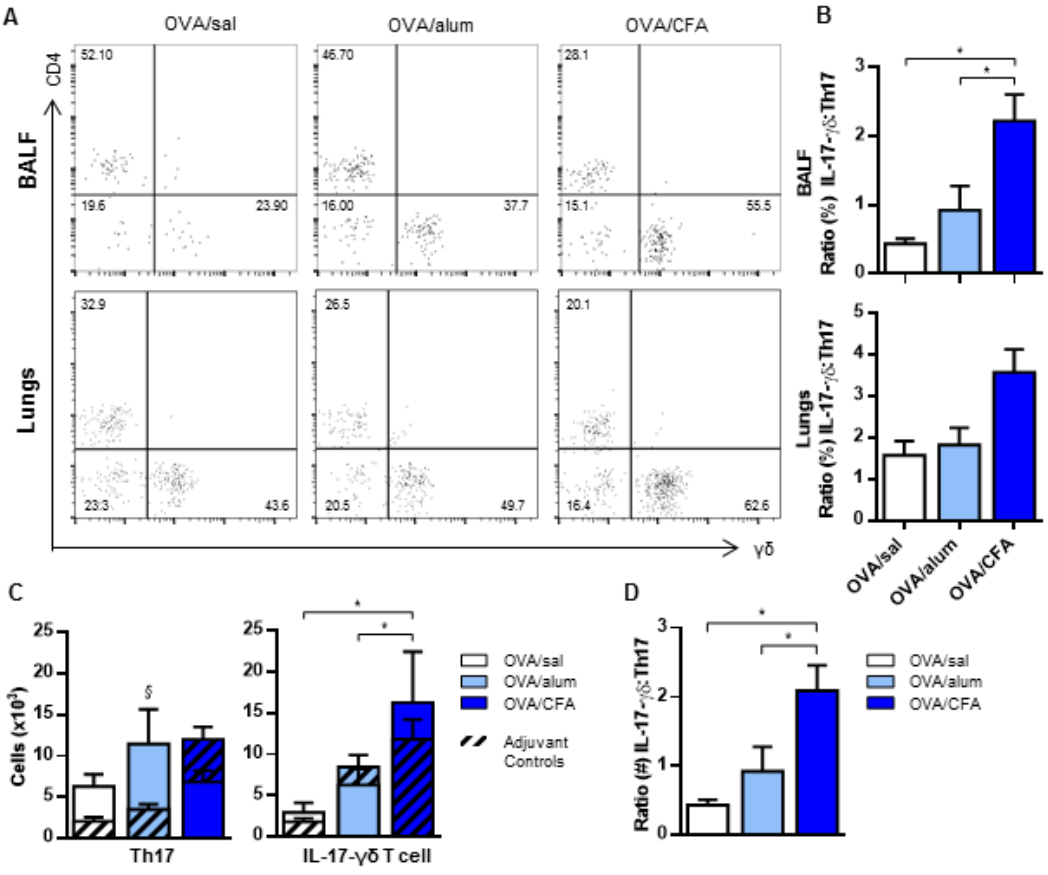
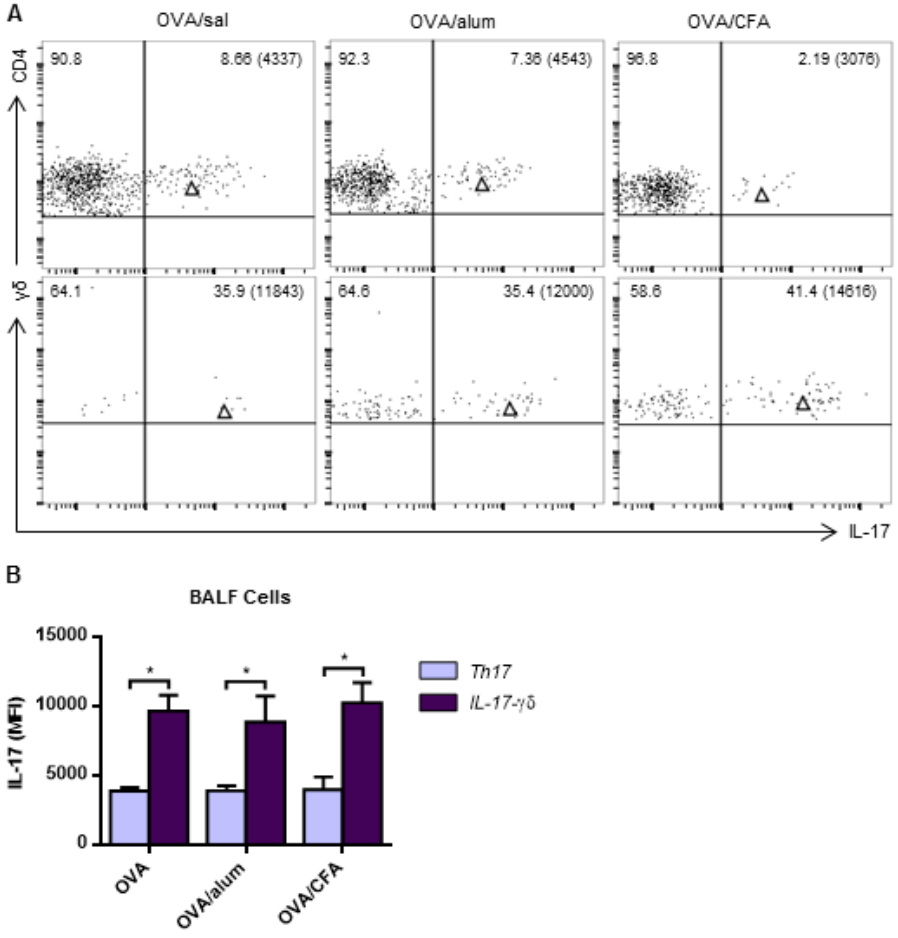


Figure 2.6: Both the frequency of IL-17$^+$ cells within the γδ T cell population and the MFI of IL-17 expressed by these cells are greater than those of CD4$^+$ T cells from the BAL fluid

BAL fluid cells were stimulated with PMA/ionomycin and triple stained with α-CD4, α-γδ TCR and α-IL-17 antibodies. (A) Representative flow cytometry plots of BAL fluid cells from OVA sensitized groups. (Δ) indicates the median fluorescence intensity (MFI) of IL-17 within the IL-17-expressing CD4$^+$ (upper panels) and γδ TCR$^+$ (lower panels) cell populations on a log scale. Flow cytometry plots, representative of 7-9 total mice from at least 2 independent experiments, are shown. (B) The MFI of IL-17 expression within the IL-17 expressing CD4$^+$ and γδ T cell populations are shown. Mean values (+SEM) from 7-9 total mice per group from at least 2 independent experiments are shown. Two-way ANOVA, Holm-Sidak test. *p<0.05 comparisons between CD4$^+$ T cells and γδ T cells.



(Fig 2.7A, 2.7B left panel) and, interestingly, IL-17$^+$ cells **(Fig 2.7A, 2.7C left panel)**, post airway challenge. There was also a positive trend for the number of IL-17-γδ T cells and IL-17$^+$ T cells to be increased in UC7-13D5-treated mice **(right panels of Fig 2.7B & C)**. The majority of the IL-17-γδ T cells in the UC7-13D5-treated mice were of the Vγ4 subset **(Fig 2.8A, B)**, which has previously been shown to inhibit AHR and airway inflammation in murine models of asthma (28). In agreement, BAL fluid eosinophils **(Fig 2.9A, B)** were significantly decreased in antibody treated mice compared to recipients of isotype control antibodies. Moreover, the frequency of BAL fluid neutrophils was also increased **(Fig 2.9B)**, which may reflect the increase in total IL-17 expressing cells in mice treated with the UC7-13D5 antibody. Finally, both respiratory resistance and elastance were diminished in OVA/CFA mice treated with the UC7-13D5 antibody, again consistent with a negative regulatory role for IL-17-γδ T cells **(Fig 2.9C)**.

Figure 2.7: Frequencies of total IL-17 expressing cells and of IL-17-γδ cells are increased in OVA/CFA sensitized mice treated with a γδ TCR stimulatory antibody

OVA/CFA sensitized mice were IV injected with a γδ TCR stimulatory antibody (UC7-13D5) or isotype control before airway challenge. BAL fluid cells were stimulated with PMA/ionomycin and stained with α-γδ TCR and α-IL-17 antibodies. (A) Representative flow cytometry plots of BAL fluid cells. The mean frequencies (left panels) and numbers (right panels) of (B) IL-17-γδ and (C) IL-17$^+$ BAL fluid cells are presented for 5 total mice per group from 2 independent experiments. (B-C) Unpaired, two-tailed t-test. *p<0.05.

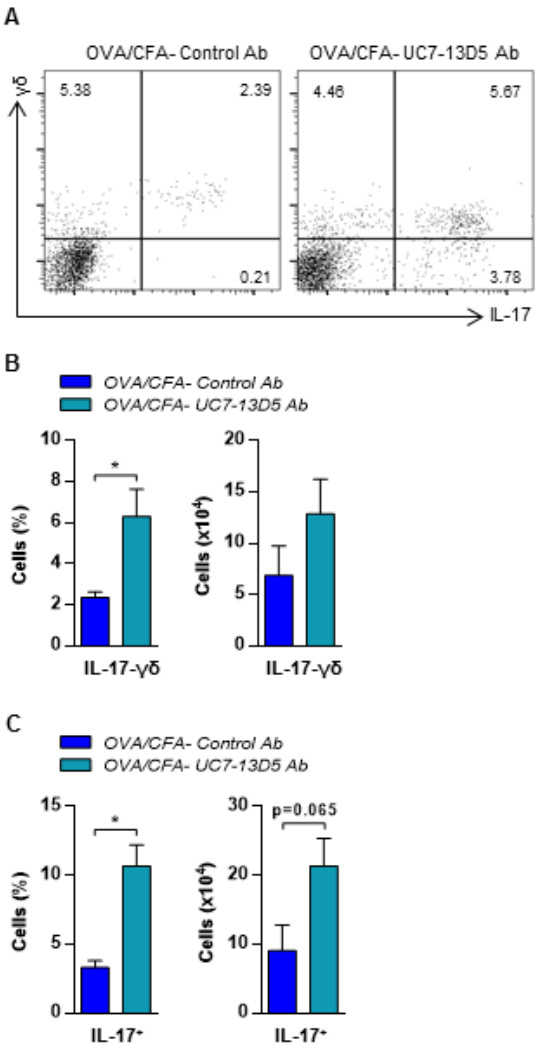


Figure 2.8: The majority of IL-17-γδ T cells in the BAL fluid of OVA/CFA sensitized mice are of the Vγ4 subset and increase in frequency with activation by the γδ TCR antibody

OVA/CFA sensitized mice were IV injected with a γδ TCR (UC7-13D5) stimulatory antibody or isotype control before airway OVA challenge. BAL fluid cells were double stained with an α-IL-17 antibody and either the α-γδ TCR or α-Vγ4 antibody. Representative flow cytometry plots of the frequencies of $\gamma\delta^{+}$IL-17^{+} (top panels) and $V\gamma4^{+}$IL-17^{+} (bottom panels) cells from recipients of (A) control or (B) UC7-13D5 antibody are shown within the total live BAL fluid cell population and are representative data from 2-3 mice per group.

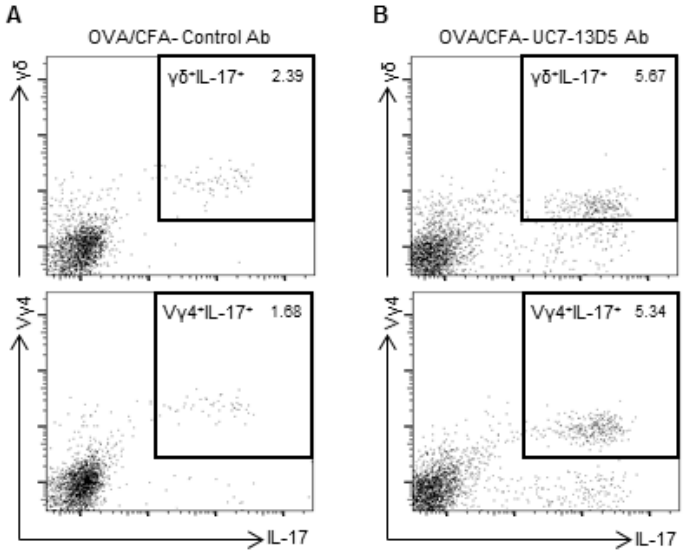
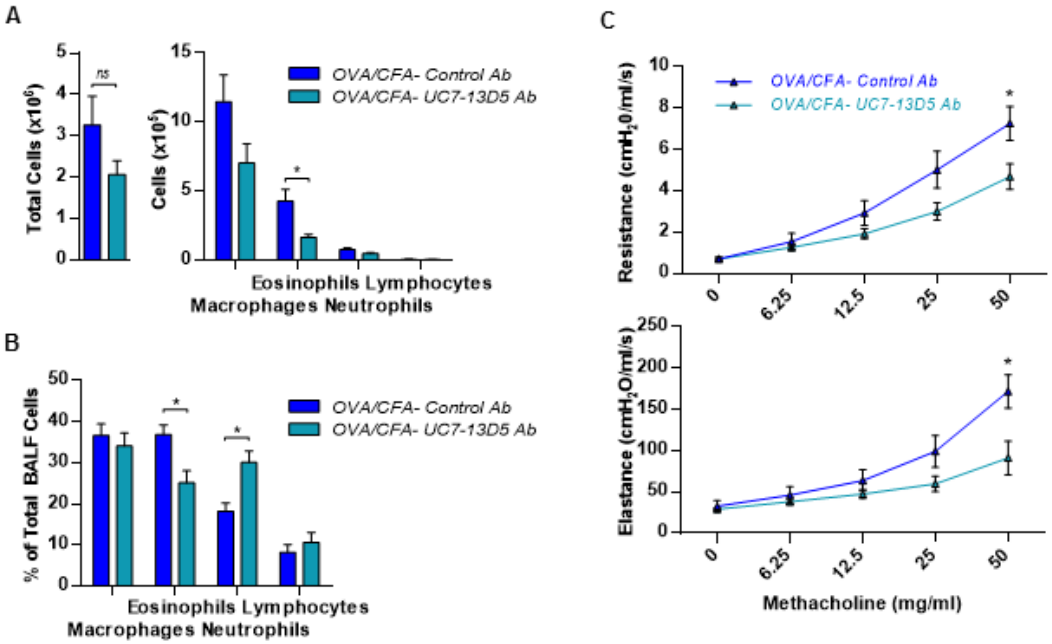


Figure 2.9: OVA/CFA sensitized mice receiving a γδ TCR stimulatory antibody, have reduced airway eosinophilia and AHR

OVA/CFA sensitized mice were IV injected with a γδ TCR (UC7-13D5) stimulatory antibody or isotype control before airway challenge. The mean (A) total cell counts and (B) frequencies of macrophages, eosinophils, neutrophils and lymphocytes (+SEM) are shown for 5 total mice per group from 2 independent experiments. (C) Total lung resistance and elastance were assessed 24h after the last airway OVA challenge. Mean (±SEM) respiratory system resistance and elastance to increasing concentrations of methacholine are shown. (A-C) Unpaired, two-tailed t-test. *p<0.05, ns= not significant.



5 Discussion

In rodent models of allergic airways disease, the types of antigen and adjuvant, as well as their concentration, route and timing of delivery can impact the nature of the ensuing immune response. Historically, the most widely used murine models of allergic asthma and rhinitis have relied upon IP injections of OVA adsorbed to an aluminum-based adjuvant (34, 35). This class of adjuvant has been used extensively in animal models of allergic airways disease for its ability to induce a Th2-biased immune response (20, 36). In contrast, CFA has been recognized as a Th17-promoting adjuvant and is comprised of a light mineral oil, a surfactant agent, and heat-killed *Mycobacterium tuberculosis* (*Mtb*) that creates an emulsion when mixed with an aqueous solution (37). A great deal of our understanding of the IL-17/CFA relationship has come from experimental autoimmune encephalomyelitis models of multiple sclerosis (38). Nevertheless, antigen sensitization with CFA has rarely been used in models of allergic airways disease. Our goal was to assess how the choice of adjuvant—alum or CFA—affected Th2 and Th17 type inflammatory responses in the airways post antigen challenge.

CFA has been widely used in experimental studies for its unsurpassed ability to enhance antibody production against a number of different antigens (37). This was confirmed in our analysis as OVA/CFA sensitized mice, post airway challenge, had significantly greater levels of serum OVA IgE compared to both OVA/sal and OVA/alum sensitized mice. Abundant data demonstrate that, independent of the mouse strain, mice IP sensitized with OVA/alum have robust airway eosinophilia post challenge (34, 35). Nevertheless, the airways of C57Bl/6 mice sensitized IP with OVA/alum or OVA/CFA can also exhibit a mixed eosinophilic/neutrophilic inflammatory profile of similar magnitude following acute antigen challenge (22). Our data

indicate that Balb/c mice IP sensitized to OVA with alum or CFA yield mixed eosinophilic/neutrophilic inflammatory profiles where equal ratios of both cell types were recovered in the BAL fluid. These data suggest that both Th2 and IL-17 responses, which are associated with airway eosinophilia (39) and neutrophilia (21), respectively, may be induced with either adjuvant. Although AHR is often correlated with airway inflammation, there is evidence from humans (40, 41), as well as murine models (42, 43), that AHR can occur independently of inflammation. Our data indicate that mice sensitized with OVA/CFA had significantly more inflammation following OVA challenge, as assessed from the BAL fluid. Nevertheless, both antigen- and adjuvant-specific increases in airways resistance and elastance were completely absent in these mice. Comparatively, OVA/alum sensitized mice exhibiting moderate inflammation, had both antigen- and adjuvant-dependent increases in AHR. These differences in airways resistance and elastance may be related to BAL fluid levels of IL-4 and IL-13, which were elevated in only the OVA/alum sensitized mice. Intratracheal instillations of IL-13 to naïve mice (44) or IL-4 to IL-13 deficient mice can induce AHR (21). Interestingly, the same studies showed IL-4 and IL-13 to be potent inducers of airway eosinophilia, whereas in our study, airway hyporesponsive, highly eosinophilic OVA/CFA sensitized mice had comparatively lower BAL fluid levels of both cytokines. We therefore considered other mediators to provide a possible explanation for the disconnect between AHR and inflammation observed in OVA/CFA mice. The ability of IL-17 to act as both a positive and negative regulator of asthma (27), as well as the ability of CFA to induce IL-17 in models of experimental autoimmune encephalomyelitis (45) made this particular cytokine a strong candidate. While IL-17 alone may not induce AHR in mice, under some circumstances it can promote Th2-dependent AHR and inflammation (26).

We identified the cellular source(s) of IL-17 in mice sensitized under different adjuvant conditions. Following OVA challenge, IL-17 was expressed predominantly by a combination of $CD4^+$ and γδ T cells in the BAL fluid and lungs **(Fig 2.5A)** of all OVA-sensitized and challenged mice. However, in OVA/sal sensitized mice, IL-17 was produced primarily by $CD4^+$ T cells. Alum or CFA, given at the time of sensitization, affected this distribution by skewing IL-17 expression toward γδ T cells. *Mtb*, an essential component of CFA, preferentially induces IL-17 expression from γδ T cells rather than $CD4^+$ T cells through activation of the Nalp3 inflammasome (45, 46). Moderate skewing of IL-17 production toward γδ T cells also occurred in mice sensitized with OVA/alum. This may reflect the ability of alum to activate the Nalp3 inflammasome (35, 47), albeit less potently than CFA, or depend on the quantity of alum used at sensitization.

Our data demonstrating that IL-17-γδ T cells, Th17 cells **(Fig 2.5C)** and total $IL\text{-}17^+$ cells **(Fig 2.4C)** recovered in the BAL fluid did not necessarily coincide with BAL fluid levels of IL-17 **(Fig 2.3C)**, are consistent with data from other groups (24, 28). For example, IL-17 levels in the BAL fluid of both airway and IP sensitized mice were undetectable at a time point when total Th17 cells, assessed by flow cytometry, were significantly higher in airway sensitized mice (24). Thus, apparent differences in IL-17 cytokine levels relative to IL-17 expressing cells may simply reflect the timing of assessment, as the ability to detect IL-17 in the BAL fluid may be quite transient (24, 28), whereas detection of cells with the potential to produce IL-17 may be more stable/long lasting.

In murine models of allergic asthma, IL-17 expression is most often associated with $CD4^+$ T cells, although γδ T cells and macrophages can also express IL-17 (24, 28, 32). Several factors, including the quantity of cytokine produced, as well as the timing and location and cell

source, likely impact the outcome of IL-17 production. For example, while Th17 cells appear to promote Th2 responses, such as eosinophil recruitment to the airways, we and others have shown that IL-17-γδ T cells actually inhibit Th2-associated AHR and/or inflammation (27). Murdoch et al specifically showed that 64% of IL-17-γδ T cells were of the $V\gamma4^+$ subset (28). This specific subset of γδ T cells actively suppresses AHR (48) and may accomplish this without affecting inflammation (49). We similarly showed a population of $IL\text{-}17^+V\gamma4^+$ cells constituting a large majority of the IL-17-γδ T cells in the BAL fluid of OVA/CFA sensitized mice **(Fig 2.8)**. Thus, the prominent IL-17-γδ T cell population recovered in OVA/CFA sensitized mice may explain, at least in part, the absence of AHR, despite the robust inflammatory response. For these same reasons, regulatory T cells are not likely to be involved in the suppression of AHR, due to their well-established role in suppressing inflammation (50).

In order to better understand how IL-17-γδ T cells regulated airway inflammatory responses in OVA/CFA sensitized mice, we treated these mice with a γδ TCR stimulatory antibody (UC7-13D5), the outcome of which was a substantial increase in the frequency of IL-17-γδ T cells accompanied by reductions in both AHR and eosinophil influx. We were not surprised to find that the increase in IL-17-γδ T cells corresponded to a decrease in AHR, in particular since the majority of the IL-17-γδ T cells induced in OVA/CFA sensitized mice appeared to be the Vγ4, inhibitory subset. Unlike the inhibition of AHR, the reduced number of airway eosinophils in UC7-13D5 treated mice was unexpected as OVA/CFA sensitized mice that followed the 4 week sensitization and challenge protocol had increased IL-17-γδ T cells in the BAL fluid **(Fig 2.5C)** and suppressed AHR **(Fig 2.3A, B)**, but had highly inflamed airways, including abundant eosinophils **(Fig 2.1B)**. γδ T cells ($IL\text{-}17^+$ and $V\gamma4^+$) are described as having either an inhibitory or no effect on airway eosinophilia, based on different OVA models of

allergic airways disease (28, 48, 49). Careful examination of the literature indicates that this may be a temporal issue. In these OVA models, γδ T cells are consistently elevated in numbers at the peak of eosinophil recruitment (24-48 hours after the last airway challenge), suggesting that IL-17-γδ T cells do not have an immediate inhibitory effect on the influx of eosinophils. However, in the days following antigen challenge, (i.e. recovery phase), IL-17-γδ T cells have specifically been shown to decrease airway eosinophil numbers and further attenuate AHR (28). Where γδ T cells are reported as having no effect on eosinophil recruitment, measurements were taken at the peak of inflammation and compared to mice that received a Vγ4 neutralizing antibody (49), which would neutralize a population of cells that has yet to influence airway eosinophil numbers. In contrast, in our studies, the UC7-13D5 antibody, that was given pre-challenge to OVA/CFA sensitized mice, stimulated and thus activated the γδ T cells, which may have allowed them to reduce airway eosinophil numbers even at 24 hours after the last airway challenge. Further studies are required to confirm these findings. IL-17-γδ T cells have been reported to increase IL-17 production from Th17 and other cell types (38, 51, 52). This may explain the increase in total IL-17$^+$ cells recovered in the BAL fluid of mice treated with the UC7-13D5 antibody. Consistent with the literature, the frequencies of neutrophils in UC7-13D5 treated mice **(Fig 2.9B)** reflected the increase in IL-17$^+$ cells **(Fig 2.7C)** (24, 53). However, the overall neutrophil cell count remained low and did not differ between UC7-13D5 and isotype control antibody treated OVA/CFA sensitized mice **(Fig 2.9A)**.

The UC7-13D5 γδ TCR antibody has previously been used to functionally deplete γδ T cells in vivo (54). Though we used comparable concentrations of this antibody, as discussed above, our data indicate that the target cells were neither neutralized, nor functionally depleted. In fact, our data are consistent with data provided by Koenecke, et al. demonstrating that γδ T

cells are activated by UC7-13D5 (33). To study γδ T cells in OVA/CFA sensitized mice, two different antibodies were used: mice were injected with the UC7-13D5 γδ TCR antibody, followed by γδ T cell detection by flow cytometry using the GL3 γδ TCR antibody. While the treatment and staining antibodies differ, our data and others (33) suggest that the GL3 and UC7-13D5 antibodies compete for the same γδ TCR epitope, as the γδ MFI was lower, but not to the extent of depletion, in BAL fluid cells recovered from UC7-13D5 antibody compared to control antibody treated mice **(Fig 2.7)**. This was also the case for the Vγ4 staining antibody (UC3-10A6 clone), but appeared to compete to a lesser degree with the UC7-13D5 antibody **(Fig 2.8)**. In either case, it is clear that the γδ T cells were not depleted. Furthermore, as discussed above, there was a significant increase in the expression of IL-17 within the γδ T cells from UC7-13D5 treated mice, while the frequency of total γδ T cells was unchanged compared to that in mice treated with the isotype control (data not shown).

Although the adjuvants, alum and CFA, significantly impacted the distribution of IL-17 expression between the $CD4^+$ and γδ T cell populations, the median fluorescence intensity (MFI) of IL-17 within each cell type was unaffected. In fact, the MFI of IL-17 in BAL fluid γδ T cells was significantly greater than in $CD4^+$ T cells for all three OVA sensitized groups. Lockhart et al discussed the frequency of IL-17 expression within the same cell populations in a murine model of *Mtb* infection (46). Although they did not directly address the MFI of IL-17, it too appeared to be higher in γδ T cells than in $CD4^+$ T cells. Therefore, the level of expression of IL-17 in this model, as well as in our model of allergic airways disease, may be applicable to other physiological or pathological lung conditions. We previously demonstrated that a low dose of IL-17 augments IL-13-induced airway inflammatory responses, while a higher dose of IL-17 attenuates these responses (27). In parallel, the "low dose" of IL-17 produced by $CD4^+$ T cells in

the current study may have augmented disease while our data suggest that the “high dose” of IL-17 produced by γδ T cells attenuated disease.

