Characterization of Immune Response in Corticosteroid-refractory Severe Asthma in Humans and Mice

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Abstract

Severe asthma (SA) remains a poorly controlled disease despite use of high doses of systemic corticosteroids (CS) although mild-moderate asthma (MMA) is responsive to low dose inhaled CS. This suggests that SA cannot be solely orchestrated by Th2 cells, which are dominant in milder disease. Analysis of broncholalveolar lavage cells isolated from MMA and SA patients revealed a significantly greater IFN-γ (Th1) immune response in the airways of severe asthmatics with lower Th2 and IL-17 responses. We modeled this complex immune response seen in human SA in mice including poor response to CS. *Ifng*-/- mice subjected to this SA model failed to mount airway hyperresponsiveness (AHR) without appreciable effect on airway inflammation. However, *Il17ra*-/- mice did not show any reduction in AHR although the mice displayed lower airway inflammation. Computer-assisted pathway analysis tools linked IFN-γ to secretory leukocyte protease inhibitor (SLPI), which is expressed by airway epithelial cells and an inverse correlation between IFN-γ and SLPI expression was detected in SA. Forced expression of SLPI in mice subjected to the SA model decreased AHR in the absence of CS, and it was further reduced when SLPI was combined with CS. Taken together, our study has identified a distinct immune response in SA highlighting a dysregulated IFN-γ-SLPI axis that impacts lung function.

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Acknowledgements

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1.0 Introduction

The human body is constantly being exposed to elements from nature, ranging from innocuous agents to harmful substances. Besides the perpetually exposed skin layer, the respiratory system (lungs) forms a constant interface with the surrounding environment. To maintain lung homeostasis, the highly evolved immune system must fight off and neutralize airway irritants. However, sometimes the body's defense mechanisms fall short of mounting an effective immune response leading to lung pathology. Lung pathologies can manifest themselves in the form of different diseases like chromic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), asthma, etc.

1.1 Asthma

Asthma is a chronic inflammatory disease of the lungs, which can make it hard for the sufferer to breathe. Asthma is characterized by reversible airflow expiratory limitation or airway hyperresponsiveness in the presence of airway inflammation (1). Asthma causes recurring periods of wheezing, chest tightness, shortness of breath, and coughing. The word comes from the Greek αα΄ζειν (aazein), which translates as "to breathe with open mouth or to pant". It first appeared in Homer's Iliad meaning short of breath, and probably was first used in a medical context by Hippocrates (2).

Asthma affects ~ 5 – 10 % of the population in many developed countries of the world(1). Approximately, 300 million people worldwide suffer from asthma. Asthma cases are on the rise worldwide, especially in developed countries. The increased incidence of asthma seen in developed countries has partly been attributed to better hygiene conditions. In 1989, Strachan proposed the hygiene hypothesis upon discovering a strong significant inverse correlation between incidences of hay fever and the number of siblings in a family. This led him to postulate that early childhood infections triggered by unhygienic conditions present in larger families offered protection against allergic diseases. This hypothesis received support from other epidemiological studies that showed neonatal exposure to endotoxin from dirty mattresses or by growing up on a farm diminished the chances of acquiring asthma in adulthood. The hygiene hypothesis functioned within the paradigm of Th1/Th2 responses; acquiring Th1 responses during childhood blunted Th2 responses in adulthood.

1.2 Asthma Phenotypes

Asthma is a heterogeneous disease owing to its complex pathology and multifactorial origin (3, 4). The heterogeneity in asthma patient population makes it harder to researchers to classify asthma. Fernando Martinez, MD, aptly summarized this when he said "It's almost as if each person is an 'n' of one (5). Clinical or physiological phenotypes relevant to asthma include those defined by level of severity (from mild to severe), the frequency of exacerbations, the presence of chronic airflow restriction, and the age of asthma onset (6). Phenotypes can also be defined on the basis of their relation to specific triggers including exercise, environmental allergens, occupational allergens and irritants, drugs (such as aspirin), and menses. Phenotypes are also categorized by their immunopathology on the basis of patterns of inflammation, specifically the presence or absence of particular inflammatory cell types - e.g., eosinophils or neutrophils (7-9) National and international guidelines are the biggest proponents of phenotyping by severity with fairly stringent criteria to define four categories of asthma severity (10)