6 Conclusions

Altogether, our data demonstrate that intraperitoneal OVA sensitization with and without the adjuvants, alum and CFA, regulated the profile of IL-17 producing cells localized to the lung post OVA challenge, providing insight into potential mechanisms by which IL-17 may negatively regulate allergic airways disease. Notwithstanding the large influx of inflammatory cells to the airways, AHR was completely absent in OVA/CFA sensitized mice post OVA challenge. This lack of AHR coincided with an increase in IL-17-γδ T cells in the BAL fluid, in line with previous data demonstrating that these cells are potent inhibitors of airway inflammatory responses (27, 28). While the dual role of IL-17 makes it a complex target for drug development, our data suggest that treatments specifically focused on enhancing the IL-17-γδ T cell population may be beneficial for asthmatics.

Having firmly established both $CD4^+$ T cells and γδ T cells as the primary cellular sources of IL-17 in allergen-induced murine models of asthma and having established an inhibitory role for activated γδ T cells expressing enhanced levels of IL-17 on AHR and inflammation (Chapter 2), we next explored the role of IL-17 on Th2-induced immune responses using a non-allergen murine model of asthma. In Chapter 3, we establish the primary cellular sources of IL-17 in an IL-13 driven, non-allergen-dependent murine model of asthma as $CD4^+$ and γδ T cells. We again demonstrate a negative regulatory role for IL-17-γδ T cells and explore activity of recombinant IL-17 delivery in this IL-13-driven model.

7 References

1. Chung, K. F., S. E. Wenzel, J. L. Brozek, A. Bush, M. Castro, et al. 2014. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur. Respir. J.* 43: 343-373.
2. Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255-281.
3. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, et al. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326: 298-304.
4. Hamid, Q., M. Azzawi, S. Ying, R. Moqbel, A. J. Wardlaw, et al. 1991. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J. Clin. Invest.* 87: 1541-1546.
5. Al-Ramli, W., D. Prefontaine, F. Chouiali, J. G. Martin, R. Olivenstein, et al. 2009. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J. Allergy Clin. Immunol.* 123: 1185-1187.
6. Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, et al. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430-438.
7. Barczyk, A., W. Pierzchala, and E. Sozanska. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 97: 726-733.

8. Van der Pouw Kraan, T. C., J. S. Van der Zee, L. C. Boeije, E. R. De Groot, S. O. Stapel, and L. A. Aarden. 1998. The role of IL-13 in IgE synthesis by allergic asthma patients. *Clin. Exp. Immunol.* 111: 129-135.

9. Saha, S. K., M. A. Berry, D. Parker, S. Siddiqui, A. Morgan, et al. 2008. Increased sputum and bronchial biopsy IL-13 expression in severe asthma. *J. Allergy Clin. Immunol.* 121: 685-691.

10. Bullens, D. M., E. Truyen, L. Coteur, E. Dilissen, P. W. Hellings, et al. 2006. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir. Res.* 7: 135.

11. Vazquez-Tello, A., R. Halwani, Q. Hamid, and S. Al-Muhsen. 2013. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J. Clin. Immunol.* 33: 466-478.

12. Laan, M., Z. H. Cui, H. Hoshino, J. Lotvall, M. Sjostrand, et al. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* 162: 2347-2352.

13. Zhao, Y., J. Yang, and Y. D. Gao. 2011. Altered expressions of helper T cell (Th)1, Th2, and Th17 cytokines in CD8(+) and gammadelta T cells in patients with allergic asthma. *J. Asthma* 48: 429-436.

14. Zhao, Y., J. Yang, Y. D. Gao, and W. Guo. 2010. Th17 immunity in patients with allergic asthma. *Int. Arch. Allergy Immunol.* 151: 297-307.

15. Cosmi, L., L. Maggi, V. Santarlasci, M. Capone, E. Cardilicchia, et al. 2010. Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J. Allergy Clin. Immunol.* 125: 222-230 e221-224.

16. Wenzel, S. E., L. B. Schwartz, E. L. Langmack, J. L. Halliday, J. B. Trudeau, et al. 1999. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am. J. Respir. Crit. Care Med.* 160: 1001-1008.

17. Fahy, J. V. 2009. Eosinophilic and neutrophilic inflammation in asthma: insights from clinical studies. *Proc Am Thorac Soc* 6: 256-259.

18. Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186: 1737-1747.

19. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, et al. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261-2263.

20. Brewer, J. M., M. Conacher, C. A. Hunter, M. Mohrs, F. Brombacher, and J. Alexander. 1999. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J. Immunol.* 163: 6448-6454.

21. McKinley, L., J. F. Alcorn, A. Peterson, R. B. Dupont, S. Kapadia, et al. 2008. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* 181: 4089-4097.

22. Zhao, J., C. M. Lloyd, and A. Noble. 2013. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol.* 6: 335-346.

23. He, R., M. K. Oyoshi, H. Jin, and R. S. Geha. 2007. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc. Natl. Acad. Sci. U. S. A.* 104: 15817-15822.

24. Wilson, R. H., G. S. Whitehead, H. Nakano, M. E. Free, J. K. Kolls, and D. N. Cook. 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 180: 720-730.

25. Bogaert, P., T. Naessens, S. De Koker, B. Hennuy, J. Hacha, et al. 2011. Inflammatory signatures for eosinophilic vs. neutrophilic allergic pulmonary inflammation reveal critical regulatory checkpoints. *Am. J. Physiol. Lung Cell Mol. Physiol.* 300: L679-690.

26. Wakashin, H., K. Hirose, Y. Maezawa, S. Kagami, A. Suto, et al. 2008. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* 178: 1023-1032.

27. Kinyanjui, M. W., J. Shan, E. M. Nakada, S. T. Qureshi, and E. D. Fixman. 2013. Dose-dependent effects of IL-17 on IL-13-induced airway inflammatory responses and airway hyperresponsiveness. *J. Immunol.* 190: 3859-3868.

28. Murdoch, J. R., and C. M. Lloyd. 2010. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing {gamma}{delta}T cells. *Am. J. Respir. Crit. Care Med.* 182: 464-476.

29. Nakano, H., M. E. Free, G. S. Whitehead, S. Maruoka, R. H. Wilson, et al. 2012. Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol.* 5: 53-65.

30. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408.

31. Lajoie, S., I. P. Lewkowich, Y. Suzuki, J. R. Clark, A. A. Sproles, et al. 2010. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat. Immunol.* 11: 928-935.

32. Song, C., L. Luo, Z. Lei, B. Li, Z. Liang, et al. 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J. Immunol.* 181: 6117-6124.

33. Koenecke, C., V. Chennupati, S. Schmitz, B. Malissen, R. Forster, and I. Prinz. 2009. In vivo application of mAb directed against the gammadelta TCR does not deplete but generates "invisible" gammadelta T cells. *Eur. J. Immunol.* 39: 372-379.

34. Conrad, M. L., A. O. Yildirim, S. S. Sonar, A. Kilic, S. Sudowe, et al. 2009. Comparison of adjuvant and adjuvant-free murine experimental asthma models. *Clin. Exp. Allergy* 39: 1246-1254.

35. Eisenbarth, S. C., O. R. Colegio, W. O'Connor, F. S. Sutterwala, and R. A. Flavell. 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453: 1122-1126.

36. Brewer, J. M., M. Conacher, A. Satoskar, H. Bluethmann, and J. Alexander. 1996. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *Eur. J. Immunol.* 26: 2062-2066.

37. Stills, H. F., Jr. 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. *ILAR J* 46: 280-293.

38. Sutton, C. E., S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31: 331-341.

39. Cohn, L., R. J. Homer, H. MacLeod, M. Mohrs, F. Brombacher, and K. Bottomly. 1999. Th2-induced airway mucus production is dependent on IL-4Ralpha, but not on eosinophils. *J. Immunol.* 162: 6178-6183.

40. Hessel, E. M., A. J. Van Oosterhout, I. Van Ark, B. Van Esch, G. Hofman, et al. 1997. Development of airway hyperresponsiveness is dependent on interferon-gamma and independent of eosinophil infiltration. *Am. J. Respir. Cell Mol. Biol.* 16: 325-334.

41. Ollerenshaw, S. L., and A. J. Woolcock. 1992. Characteristics of the inflammation in biopsies from large airways of subjects with asthma and subjects with chronic airflow limitation. *Am. Rev. Respir. Dis.* 145: 922-927.

42. Tomkinson, A., G. Cieslewicz, C. Duez, K. A. Larson, J. J. Lee, and E. W. Gelfand. 2001. Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am. J. Respir. Crit. Care Med.* 163: 721-730.

43. Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, et al. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* 183: 109-117.

44. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, et al. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258-2261.

45. Lalor, S. J., L. S. Dungan, C. E. Sutton, S. A. Basdeo, J. M. Fletcher, and K. H. Mills. 2011. Caspase-1-processed cytokines IL-1beta and IL-18 promote IL-17 production by gammadelta and CD4 T cells that mediate autoimmunity. *J. Immunol.* 186: 5738-5748.

46. Lockhart, E., A. M. Green, and J. L. Flynn. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J. Immunol.* 177: 4662-4669.

47. Franchi, L., and G. Nunez. 2008. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. *Eur. J. Immunol.* 38: 2085-2089.

48. Jin, N., C. Taube, L. Sharp, Y. S. Hahn, X. Yin, et al. 2005. Mismatched antigen prepares gamma delta T cells for suppression of airway hyperresponsiveness. *J. Immunol.* 174: 2671-2679.

49. Hahn, Y. S., C. Taube, N. Jin, K. Takeda, J. W. Park, et al. 2003. V gamma 4+ gamma delta T cells regulate airway hyperreactivity to methacholine in ovalbumin-sensitized and challenged mice. *J. Immunol.* 171: 3170-3178.

50. Faustino, L., D. M. da Fonseca, M. C. Takenaka, L. Mirotti, E. B. Florsheim, et al. 2013. Regulatory T cells migrate to airways via CCR4 and attenuate the severity of airway allergic inflammation. *J. Immunol.* 190: 2614-2621.

51. Sutton, C. E., L. A. Mielke, and K. H. Mills. 2012. IL-17-producing gammadelta T cells and innate lymphoid cells. *Eur. J. Immunol.* 42: 2221-2231.

52. Roark, C. L., Y. Huang, N. Jin, M. K. Aydintug, T. Casper, et al. 2013. A canonical Vgamma4Vdelta4+ gammadelta T cell population with distinct stimulation requirements which promotes the Th17 response. *Immunol. Res.* 55: 217-230.

53. Oboki, K., T. Ohno, H. Saito, and S. Nakae. 2008. Th17 and allergy. *Allergol Int* 57: 121-134.

54. Pollinger, B., T. Junt, B. Metzler, U. A. Walker, A. Tyndall, et al. 2011. Th17 cells, not IL-17+ gammadelta T cells, drive arthritic bone destruction in mice and humans. *J. Immunol.* 186: 2602-2612.

CHAPTER 3: Dose-dependent effects of IL-17 on IL-13-induced airway inflammatory responses and airway hyperresponsiveness

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1 Abstract

The Th2 cytokine IL-13 regulates several aspects of the asthmatic phenotype, including airway inflammation, airway hyperresponsiveness, and mucus production. The Th17 cytokine IL-17A is also implicated in asthma and has been shown to both positively and negatively regulate Th2-dependent responses in murine models of allergic airways disease. Our objective in this study was to better understand the role of IL-17 in airway inflammation by examining how IL-17 modifies IL-13–induced airway inflammatory responses. We treated BALB/c mice intranasally with IL-13 or IL-17 alone or in combination for 8 consecutive days, after which airway hyperresponsiveness, inflammatory cell influx into the lung, and lung chemokine/cytokine expression were assessed. As expected, IL-13 increased airway inflammation and airway hyperresponsiveness. IL-13 also increased numbers of IL-17–producing $CD4^+$ and γδ T cells. Treating mice with a combination of IL-13 and IL-17 reduced infiltration of IL-17^+ γδ T cells, but increased the number of infiltrating eosinophils. In contrast, coadministration of IL-13 with a higher dose of IL-17 decreased all IL-13–induced inflammatory responses, including infiltration of both IL-17^+CD4^+ and γδ T cells. To examine the inhibitory activity of IL-17–expressing γδ T cells in this model, these cells were adoptively transferred into naive recipients. Consistent with an inhibitory role for γδ T cells, IL-13–induced infiltration of eosinophils, lymphocytes, and IL-17^+CD4^+ T cells was diminished in recipients of the γδ T cells. Collectively, our data indicate that allergic airway inflammatory responses induced by IL-13 are modulated by both the quantity and the cellular source of IL-17.

2 Introduction

Several features of allergic airways disease are mediated by the Th2 cytokine IL-13, including airway inflammation, airway hyperresponsiveness (AHR), and mucus production (1-3). Loss of IL-13 expression or blockade of IL-13 activity diminishes each of these responses in murine models of allergic airways disease (1, 3). These proinflammatory effects of IL-13 are mediated by the IL-13 receptor, which, upon ligation, induces tyrosine phosphorylation of the STAT6 transcription factor (1, 4). Once phosphorylated, STAT6 dimerizes and translocates to the nucleus where it binds to DNA and initiates expression of a number of genes, including chemokines such as CCL11 (eotaxin-1) and CCL17 (thymus and activation–regulated chemokine), which then promote eosinophil and leukocyte infiltration into the lung (5-8).

The Th17 cytokine IL-17A (hereafter referred to as IL-17) is also implicated in asthma. IL-17 levels are elevated in the bronchoalveolar lavage fluid (BALF), lungs, and sputum of asthmatics (9-11). More recently, IL-17 has also been implicated specifically in severe asthma (12-15). Elevated levels of IL-17 are thought to participate in increased airway neutrophilia in this group of asthmatics (13, 16, 17) owing to IL-17–mediated production of chemokines that promote neutrophil recruitment (18-21). The role of IL-17 in murine models of allergic airways disease is unclear. Abundant data demonstrate a proinflammatory role for IL-17 and/or Th17 cells in allergic airways disease. For example, IL-17–producing T cells induce airway neutrophilia in mice (22-24). These cells also positively regulate Th2-driven AHR and airway eosinophilia (23, 24). Neutralizing IL-17 diminishes Th2-driven allergic airways disease (25-27), and mice lacking IL-17 have reduced airway inflammatory responses upon antigen (Ag) challenge (28). Whereas $CD4^+$ T cells producing IL-17 are most often implicated in these

responses, IL-17 produced by other cells, such as macrophages, may also promote allergic airways disease in these models (27).

In contrast to these data demonstrating that IL-17 promotes allergic airways disease, there is also evidence that IL-17 can play a protective role. Adoptive transfer of IL-17–producing γδ T cells into mice with ongoing allergic inflammation decreases both AHR and the duration of airway eosinophilia, whereas IL-17–deficient γδ cells are unable to promote resolution of the allergic inflammatory response (29). Similarly, intranasal administration of IL-17 during Ag challenge of sensitized mice decreases AHR and diminishes airway eosinophilia (30). To date, the mechanism by which IL-17 participates in the resolution of inflammatory events remains unclear.

The aim of our study was to investigate the effects of IL-17 on Th2-biased inflammatory responses in the airways. To this end, we established an acute model of airway inflammation in which we treated naive BALB/c mice with IL-13 in combination with two different doses of IL-17. IL-13 alone induced several hallmarks of allergic airways disease (i.e., AHR, airway inflammation, and mucus production) as well as increased mRNA and protein levels of chemokines that promote airway inflammation. We also found increased IL-17 mRNA expression that was accompanied by an increase in the number of IL-17–producing $CD4^+$ and γδ T cells. These IL-13–induced responses were variably affected by co-administration of IL-17: lower doses of IL-17 enhanced eosinophil recruitment to the airways, at the same time selectively reducing the number of IL-17–expressing γδ T cells recruited to the BALF, whereas higher doses inhibited airway eosinophil recruitment, chemokine production, AHR, and both subsets of IL-17–expressing T cells. Consistent with an inhibitory role for γδ T cells, adoptive transfer of these cells reduced airway inflammation. Our data suggest that IL-13–induced allergic

responses in the airways are shaped by the overall amount of IL-17 present as well as by the cell types producing this cytokine.

3 Materials and methods

Animals and cytokine treatment

Wild-type (WT) BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Montreal, QC, Canada). C57BL/6 IL-17 knockout (IL-17$^{-/-}$) mice were a gift from Dr. Yoichiro Iwakura (University of Tokyo). All animals, including TLR4$^{-/-}$ mice, were bred at the Meakins-Christie Laboratories Animal Facility. Animal studies were approved by the McGill University Animal Care Committee and were performed following the guidelines of the Canadian Council on Animal Care. For cytokine treatment, mice were briefly anesthetized with isoflurane prior to intranasal administration of cytokines or control saline in a total volume of 30 μl. Mice received IL-13 (Life Technologies, Burlington, ON, Canada) or IL-17 (BioLegend, San Diego, CA) alone (0.5 μg/d for 8 d) or IL-13 plus IL-17 (either 0.5 μg/d or 1.5 μg/d for 8 d). Mice were sacrificed 48 h following cytokine administration. For adoptive transfer experiments, mice were treated for 8 d with IL-13. One day after the final treatment, mice were sacrificed with an overdose of sodium pentobarbital and lungs were harvested. Following enzymatic digestion with Liberase (100 μg/ml; Roche Applied Science, Laval, QC, Canada), in combination with collagenase XI (250 μg/ml), hyaluronidase 1a (1 mg/ml), and DNase I (200 μg/ml; Sigma-Aldrich, Oakville, ON, Canada) for 1 h at 37°C (31), CD3$^+$ cells were purified by negative magnetic selection with the EasySep mouse T cell enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) and labeled with anti–γδ TCR-FITC (see below). Subsequently, propidium iodide−, γδ$^+$ T cells were FACS-sorted and adoptively transferred, via IP injection, into naive recipient mice, which were then exposed to IL-13 for each of the next 8 d as above. Control animals received saline. More

than 95% of purified cells were γδ T cells. All mice were sacrificed 48 h following cytokine administration.

Analysis of AHR and airway inflammation

To assess AHR, animals were anesthetized using xylazine and sodium pentobarbital and paralyzed with pancuronium bromide. AHR in response to increasing doses of methacholine was measured as previously described (32). Briefly, anesthetized and paralyzed mice were attached to a computer-controlled small-animal ventilator (flexiVent; Scireq, Montreal, QC, Canada). The baseline respiratory system resistance was measured before recording the maximal resistance obtained with increasing doses of nebulized methacholine. Heart rate was monitored throughout the procedure to ensure adequate anesthesia. Afterward, the lungs were lavaged twice with 1 ml PBS. Cells were recovered by centrifugation of the BALF and the supernatant was collected and stored at −80°C for chemokine analysis. Cells from both lavage fractions were pooled and counted before being spun onto microscope slides. Cells were then stained using Diff-Quick (Fisher Scientific, Ottawa, ON, Canada), and differential cell counts were obtained by counting three to six fields comprising 300 cells per field.

Intracellular cytokine staining of BALF inflammatory cells

BALF cells recovered from groups of three to four mice were stimulated for 4 h in complete RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (Life Technologies) with 50 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and GolgiStop

(BD Biosciences, Mississauga, ON, Canada). Cells were then fixed, permeabilized, and stained with anti–CD4-allophycocyanin, anti–γδ TCR-FITC, and anti–IL-17-PE Abs (BD Biosciences).

RNA purification and real-time PCR analysis

Following collection of BALF, lungs were dissected away from the trachea and main bronchi and stored in RNAlater (Life Technologies). Total cellular RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's protocol and reverse transcribed using SuperScript II reverse transcriptase (Life Technologies). Real-time quantitative PCR was performed using the Applied Biosystems PCR system (Life Technologies) and AB Power SYBR Green mix (Life Technologies). Cycle threshold values were determined using AB Step One software (Life Technologies) and normalized to GAPDH. The comparative cycle threshold method was then used to calculate the relative gene expression levels (33).

Quantitative analysis of lung chemokines

BALF chemokine levels were assessed using the mouse Q-Plex chemokine kit from Quansys Biosciences (Logan, UT) as per the manufacturer's instructions.

Statistical analysis

Results are expressed as the mean ± SEM. In comparisons of only two groups, statistical significance was measured using the Student t test. For experiments comparing more than two groups, statistical significance was measured by ANOVA followed by a post hoc Student–Newman–Keuls test for pairwise comparisons. A p value <0.05 was considered significant.

4 Results

Intranasal delivery of rIL-13 induces AHR, airway inflammation, and chemokine/cytokine expression

To better understand the role of IL-17 in allergic airway inflammation we first established an acute model of IL-13–induced airway disease in which 0.5 μg IL-13 was administered intranasally to anesthetized mice for 8 consecutive days. Airway resistance to methacholine, quantified 48 h after the last IL-13 administration, was increased in mice treated with IL-13 compared with control animals that received saline alone **(Fig 3.1A)**. The increased respiratory system resistance was accompanied by an influx of eosinophils, neutrophils, and lymphocytes into the bronchoalveolar compartment of the lung **(Fig 3.1B)**. These results are consistent with previously reported acute models of IL-13–induced airway inflammation (1-4). Administration of IL-13 also induced expression of the mucin gene MUC5AC (2, 34-36) **(Fig 3.1C)** and increased goblet cell mucus production (assessed by periodic acid–Schiff staining; data not shown). Consistent with the ability of IL-13 to increase expression of chemokines that regulate eosinophil and lymphocyte chemotaxis (5, 37-39), the levels of mRNA encoding CCL11 (eotaxin-1), CCL17 (thymus and activation–regulated chemokine), and CCL22 (macrophage-derived chemokine) were also increased **(Fig 3.1C)**. Levels of mRNA encoding CCL5 (RANTES) and CCL24 (eotaxin-2) were not significantly increased in the lungs of IL-13–treated mice.

Mice treated with IL-13 had increased numbers of Gr-1^{high} neutrophils (40) in the BALF **(Fig 3.2A, B)**. One potential mechanism by which neutrophils are recruited into the airways is via IL-17–induced production of two CXC chemokines (CXCL1 and CXCL5) (19, 20, 41).

Figure 3.1: Intranasal delivery of recombinant IL-13 induces AHR, airway inflammation, and chemokine/cytokine expression

(A) Respiratory resistance to methacholine assessed 48 h following the last dose of IL-13. Control mice were treated with saline (SAL). Mean respiratory resistance to methacholine (±SEM) from a combination of three independent experiments using three to five mice per group in each experiment is shown. (B) BALF differential cell counts from SAL (open bars)- and IL-13 (filled bars)–treated mice. Cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes ± SEM are shown. BALF cell counts are from one representative experiment out of at least three in which three to four mice per group were used. (C) Relative expression of MUC5AC and chemokine genes as assessed by real-time PCR following SAL (empty bars) or IL-13 (black bars) treatment. The relative gene expression levels in IL-13–treated mice compared with SAL are presented as the means ± SEM from at least three experiments using three to five mice per group in each experiment. *$p < 0.05$ SAL versus IL-13.

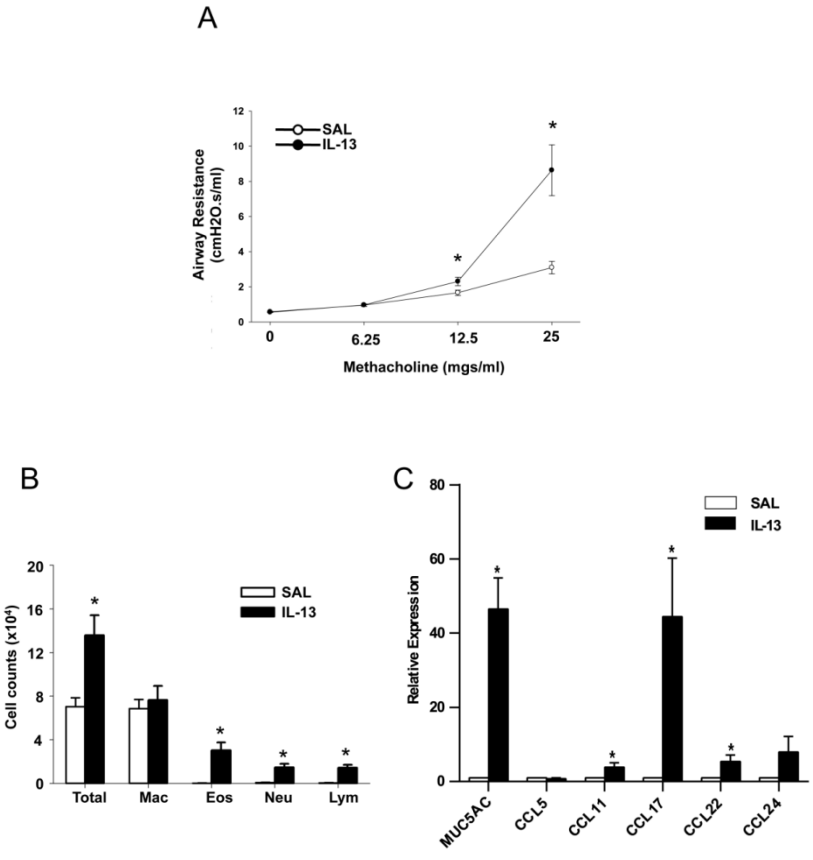
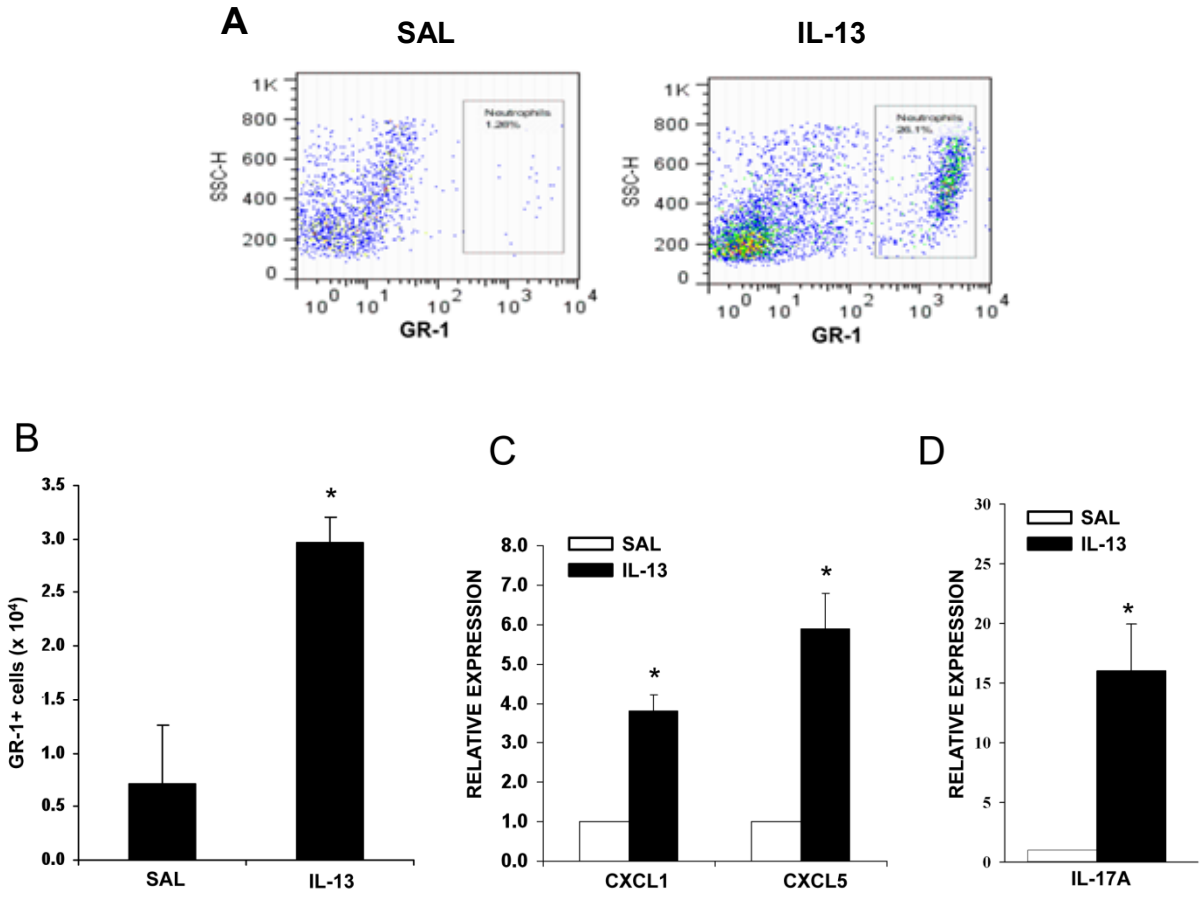


Figure 3.2: In vivo administration of IL-13 leads to increases in neutrophils, neutrophil chemokines, and IL-17

(A) Cells recovered from the BALF of mice treated with saline (SAL) (top panel) or IL-13 (bottom panel) were labeled for flow cytometry using GR-1 Abs. (B) Mean cell counts for GR-1high cells ± SEM from two experiments using three to five mice per group in each experiment are shown. (C and D) Relative expression of CXCL1 and CXCL5 (C) or IL-17 (D) assessed by real-time PCR following SAL (open bars) or IL-13 (filled bars) treatment. The relative gene expression levels in IL-13–treated mice are presented as the means ± SEM from at least three experiments using three to five mice per group in each experiment. *$p < 0.05$ SAL versus IL-13.



Interestingly, mRNA levels of each of these chemokines **(Fig 3.2C)**, as well as IL-17 **(Fig 3.2D)**, were increased in the lungs of IL-13–treated mice. Consistent with the elevated levels of IL-17 mRNA, we identified two primary populations of cells producing IL-17 in IL-13–treated mice. Characterization of these IL-17–producing cells indicated that they were $CD4^+$ and γδ T cells **(Fig 3.3A)**. Although IL-17–producing cells could be detected in control mice, larger numbers were present following IL-13 treatment **(Fig 3.3B, C; see also below)**. In an attempt to further define these IL-17–producing cells, expression of IL-22, IL-4, and IFN-γ was assessed by intracellular cytokine staining, but none of these cytokines could be detected (data not shown). Moreover, to examine the possibility that LPS in the IL-13 preparation was contributing to the increases in IL-17–expressing T cells, responses to IL-13 were examined in TLR4 knockout mice. IL-13 not only retained the ability to induce IL-17–expressing $CD4^+$ and γδ T cell subsets in these mice, but their induction was also enhanced **(Fig 3.3B, C)**. IL-13 also retained the ability to induce airway inflammation in $TLR4^{-/-}$ mice **(Fig 3.4A)**. Whereas eosinophil and neutrophil numbers did not differ, macrophage and lymphocyte numbers were enhanced in TLR4 knockout mice treated with IL-13. IL-13–induced AHR did not differ between IL-13–treated WT and TLR4 knockout mice **(Fig 3.4B)**.

In vivo administration of IL-13 and low-dose IL-17 leads to enhanced airway inflammation

To better understand how IL-17 interacts with IL-13 to modulate airway inflammatory responses, mice were treated with IL-13 alone, IL-17 alone, or the combination of both cytokines, and AHR, inflammatory cell influx, and chemokine expression were assessed. Unlike IL-13, IL-17 was unable to induce AHR. IL-13 alone, as well as in combination with IL-17, induced comparable degrees of airway hyperresponsiveness to methacholine **(Fig 3.5A)**.

Nevertheless, total cell numbers recovered in the BALF were greater in mice treated with the combination of IL-13 and IL-17 compared with IL-13 alone **(Fig 3.5B)**. Significantly, the numbers of both eosinophils and lymphocytes recovered from IL-13/IL-17–treated mice were increased compared with animals treated with IL-13 alone. Although IL-17 alone did not induce eosinophil or neutrophil recruitment into the airways, it did induce an increase in macrophages, consistent with previous data showing that IL-17 promotes macrophage recruitment and survival in the airways (42).

Assessment of MUC5AC and chemokine expression revealed that IL-13–induced increases in MUC5AC and CCL17 mRNA remained elevated in animals treated with both IL-13 and IL-17 **(Fig 3.5C)**. Conversely, mRNA levels of CCL22 and CCL24 were decreased in mice treated with both IL-13 and IL-17. Although IL-17 alone had no effect on mRNA levels of CCL17, CCL22, and CCL24, there was a trend toward increased mRNA levels of MUC5AC, consistent with previous in vitro data (43).

At this point, our data suggested that IL-17 had only minor effects on IL-13–induced gene expression and AHR, but enhanced IL-13–induced airway inflammation (by increasing recruitment of eosinophils and lymphocytes into the BALF). However, IL-17 potently inhibited its own expression induced by IL-13: IL-17 mRNA levels were decreased to basal levels in animals treated with the combination of IL-13 and IL-17 compared with those treated with IL-13 alone **(Fig 3.5D)**. Given the dramatic decrease in IL-17 mRNA levels in these mice, we examined the effect of coadministration of IL-17 on CD4 and γδ T cell–specific IL-17 expression. There was no difference in the frequency of infiltrating $IL\text{-}17^{+}CD4^{+}$ T cells between animals treated with IL-13 or the combination of IL-13 and IL-17 **(Fig 3.6)**. In contrast, the IL-13–induced increase in the frequency of IL-17–producing γδ T cells was dramatically decreased

Figure 3.3: In vivo administration of IL-13 leads to increases in IL-17–producing T cells in the airways of WT and TLR4$^{-/-}$ mice

(A) Cells recovered from the BALF of saline (SAL) (top panel)- or IL-13 (bottom panel)–treated WT mice were labeled for flow cytometry using fluorescent Abs against IL-17, CD4, and γδ TCR. Left panels contain frequencies of CD4$^+$ and IL-17$^+$ cells. Right panels contain frequencies of γδ TCR$^+$ and IL-17$^+$ cells. Flow cytometry plots, representative of at least three independent experiments where BALF cells from three to five mice per group were pooled, are shown. (B and C) Number of IL-17–producing CD4 (B) or γδ (C) T cells recovered from the BALF of cytokine-treated mice. Data are presented as the number of IL-17$^+$ cells ± SEM from two independent experiments using three to four mice per group in each experiment. *$p < 0.05$ SAL versus IL-13.

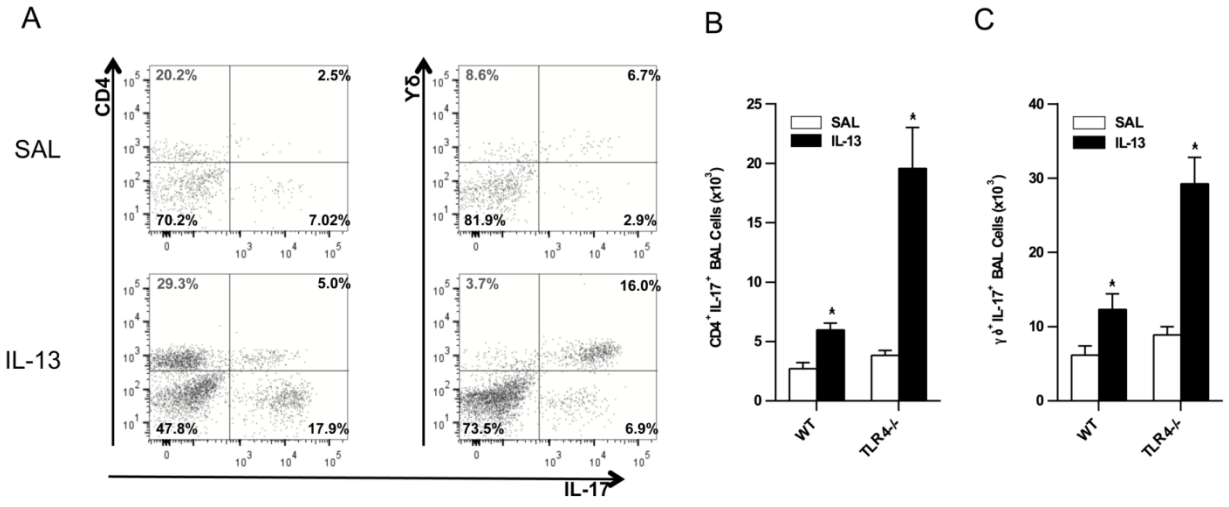


Figure 3.4: Lack of TLR4 augments IL-13-induced airway inflammation, but not AHR

IL-13 (0.5µg) was administered intranasally to TLR4$^{-/-}$ or wild-type mice. A, BAL fluid differential cell counts from SAL treated wild type (empty bars), SAL treated TLR4$^{-/-}$ (light grey), IL-13 treated wild-type (black bars) and IL-13 treated TLR4$^{-/-}$ (dark grey bars) mice are shown. Mean cell counts for total cells, macrophages, eosinophils, neutrophils and lymphocytes ± SEM from two independent experiments using 3-4 mice per group in each experiment are shown. B, Respiratory resistance to methacholine assessed 48h following the last dose of IL-13. Control animals were treated with SAL. Mean respiratory resistance to methacholine ± SEM from two independent experiments using 3-4 mice per group in each experiment is shown. * $p<0.05$ wt SAL vs. wt IL-13, # $p<0.05$, TLR4$^{-/-}$ SAL vs. TLR4$^{-/-}$ IL-13, § $p<0.05$ wt IL-13 vs. TLR4$^{-/-}$ IL-13.

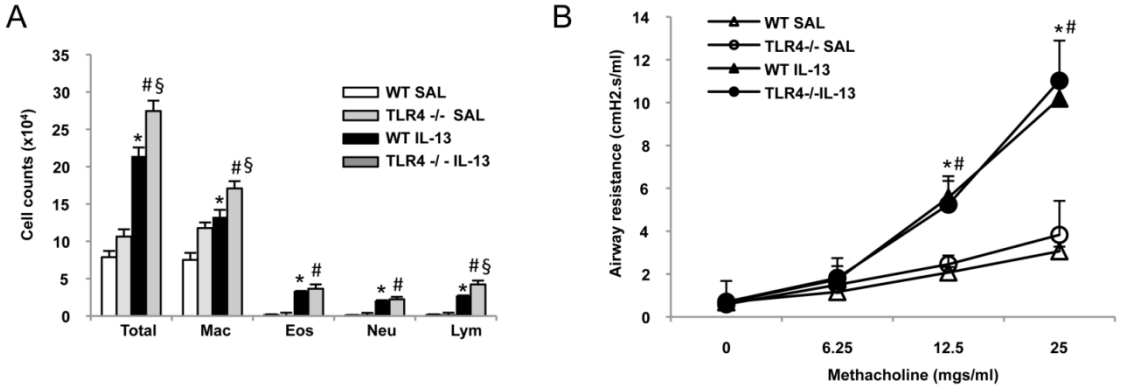


Figure 3.5: In vivo administration of both IL-13 and IL-17 leads to enhanced airway inflammation

Mice were treated with saline (SAL), IL-13 (0.5 μg), IL-17 (0.5 μg), or IL-13/17 (0.5 μg each). (A) Respiratory resistance to methacholine assessed following treatment with IL-13 and/or IL-17. Mean respiratory resistance to methacholine ± SEM from a combination of three independent experiments using three to five mice per group in each experiment is shown. (B) BALF differential cell counts from SAL (white bars)-, IL-13 (black bars)-, IL-17 (dark gray bars)–, and IL-13/17 (light gray bars)–treated mice. Cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes ± SEM are shown. Cell counts are from one representative experiment out of at least three in which three to four animals per group were used. (C and D) Relative expression of MUC5AC and chemokine genes (C) or IL-17 (D) assessed by real-time PCR following IL-13 and/or IL-17 treatment. Relative gene expression levels in IL-13–treated animals compared with SAL are presented as the means ± SEM from at least three experiments using three to five mice per group in each experiment. *$p < 0.05$ SAL versus IL-13, §$p < 0.05$ IL-13 versus IL-13/IL-17.

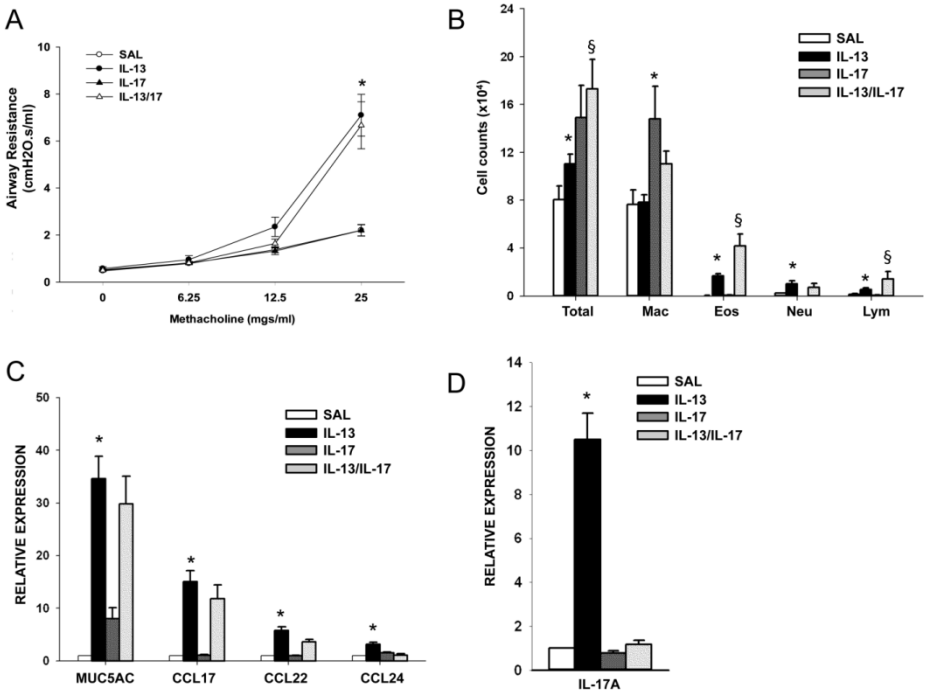
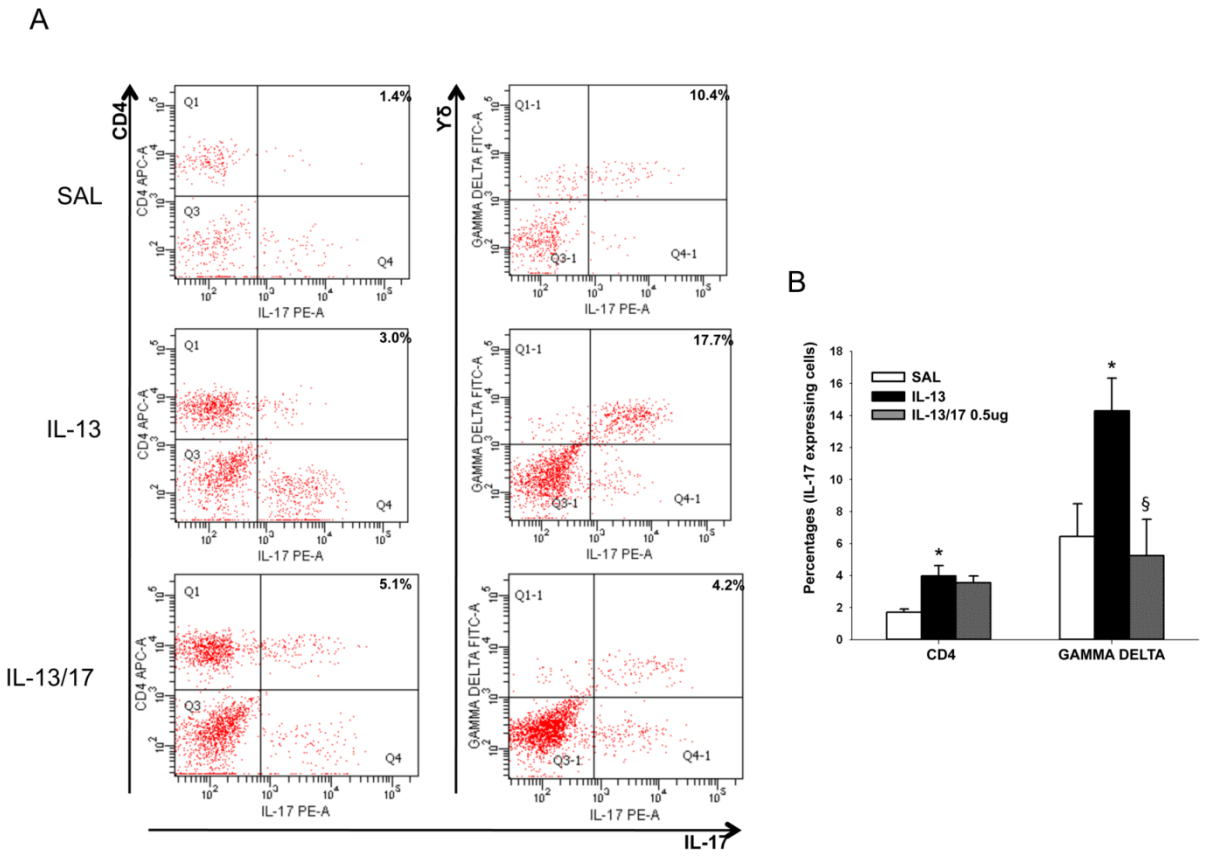


Figure 3.6: Administration of IL-13 and IL-17 leads to a decrease in IL-17–producing γδ T cells but not CD4+ T cells in the airway

(A) Cells recovered from the BALF of saline (SAL)-, IL-13–, or IL-13/17–treated mice were assessed for expression of CD4, γδ TCR, and IL-17. Left panels contain frequencies of CD4+ and IL-17$^+$ cells. Right panels contain frequencies of γδ TCR$^+$ and IL-17$^+$ cells. Flow cytometry plots, representative of at least three independent experiments where BALF cells from three to five mice per group were pooled, are shown. (B) Percentage of CD4 and γδ IL-17–producing cells recovered from mice treated with SAL (white bars), IL-13 (black bars), or IL-13/17 (light gray bars). Data are presented as the percentage of IL-17$^+$ cells ± SEM from at least three independent experiments using BALF cells pooled from three to five mice per group. *p < 0.05 SAL versus IL-13, §p < 0.05 IL-13 versus IL-13/IL-17.



by coadministration of IL-17 **(Fig 3.6)**. The decrease in γδ T cells was inversely correlated with the increased airway inflammation, in agreement with a previous study demonstrating that IL-17–producing γδ T cells promote resolution of airway inflammation (29).

The proinflammatory versus protective effects of IL-17 are dose dependent

Treatment with a combination of IL-13 and IL-17 at the 0.5 μg dose led to a modest proinflammatory effect in comparison with treatment with IL-13 alone **(Fig 3.5B)**. We also treated mice with a higher dose of IL-17 (1.5 μg). Surprisingly, IL-13–induced AHR was decreased in mice treated with IL-17 at the higher dose compared with animals treated with IL-13 alone **(Fig 3.7A)**. IL-13–induced inflammatory cell influx into the lung was also decreased in animals treated with IL-13 in combination with the higher dose of IL-17 **(Fig 3.7B)**. This decrease was attributable to fewer macrophages and fewer eosinophils recovered in the BALF. The IL-13–induced increase in IL-17$^+$ γδ T cells recovered in the BALF was abrogated with the lower dose of IL-17 and this did not change upon delivery of the higher dose of IL-17 **(Fig 3.7C)**. Additionally, although we found that the number of IL-17$^+$ CD4$^+$ T cells was unaffected in animals treated with IL-13 and IL-17 at the lower dose, this increase was also abrogated in mice treated with IL-13 and the higher dose of IL-17 **(Fig 3.7D)**.

Higher dose IL-17 decreases IL-13–induced production of chemokines

As shown in Figs. 3.1 and 3.5, IL-13 treatment alone increased mRNA levels of several eosinophil chemokines. Because inflammation was differentially modulated by the two different doses of IL-17, chemokines recovered in the BALF of mice treated with IL-13 alone or with IL-17 were quantified. BALF levels of CCL5, CCL11, CCL17, and CCL22 were elevated in IL-13–

treated mice compared with saline-treated control mice **(Fig 3.8)**, and levels in mice treated with IL-13 and the lower dose of IL-17 did not significantly differ. In contrast, animals treated with a combination of IL-13 and IL-17 at the higher dose had significantly lower levels of CCL11, CCL17, and CCL22, with no changes detected in CCL5 **(Fig 3.8)**.

Adoptive transfer of γδ T cells from IL-13–treated mice diminishes IL-13–induced airway inflammation

The loss of IL-17–expressing γδ T cells in mice treated with IL-13 and the lower dose of IL-17 correlated with enhanced airway inflammation **(Figs 3.5B, 3.7B)**. To more directly assess the activity of γδ T cells on IL-13–induced airway inflammatory responses, these cells were purified from the lungs of IL-13–treated mice. γδ T cells (10,000 or 50,000) were delivered i.p. to naive recipients, which were then treated with IL-13 as above. Consistent with an inhibitory role for γδ T cells, total inflammatory cell influx was decreased in recipients of γδ T cells, with significant reductions in eosinophils and lymphocytes with the higher dose of γδ T cells **(Fig 3.9A)**. Although inflammation was reduced, adoptively transferred γδ T cells had no effect on IL-13–induced AHR **(Fig 3.9B)**. Whereas the frequency of total lung γδ T cells did not differ in recipients of exogenous γδ T cells (data not shown), the frequency of IL-17–expressing γδ T cells in the lung increased in a dose-dependent manner, likely due to trafficking of the adoptively transferred γδ T cells to the lung **(Fig 3.10A)**. Notably, IL-13–induced increases in BALF numbers of total IL-17–expressing cells **(Fig 3.9C)**, as well as $CD4^+$ T cells expressing IL-17 **(Fig 3.9D)**, were reduced in recipients of adoptively transferred γδ T cells. Similar reductions in BALF γδ T cells expressing IL-17 were not found **(Fig 3.10B)**. However, these cells may represent a mixture of the adoptively transferred γδ T cells and endogenous γδ T cells induced by

Figure 3.7: Administration of IL-13 in combination with a high dose of IL-17 (1.5 µg) leads to the inhibition of AHR and airway inflammation

(A) AHR measured in mice treated with IL-13 alone or IL-13 in combination with a high dose of IL-17 (1.5 µg). Mean respiratory resistance to methacholine ± SEM from a combination of three independent experiments using three to five mice per group in each experiment is shown. (B) BALF differential cell counts from saline (SAL) (white bars)-, IL-13 (black bars)-, IL-13/17 (0.5 µg) (dark gray bars)–, and IL-13/17 (1.5 µg) (light gray bars)–treated mice. Cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes ± SEM are shown. BALF cell counts are from one representative experiment out of at least three in which three to four animals per group were used. (C and D) Number of IL-17–producing γδ (C) or CD4 (D) T cells recovered from the BALF of cytokine-treated mice. Data are presented as the number of IL-17+ cells ± SEM from at least three experiments using cells pooled from three to five mice per group in each experiment. *$p < 0.05$ SAL versus IL-13, §$p < 0.05$ IL-13 versus IL-13/IL-17 (0.5 µg), #$p < 0.05$ IL-13 versus IL-13/IL-17 (1.5 µg).

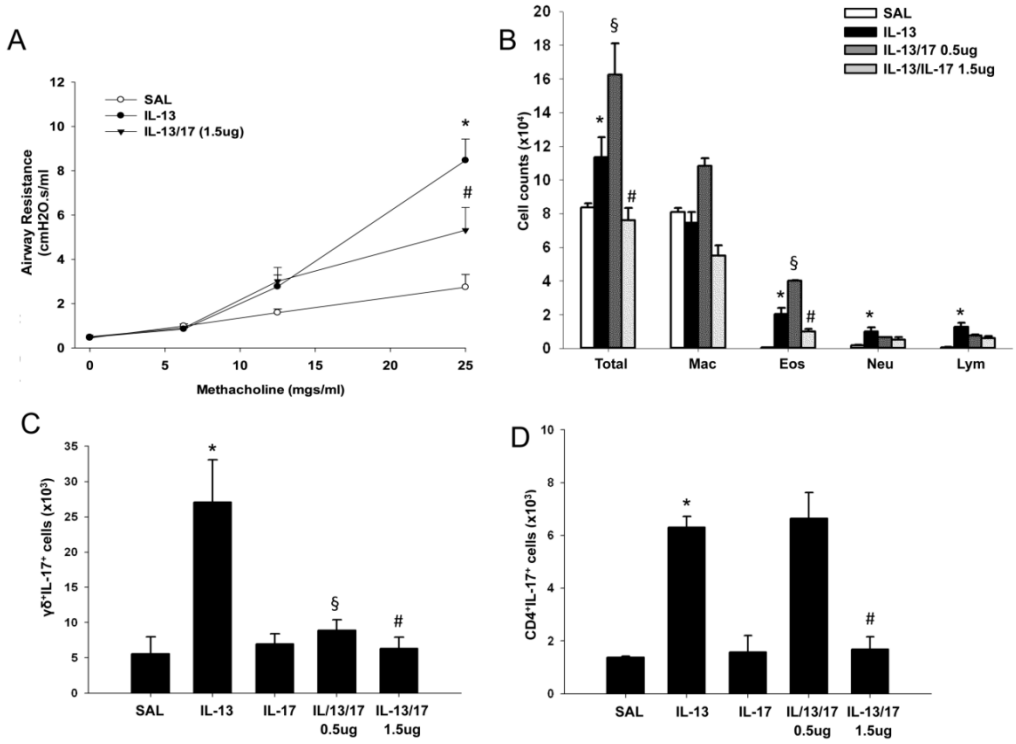


Figure 3.8: Administration of IL-13 in combination with a high dose of IL-17 (1.5 μg) leads to decreased chemokine production

(A–D) BALF chemokines (CCL5, CCL11, CCL17, and CCL22) were quantified using a Quansys chemokine multiplex kit. Mean chemokine levels ± SEM from two experiments using BALF from four mice per group in each experiment are graphed. *p < 0.05 SAL versus IL-13, #p < 0.05 IL-13 versus IL-13/IL-17 (1.5 μg).

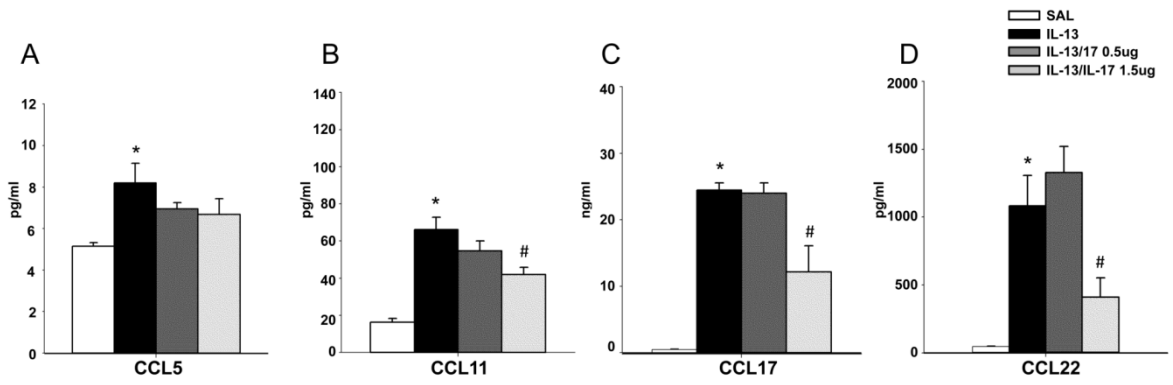


Figure 3.9: Adoptively transferred γδ T cells diminish IL-13–induced airway inflammatory responses

(A) BALF differential cell counts from mice treated with saline (SAL) (white bars), IL-13 (black bars), IL-13 plus low-dose γδ T cells (IL-13 + γδ lo) (dark gray bars), or IL-13 + high-dose γδ T cells (IL-13 + γδ hi) (light gray bars). Mean cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes ± SEM from two independent experiments using three mice per group in each experiment are shown. (B) AHR measured in mice treated with SAL, IL-13, IL-13 plus low-dose γδ T cells (IL-13 + γδ lo), or IL-13 plus high-dose γδ T cells (IL-13 + γδ hi). Mean respiratory resistance to methacholine ± SEM from two independent experiments using three mice per group in each experiment is shown. (C and D) Number of total IL-17–producing cells (C) or IL-17–producing CD4 T cells (D) recovered from the BALF of mice treated with SAL (white bars), IL-13 (black bars), IL-13 plus low-dose γδ T cells (dark gray bars) (γδ lo), or IL-13 plus high-dose γδ T cells (light gray bars) (γδ hi). Data are presented as the number of IL-17+ cells ± SEM from two independent experiments using a total of six mice per group. *$p < 0.05$ SAL versus IL-13, §$p < 0.05$ IL-13 versus γδ lo, %$p < 0.05$ IL-13 versus γδ hi, #$p < 0.05$ SAL versus γδ hi.

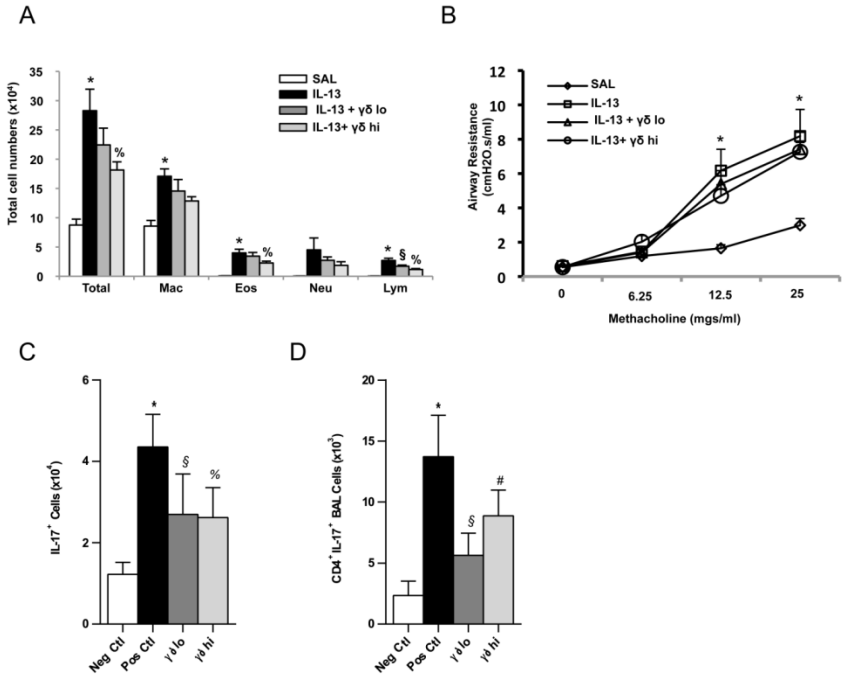
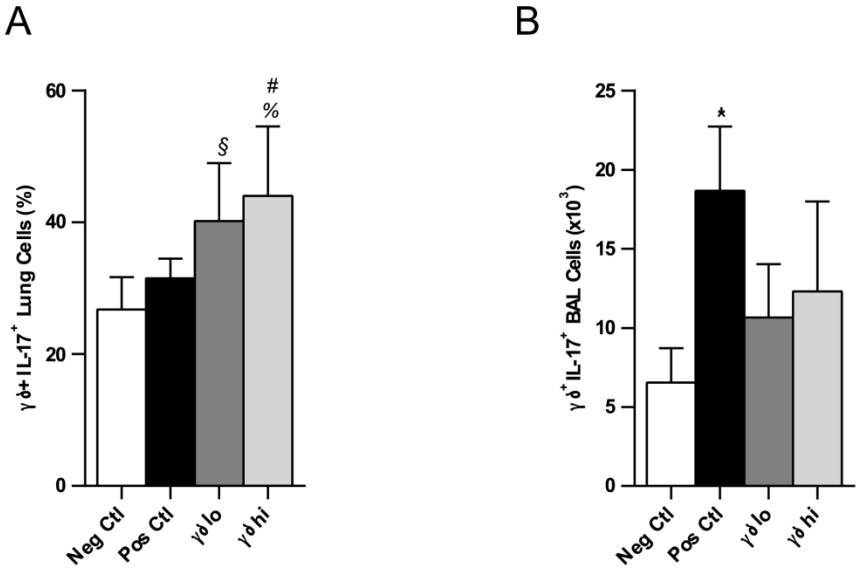


Figure 3.10: Frequency or IL-17 expressing γδ T cells in the lung and BAL fluid of IL-13 treated mice following adoptive transfer of γδ T cells

A, Frequency of IL-17 producing γδ T cells in lung homogenates. B, Number of IL-17 producing γδ CD4 T cells recovered from the BAL fluid. Mice were treated with SAL (empty bars), IL-13 (black bars), IL-13 $^{+}$ low dose γδ T cells (dark gray bars) (γδ lo), or IL-13 $^{+}$ high dose γδ T cells (light grey bars) (γδ hi). Data are presented as the frequency (A) or number (B) of IL-17 positive γδ T cells ± SEM from two independent experiments using a total of 6 mice per group. * $p<0.05$ SAL vs. IL-13; § $p<0.05$ IL-13 vs. γδ lo; % $p<0.05$ IL-13 vs. γδ hi; # $p<0.05$ SAL vs. γδ hi.



IL-13. These reductions in BALF eosinophils and lymphocytes as well as IL-17–expressing $CD4^+$ T cells in recipients of adoptively transferred γδ T cells provide further support for a negative regulatory role for γδ T cells expressing IL-17 in allergic airways disease.

5 Discussion

IL-17 has been linked to a number of autoimmune and allergic diseases (44-46). Clinical data have associated Th17 cells, IL-17, and increased neutrophilia with severe asthma. However, the role of IL-17 in murine models is controversial, as IL-17 has been shown to both promote and inhibit pathogenesis in models of allergic airways disease (47). Although the reasons for these conflicting data may be differences in conditions of Ag sensitization and/or mouse strain, to our knowledge, we show in this study for the first time a dose-dependent effect of IL-17 in the modulation of airway inflammation. At lower doses, IL-17 augmented IL-13–induced airway inflammation, promoting influx of greater numbers of eosinophils. At higher doses, IL-17 negatively regulated not only its own expression in both CD4 and γδ T cells, but also that of a number of key inflammatory chemokines. At higher doses, IL-17 powerfully inhibited IL-13–induced AHR, airway inflammation, and chemokine production.

Some of the confusion regarding the precise role of IL-17 in asthma may be due to the fact that IL-17 is expressed by different cells and/or in combination with different cytokines. In the lung, for instance, IL-17 can be produced by CD4 T cells (48), γδ T cells (29), and macrophages (27). Although Th17 cells, which can also produce IL-17F and IL-22 (19, 24, 49, 50), are often thought of as the main producers of IL-17, other effector T cell subsets, including Th1 (51), Th2 (48), and Th0 (51), all have the capacity to produce IL-17 under some conditions. These subsets, in which IL-17 is produced in combination with other classically defined Th1 or Th2 cytokines, have also been implicated in asthma pathogenesis in humans and in murine models. In contrast, IL-17–producing γδ T cells, but not CD4 T cells, have the ability to promote resolution of allergic airway inflammation in mice (29). Our data demonstrating inhibition of airway

inflammatory cell influx by adoptively transferred γδ T cells support an anti-inflammatory role for these cells as well. Thus, the cellular context in which IL-17 is produced is able to modulate the inflammatory outcome. An additional complication is the fact that in mice different profiles of T cell subsets may be generated, depending on the strain of mouse (52) and/or conditions of Ag sensitization (25, 53). In our own experiments, we found significant increases in IL-17–producing γδ cells in response to IL-13 delivery in BALB/c mice, whereas far fewer of these cells were recovered in C57BL/6 mice treated with IL-13 (data not shown).

To bypass the complexity inherent in Ag-dependent allergy models, we treated mice directly with recombinant IL-13 and/or IL-17 to assess how these cytokines interact in vivo. IL-13 is considered the primary effector cytokine in Th2-biased responses. As well, we examined the dose-dependent effects of IL-17 on IL-13–induced inflammatory responses in vivo. We found that administration of IL-13 promoted increases in both CD4 and γδ T cells expressing IL-17. These cells are likely effector/memory T cells, as they did not express CD62L (data not shown). Cytokine-induced cytokine production by CD4 T cells in the absence of Ag stimulation has been previously demonstrated (54, 55) and represents a link between the innate and adaptive immune systems whereby T cells produce effector cytokines in response to innate stimuli. For example, treatment of $CD4^+$ and γδ T cells with the combination of IL-1β or IL-18 and IL-23 is sufficient to induce IL-17 production (54-56). With regard to IL-13, previous studies have shown that IL-13 overexpression in the lung may lead to a cytokine environment conducive to the generation of IL-17–producing cells. Fulkerson and colleagues (57) used a transgenic system in which IL-13 expression was targeted to the lung using a reversible doxycycline system. Doxycycline exposure induced lung expression of IL-13, which led to an increase in IL-1β, TGF-β, and IL-6, all of which promote IL-17 production. Although IL-17 production was not reported in these

mice, our own data demonstrate that delivery of IL-13 to the lung induced production of IL-17 via pathways that were at least partially dependent on caspase-1 activation and IL-1β production (data not shown). To rule out the possibility that LPS present in the IL-13 preparations we used was contributing to the enhanced production of IL-17, we assessed responses in $TLR4^{-/-}$ mice. None of the outcome measures we assessed was diminished in the absence of TLR4. In fact, our data indicate that TLR4 may actually negatively regulate IL-13–induced IL-17 expression, as production of both IL-17–expressing $CD4^+$ and γδ T cells was increased, as was the influx of macrophages and lymphocytes into the lung, in IL-13–treated $TLR4^{-/-}$ mice.

Our data demonstrating that the lower dose of IL-17 enhanced Th2-dependent airway inflammation are compatible with those of Wakashin et al. (23) and Wilson et al. (53), all of whom showed that Th17 cells enhance Th2-dependent airway inflammation and AHR. Wakashin et al. speculated that the enhanced inflammation was due to increased production of eotaxins 1 and 2, based on increased mRNA levels. In our studies, the increased eosinophil recruitment to the airways in mice treated with both IL-13 and IL-17 did not correlate with enhanced expression or production of a number of chemokines **(Figs 3.5, 3.8)**. Thus, the precise mechanism by which IL-17 enhanced IL-13–induced eosinophil recruitment is not clear.

Although our data indicate that the lower dose of IL-17 enhanced BALF eosinophil numbers, it did not modify IL-13–induced AHR in BALB/c mice. These data differ from those of Wills-Karp and colleagues (26) who demonstrated that IL-17 enhanced AHR induced by IL-13 in A/J mice. This may be due to differences in the strain of mice and/or the conditions of cytokine administration. A/J mice received 15 μg of each cytokine during the course of 7 d whereas in our study mice received only 4 μg of each cytokine during the course of 8 d. We also examined the effect of administering larger amounts of IL-17 to BALB/c mice, increasing the

dose from 4 to 12 μg, again during the course of 8 d. Not only were IL-13–induced inflammation and AHR not enhanced, they were actually inhibited. These diminished responses in BALB/c mice treated with the higher dose of IL-17 corresponded well with the enhanced responses in C57BL/6 mice lacking IL-17, where AHR as well as BALF numbers of macrophages, eosinophils, and lymphocytes were increased **(Fig 3.11)**. Taken together, these data suggest that, at least under some conditions, IL-17 is an effective inhibitor of IL-13–induced airway inflammatory responses, in agreement with previously published data (29, 30).

One interesting possibility that arose from our data is the fact that the IL-13–induced increase in IL-17–producing cells was altered by coadministration of IL-17. Lower doses of IL-17 abolished the IL-13–induced increases in γδ T cells producing IL-17. As these cells were recently shown to promote resolution of airway inflammation in an OVA model, the enhanced eosinophilia we see may be due to the decreased numbers of these cells. However, at higher doses of IL-17, the IL-13–induced increases in IL-17–producing $CD4^+$ and γδ T cells were both reduced to baseline levels. These marked changes were accompanied by decreases in chemokine levels, BALF inflammation, and AHR, suggesting that at higher doses IL-17 can inhibit IL-13–induced responses in many cell types, effectively reducing inflammation. Given that high doses of IL-17 enhanced IL-13–induced responses in A/J mice (26), there appears to be a fine balance in the outcome of inflammation when both IL-13 and IL-17 are present.

The exact molecular mechanisms by which IL-17 modulates allergic airway inflammation remain to be identified. Data from Schnyder-Candrian (30) and colleagues suggested that IL-17 inhibits DC activity in the lungs, thereby modifying T cell activation and recruitment (30). However, as our system using recombinant cytokines lacked an obvious Ag, it seems unlikely that altered dendritic cell/T cell interactions were responsible for the altered responses we have

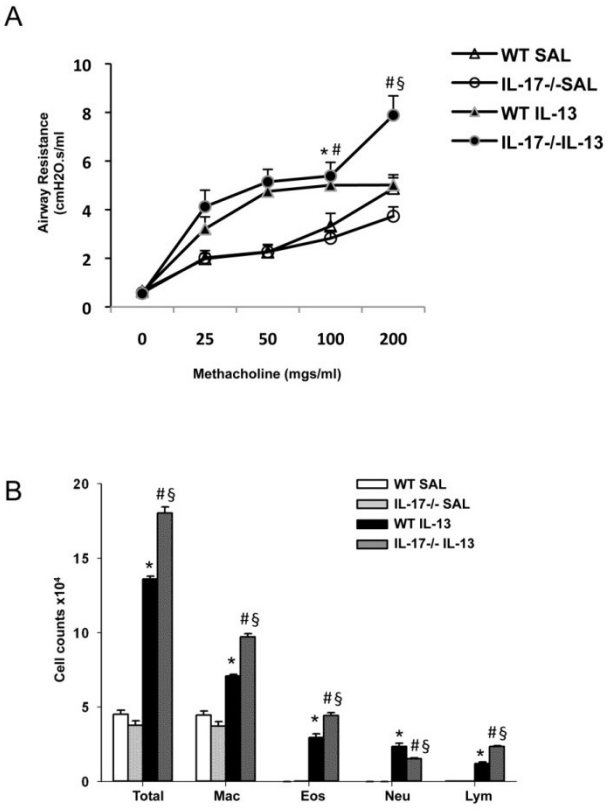
defined. Rather, our data suggested that eosinophilic airway inflammation was enhanced when γδ T cells expressing IL-17 were absent (in mice treated with IL-13 and the lower dose of IL-17). To more directly assess the role of γδ T cells in IL-13–induced airway inflammatory responses, including production of IL-17–expressing cells, we adoptively transferred purified γδ T cells from IL-13–treated mice into naive mice that were then treated with IL-13. IL-13–induced inflammatory cell influx was reduced in these mice, although AHR was unaffected. Recipients of γδ T cells also had fewer total IL-17–expressing cells as well as $CD4^+$ T cells expressing IL-17, although significant reductions were not found in IL-17–expressing γδ T cells, likely due to the fact that this cell population was comprised of endogenous γδ T cells and those that were adoptively transferred. Mills and colleagues (55) had previously shown that IL-17–expressing γδ T cells promoted production of IL-17 from Ag-stimulated $CD4^+$ T cells. Our data suggest that in the absence of Ag stimulation, γδ T cells may have inhibitory activity toward $CD4^+$ T cells.

Collectively, our data demonstrate that the levels of IL-17 modulate IL-13–induced inflammation in a dose-dependent manner where lower doses promote inflammation and higher doses prevent/protect against disease. They also provide evidence that γδ T cells expressing IL-17 may play a protective role in allergic airway inflammation. Therefore, efforts to modulate IL-17 levels during inflammation should be carefully considered since the effects could be either pathogenic or protective.

Chapters 2 & 3 of the thesis examined the role of IL-17 delivered by γδ T cells and as a recombinant protein. These studies established γδ T cells and/or $CD4^+$ T cells as the primary cellular source of IL-17. Chapter 4 focuses on exploring the role of IL-17, delivered specifically by $CD4^+$ T cells, on Th2 immune responses in an OVA-dependent adoptive transfer murine model of allergic airways disease.

Figure 3.11: Lack of IL-17 augments IL-13-induced AHR and airway inflammation

IL-13 (0.5μg) was administered intranasally to C57Bl/6 IL-17$^{-/-}$ or wild-type littermates A, Respiratory resistance to methacholine assessed 48h following the last dose of IL-13. Control animals were treated with SAL. Mean respiratory resistance to methacholine ± SEM from a combination of three independent experiments using 3-5 mice per group in each experiment is shown. B, BAL fluid differential cell counts from SAL treated wild type (empty bars), SAL treated IL-17$^{-/-}$ (light grey), IL-13 treated wild type (black bars) and IL-13 treated IL-17$^{-/-}$ (dark grey bars) mice are shown. BAL fluid cell counts are from one representative experiment out of five in which a minimum of 5 mice per group were used. Cell counts for total cells, macrophages, eosinophils, neutrophils and lymphocytes ± SEM are shown. * $p<0.05$ wt SAL vs. wt IL-13, # $p<0.05$, IL-17$^{-/-}$ SAL vs. IL-17$^{-/-}$ IL-13, § $p<0.05$ wt IL-13 vs. IL-17$^{-/-}$ IL-13.



6 Disclosures

The authors have no financial conflicts of interest.

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8 References

1. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, et al. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261-2263.
2. Kuperman, D. A., X. Huang, L. L. Koth, G. H. Chang, G. M. Dolganov, et al. 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* 8: 885-889.
3. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, et al. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258-2261.
4. Yang, M., S. P. Hogan, P. J. Henry, K. I. Matthaei, A. N. McKenzie, et al. 2001. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. *Am. J. Respir. Cell Mol. Biol.* 25: 522-530.
5. Hoeck, J., and M. Woisetschlager. 2001. Activation of eotaxin-3/CCLl26 gene expression in human dermal fibroblasts is mediated by STAT6. *J. Immunol.* 167: 3216-3222.
6. Matsukura, S., C. Stellato, S. N. Georas, V. Casolaro, J. R. Plitt, et al. 2001. Interleukin-13 upregulates eotaxin expression in airway epithelial cells by a STAT6-dependent mechanism. *Am. J. Respir. Cell Mol. Biol.* 24: 755-761.
7. Medoff, B. D., E. Seung, S. Hong, S. Y. Thomas, B. P. Sandall, et al. 2009. CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J. Immunol.* 182: 623-635.

8. Neilsen, C. V., and P. J. Bryce. 2010. Interleukin-13 directly promotes oesophagus production of CCL11 and CCL24 and the migration of eosinophils. *Clin. Exp. Allergy* 40: 427-434.

9. Chakir, J., J. Shannon, S. Molet, M. Fukakusa, J. Elias, et al. 2003. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J. Allergy Clin. Immunol.* 111: 1293-1298.

10. Doe, C., M. Bafadhel, S. Siddiqui, D. Desai, V. Mistry, et al. 2010. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. *Chest* 138: 1140-1147.

11. Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, et al. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430-438.

12. Agache, I., C. Ciobanu, C. Agache, and M. Anghel. 2010. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir. Med.* 104: 1131-1137.

13. Al-Ramli, W., D. Prefontaine, F. Chouiali, J. G. Martin, R. Olivenstein, et al. 2009. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J. Allergy Clin. Immunol.* 123: 1185-1187.

14. Bullens, D. M., E. Truyen, L. Coteur, E. Dilissen, P. W. Hellings, et al. 2006. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir. Res.* 7: 135.

15. Zhao, Y., J. Yang, Y. D. Gao, and W. Guo. 2010. Th17 immunity in patients with allergic asthma. *Int. Arch. Allergy Immunol.* 151: 297-307.

16. Fahy, J. V., K. W. Kim, J. Liu, and H. A. Boushey. 1995. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J. Allergy Clin. Immunol.* 95: 843-852.

17. Shannon, J., P. Ernst, Y. Yamauchi, R. Olivenstein, C. Lemiere, et al. 2008. Differences in airway cytokine profile in severe asthma compared to moderate asthma. *Chest* 133: 420-426.

18. Hellings, P. W., A. Kasran, Z. Liu, P. Vandekerckhove, A. Wuyts, et al. 2003. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 28: 42-50.

19. Liang, S. C., A. J. Long, F. Bennett, M. J. Whitters, R. Karim, et al. 2007. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J. Immunol.* 179: 7791-7799.

20. Liu, Y., J. Mei, L. Gonzales, G. Yang, N. Dai, et al. 2011. IL-17A and TNF-alpha exert synergistic effects on expression of CXCL5 by alveolar type II cells in vivo and in vitro. *J. Immunol.* 186: 3197-3205.

21. Zrioual, S., R. Ecochard, A. Tournadre, V. Lenief, M. A. Cazalis, and P. Miossec. 2009. Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J. Immunol.* 182: 3112-3120.

22. McKinley, L., J. F. Alcorn, A. Peterson, R. B. Dupont, S. Kapadia, et al. 2008. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* 181: 4089-4097.

23. Wakashin, H., K. Hirose, Y. Maezawa, S. Kagami, A. Suto, et al. 2008. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* 178: 1023-1032.

24. Wilson, N. J., K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, et al. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat. Immunol.* 8: 950-957.

25. He, R., H. Y. Kim, J. Yoon, M. K. Oyoshi, A. MacGinnitie, et al. 2009. Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13. *J. Allergy Clin. Immunol.* 124: 761-770 e761.

26. Lajoie, S., I. P. Lewkowich, Y. Suzuki, J. R. Clark, A. A. Sproles, et al. 2010. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat. Immunol.* 11: 928-935.

27. Song, C., L. Luo, Z. Lei, B. Li, Z. Liang, et al. 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J. Immunol.* 181: 6117-6124.

28. Nakae, S., Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, et al. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 17: 375-387.

29. Murdoch, J. R., and C. M. Lloyd. 2010. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing {gamma}{delta}T cells. *Am. J. Respir. Crit. Care Med.* 182: 464-476.

30. Schnyder-Candrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, et al. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203: 2715-2725.

31. Nakano, H., M. E. Free, G. S. Whitehead, S. Maruoka, R. H. Wilson, et al. 2012. Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol.* 5: 53-65.

32. McCusker, C. T., Y. Wang, J. Shan, M. W. Kinyanjui, A. Villeneuve, et al. 2007. Inhibition of experimental allergic airways disease by local application of a cell-penetrating dominant-negative STAT-6 peptide. *J. Immunol.* 179: 2556-2564.

33. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408.

34. Pope, S. M., P. C. Fulkerson, C. Blanchard, H. S. Akei, N. M. Nikolaidis, et al. 2005. Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation. *J. Biol. Chem.* 280: 13952-13961.

35. Therien, A. G., V. Bernier, S. Weicker, P. Tawa, J. P. Falgueyret, et al. 2008. Adenovirus IL-13-induced airway disease in mice: a corticosteroid-resistant model of severe asthma. *Am. J. Respir. Cell Mol. Biol.* 39: 26-35.

36. Zhen, G., S. W. Park, L. T. Nguyenvu, M. W. Rodriguez, R. Barbeau, et al. 2007. IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am. J. Respir. Cell Mol. Biol.* 36: 244-253.

37. Hoeck, J., and M. Woisetschlager. 2001. STAT6 mediates eotaxin-1 expression in IL-4 or TNF-alpha-induced fibroblasts. *J. Immunol.* 166: 4507-4515.

38. Liddiard, K., J. S. Welch, J. Lozach, S. Heinz, C. K. Glass, and D. R. Greaves. 2006. Interleukin-4 induction of the CC chemokine TARC (CCL17) in murine macrophages is mediated by multiple STAT6 sites in the TARC gene promoter. *BMC Mol. Biol.* 7: 45.

39. Wirnsberger, G., D. Hebenstreit, G. Posselt, J. Horejs-Hoeck, and A. Duschl. 2006. IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. *Eur. J. Immunol.* 36: 1882-1891.

40. Nabe, T., F. Hosokawa, K. Matsuya, T. Morishita, A. Ikedo, et al. 2011. Important role of neutrophils in the late asthmatic response in mice. *Life Sci.* 88: 1127-1135.

41. Sun, D., M. Novotny, K. Bulek, C. Liu, X. Li, and T. Hamilton. 2011. Treatment with IL-17 prolongs the half-life of chemokine CXCL1 mRNA via the adaptor TRAF5 and the splicing-regulatory factor SF2 (ASF). *Nat. Immunol.* 12: 853-860.

42. Sergejeva, S., S. Ivanov, J. Lotvall, and A. Linden. 2005. Interleukin-17 as a recruitment and survival factor for airway macrophages in allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 33: 248-253.

43. Chen, Y., P. Thai, Y. H. Zhao, Y. S. Ho, M. M. DeSouza, and R. Wu. 2003. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J. Biol. Chem.* 278: 17036-17043.

44. Alcorn, J. F., C. R. Crowe, and J. K. Kolls. 2010. TH17 cells in asthma and COPD. *Annu. Rev. Physiol.* 72: 495-516.

45. Chen, Z., and J. J. O'Shea. 2008. Th17 cells: a new fate for differentiating helper T cells. *Immunol. Res.* 41: 87-102.

46. Ghoreschi, K., A. Laurence, X. P. Yang, K. Hirahara, and J. J. O'Shea. 2011. T helper 17 cell heterogeneity and pathogenicity in autoimmune disease. *Trends Immunol.* 32: 395-401.

47. Finkelman, F. D., S. P. Hogan, G. K. Hershey, M. E. Rothenberg, and M. Wills-Karp. 2010. Importance of cytokines in murine allergic airway disease and human asthma. *J. Immunol.* 184: 1663-1674.

48. Wang, Y. H., K. S. Voo, B. Liu, C. Y. Chen, B. Uygungil, et al. 2010. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J. Exp. Med.* 207: 2479-2491.

49. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, et al. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6: 1123-1132.

50. Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, et al. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203: 2271-2279.

51. Aarvak, T., M. Chabaud, P. Miossec, and J. B. Natvig. 1999. IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. *J. Immunol.* 162: 1246-1251.

52. Fei, M., S. Bhatia, T. B. Oriss, M. Yarlagadda, A. Khare, et al. 2011. TNF-alpha from inflammatory dendritic cells (DCs) regulates lung IL-17A/IL-5 levels and neutrophilia versus eosinophilia during persistent fungal infection. *Proc. Natl. Acad. Sci. U. S. A.* 108: 5360-5365.

53. Wilson, R. H., G. S. Whitehead, H. Nakano, M. E. Free, J. K. Kolls, and D. N. Cook. 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 180: 720-730.

54. Lalor, S. J., L. S. Dungan, C. E. Sutton, S. A. Basdeo, J. M. Fletcher, and K. H. Mills. 2011. Caspase-1-processed cytokines IL-1beta and IL-18 promote IL-17 production by gammadelta and CD4 T cells that mediate autoimmunity. *J. Immunol.* 186: 5738-5748.

55. Sutton, C. E., S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31: 331-341.

56. Guo, L., G. Wei, J. Zhu, W. Liao, W. J. Leonard, et al. 2009. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc. Natl. Acad. Sci. U. S. A.* 106: 13463-13468.

57. Fulkerson, P. C., C. A. Fischetti, L. M. Hassman, N. M. Nikolaidis, and M. E. Rothenberg. 2006. Persistent effects induced by IL-13 in the lung. *Am. J. Respir. Cell Mol. Biol.* 35: 337-346.

CHAPTER 4: CD4$^+$ T cell specific IL-17 expression: dose-dependent effects of IL-17 in allergic airways disease

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1 Abstract

PURPOSE: Clinical data have associated IL-17 with severe asthma. Murine models suggest a possible dual role for IL-17 in allergic asthma as both a positive and negative regulator. The objective of these experiments was to assess how $CD4^+$ T cell specific expression of IL-17 modifies OVA-induced airway inflammatory responses using a murine adoptive transfer model. METHODS: $CD4^+$ T cells from OVA-sensitized donor BALB/c mice, re-stimulated with OVA ex vivo, were transduced with recombinant retroviruses encoding EGFP ± IL-17. Following transduction, $CD4^+$ T cells were purified and adoptively transferred intraperitoneally into naïve mice that were subsequently challenged intranasally with OVA each day for five days. RESULTS: $CD4^+$ T cells were efficiently transduced with retroviruses. Following adoptive transfer and airway OVA challenge, bronchoalveolar lavage fluid (BALF) levels of IL-17 positively correlated with airway resistance. Moderate increases in IL-17 were sufficient to enhance OVA-induced BALF neutrophil influx, whereas higher levels induced airway hyperresponsiveness (AHR); enhanced BALF macrophage, eosinophil, neutrophil and lymphocyte numbers; and increased BALF Th2 cytokine and serum IL-17 levels. Following OVA challenge, both transduced and host $CD4^+$ T cells were recovered in significantly greater numbers from recipients producing larger amounts of IL-17 and increases in T cell numbers corresponded to increases in BALF CCL17. Nevertheless, when delivered as a recombinant protein, IL-17 either suppressed or had no effect on OVA-induced responses. CONCLUSIONS: These data suggest $CD4^+$ T cell-specific IL-17 delivery promotes OVA-induced, Th2-biased allergic airway responses and that the relative threshold levels of IL-17 required to induce these responses differ.

2 Introduction

Severe asthma affects 5-10% of the estimated 300 million asthmatics worldwide (1, 2). Compared to the mild and moderate asthma phenotypes, severe asthmatics show a greater reduction in lung function, more frequent exacerbations and more importantly, are poor responders or are unresponsive to high dose inhaled corticosteroids even in combination with a secondary controller (3). Interleukin-17A (hereafter referred to as IL-17) is the primary effector cytokine associated with the Th17 subset of T helper cells and is linked to the severe asthma phenotype (4). Th17 cells and/or IL-17 are increased in the peripheral blood, serum, sputum, bronchoalveolar lavage (BAL) and lungs of severe asthmatics compared to mild, moderate and/or non-asthmatic controls (5-7). IL-17 expression is positively correlated with AHR, neutrophilia and corticosteroid insensitivity in moderate and severe human asthmatics (8-11). Similarly, in mice, adoptive transfer of Th17 cells induces airway hyperresponsiveness (AHR) and increases neutrophilia, both of which are resistant to treatment with the inhaled corticosteroid, dexamethasone (12).

Th2 cells have long been considered the driving force behind the asthmatic response (13). Th2-biased inflammation, largely governed by the cytokines IL-4, IL-5 and IL-13, is associated with AHR, eosinophilia and IgE production in both humans and in animal models (14) of allergic airways disease and is positively correlated with asthma severity (15, 16). Accumulating evidence from murine models indicates that IL-17 can both positively and negatively regulate allergic airways disease (17-20).When delivered by Th17 cells, IL-17 has largely been shown to enhance Th2-induced airway inflammatory responses (12, 21, 22). In contrast, IL-17-expressing γδ T cells both negatively regulate and also promote resolution of Th2-biased airway

inflammation (17-19)**.** Interestingly, the conclusions from studies addressing the role of IL-17 as a recombinant protein, without a specific cell origin, are contradictory (18-20, 23, 24). Thus, it remains unclear whether the cell source and/or the amount of IL-17 produced by each cell type, defines IL-17 as a positive or negative regulator of asthma. Furthermore, differences in threshold quantities of IL-17 required to enhance (or suppress) specific Th2-induced features of allergic airways disease, including AHR, inflammation and cytokine production, remain poorly defined.

The primary aim of this study was to examine how different levels of IL-17 produced by gene-modified, non-transgenic $CD4^+$ T cells, presented in the context of Th2, influence airway inflammatory responses, including AHR (25, 26). While moderate increases in BAL fluid (BALF) levels of IL-17 selectively increased neutrophil influx into the lungs, higher levels were required to increase serum IL-17; BALF levels of IL-4, IL-5 and IL-13; and to induce AHR. Reflecting the increase in chemokine expression, BALF macrophages, eosinophils, neutrophils and lymphocytes were also enhanced in these mice. Moreover, BALF levels of IL-17 originating from adoptively transferred $CD4^+$ T cells positively correlated with total lung resistance; total inflammatory cells recovered; as well as BALF levels of CCL17 (TARC), a potent Th2 cell chemoattractant (27). In spite of these increases, recombinant IL-17, when added to cultured lung cells stimulated with OVA, or when delivered *in vivo* with OVA, failed to enhance, and even suppressed, CCL17 levels.

3 Materials and methods

Animals

BALB/c mice (Charles River Laboratories, Montreal, QC) and DO11.10 OVA TCR transgenic mice (Jackson Laboratories, Bar Harbor, ME) were bred at the Meakins-Christie Laboratories Animal Facility and used for experimentation at ages 6-10 weeks. Animal studies were approved by the McGill University Animal Care Committee and performed following the Canadian Council on Animal Care guidelines.

Recombinant retrovirus isolation

Phoenix-Ecotropic (Phoenix-Eco) retrovirus packaging cells (ATCC Manassas, VA) were stably transfected with the IL-17pAP2 retrovector containing the murine IL-17A open reading frame upstream and the enhanced green fluorescence protein (EGFP) open reading frame downstream of the internal ribosomal entry site. Alternatively, Phoenix-Eco cells were transfected with the control pAP2 retrovector encoding EGFP only (28). The pAP2 and IL-17pAP2 Phoenix cell lines were cultured in 150mm cell culture plates with 20ml of RPMI media containing 1% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA) and 50µg/ml β-mercaptoethanol. At 80-90% confluence, the media was replaced and 24 hours later the retrovirus-rich media (supernatant) was harvested and replaced with fresh media for a second harvest from the same plate, an additional 24 hours later. The supernatants were immediately filtered (0.45µM) and concentrated 10 fold using the Ultracel® PL-100, Centricon® Plus-70 (Merck Millipore Ltd, Cork, Ireland) device, after which aliquots of the concentrated retrovirus were kept frozen at -80ºC (26).

Retroviral transductions

Donor BALB/c mice were sensitized intraperitoneally (IP) on days 0 & 7 with 100µg OVA (Sigma-Aldrich, St. Louis, MO) in a 10% solution of Imject Alum (Thermo Scientific, Rockford, IL) (**Fig 4.1**). A single cell suspension of splenocytes was prepared by mechanical separation of spleens harvested from donor mice on day 14. Following red blood cell lysis, splenocytes were plated at 5 x 106 cells/ml in RPMI media containing 1% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (Life Technologies) and 50µg/ml β-mercaptoethanol and stimulated with OVA at a concentration of 100µg/ml on day 0 of cell culture. Proliferating T cells were targeted for transduction, twice on day 1 (separated by 6 hours) and once on day 2, by removing the top acellular layer of media (or retroviral supernatant) and replacing it with a 1ml aliquot of concentrated retroviral supernatant for every 5 x 106 cells plated and centrifuging cells and retroviruses at 455xg for 45 minutes at 37ºC. On day 3, fresh media was added, 500µl for every milliliter of supernatant. Cells were harvested on day 5.

Adoptive transfer, IL-17 delivery, and airway OVA challenges

$CD4^+$ T cells were isolated from retrovirally-exposed splenocytes using the EasySep mouse $CD4^+$ T cell enrichment kit (Stemcell Technologies, Vancouver, BC). The purity and transduction efficiency (based on EGFP expression) of $CD4^+$ T cells were analyzed by flow cytometry. 1.25 – 2.5 x 106 total $CD4^+$ T cells were adoptively transferred into naïve BALB/c recipients by IP injection, after which mice were lightly anaesthetized with isoflurane and challenged with 30µl of saline containing 50µg of OVA, followed by four additional daily OVA challenges. Mice were sacrificed 48 hours after the last airway challenge. For experiments examining the effect of recombinant IL-17 delivery to OVA-induced airway inflammatory

responses, naïve DO11.10 mice were lightly anaesthetized with isoflurane before intranasal exposure to 0.1µg or 1.5µg of recombinant IL-17 (Biolegend, San Diego, CA) in 30µl of sterile PBS or PBS alone at least 1 hour prior to intranasal instillation of 100µg OVA in 15µl sterile PBS or PBS alone for 4 consecutive days. Mice were sacrificed 24 hours after the last OVA exposure.

Analysis of airway hyperresponsiveness & inflammation

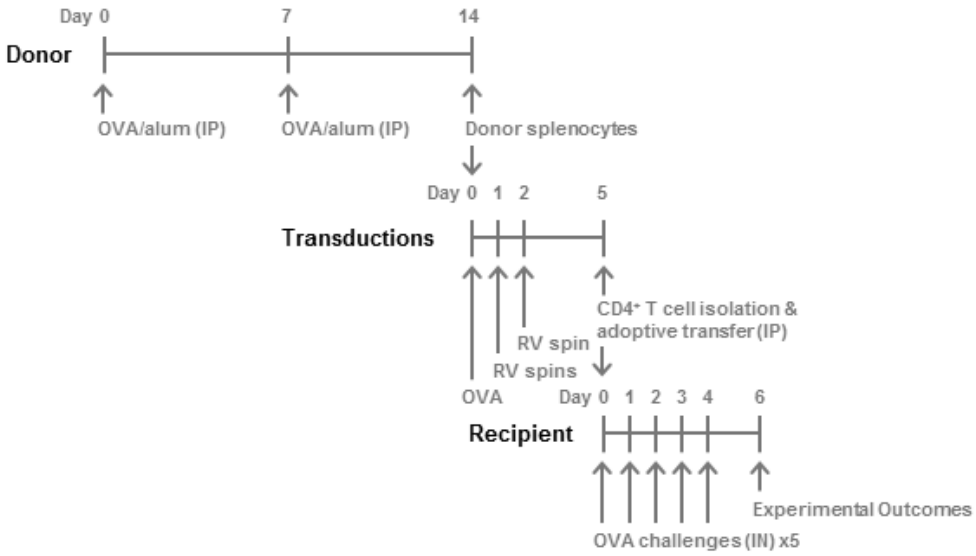
To examine changes in lung function using the flexiVent small animal ventilator (SCIREQ, Montreal, QC), mice were deeply anaesthetized with a cocktail of xylaxine and sodium pentobarbital, followed by an injection of the paralyzing agent, pancuronium bromide. Heart rate was monitored to ensure adequate anesthesia throughout the procedure. Baseline respiratory resistance was recorded and then mice were exposed to increasing concentrations of aerosolized methacholine in order to obtain maximal resistance values at each dose. Following assessment of AHR, BALs were performed. Cells recovered from 2- 1 ml PBS lavages of the airways were pooled and red blood cells lysed before counting total cells. A fraction of the cells was centrifuged onto glass slides and stained using Diff-Quick (Fisher Scientific, Ottawa, ON) from which differential cell counts, based on a 300 cell count from five to ten fields, were obtained. The remaining cells were used for FACS analysis. BALF from the first lavage was frozen at -80°C for later cytokine/chemokine protein analysis.

Preparation of lung cells

A single cell suspension of lung cells was obtained from the left lung of each mouse by mincing and incubating the lung in 1ml of serum-free DMEM (Life Technologies, Carlsbad, CA)

Figure 4.1: Protocol for the adoptive transfer of retrovirally transduced $CD4^+$ T cells

Donor BALB/c mice were IP OVA sensitized on days 0 & 7 and sacrificed on day 14. Splenocytes from donor mice were stimulated with OVA on day 0 of cell culture, then centrifuged twice on day 1 (6 hours apart) and once on day 2, with concentrated pAP2 or IL-17pAP2 retroviral supernatant. OVA-specific $CD4^+$ T cells proliferating in response to OVA stimulation were targeted for transduction. On day 5 of cell culture, $CD4^+$ T cells were enriched by magnetic selection and adoptively transferred into naïve BALB/c recipients. Immediately following adoptive transfer, mice were challenged with OVA, followed by four additional daily challenges. Recipients were sacrificed 48 hours after the last airway challenge to assess allergic airways disease outcomes. IP= intraperitoneal, IN= intranasal & RV= retroviral



containing Liberase TM (100µg/ml; Roche, Indianapolis, IN) combined with collagenase XI (250µg/ml), hyaluronidase 1a (1mg/ml) and DNase I (200µg/ml, Sigma-Aldrich) for 1h at 37°C. The reaction was stopped with a 20mM final concentration of EDTA (29). Red blood cells were lysed following enzymatic digestion.

Flow cytometric analysis

Single cell preparations from BALF and lung were incubated for 4h in 1ml of RPMI media containing 1% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (Life Technologies); 0.67 µl/ml GolgiStop from the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA); and 1µg/ml of α-CD3 stimulating Ab. Surface and intracellular cytokine staining were performed according to the kit protocol. Cells were stained with α-CD4-Pacific Blue and α-IL-17-AlexaFluor 647 (BD Biosciences) and transduced cells detected by expression of EGFP encoded by the retroviral vector. Stained cells were processed using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

In vitro lung cell culture and stimulation

BALB/c mice were IP sensitized on days 0 & 7 with 100µg OVA (Sigma-Aldrich) in a 10% solution of Imject Alum (Thermo Scientific), challenged with 50µg OVA in 30µl sterile PBS on days 28-30 and sacrificed on day 32. A single suspension of lung cells was obtained as described above and then cells were cultured in RPMI media containing 1% penicillin/streptomycin, 50µg/ml β-mercaptoethanol and 10% heat-inactivated fetal bovine serum (0 hr time point). Cells were additionally stimulated with 100µg/ml OVA alone or in combination with 10 fold

increasing concentrations of recombinant IL-17A (Biolegend) ranging from 10pg/ml to 1µg/ml for 4, 12 and 36 hrs. Supernatants were collected and stored at -80°C for future analysis of CCL17 levels.

Detection of serum IL-17

Preceding removal of the lungs, the chest cavity was opened and blood collected by cardiac puncture. After 30 minutes, serum was separated from blood by centrifugation and stored at -80°C for future analysis. Serum IL-17 levels were measured using the Mouse IL-17 ELISA Ready-SET-Go kit (eBioscience, San Diego, CA), according to the manufacturer's instructions.

Quantitative analysis of mediators from BALF and cell culture supernatants

BALF IL-13 levels were quantified using the Mouse IL-13 ELISA Ready-SET-Go kit (eBioscience). BALF CCL17 from DO11.10 mice and supernatants from cultured BALB/c lung cells were quantified using the CCL17 Duoset kit (R&D Systems, Minneapolis, MN). All other mediators were quantified with the Q-View Imager using the 16-plex mouse cytokine screen and a custom 9-plex chemokine kit (Quansys Biosciences, Logan, UT).

Statistical analysis

Two-way, unpaired t-tests were performed for single comparisons. Data are expressed as the mean +SEM. Analysis of CCL17 levels from BALB/c cultured lung cell supernatants and BALF samples from DO11.10 mice were performed using a one-way ANOVA followed by a Holm-Sidak post-hoc test. Data are expressed as the mean +SEM. Pearson correlations were performed to assess relationships between respiratory resistance, total BALF cell numbers and BALF levels

of CCL17 against BALF IL-13 and/or IL-17 levels. p-values $\leqslant 0.05$ were considered statistically significant. All data were analyzed using GraphPad Prism 6 (La Jolla, CA).

4 Results

Efficient retroviral transduction of $CD4^+$ T cells can be achieved to express IL-17 protein

In order to examine the effects of antigen-induced expression of IL-17 by $CD4^+$ T cells in the context of a Th2-biased airway immune response, we first generated stable cell lines that produced retrovirus encoding both IL-17 and EGFP (IL-17pAP2) or, as a negative control, EGFP only (pAP2). These retroviral packaging cell lines produced both retrovirus, as well as EGFP ± IL-17. Frequencies of EGFP expressing cells were similar in IL-17pAP2 (34.0% ± 2.6) and pAP2 (33.7% ± 3.5) packaging cell lines **(Fig 4.2A, B)**. Internal Ribosomal Entry Site-regulated co-expression of IL-17 and EGFP resulted in an IL-17 expressing $EGFP^+$ population of cells in IL-17pAP2 transfected cells that was absent in pAP2 transfected cells **(Fig 4.2C)**.

IL-17pAP2 and pAP2 retroviruses, harvested from their respective packaging cell lines, were concentrated and incubated with OVA-stimulated splenocytes harvested from OVA-sensitized donor BALB/c mice **(Fig 4.1)** (25, 26, 30). $CD4^+$ T cells were successfully transduced by either the pAP2 or IL-17pAP2 retroviruses as indicated by their respective EGFP and EGFP/IL-17 expressing cells **(Fig 4.3A)**. Based on EGFP expression of pAP2 (32.5% ± 4.6) and IL-17pAP2 (36.2% ± 1.8) retrovirally exposed $CD4^+$ T cells **(Fig 4.3B)**, similar transduction efficiencies were achieved. Only $CD4^+$ T cells exposed to IL-17pAP2 retrovirus co-expressed EGFP and IL-17 **(Fig 4.3C)**.

Figure 4.2: Phoenix-Eco packaging cells, stably transfected with the IL-17pAP2 or pAP2 retrovectors produce IL-17 and/or EGFP, respectively

The Phoenix Ecotropic packaging cell line was transfected stably with either the pAP2 or IL-17pAP2 retrovector. Transfected cells expressing endogenous EGFP were stained with an α-IL-17 antibody. (A) Representative flow cytometry plots of Phoenix-Eco cells, first gated on live cells, are shown as EGFP versus IL-17 and are representative of three independent experiments. Frequencies of Phoenix-Eco cells expressing (B) EGFP and (C) $EGFP^{+}IL\text{-}17^{+}$ are shown. (B-C) Mean values (+SEM) from 3 independent experiments are shown. Two-tailed, unpaired t-tests were performed, *$p<0.05$ and ns= not significant.

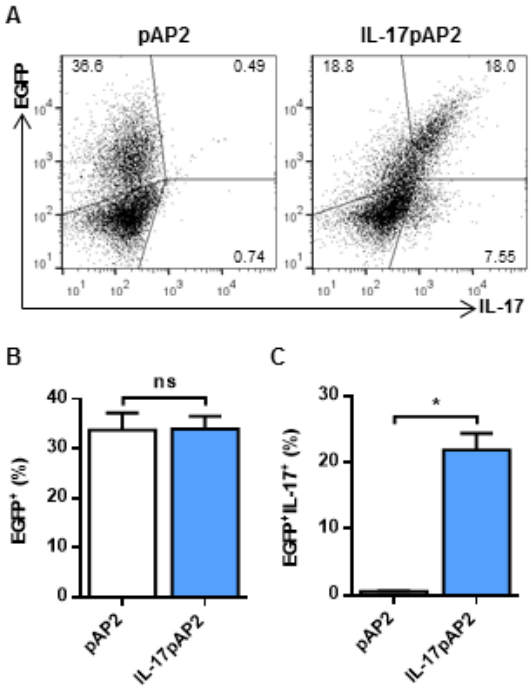
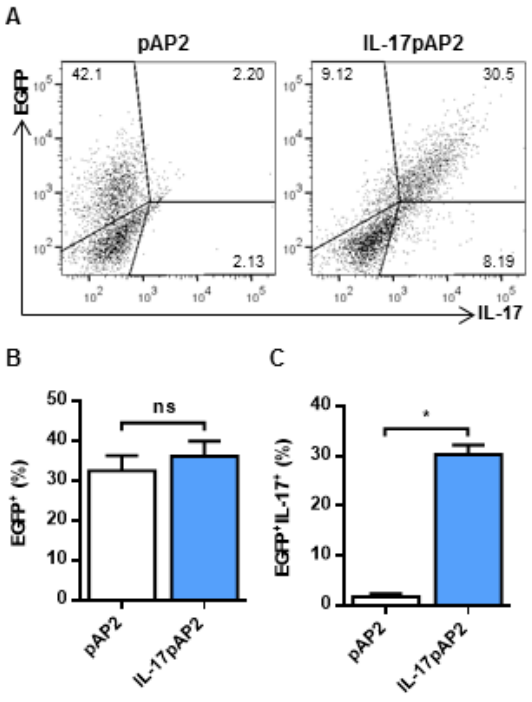


Figure 4.3: pAP2 and IL-17pAP2 retroviruses transduce CD4$^+$ T cells with equal efficiency

CD4$^+$ T cells from OVA sensitized BALB/c mice were targeted for transduction with the pAP2 (control) retrovirus encoding EGFP or the IL-17pAP2 retrovirus encoding both EGFP and IL-17. Splenocytes transduced with pAP2 or IL-17pAP2 retrovirus were stained with α-IL-17 and α-CD4 antibodies. (A) Representative flow cytometry plots show CD4$^+$ splenocytes pre-gated on live cells and presented as EGFP versus IL-17. The mean frequencies of CD4$^+$ T cells that were (B) EGFP$^+$ and (C) EGFP$^+$IL-17$^+$ are shown. (B-C) Data are presented as the mean (+SEM) of at least 4 independent experiments from a minimum of 4 pooled spleens per experiment. A two-tailed, unpaired t-test was performed. *$p<0.05$, ns= not significant.



BALF IL-17 levels correlate with respiratory resistance and augment allergic airways disease

Following transductions, $CD4^+$ T cells (comprised of a mixture of transduced and non-transduced T cells) were purified and adoptively transferred by intraperitoneal (IP) injection into naïve BALB/c recipients **(Fig 4.1)** that were then intranasally challenged with OVA to enhance airway recruitment and re-activation of adoptively transferred, OVA-specific $CD4^+$ T cells. Mice that received IL-17pAP2 transduced $CD4^+$ T cells presented with a wide range of BALF IL-17 levels, which suggests variable activation and/or recruitment of adoptively transferred cells **(Fig 4.4A).** The range in BALF IL-17 levels allowed us to correlate IL-17 levels with different experimental outcomes. BALF IL-17 levels, unlike IL-13, were highly correlated to respiratory resistance **(Fig 4.4B)** and showed a strong, but not statistically significant correlation ($p=0.058$) to total airway inflammatory cell numbers **(Fig 4.4C)**.

Recipients of IL-17pAP2 transduced $CD4^+$ T cells were further subdivided into 2 groups according to BALF levels of IL-17, based on data from a study by Barczyk et al. (11) who did not observe significant differences in sputum IL-17 levels in normal subjects compared to asthmatics until they were compared to a sub-population of bronchial hyperreactive asthmatics. In our experiments, some recipient mice had relatively low levels of IL-17, averaging 9.4 ± 1.5 pg/ml (labeled IL-17pAP2-lo), while others had IL-17 levels that were orders of magnitude greater, averaging 629.6 ± 322 pg/ml (identified hereafter as IL-17pAP2-hi) **(Fig 4.5A)**. For comparison, recipients of pAP2 transduced $CD4^+$ T cells had BALF IL-17 levels averaging 5.9 ± 0.5 pg/ml **(Fig 4.6A)**. Despite having comparatively lower BALF levels of IL-17, IL-17pAP2-lo mice still maintained significantly higher levels of IL-17 in comparison to OVA airway challenged pAP2 mice **(Fig 4.6A)**. The level of IL-17 in the IL-17pAP2-lo mice did not

influence BALF levels of Th2 cytokines, IL-4, IL-5 and IL-13, nor did it affect AHR **(Fig 4.6B, C)**. However, significantly fewer macrophages and significantly more neutrophils were recovered in the BALF of IL-17pAP2-lo compared to pAP2 mice **(Fig 4.6D)**, providing evidence that even small changes in IL-17 can modulate macrophage and neutrophil influx into the airways.

Significantly higher levels of IL-17 were present in the BALF of IL-17pAP2-hi mice compared to IL-17pAP2-lo mice **(Fig 4.5A)**. Both respiratory resistance and elastance were increased in IL-17pAP2-hi mice compared to IL-17pAP2-lo mice **(Fig 4.5B)**. Although BALF levels of the Th1 cytokine, IFN-γ, did not differ **(Fig 4.5C)**, higher BALF levels of the Th2 cytokines, IL-4, IL-5 and IL-13 **(Fig 4.5D)** were detected in IL-17pAP2-hi mice. Additionally, IL-17pAP2-hi mice had significantly greater levels of serum IL-17 **(Fig 4.5E)**, which is also an independent risk factor for severe asthma in humans (31, 32).

Consistent with elevated BALF levels of IL-17 and Th2-related cytokines, BALF macrophages, eosinophils, neutrophils and lymphocytes were all significantly increased in IL-17pAP2-hi mice **(Fig 4.7A)**. Moreover, BALF levels of macrophage/monocyte chemoattractant CCL1 (T cell activation-3), but not CCL2 (monocyte chemotactic protein-1) **(Fig 4.7B)**; eosinophil chemoattractant CCL11 (eotaxin-1) and neutrophil chemoattractant CXCL1 **(Fig 4.7C)** were significantly increased in IL-17pAP2-hi mice as were CCL5 (RANTES), CCL17 (thymus and activation regulated chemokine), CCL22 (macrophage-derived chemokine) and CXCL10 (interferon-γ inducible protein 10) **(Fig 4.7D)**. These data suggest that $CD4^+$ T cell-derived IL-17 may promote production of a number of different chemokines, thereby enhancing airway inflammation in allergic airways disease.

Figure 4.4: IL-17 levels correlate with AHR

$CD4^+$ T cells from OVA sensitized donors, transduced with the IL-17pAP2 retrovirus were adoptively transferred into naïve recipients. Following 5 days of IN challenges, the BALF was collected for analysis. (A) Mean BALF IL-17 levels (±SEM) are shown. Data are from n=20 mice total, from a minimum of 3 independent experiments for each group. A two-tailed, unpaired t-test was performed, *p<0.05. BALF IL-13 and IL-17 linear relationships to (B) respiratory resistance and (C) total cells from the BALF were assessed by determining the Pearson correlation coefficient, 'r'. Data are from n=14-20 mice from at least 7 independent experiments. *p<0.05.

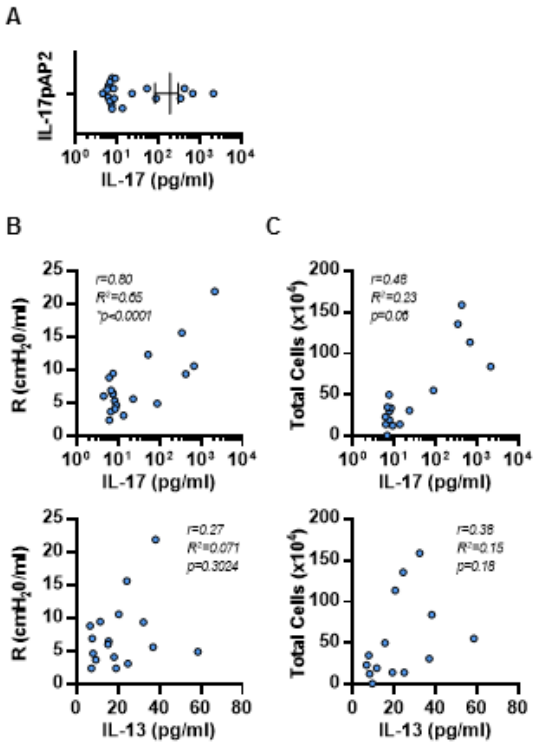


Figure 4.5: IL-17pAP2-hi mice with higher BALF levels of IL-17 have enhanced AHR, and increased BALF Th2 cytokine and serum IL-17 levels

Naïve mice received IL-17pAP2 transduced $CD4^+$ T cells from OVA sensitized donor mice, followed by OVA airway challenges. (A) Mean BALF IL-17 levels (+SEM) from high (IL-17pAP2-hi) and low (IL-17pAP2-lo) IL-17 expressers are shown (B) Respiratory resistance and elastance were obtained 48 hours after the last challenge. BALF levels of the (C) Th1 cytokine, IFN-γ and (D) Th2 cytokines, IL-4, IL-5 and IL-13 are shown. (E) Serum levels of IL-17 were quantified. Mean values (+SEM) from 6-14 total mice per group from at least 7 independent experiments are shown. Two-tailed, unpaired t-tests were performed, *p<0.05 and ns= not significant.

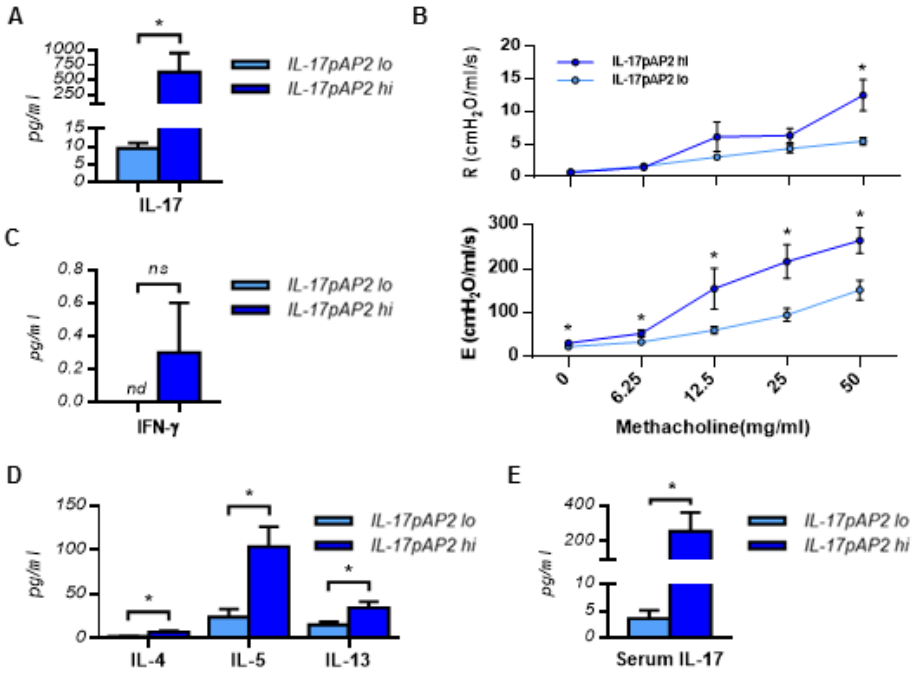


Figure 4.6: A small but significant increase in BALF IL-17 is sufficient to induce airway neutrophilia, but not Th2 inflammatory responses

IL-17pAP2-lo mice were compared to pAP2 mice. BALF levels of (A) IL-17 and (B) Th2 cytokines, IL-4, IL-5 and IL-13 were quantified. (C) AHR and (D) BALF inflammatory cell profiles were also assessed. (A-D) Mean values (+SEM) are shown for 11-16 mice from at least 5 independent experiments. Two-tailed, unpaired t-tests were performed, *p≤0.05.

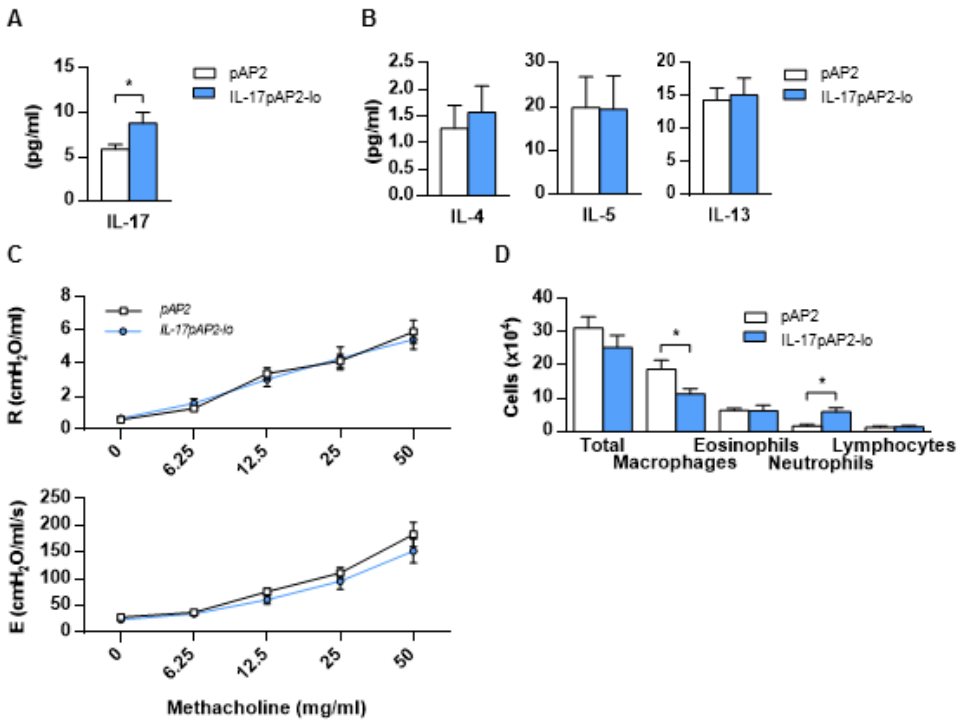
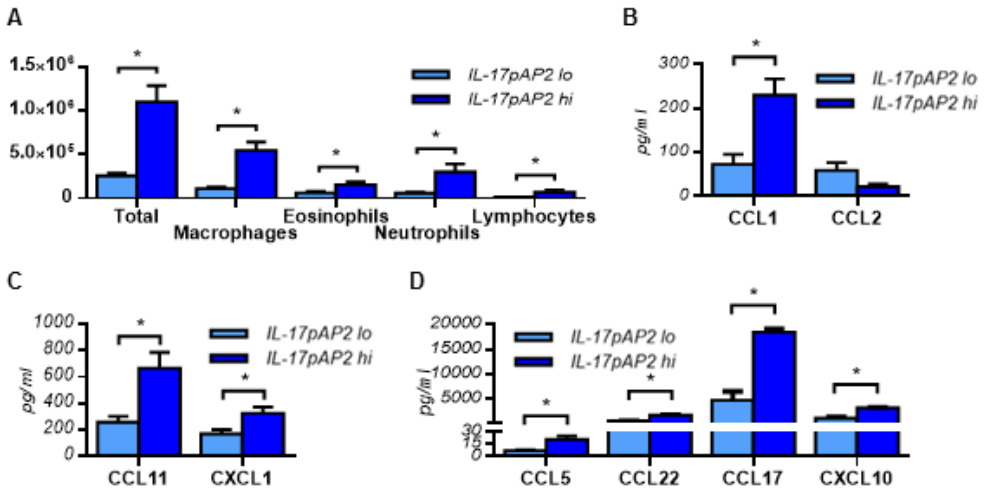


Figure 4.7: IL-17pAP2-hi mice have enhanced airway inflammation associated with increased BALF chemokine expression

Naïve mice received IL-17pAP2 transduced $CD4^+$ T cells from OVA sensitized donor mice, followed by OVA airway challenges. **(A)** Total cells, macrophages, eosinophils, neutrophils and lymphocytes were quantified from the BALF of IL-17pAP2-hi and IL-17pAP2-lo mice. **(B)** Macrophage/monocyte associated cytokines CCL1 & CCL2; **(C)** eosinophil chemoattractant CCL1 & neutrophil chemoattractant CXCL1; and **(D)** T cell chemoattractants CCL5, CCL17, CCL22 and CXCL10 are shown for IL-17pAP2-hi and IL-17pAP2-lo mice. Mean values (+SEM) from 5-11 mice per group from at least 3 independent experiments are shown. Two-tailed, unpaired t-tests were performed, *$p<0.05$.



Following adoptive transfer and airway OVA challenge, IL-17pAP2 transduced $CD4^+$ T cells expressing EGFP were present in the lungs of both IL-17pAP2-lo and IL-17pAP2-hi mice **(Fig 4.8A)**. Greater frequencies of $CD4^+$ T cells from the BALF and lungs of IL-17pAP2-hi mice were $EGFP^+$ compared to IL-17pAP2-lo mice **(Fig 4.8B)**. Total $CD4^+EGFP^+$ T cell numbers were similarly increased in the BALF of IL-17pAP2-hi mice **(Fig 4.8C)**. Additionally, total $CD4^+$ T cells, inclusive of host and adoptively transferred cells, were increased in the BALF of IL-17pAP2-hi compared to IL-17pAP2-lo mice **(Fig 4.8D)**. Altogether, the data suggest that the wide range of IL-17 detected in the BALF is due, at least in part, to differential recruitment of adoptively transferred IL-17pAP2 transduced $CD4^+$ T cells and suggest that the increased IL-17 produced by these cells may establish a positive feedback loop that *further* enhances recruitment of both host and adoptively transferred $CD4^+$ T cells.

BALF IL-17 levels correlate with CCL17 *when sourced from $CD4^+$ T cells*, whereas exogenous IL-17 negatively regulates *in vitro,* antigen-induced CCL17 production

Post-airway challenge, BALF IL-17 levels from recipients of IL-17pAP2 transduced $CD4^+$ T cells were highly correlated with levels of BALF CCL17, suggesting that $CD4^+$ T cell-derived IL-17 may positively regulate CCL17 production **(Fig 4.9A)**. Conversely, Schnyder-Candrian et al. demonstrated that recombinant IL-17 inhibits inflammation and diminishes CCL17 levels in the BALF and lung (19). To clarify how IL-17 modulates OVA-induced CCL17, we examined how different doses of IL-17 modulated CCL17 production from OVA-stimulated lung cells in culture. Concentrations of recombinant IL-17 spanning five decades (10pg/ml to 1μg/ml) were used to examine dose-dependent effects of IL-17 on CCL17 production in this experimental system. OVA-induced CCL17 production increased steadily over

the course of 36 hours. Nevertheless, IL-17 levels as low as 100pg/ml to as high as 100ng/ml significantly reduced OVA-induced CCL17 production **(Fig 4.9B)**.

Exogenous IL-17 does not alter *in vivo* OVA-induced airway inflammation or CCL17 production

A related experiment was designed to examine the ability of IL-17 to enhance OVA-induced airway inflammatory responses, including CCL17 production, when delivered to naïve DO11.10 mice exposed to antigen for the first time. Iwakura and colleagues had shown previously that OVA-induced responses were reduced in IL-17 knockout mice exposed to OVA under similar conditions (33). Thus, recombinant IL-17 (0.1 or 1.5µg) was delivered to naïve DO11.10 mice prior to each of four OVA challenges. As expected, $CD4^+$ T cells and total inflammatory cells were increased in the BALF of all OVA-challenged mice compared to the saline controls **(Fig 4.9C)**. However, the low and high doses of IL-17 neither enhanced nor suppressed these OVA-induced inflammatory responses. Although the OVA-challenged mice that received the high dose of IL-17 had significantly greater levels of BALF CCL17 compared to saline-challenged mice, IL-17 did not significantly enhance OVA-induced CCL17, when compared to mice challenged with OVA alone. Thus, delivery of recombinant IL-17 did not modulate OVA-induced inflammatory responses in naïve mice.

Figure 4.8: Adoptively transferred and total CD4$^+$ T cells are more efficiently recruited to the airways of IL-17pAP2-hi mice

BALF and lung cells from recipients of IL-17pAP2 transduced CD4$^+$ T cells were stimulated with α-CD3 antibody before staining with α-CD4 and α-IL-17 antibodies. **(A)** Representative flow cytometry plots of BALF and lung cells from IL-17pAP2-lo and IL-17pAP2-hi mice are shown. CD4$^+$ T cells, pre-gated on live cells, are presented as EGFP versus IL-17. **(B)** The frequencies of BALF and lung CD4$^+$ T cells expressing EGFP are shown. **(C)** The total numbers of BALF CD4$^+$EGFP$^+$ cells were calculated from total CD4$^+$ T cell counts and the BALF frequencies of EGFP$^+$ cells from Figure 6B. **(D)** The total numbers of BALF CD4$^+$ T cells (host and adoptively transferred) were calculated from total cell counts and BALF frequencies of CD4$^+$ cells. **(B-D)** Mean values (±SEM) are for 6-9 mice per group, from at least 3 independent experiments. Two-tailed, unpaired t-tests were performed, *p<0.05.

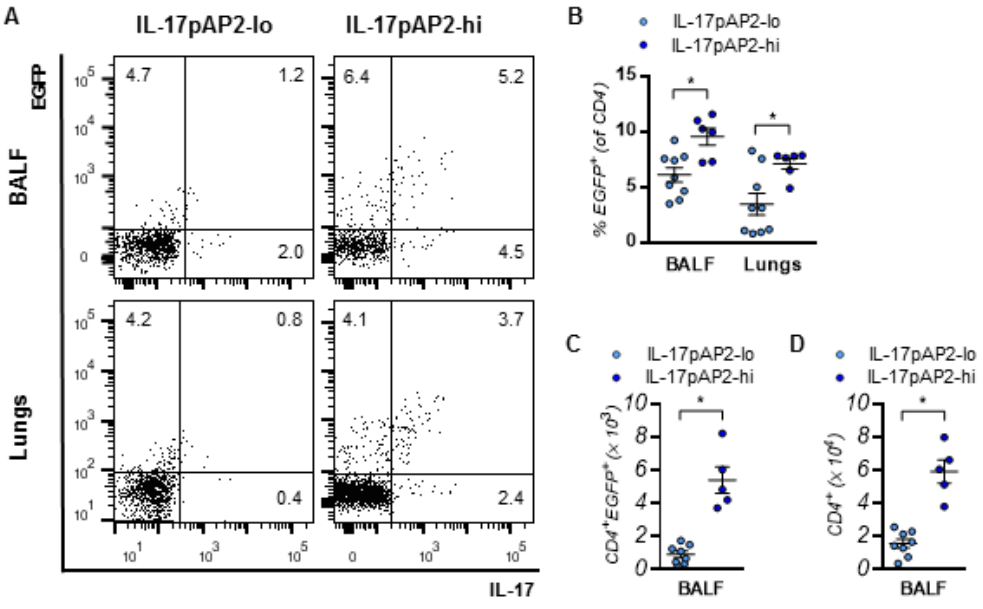
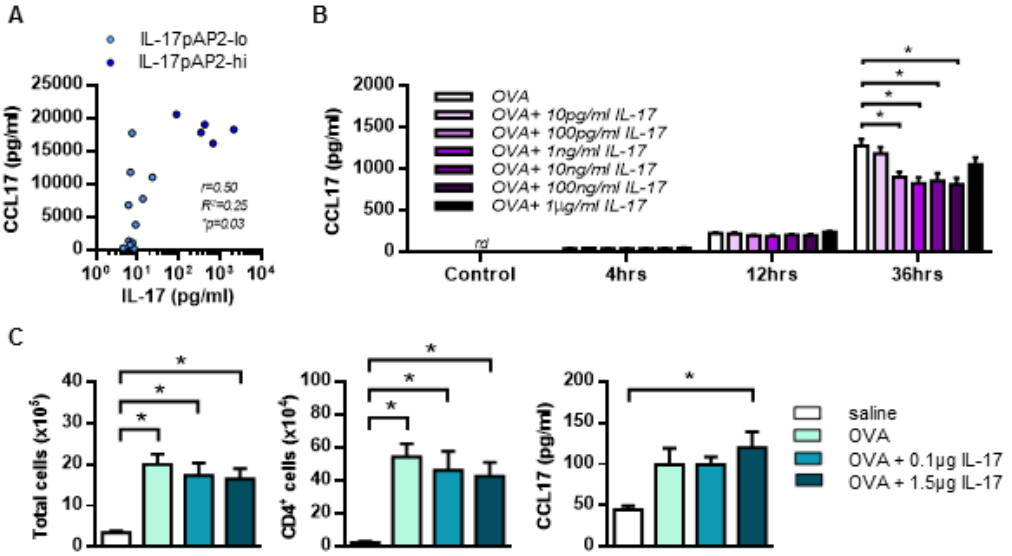


Figure 4.9: IL-17 originating from CD4$^+$ T cells positively regulates *in vivo* BALF CCL17 expression, whereas exogenous IL-17 has no effect or negatively regulates OVA-induced CCL17 expression

Following OVA airway challenges, recipients of IL-17pAP2 transduced CD4$^+$ T cells were assessed for BALF levels of IL-17 & CCL17. (A) The linear relationship between BALF IL-17 and CCL17 was assessed by determining the Pearson correlation coefficient, 'r'. Data are from n=19 mice from at least 7 independent experiments. *p<0.05. (B) Single cell suspensions of lung cells from OVA sensitized and challenged BALB/c mice were re-stimulated with OVA, ± IL-17 (ranging from 10pg/ml to 1µg/ml), for 4, 12 and 36 hours or left unstimulated (control- 0hr). The figure shows data from the lungs of 7-8 mice and is representative of 2 independent experiments. A one-way ANOVA was performed, followed by a Holm-Sidak post-hoc test comparing values to the OVA group at each time point, *p<0.05. (C) DO11.10 mice were OVA challenged with saline, with OVA alone, or OVA +IL-17 (0.1 or 1.5µg). Total cells, CD4$^+$ T cells and CCL17 levels were assessed from the BALF. Data from 5-6 mice are shown from 2 independent experiments. A one-way ANOVA was performed, followed by a Holm-Sidak post-hoc test. *p<0.05



5 Discussion

Asthma is a complex disease associated with both Th2 and Th17 immune responses with murine studies consistently showing IL-17 enhancement of Th2 responses, when sourced from T helper cells (20, 21, 23, 34). Nevertheless, human and murine studies examining Th17/IL-17 effects on Th2 driven allergic airways responses are limited in number. Wang et al. specifically describe a Th2 subset of memory/effector cells co-expressing IL-17 that induces BALF cytokine and inflammatory profiles similar to recipients of a mix of classic Th2 and Th17 cells (34). We used a modified OVA sensitization protocol to generate $CD4^+$ T cells with a predominant Th2 phenotype (35) combined with a previously established protocol of concentrating retroviral supernatants by centrifugal filtration (26) to efficiently transduce OVA-stimulated $CD4^+$ T cells with recombinant retrovirus encoding IL-17. This allowed us to study the role of antigen-induced IL-17 production by $CD4^+$ T cells in the context of a Th2-biased inflammatory response. Our data demonstrated that, when sourced from $CD4^+$ T cells, BALF IL-17 levels positively correlated with respiratory resistance, BALF CCL17 levels and nearly correlated with total airway inflammatory cell numbers ($p<0.058$) **(Fig 4.4)**. Interestingly, BALF levels of IL-13, a cytokine that is strongly associated with AHR, did not correlate with peak respiratory resistance values. However, IL-17, which did correlate with AHR, can synergize with IL-13 to enhance AHR (23) and can, under some circumstances, induce AHR independently of IL-13 (12). Moreover, IL-4, whose levels were significantly increased in IL-17pAP2-hi compared to IL-17pAP2-lo mice, has also been shown to positively regulate AHR, independently of IL-13 (36). In murine models of allergic airways disease large quantities of transgenic cells or recombinant proteins are commonly delivered to ensure modulation of the pathological features under study.

We and others have directly instilled as little as 0.1µg and as much as 15µg of recombinant IL-17 to murine airways, approximately half of which is likely localized to the lower airways (37), which would equate to approximately 75-11,000 fold more IL-17 than the average level detected in the BALF of the IL-17pAP2-hi mice (17-20, 23).

We subdivided recipients of IL-17pAP2 transduced $CD4^+$ T cells into two groups according to BALF levels of IL-17. Our approach was based on a study by Barczyk et al. who found that while IL-17 levels in induced sputum did not differ between healthy controls and patients with asthma, chronic obstructive pulmonary disease or chronic bronchitis (11), IL-17 was significantly elevated in subpopulations of patients exhibiting bronchial hyperreactivity (11). Similarly, the IL-17pAP2-lo mice were hyporesponsive to methacholine challenges and had only slightly elevated BALF IL-17 levels **(Fig 4.6A & C)**, whereas the IL-17pAP2-hi mice were hyperresponsive and had dramatically elevated BALF IL-17 levels. We defined IL-17pAP2-lo mice as those in which the level of BALF IL-17 fell within 3 standard deviations of the mean (9.4 ± 1.5 pg/ml). The value of each sample also factored into a calculated average maintained at less than 2 fold the average pAP2 mouse (5.9 ± 0.5 pg/ml), a group that was similarly hyporesponsive. Consequently, BALF levels of IL-17 for each IL-17pAP2-hi mouse were at least 5 fold higher than the average IL-17pAP2-lo mouse.

It is likely that the increased allergic airways responses, including airway inflammation and BALF levels of Th2 cytokines **(Fig. 4.5, 4.7)** in IL-17pAP2-hi mice are in response to the increase in IL-17 production and not the result of retroviral targeting or increased activation of $CD4^+$ T cells for several reasons. Within each experiment, the same pool of splenocytes was incubated with either the pAP2 or IL-17pAP2 retroviruses, which have similar transduction efficiencies **(Fig 4.3)**. Transducing pooled splenocytes ensured that both retroviruses targeted the

same set of $CD4^+$ T cells within each experiment. If preferential targeting and/or activation of $CD4^+$ T cells was responsible for the increased allergic airway responses in the IL-17pAP2-hi mice, we reasoned that higher levels of T helper cytokines and chemokines would also be present in the BALF of recipients of pAP2 transduced T cells from the same pooled splenocyte preparations. In fact, similar BALF levels of IL-4 (0.7±0.5 vs. 1.5±0.6pg/ml), IL-5 (3.9±2.0 vs. 26±9.0pg/ml) and chemokines CCL5, CCL11 and CCL17 (data not shown), as well as significantly lower BALF levels of IL-13 (8.1±1.6 vs. 17±1.7pg/ml) were present in recipients of pAP2 transduced T cells that originated from pooled cells that produced the IL-17pAP2-hi mice, compared to recipients of pAP2 transduced T cells that came from the same pooled cells that produced the IL-17pAP2-lo mice. Equally important, BALF levels of IL-17 did not differ between these groups (4.4±0.8 vs. 6.5±0.6pg/ml), providing further evidence that the increased BALF levels of IL-17 in the IL-17pAP2-hi mice were produced from the retroviral IL-17 gene insert.

Severe asthmatics have greater serum levels of IL-17 compared to mild and/or moderate asthmatics and healthy controls (32, 38) with high levels of serum IL-17 being an independent risk factor for severe asthma (31, 32). Accordingly, following OVA airway challenge, the highest levels of IL-17 were present in the serum of airway hyperresponsive and inflamed IL-17pAP2-hi mice **(Fig 4.5E)**.

Following OVA airway challenge, IL-17pAP2 transduced $CD4^+$ T cells were recruited to the airways and lungs and at higher frequencies in mice with the highest BALF levels of IL-17 (IL-17pAP2-hi) **(Fig 4.8)**. IL-17pAP2-hi mice additionally recruited more total $CD4^+$ T cells (both adoptively transferred and host). Recruitment of host $CD4^+$ T cells is believed to be an indirect response to antigen challenge, as it is unlikely that there was sufficient time for the

recipient mice to mount an adaptive immune response to OVA during the 5-day period of airway OVA challenges (25). The IL-17 produced by the adoptively transferred, OVA-specific $CD4^+$ T cells initially recruited in response to the OVA airway challenges likely upregulated expression of T cell related chemoattractants CCL5, CCL17, CCL22 and CXCL10 **(Fig 4.7D)**, that in turn contributed to the recruitment of additional adoptively transferred ($EGFP^+$), as well as host ($EGFP^-$) $CD4^+$ T cells, which made up the majority of $CD4^+$ T cells recovered from the BALF **(Fig 4.8B)**. Moreover, Th2 cells are direct producers of CCL17 (39). Therefore, it is likely that the adoptively transferred $CD4^+$ T cells in our study also produced CCL17 that participated in a positive feedback loop promoting further Th2 cell recruitment. The enhanced recruitment of adoptively transferred cells in IL-17pAP2-hi mice also explains, in part, the higher BALF levels of IL-17. However, there was only a 6-fold increase in total $EGFP^+CD4^+$ T cells **(Fig 4.8C)**, which does not explain the more than 60-fold higher BALF levels of IL-17 **(Fig 4.5A)**. Thus, the primary increase in IL-17 must come from the activation of IL-17pAP2-transduced cells, in response to the OVA challenges.

A simplified *in vitro* experiment was designed to confirm the role of IL-17 in regulating CCL17 expression by preparing lung cells from OVA sensitized and challenged mice and re-stimulating them with OVA and varying concentrations of IL-17. Despite using 10-fold increasing concentrations, IL-17 did not positively regulate CCL17 expression, but had a negative effect across several doses **(Fig 4.9B)**. This negative effect of IL-17 coincided with results from a similar experiment covering a smaller range of IL-17 concentrations (19). We used a wider range of IL-17 concentrations as our published data showed positive *and* negative effects of IL-17 on IL-13-induced airways responses that were dose dependent (18). To determine whether these apparently contradictory results were due to the *in vitro* versus *in vivo*

environment, or the timing of IL-17 delivery relative to antigen exposure, we examined how IL-17 modulated OVA-induced CCL17 levels and airway inflammation when delivered *in vivo* to naive mice at the time of airway antigen exposure. DO11.10 (OVA T cell receptor transgenic) mice were exposed to OVA in combination with either a low or high dose of IL-17 **(Fig 4.9C)**. However, even in this *in vivo* setting, both concentrations of recombinant IL-17 failed to alter OVA-induced inflammatory responses and CCL17 levels in the BALF.

Consistent with the published literature, our data indicate that experimental conditions have a significant effect on whether IL-17 has a positive, negative or even no effect on CCL17 expression and airway inflammatory responses. Despite these variable responses, IL-17-altered CCL17 levels consistently coincide with AHR and airway inflammatory responses. Thus, elevations in CCL17 levels (in response to $CD4^+$ T cell delivered IL-17) coincided with increases in AHR and inflammation **(Fig 4.5B & 4.7A)**, while unaltered CCL17 levels (in response to recombinant IL-17 delivery) in OVA-challenged DO11.10 mice coincided with airway inflammatory responses that were unaffected **(Fig 4.9C)**. Even CCL17 expression, attenuated upon delivery of IL-17 by γδ T cells or augmented upon neutralizing IL-17-γδ T cells, coincides with changes to AHR and airway inflammation (17). Altogether, these data indicate that a central point downstream of IL-17, but upstream of CCL17 is pivotal to committing IL-17 as a positive or negative regulator of allergic airways disease. Further elucidation of the mechanism(s) linking IL-17 to chemokine production could identify improved targets within the IL-17 pathway for future drug development in the treatment of asthma since targeting IL-17 could impair treatment or worsen asthma symptoms as easily as it could alleviate them.

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7 Disclosures

The authors have no conflicts of interest to disclose.

8 References

1. GINA. 2014. Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma, Vancouver, USA. 1-134.
2. Moore, W. C., E. R. Bleecker, D. Curran-Everett, S. C. Erzurum, B. T. Ameredes, et al. 2007. Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. *J. Allergy Clin. Immunol.* 119: 405-413.
3. Chung, K. F., S. E. Wenzel, J. L. Brozek, A. Bush, M. Castro, et al. 2014. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur. Respir. J.* 43: 343-373.
4. Oboki, K., T. Ohno, H. Saito, and S. Nakae. 2008. Th17 and allergy. *Allergol Int* 57: 121-134.
5. Zhao, Y., J. Yang, Y. D. Gao, and W. Guo. 2010. Th17 immunity in patients with allergic asthma. *Int. Arch. Allergy Immunol.* 151: 297-307.
6. Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, et al. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430-438.
7. Al-Ramli, W., D. Prefontaine, F. Chouiali, J. G. Martin, R. Olivenstein, et al. 2009. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J. Allergy Clin. Immunol.* 123: 1185-1187.

8. Bullens, D. M., E. Truyen, L. Coteur, E. Dilissen, P. W. Hellings, et al. 2006. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir. Res.* 7: 135.

9. Vazquez-Tello, A., R. Halwani, Q. Hamid, and S. Al-Muhsen. 2013. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J. Clin. Immunol.* 33: 466-478.

10. Laan, M., Z. H. Cui, H. Hoshino, J. Lotvall, M. Sjostrand, et al. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* 162: 2347-2352.

11. Barczyk, A., W. Pierzchala, and E. Sozanska. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 97: 726-733.

12. McKinley, L., J. F. Alcorn, A. Peterson, R. B. Dupont, S. Kapadia, et al. 2008. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* 181: 4089-4097.

13. Holgate, S. T. 2012. Innate and adaptive immune responses in asthma. *Nat. Med.* 18: 673-683.

14. Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255-281.

15. Shi, Y. H., G. C. Shi, H. Y. Wan, X. Y. Ai, H. X. Zhu, et al. 2013. An increased ratio of Th2/Treg cells in patients with moderate to severe asthma. *Chin. Med. J.* 126: 2248-2253.

16. Woodruff, P. G., B. Modrek, D. F. Choy, G. Jia, A. R. Abbas, et al. 2009. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am. J. Respir. Crit. Care Med.* 180: 388-395.

17. Murdoch, J. R., and C. M. Lloyd. 2010. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing {gamma}{delta}T cells. *Am. J. Respir. Crit. Care Med.* 182: 464-476.

18. Kinyanjui, M. W., J. Shan, E. M. Nakada, S. T. Qureshi, and E. D. Fixman. 2013. Dose-dependent effects of IL-17 on IL-13-induced airway inflammatory responses and airway hyperresponsiveness. *J. Immunol.* 190: 3859-3868.

19. Schnyder-Candrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, et al. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203: 2715-2725.

20. Wilson, R. H., G. S. Whitehead, H. Nakano, M. E. Free, J. K. Kolls, and D. N. Cook. 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 180: 720-730.

21. Wakashin, H., K. Hirose, Y. Maezawa, S. Kagami, A. Suto, et al. 2008. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* 178: 1023-1032.

22. Kudo, M., A. C. Melton, C. Chen, M. B. Engler, K. E. Huang, et al. 2012. IL-17A produced by alphabeta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat. Med.* 18: 547-554.

23. Lajoie, S., I. P. Lewkowich, Y. Suzuki, J. R. Clark, A. A. Sproles, et al. 2010. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat. Immunol.* 11: 928-935.

24. Nakada, E. M., J. Shan, M. W. Kinyanjui, and E. D. Fixman. 2014. Adjuvant-dependent regulation of interleukin-17 expressing inverted question mark inverted question mark T

cells and inhibition of Th2 responses in allergic airways disease. *Respiratory research* 15: 90.

25. Kinyanjui, M. W., M. Tamaoka, and E. D. Fixman. 2007. T-helper type 2-dependent early recruitment of antigen non-specific CD4+ T cells in experimental asthma. *Clin. Exp. Allergy* 37: 1455-1466.

26. Kinyanjui, M. W., D. Ramos-Barbon, A. Villeneuve, and E. D. Fixman. 2006. Enhanced transduction of antigen-stimulated T lymphocytes with recombinant retroviruses concentrated by centrifugal filtration. *J. Immunol. Methods* 314: 80-89.

27. Sallusto, F., D. Lenig, C. R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* 187: 875-883.

28. Galipeau, J., H. Li, A. Paquin, F. Sicilia, G. Karpati, and J. Nalbantoglu. 1999. Vesicular stomatitis virus G pseudotyped retrovector mediates effective in vivo suicide gene delivery in experimental brain cancer. *Cancer Res.* 59: 2384-2394.

29. Nakano, H., M. E. Free, G. S. Whitehead, S. Maruoka, R. H. Wilson, et al. 2012. Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol.* 5: 53-65.

30. Costa, G. L., J. M. Benson, C. M. Seroogy, P. Achacoso, C. G. Fathman, and G. P. Nolan. 2000. Targeting rare populations of murine antigen-specific T lymphocytes by retroviral transduction for potential application in gene therapy for autoimmune disease. *J. Immunol.* 164: 3581-3590.

31. Agache, I., C. Ciobanu, C. Agache, and M. Anghel. 2010. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir. Med.* 104: 1131-1137.

32. Chien, J. W., C. Y. Lin, K. D. Yang, C. H. Lin, J. K. Kao, and Y. G. Tsai. 2013. Increased IL-17A secreting CD4+ T cells, serum IL-17 levels and exhaled nitric oxide are correlated with childhood asthma severity. *Clin. Exp. Allergy* 43: 1018-1026.

33. Nakae, S., Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, et al. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 17: 375-387.

34. Wang, Y. H., K. S. Voo, B. Liu, C. Y. Chen, B. Uygungil, et al. 2010. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J. Exp. Med.* 207: 2479-2491.

35. Mattes, J., M. Yang, A. Siqueira, K. Clark, J. MacKenzie, et al. 2001. IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. *J. Immunol.* 167: 1683-1692.

36. Perkins, C., M. Wills-Karp, and F. D. Finkelman. 2006. IL-4 induces IL-13-independent allergic airway inflammation. *J. Allergy Clin. Immunol.* 118: 410-419.

37. Southam, D. S., M. Dolovich, P. M. O'Byrne, and M. D. Inman. 2002. Distribution of intranasal instillations in mice: effects of volume, time, body position, and anesthesia. *Am. J. Physiol. Lung Cell Mol. Physiol.* 282: L833-839.

38. Alyasin, S., M. H. Karimi, R. Amin, M. Babaei, and S. Darougar. 2013. Interleukin-17 gene expression and serum levels in children with severe asthma. *Iran J. Immunol.* 10: 177-185.

39. Wirnsberger, G., D. Hebenstreit, G. Posselt, J. Horejs-Hoeck, and A. Duschl. 2006. IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. *Eur. J. Immunol.* 36: 1882-1891.

CHAPTER 5: DISCUSSION

The overall objective of this thesis was to determine the role of IL-17 on Th2-induced immune responses in murine models of asthma. We hypothesized that adjuvants modulate allergic airway responses and the cellular profile of IL-17 producing cells in the airways and lungs. We also hypothesized that the source of IL-17 would have a profound effect on whether IL-17 acted as a positive or negative regulator of allergic airway responses. We utilized different methodologies to study the role(s) of IL-17, by instilling the cytokine directly into the airways and by increasing expression from specific cellular sources adoptively transferred into and/or localized to the airways of mice. To modulate IL-17 expression in an OVA model of allergic airways disease, we IP OVA sensitized mice with or without the adjuvants, alum and CFA, followed by airway challenges and showed significant adjuvant-dependent modulation of AHR and Th2 responses, in addition to a shift in the frequencies of BALF IL-17-expressing cells from primarily Th17 to IL-17-γδ T cells (Chapter 2). As part of this study, we also utilized a γδ TCR stimulatory antibody to assess the role of IL-17-γδ T cells on allergic airway responses induced in IP OVA/CFA sensitized and challenged mice. In another model, we examined the role of IL-17 on IL-13-induced responses. We co-administered two different doses of recombinant IL-17 with recombinant IL-13 into the nasal passages of anaesthetized mice and found that lower doses of IL-17 enhanced IL-13-induced eosinophilic and lymphocytic inflammation, while higher doses of IL-17 attenuated IL-13-induced AHR and eosinophilia (Chapter 3). As part of these studies, we adoptively transferred lung γδ T cells stimulated to express increased levels of IL-17, and demonstrated that these cells attenuated IL-13-induced eosinophilic and lymphocytic

inflammation. Finally, in a third model, we induced IL-17 expression in OVA-stimulated $CD4^+$ T cells using recombinant retroviruses. Upon IP adoptive transfer into naïve mice, these cells were recruited to the lungs by OVA airway challenge. Our data demonstrate that moderate increases in BALF levels of IL-17 produced by retrovirally transduced and adoptively transferred $CD4^+$ T cells, were sufficient to induce airway neutrophilia, while higher BALF levels of IL-17 were associated with robust AHR and inflammatory responses (Chapter 4).

1 Dual role of IL-17 in human asthma

Although data from several laboratories, including our own, have established both positive and negative regulatory effects attributable to IL-17 in murine models of allergic airways disease (1-4), it is not clear whether this dual role is applicable to human asthma. Firstly, much of the human data associating IL-17 to asthma either does not state the cellular source producing IL-17 or attributes its source to Th17 cells, without conclusively localizing IL-17 to airway or lung $CD4^+$ T cells (5-8). This opens up the possibility that γδ T cells or other cell types could be important sources of IL-17 in asthma. Even those human asthma studies that localize IL-17 to $CD4^+$ T cells typically do not consider secondary sources of IL-17 (9, 10). This is important because the IP adoptive transfer of small numbers of γδ T cells is sufficient to reduce inflammatory responses in rodent models of asthma. For example, as few as 35 000 γδ T cells transferred into OVA sensitized and challenged rats (11) or 50 000 γδ T cells transferred into IN IL-13 exposed mice (Chapter 3) (12) attenuated airway eosinophilia and/or AHR in recipients. Thus far, few studies have assessed a potential contribution of IL-17-expressing γδ T cells in human asthmatics. One group reported a decreased ratio of IFN-γ:IL-17-expressing γδ T cells in the peripheral blood of allergic asthmatics, compared to healthy controls (13). Xuekun et al. also reported an increase in both Th17 and IL-17-γδ T cells in the peripheral blood of patients with allergic rhinitis, compared to control subjects (14). Both studies identify a population of IL-17-γδ T cells that is increased in patients with allergic airways disease, albeit in the peripheral blood, but do not address the functional significance of this increase. Nevertheless, the frequency and number of $IL\text{-}17^+$ cells and the levels of serum, BALF and sputum IL-17 correlate with asthma

severity, associating IL-17 responses to the pathogenesis and/or resolution of asthma (5-7, 15, 16).

2 Factors influencing the dual role of IL-17

Whereas Th17 cells are associated with positive regulation and IL-17-γδ T cells are associated with negative regulation of allergic airways disease in mice, it remains to be seen whether it is the IL-17 released by Th17 cells and IL-17-γδ T cells or the $CD4^+$ T cell and γδ T cells (that happen to express IL-17) that establishes function. In favour of the cytokine, are data demonstrating that lower (0.5μg) and higher (1.5μg) doses of IL-17 have opposing effects on IL-13-induced responses, independent of the cellular source (12). In addition, Murdoch et al. used an OVA model, in which the adoptive transfer of IL-17-γδ T cells into IP OVA/alum sensitized and challenged mice, during the resolution phase, decreases lung resistance, airway eosinophil and Th2 cell recruitment, and BALF levels of IL-4 and IL-13, responses that were similarly attenuated with recombinant IL-17, but not Th17 cells or $IL\text{-}17^{-/-}$ γδ T cells (2). However, the inability of transferred Th17 cells to attenuate allergic airway responses favours the cell type, rather than the cytokine, as the prominent factor influencing IL-17 function.

Interestingly, the cellular source and dose of IL-17 may be related. The adoptive transfer of Th17 cells into mice, during the resolution phase of an OVA model of allergic airways disease, does not augment BALF IL-17 levels, whereas the transfer of IL-17-γδ T cells is associated with a slight, albeit an insignificant increases in IL-17 (2). This could indicate that IL-17-γδ T cells release larger amounts of IL-17 compared to Th17 cells, To further support this hypothesis, we showed the MFI of IL-17 expressed by IL-17-γδ T cells is consistently higher in IL-17-γδ T cells than in Th17 cells (Chapter 2). Moreover, serum levels of IL-17, in patients with allergic rhinitis, correlate with the frequency of peripheral blood IL-17-γδ T cells, but not Th17 cells (14).

The ‘preferred’ location, within the lungs, of each IL-17-expressing cell type may also influence the biological response to this cytokine. Generally, γδ T cells are localized to the mucosal region of the lungs where they are able to quickly respond to infection as part of the innate arm of the immune response (17, 18), while $CD4^+$ T cells are adaptive immune cells recruited to the airways and lungs and have been found in contact with ASM cells, in a rat OVA model of asthma (19, 20), where they are thought to promote proliferation, reduce apoptosis and modulate ASM cell function, by enhancing the shortening velocity of ASM.

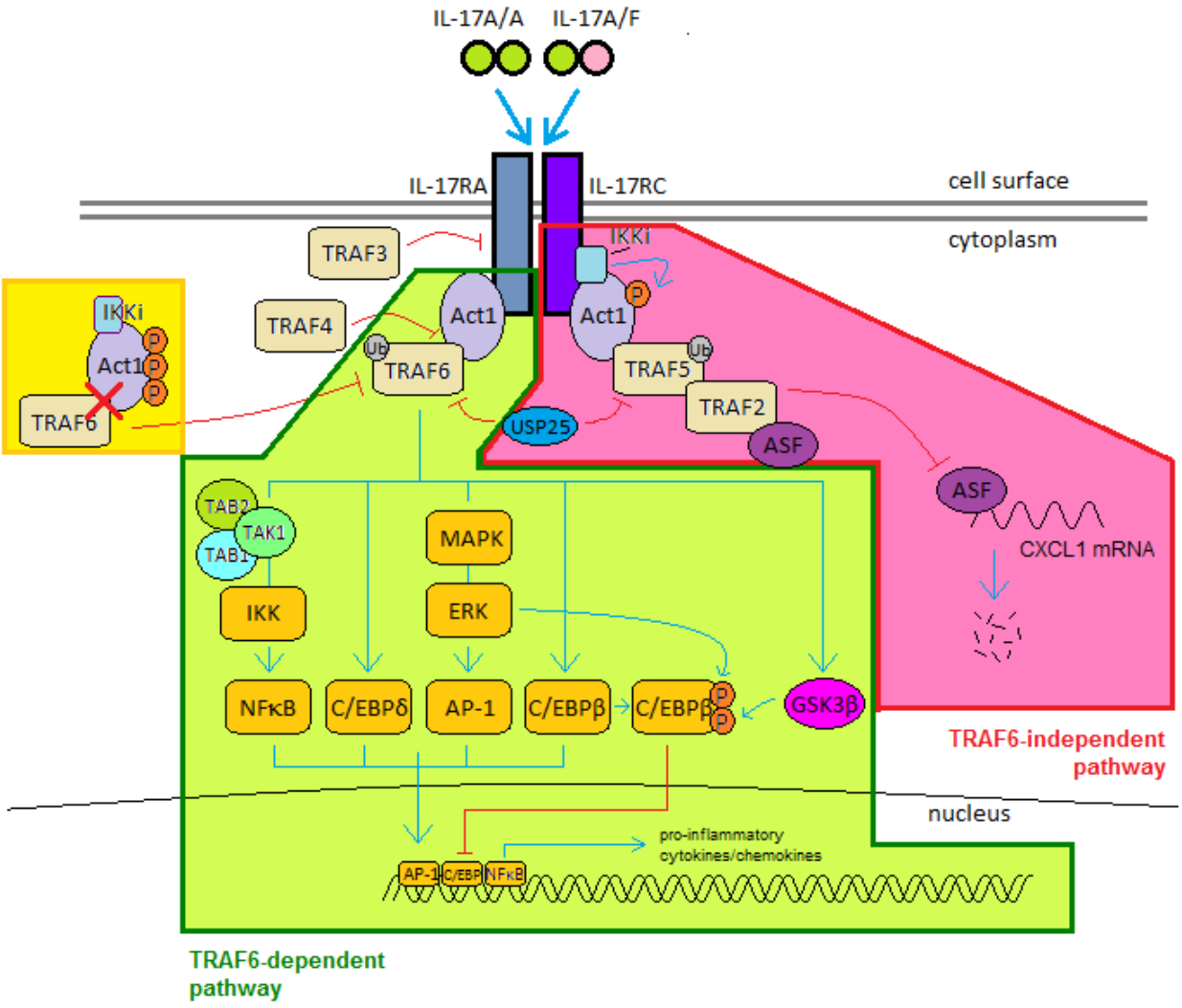
3 Different pathways downstream of the IL-17 receptor may be involved in establishing IL-17 as a positive or negative regulator of allergic airways disease

IL-17 binds the IL-17R as a homodimer (IL-17A/A) or as a heterodimer with IL-17F (IL-17A/F) (21, 22). The IL-17R consists of two subunits, the ubiquitously expressed IL-17RA subunit and the IL-17RC subunit. Upon ligand binding, the IL-17R subunits heterodimerize and activate two main pathways: the tumor necrosis factor receptor-associated factor (TRAF)6-dependent pathway involving NFκB, MAPKs and the CCAAT-enhancer-binding proteins (C/EBPs), C/EBPδ and C/EBPβ (**Fig 5.1,** green box); and the TRAF6-independent pathway involving TRAF2 and TRAF5, which stabilizes mRNAs induced by TNFα, such as CXCL1 (**Fig 5.1**, pink box) (21, 22). In the TRAF6-dependent pathway, IL-17 signaling recruits the adaptor molecule Act1 to the IL-17R, followed by TRAF 6, which activates NFκB and c-Jun N-terminal kinase (JNK) signaling pathways (22). Act1 contains a U-box like E3 ligase, which ubiquitinates TRAF6 that leads to the recruitment of the TAK1 complex (TAK1-TAB1-TAB2), followed by activation of IKK and NFκB and the positive regulation of pro-inflammatory cytokine and chemokine expression. The TRAF6-independent pathway also recruits Act1, followed by inducible kinase (IKKi) that phosphorylates the Ser-311 residue on Act-1, allowing for the ubiquitination of and recruitment of TRAF5, followed by TRAF2 (23). These mediators then recruit alternative splicing factor (ASF), which forms the complex, Act1-TRAF5/TRAF2-ASF, which prevents ASF from binding to and cleaving mRNA encoding CXCL1.

Additional proteins activated by the IL-17R keep the pro-inflammatory pathway under tight control: TRAF3 binds directly to the IL-17R to prevent Act1 binding; TRAF4 competes with

Figure 5.1: Regulation of the IL-17 receptor pathway

The IL-17 homodimer (IL-17A/A) and heterodimer (IL-17A/F) bind the IL-17R, consisting of the IL-17RA and IL-17RC subunits. Upon ligand binding, the IL-17R undergoes a conformational change, recruiting the adaptor molecule, Act1, activating the TRAF6-dependent (green box) and/or –independent (pink box) pathways. In the TRAF6-dependent pathway, TRAF6 is poly-ubiquitinated by Act1, initiating the recruitment of the TAK1 complex (TAK1-TAB1-TAB2), thus activating IKK and NFκB, which regulate expression of a number of pro-inflammatory cytokines and chemokines. TRAF6 also activates the MAPK pathway, involving ERK and AP-1; as well as the C/EBP proteins, C/EBPβ and C/EBPδ, which are all positive regulators of pro-inflammatory-related gene expression, including IL-6. The TRAF6-independent pathway involves recruitment of IKKi, which phosphorylates Act1, recruiting/ubiquitinating TRAF5 and recruiting TRAF2 to form a complex with ASF. As a complex, ASF is prevented from degrading transcripts like CXCL1, thus stabilizing mRNA expression. The IL-17 pathway is also regulated by TRAF3, which prevents Act1 from being recruited to the IL-17R, and TRAF4, which competes with TRAF6 to bind Act1. USP25 is also known to de-ubiquitinate TRAF5 and TRAF6. Chronic IL-17 receptor signaling can lead to IKKi phosphorylation of additional serine residues on Act1 (yellow box), thus preventing TRAF6 signaling. IL-17 also induces activation of ERK and GSK3β, which can phosphorylate threonine residues on C/EBPβ, converting it from a positive to a negative regulator of IL-17-induced responses.


IL-17A/A
IL-17A/F
IL-17RA
IL-17RC
cell surface
cytoplasm
TRAF3
TRAF4
Act1
TRAF6
IKKi
P
Ub
TRAF5
TRAF2
ASF
USP25
TAB2
TAK1
TAB1
MAPK
IKK
ERK
NFκB
C/EBPδ
AP-1
C/EBPβ
GSK3β
CXCL1 mRNA
TRAF6-independent pathway
nucleus
pro-inflammatory cytokines/chemokines
TRAF6-dependent pathway

TRAF6 to bind Act1; and USP25 de-ubiquitinates TRAF5 and TRAF6 (22). Altogether, TRAF3, TRAF4 and USP25 regulate IL-17 signaling pathways through a negative feedback loop, thus preventing an aberrant pro-inflammatory response from being established (22). In addition, persistent stimulation of the IL-17R decreases Act1 protein levels, without affecting mRNA levels, by first phosphorylating and then recruiting Act1 to a ubiquitin ligase complex for degradation (22). IL-17 also induces activation of IKKi and other kinases that can phosphorylate 3 additional serine residues on Act1 to inhibit it from binding TRAF6 (**Fig 5.1**, yellow box), thus inhibiting the NFκB pathway (22, 24).

Ge et al. were the first to describe C/EBPβ as a negative regulator of the IL-17R, as opposed to identifying mediators involved in regulating the pro-inflammatory TRAF6-dependent and -independent pathways (22, 25). C/EBPβ positively regulates pro-inflammatory cytokine and chemokine expression, but when phosphorylated on Thr-179 and Thr-188 residues by glycogen synthase kinase (GSK)-3β and extracellular-signal-regulated kinases (ERK), respectively, it negatively regulates IL-17-induced gene expression (**Fig 5.1**, green box) (25). Moreover, GSK-3β and ERK are activated by IL-17, thus completing the negative regulatory pathway of IL-17 (21, 25).

The signaling pathways downstream of the IL-17R may explain much of the positive and negative effects induced by IL-17 in allergic airways disease. For example, irrespective of the source, dose or whether it acts as a positive or negative regulator, IL-17 is consistently and closely associated with airway neutrophilia (3, 4, 26). This is likely due to IL-17R signaling that activates the TRAF6-dependent pathway, thereby upregulating expression of pro-inflammatory cytokines/chemokines including the potent neutrophil chemoattractant, CXCL1. It also activates

the TRAF-6-independent pathway, which would stabilize the CXCL1 mRNA by forming the complex that prevents ASF-dependent mRNA cleavage (22).

The downstream IL-17R signaling pathways may also explain the dual roles associated with IL-17 as both a positive and negative regulator of airway immune responses in murine models of asthma. For example, we observed dose-dependent effects of IL-17 on IL-13-induced responses (Chapter 3) with the lower dose of IL-17 positively regulating and the higher dose of IL-17 negatively regulating IL-13-induced responses (12). We speculate that the lower dose of IL-17 is sufficient to activate the conventional TRAF6-dependent pathway, thus activating NFκB, AP-1 and C/EBP proteins that would positively regulate expression of pro-inflammatory cytokines and chemokines (22). However, excessive and chronic stimulation of the IL-17R, provided by the higher dose of IL-17, would lead to the excessive phosphorylation of Thr residues on C/EBPβ via GSK-3β and ERK, thus converting C/EBPβ from a positive to a negative regulator of IL-17R signaling (25).

Furthermore, our studies indicate that Th17 cells positively regulate (Chapter 4), while IL-17-γδ T cells negatively regulate (Chapters 2 & 3) Th2 inflammatory responses in murine models of allergic airways disease. Similar to our study on the dose-dependent effects of IL-17 (Chapter 3), the positive and negative regulatory roles of Th17 and γδ T cells may reflect the amount of IL-17 produced by these cells. We showed in **Fig 2.6** from Chapter 2 that the MFI of IL-17 expressed by BALF Th17 cells is significantly lower than that in IL-17-γδ T cells. Murdoch et al. also showed that γδ T cells from wild type, but not IL-17-deficient mice helped improve inflammatory responses when adoptively transferred into OVA sensitized and challenged mice at the peak of inflammation (2). This once again highlights the IL-17 produced by γδ T cells in this model, rather than the γδ T cell itself as the factor that is responsible for the

suppressive activity of IL-17-γδ T cells. Finally, BALF levels of IL-17 in recipients of IL-17pAP2 transduced $CD4^+$ T cells were shown to positively correlate with AHR (Chapter 4, **Fig 4.4**). In fact, most studies associate increasing levels of sputum, BALF and serum IL-17 and IL-17^+ cells with asthma severity. However, as was briefly presented in the Chapter 4 discussion, despite the high levels of BALF IL-17 in some recipients of the IL-17pAP2 transduced $CD4^+$ T cells, even the average BALF level of IL-17 from IL-17pAP2-hi recipients was still orders of magnitude less than the amount of recombinant IL-17 commonly administered to mice in allergic airways disease studies (1, 4, 12). Altogether, the data indicate that increasing numbers of IL-17^+ cells and levels of IL-17 expression may be associated with allergic airways disease severity via the increased activation of the pro-inflammatory pathways downstream of the IL-17R; however, past a point of excessive or chronic IL-17 stimulation, we speculate that the Thr residues on C/EBPβ are phosphorylated, thus switching the transcription factor from acting as a positive to a negative regulator of allergic airway responses.

4 The disconnect between BALF cytokine/chemokine expression and inflammation in IP OVA/CFA sensitized and challenged mice

In a typical murine model of allergic airways disease, BALF levels of Th2 (and/or Th17) cytokines and chemokines are increased in sensitized mice, following airway antigen challenge, which coincides with an influx of inflammatory cells into the airways (27, 28). As discussed in Chapter 1 of the thesis, Th2 cytokines: IL-4, IL-5 and IL-13, as well as IL-17, are established pro-inflammatory mediators regulating airway inflammation via the upregulated expression of chemokines. Nevertheless, our IP OVA/CFA sensitized and challenged mice (Chapter 2); having a robust inflammatory response characterized by a significant influx of macrophages, eosinophils, neutrophils and lymphocytes; had significantly lower levels of Th2 and Th17 cytokines than one or both of the IP OVA/sal and OVA/alum sensitized and challenged groups. In addition, OVA/CFA mice did not differ in the relative expression of chemokines in the lungs compared to OVA/sal sensitized mice.

The apparent lack of cytokine and chemokine induction in IP OVA/CFA sensitized and challenged mice in the face of a robust airway inflammatory response may be confusing. However, these discrepancies suggest the successful induction of pro-inflammatory mediators that would have led to the recruitment of inflammatory cells as was observed in our OVA/CFA mice. This response would likely activate regulatory mechanism(s) to prevent the inflammatory response from getting out of control by down-regulating expression of the pro-inflammatory mediators that initiated inflammation. More specifically, we hypothesize that the influx of eosinophils and neutrophils into the airways of IP OVA/CFA sensitized and challenged mice leads to their degranulation and release of histamine, matrix metalloproteases, oxygen species,

polymorphonuclear-derived serine proteases like elastase and cathespin G, as well as other mediators that cause airway damage (29). Although serine proteases are known to cleave pro-forms of cytokines into their biologically active forms, there is growing evidence that they also cleave active cytokines into inactive fragments (29, 30). Moreover, they are also known to cleave cytokine receptors from the cellular membrane to form soluble receptors that bind their respective cytokine, protecting them from degradation and/or preventing them from signaling (29, 31). In any case, both mechanisms could explain the low levels of BALF cytokines detected in the airways of our OVA/CFA sensitized mice.

The highly inflamed airways of IP OVA/CFA sensitized and challenged mice and the hypothesized proteolytic environment that might be expected to follow a large influx of eosinophils and neutrophils may also provide an explanation for the disjointed BALF IL-17 and IL-17$^+$ BALF cell data. OVA/CFA mice had lower BALF levels of IL-17, yet they had higher total numbers of IL-17$^+$ BALF cells compared to OVA/sal and OVA/alum sensitized mice. The IL-17 detected by flow cytometry from BALF cells would have been produced and protected within the environment provided by the cell, whereas the IL-17 detected from the BALF is vulnerable to degradation by extracellular proteases. We alternatively considered the differential production of IL-17 by Th17 and IL-17-γδ T cells as a possible explanation for the discrepancy, especially considering the difference in the ratio of the frequencies of IL-17-γδ T cells:Th17 cells in OVA/CFA compared to OVA/sal and OVA/alum sensitized mice; however, the MFI of IL-17 in the IL-17-γδ T cells was at least twice as high as the Th17 cells. Thus, although we did not measure proteolytic activity in the airways and lungs of our mice, we hypothesize that this activity is significantly enhanced in OVA/CFA sensitized and challenged mice, resulting in the

degradation of those cytokines and chemokines that would have initiated recruitment of airway inflammatory cells.

5 IL-17-expressing Th2 cells are associated with severe asthma

Here we examine IL-17 effects on Th2 responses in asthma and detail our current understanding of a minor population of IL-17-expressing Th2 cells as they apply to allergic airways disease. Although some studies indicate that the adoptive transfer of Th17 cells is sufficient to induce airway recruitment of neutrophils upon antigen/allergen challenge (3, 32), several effects of IL-17 appear to be indirect, augmenting and attenuating allergic airway responses through the diminution or enhancement of antigen-induced Th2 responses (1, 2, 27, 32). For example, OVA-specific Th17 cells, on their own, may (3) or may not (32) have a moderate effect on AHR, and do not affect total BALF cell counts (32) following adoptive transfer and airway challenge of naïve mice, but are able to significantly enhance the same responses induced by the co-transfer of Th2 cells (32). Furthermore, dependent upon the asthma model, the absence of IL-17 signaling can significantly enhance or inhibit Th2 responses, including Th2 cytokine production and eosinophil recovery in the BALF (1, 33).

In addition to the organism, Th2 and IL-17 immune responses are present at the level of the single cell. Populations of $CD4^+$ T cells simultaneously expressing IL-17 and IL-4 or IL-13 have been identified in asthmatics and in animal models of allergic airways disease. A group of these cells, referred to here as IL-17-Th2 cells (or Th2/Th17 cells), is increased in frequency in the peripheral blood and BALF of asthmatics and similarly induced in the lungs of mice with allergic airways disease (34-36). Unlike classical Th2 and Th17 cells, IL17-Th2 cells co-express IL-4 and IL-17, in addition to co-expressing RORγt and GATA-binding protein 3 (GATA3), the master transcription factor regulating differentiation of Th2 cells (34-36). There is yet no consensus on the origin of these cells, as IL-17 and IL-4 can be induced in Th2 and Th17 cells,

respectively, under appropriate Th17 and Th2-skewing conditions, despite already being 'committed' to another T helper lineage (34, 35, 37). In favour of a Th17 origin, Cosmi et al. show evidence that human Th2 cells do not express IL-1 or IL-23 receptor and are therefore unresponsive to Th17 skewing conditions mounted by IL-1β and IL-23 stimulation, whereas Th17 cells express sufficient surface IL-4 receptor to phosphorylate and activate downstream STAT6, which promotes Th2-related gene expression, including IL-4 and IL-13 (35).

Murine recipients of IL-17-Th2 cells have greater total cells, eosinophils, neutrophils, macrophages and lymphocytes in their BALF and have increased goblet cell metaplasia compared to recipients of classic Th2 or Th17 cells only, similar to recipients of a mix of classical Th2 and Th17 cells, (34). Interestingly, under chronic conditions, the small population of IL-17-Th2 cells become the major T cell source of IL-17 in mice (34) and unlike classic Th2 cells but similar to classic Th17 cells in mice and in humans, IL-17-Th2 cells from the BALF of human asthmatics are resistant to *ex vivo* DEX treatment (3, 36). In a study by Irvin et al., the authors forego *ex vivo* stimulation of peripheral blood cells for a more physiologically representative group of unstimulated BALF cells to show a significantly higher frequency of IL-17-Th2 cells in the airways of asthmatics versus healthy controls, as well as showing a positive correlation between the frequency of total BALF IL-17-Th2 cells with BALF levels of IL-17, AHR, eosinophil and lymphocyte numbers and a negative correlation with FEV_1 (36, 37). Finally, Brandt et al. looking at a population of IL-17-Th2 cells that co-express IL-17 and IL-13 in a diesel exhaust particle enhanced HDM model of asthma (38), found that the frequency of these cells is significantly increased in the BALF of HDM exposed mice with simultaneous exposure to diesel exhaust particles and is associated with increased AHR and inflammation, which are attenuated by neutralizing IL-17.

The ultimate aim of our studies was to better understand the relationship between IL-17 and Th2 responses in allergic airways disease, which was accomplished, in part, by retrovirally transducing target antigen-specific $CD4^+$ T cells, with a Th2 background, for adoptive transfer (Chapter 4). This allowed us to recruit antigen-specific $CD4^+$ T cells to the airways and induce IL-17 expression from these cells, upon antigen stimulation. Upon reading the studies describing the novel Th2/Th17 (or IL-17-Th2) subset of T helper cells and their association with allergic airways disease (34-36, 38), it became clear that a more thorough characterization of the cytokines produced by the transduced T cells in our model would have strengthened our ability to understand the link between IL-17 and Th2 (and possibly Th1) cytokines expressed by the $CD4^+$ T cells destined for adoptive transfer. However, even without this information our data demonstrate that $CD4^+$ T cell specific IL-17 production promotes Th2 responses in allergic airways disease.

6 Future Directions

The chapters of this thesis primarily focused on Th17 and IL-17-γδ T cell populations and their roles in allergic airways disease. IL-17-γδ T cells were identified as the major source of IL-17 in naïve mice and in recipients of recombinant IL-13 (Chapter 3), whereas both cell types combined to represent the primary sources of IL-17 in mice IP sensitized to OVA, with and without adjuvant (Chapter 2). Nevertheless, there are several major and minor sources of IL-17 in the lungs including ILC3s and alveolar macrophages. Although ILC3s are more relevant to obesity-induced models of asthma (39) and while alveolar macrophages were identified as the primary source of IL-17 in a single study (23), it may be interesting to determine their relative contributions to IL-17 production and their impact on allergic airway responses in our murine models of asthma and whether they are similarly manipulated by the use of adjuvant at sensitization.

We observed (Chapter 2) and then discussed (Chapter 5, section 5) a disconnect between the robust inflammatory response and low level cytokine/chemokine induction in the airways and lungs of IP OVA/CFA sensitized and challenged mice. We hypothesized that these mediators were upregulated following airway challenge, resulting in the influx of inflammatory cells, such as eosinophils and neutrophils,that degranulated, creating a proteolytic environment that cleaved the very mediators that regulated their recruitment, into inactive fragments, resulting in lower levels of cytokine/chemokine expression detected in the BALF of these highly inflamed mice. It would be interesting to follow up on this hypothesis by measuring the expression of various proteases in the BALF and lungs, including the polymorphonuclear-derived serine proteases, elastase, cathespin G and proteinase 3.

As of the date of the thesis submission, we found 3 research articles that identified a minor population of IL-17-expressing Th2 cells that was significantly increased in both murine models of allergic airways disease and in humans with asthma (34-36). As this minor population becomes the major IL-17-producing cellular source of IL-17 in a murine model (34), further investigation into the role of IL-17-Th2 cells is necessary. The role of IL-17-Th2 cells could be effectively addressed using the IL-17pAP2-transduced OVA-specific $CD4^+$ T cell model of allergic airways disease established in Chapter 4, and requires co-staining of transduced cells for both IL-4 and IL-17, something that was not performed in the earlier study (Chapter 4), and adoptively transferring these IL-17-Th2 cells into naïve recipients following airway challenge. Allergic airway responses in these mice can be compared to recipients of a mix of conventional IL-4^+CD4^+ (Th2) and IL-17^+CD4^+ (Th17) cells and to recipients of a pure population of Th2 cells.

However, the most interesting aspect of the IL-17 response in allergic airways disease that requires examination are the mechanisms involved and the point in the IL-17 signaling pathway when the role of IL-17 is established and therefore committed as either a positive or negative regulator of airways disease. I discussed possible mechanisms involved in determining these responses in Chapter 5, section 3 and **Fig 5.1**. Using the murine model of asthma established in Chapter 4, in which two different doses of IL-17 administered to naïve mice positively and negatively regulated IL-13-induced responses, the differential activation of the TRAF6-dependent and –independent pathways, as well as the status of phosphorylation of C/EBPβ could be investigated, thus implicating or eliminating their involvement in establishing IL-17 as a positive or negative regulator of asthma.

7 References

1. Schnyder-Candrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, et al. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203: 2715-2725.
2. Murdoch, J. R., and C. M. Lloyd. 2010. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing {gamma}{delta}T cells. *Am. J. Respir. Crit. Care Med.* 182: 464-476.
3. McKinley, L., J. F. Alcorn, A. Peterson, R. B. Dupont, S. Kapadia, et al. 2008. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* 181: 4089-4097.
4. Lajoie, S., I. P. Lewkowich, Y. Suzuki, J. R. Clark, A. A. Sproles, et al. 2010. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat. Immunol.* 11: 928-935.
5. Al-Ramli, W., D. Prefontaine, F. Chouiali, J. G. Martin, R. Olivenstein, et al. 2009. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J. Allergy Clin. Immunol.* 123: 1185-1187.
6. Sun, Y. C., Q. T. Zhou, and W. Z. Yao. 2005. Sputum interleukin-17 is increased and associated with airway neutrophilia in patients with severe asthma. *Chin. Med. J.* 118: 953-956.
7. Barczyk, A., W. Pierzchala, and E. Sozanska. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 97: 726-733.

8. Bullens, D. M., E. Truyen, L. Coteur, E. Dilissen, P. W. Hellings, et al. 2006. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir. Res.* 7: 135.

9. Cosmi, L., R. De Palma, V. Santarlasci, L. Maggi, M. Capone, et al. 2008. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J. Exp. Med.* 205: 1903-1916.

10. Zhao, Y., J. Yang, Y. D. Gao, and W. Guo. 2010. Th17 immunity in patients with allergic asthma. *Int. Arch. Allergy Immunol.* 151: 297-307.

11. Isogai, S., A. Athiviraham, R. S. Fraser, R. Taha, Q. Hamid, and J. G. Martin. 2007. Interferon-gamma-dependent inhibition of late allergic airway responses and eosinophilia by CD8+ gammadelta T cells. *Immunology* 122: 230-238.

12. Kinyanjui, M. W., J. Shan, E. M. Nakada, S. T. Qureshi, and E. D. Fixman. 2013. Dose-dependent effects of IL-17 on IL-13-induced airway inflammatory responses and airway hyperresponsiveness. *J. Immunol.* 190: 3859-3868.

13. Zhao, Y., J. Yang, and Y. D. Gao. 2011. Altered expressions of helper T cell (Th)1, Th2, and Th17 cytokines in CD8(+) and gammadelta T cells in patients with allergic asthma. *J. Asthma* 48: 429-436.

14. Xuekun, H., Y. Qintai, C. Yulian, and Z. Gehua. 2014. Correlation of gammadelta-T-cells, Th17 cells and IL-17 in peripheral blood of patients with allergic rhinitis. *Asian Pac. J. Allergy Immunol.* 32: 235-239.

15. Agache, I., C. Ciobanu, C. Agache, and M. Anghel. 2010. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir. Med.* 104: 1131-1137.

16. Chien, J. W., C. Y. Lin, K. D. Yang, C. H. Lin, J. K. Kao, and Y. G. Tsai. 2013. Increased IL-17A secreting CD4+ T cells, serum IL-17 levels and exhaled nitric oxide are correlated with childhood asthma severity. *Clin. Exp. Allergy* 43: 1018-1026.

17. Chien, Y. H., C. Meyer, and M. Bonneville. 2014. gammadelta T cells: first line of defense and beyond. *Annu. Rev. Immunol.* 32: 121-155.

18. Augustin, A., R. T. Kubo, and G. K. Sim. 1989. Resident pulmonary lymphocytes expressing the gamma/delta T-cell receptor. *Nature* 340: 239-241.

19. Ramos-Barbon, D., J. F. Presley, Q. A. Hamid, E. D. Fixman, and J. G. Martin. 2005. Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma. *J. Clin. Invest.* 115: 1580-1589.

20. Matusovsky, O. S., E. M. Nakada, L. Kachmar, E. D. Fixman, and A. M. Lauzon. 2014. CD4+ T cells enhance the unloaded shortening velocity of airway smooth muscle by altering the contractile protein expression. *J. Physiol.* 592: 2999-3012.

21. Shen, F., N. Li, P. Gade, D. V. Kalvakolanu, T. Weibley, et al. 2009. IL-17 receptor signaling inhibits C/EBPbeta by sequential phosphorylation of the regulatory 2 domain. *Science signaling* 2: ra8.

22. Song, X., and Y. Qian. 2013. The activation and regulation of IL-17 receptor mediated signaling. *Cytokine* 62: 175-182.

23. Song, C., L. Luo, Z. Lei, B. Li, Z. Liang, et al. 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J. Immunol.* 181: 6117-6124.

24. Qu, F., H. Gao, S. Zhu, P. Shi, Y. Zhang, et al. 2012. TRAF6-dependent Act1 phosphorylation by the IkappaB kinase-related kinases suppresses interleukin-17-induced NF-kappaB activation. *Mol. Cell. Biol.* 32: 3925-3937.

25. Ge, D., R. T. Dauchy, S. Liu, Q. Zhang, L. Mao, et al. 2013. Insulin and IGF1 enhance IL-17-induced chemokine expression through a GSK3B-dependent mechanism: a new target for melatonin's anti-inflammatory action. *J. Pineal Res.* 55: 377-387.

26. Oboki, K., T. Ohno, H. Saito, and S. Nakae. 2008. Th17 and allergy. *Allergol Int* 57: 121-134.

27. Wilson, R. H., G. S. Whitehead, H. Nakano, M. E. Free, J. K. Kolls, and D. N. Cook. 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 180: 720-730.

28. Conrad, M. L., A. O. Yildirim, S. S. Sonar, A. Kilic, S. Sudowe, et al. 2009. Comparison of adjuvant and adjuvant-free murine experimental asthma models. *Clin. Exp. Allergy* 39: 1246-1254.

29. Bank, U., and S. Ansorge. 2001. More than destructive: neutrophil-derived serine proteases in cytokine bioactivity control. *J. Leukoc. Biol.* 69: 197-206.

30. Bank, U., B. Kupper, D. Reinhold, T. Hoffmann, and S. Ansorge. 1999. Evidence for a crucial role of neutrophil-derived serine proteases in the inactivation of interleukin-6 at sites of inflammation. *FEBS Lett.* 461: 235-240.

31. Bank, U., D. Reinhold, C. Schneemilch, D. Kunz, H. J. Synowitz, and S. Ansorge. 1999. Selective proteolytic cleavage of IL-2 receptor and IL-6 receptor ligand binding chains by neutrophil-derived serine proteases at foci of inflammation. *J. Interferon Cytokine Res.* 19: 1277-1287.

32. Wakashin, H., K. Hirose, Y. Maezawa, S. Kagami, A. Suto, et al. 2008. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* 178: 1023-1032.

33. Newcomb, D. C., M. G. Boswell, S. Reiss, W. Zhou, K. Goleniewska, et al. 2013. IL-17A inhibits airway reactivity induced by respiratory syncytial virus infection during allergic airway inflammation. *Thorax* 68: 717-723.

34. Wang, Y. H., K. S. Voo, B. Liu, C. Y. Chen, B. Uygungil, et al. 2010. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J. Exp. Med.* 207: 2479-2491.

35. Cosmi, L., L. Maggi, V. Santarlasci, M. Capone, E. Cardilicchia, et al. 2010. Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J. Allergy Clin. Immunol.* 125: 222-230 e221-224.

36. Irvin, C., I. Zafar, J. Good, D. Rollins, C. Christianson, et al. 2014. Increased frequency of dual-positive T2/T17 cells in bronchoalveolar lavage fluid characterizes a population of patients with severe asthma. *J. Allergy Clin. Immunol.* 134: 1175-1186.

37. Bhakta, N. R., and D. J. Erle. 2014. IL-17 and "T2-high" asthma: Adding fuel to the fire? *J. Allergy Clin. Immunol.* 134: 1187-1188.

38. Brandt, E. B., M. B. Kovacic, G. B. Lee, A. M. Gibson, T. H. Acciani, et al. 2013. Diesel exhaust particle induction of IL-17A contributes to severe asthma. *J. Allergy Clin. Immunol.* 132: 1194-1204 e1192.

39. Kim, H. Y., H. J. Lee, Y. J. Chang, M. Pichavant, S. A. Shore, et al. 2014. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat. Med.* 20: 54-61.