Genetic and lung-specific biomarkers have been proposed to distinguish different phenotypes of asthma based on severity, however, few have been proven experimentally (6). A recent study that employed unsupervised phenotyping of asthma characteristics using machine-learning approaches showed that asthma subjects with the lowest lung function exhibited persistent airway inflammation and were all on systemic CS use (11). Both clinical and statistical approaches are now being used to define asthma subtypes (12).

1.3 Airway Inflammation in Asthma

At least three phenotypes of asthma have been proposed based on the type of airway infiltrate observed: eosinophilic, neutrophilic and paucigranulocytic (8, 9, 13-15). (8). These phenotypes are becoming increasingly associated with distinct clinical and physiological inflammatory and repair processes (8, 13-15).

Eosinophils form the major inflammatory cell type in more than 50% of asthma cases. Eosinophils secrete cysteinyl leukotrienes, powerful inflammatory molecules that induce constriction of the bronchioles, mucus hypersecretion, and eosinophil activation (16-19).

Neutrophils have been associated with asthma severity for several years (20, 21). Neutrophilic asthma has been reported in autopsies of patients who died soon after the onset of a

severe exacerbation (21-23). Lung neutrophilia has been associated with lower lung function, more trapping of air and airway remodeling (8, 24-27). Sputum neutrophilia was associated with severely obstructed, incompletely reversible asthma and highest healthcare usage with systemic CS use in a Severe Asthma Research Program (SARP) cluster (28). Neutrophilia has been shown to co-exist with eosinophilia and this group of asthmatics was identified with the most severe form asthma(29, 30). Presence of a mixed inflammatory phenotype suggests involvement of pathways in addition to Th2 lineage, including Th1, Th17 and even innate immunity (31-33). The role of non-Th2 pathways in asthma pathogenesis is not well understood. It has been suggested that non-Th2 asthma affects 50% or more corticosteroid-naïve individuals (34, 35).

Even though asthma has been thought of as an inflammatory disease, studies suggest that asthma can exist independently of an obvious influx of inflammatory cells like eosinophils, neutrophils and lymphocytes (8, 9). In such asthma cases, the pathogenesis of thought to involve airway structural cells such as epithelial cells and smooth muscle cells.

1.4 Airway inflammation and AHR

In a study correlating AHR with airway inflammation, the ED50 dose of dexamethasone required to inhibit AHR was higher than that needed to inhibit eosinophilia (36). Studies utilizing antibodies against IL-16 in mice (37), IL-10-deficient mice (38) and mast-cell-deficient mice show that by lowering the allergic challenge eosinophilia was lost but AHR remained. Using clinical asthma samples, it was suggested that no relevant correlation between AHR and the number of inflammatory cells in sputum or bronchoalveolar lavage existed (39).

In a neutrophilic model of asthma, depletion of neutrophils to baseline levels did not reduce AHR thus showing that AHR was not dependent on the neutrophilic influx (40).

1.5 Severe Asthma

Per asthma patient, the healthcare-related costs attributed for the welfare of SA patients are six times higher than those for mild-moderate asthma patients (41). Because of frequent hospitalizations and need for emergency care, up to 50% of healthcare costs for all asthma is spent on severe asthmatics in the US and in Europe (42, 43).

According to the ATS/ERS definition (10) severe asthma only includes patients with refractory asthma and those in whom treatment of comorbidities such as severe sinus disease or

obesity remains incomplete. Severe asthma is defined as ‘‘asthma which requires treatment with high dose inhaled corticosteroids (ICS) plus a second controller (and/or systemic corticosteroids) to prevent it from becoming ‘uncontrolled’ or which remains ‘uncontrolled’ despite this therapy.’’

Uncontrolled asthma defined as at least one of the following:

1) Poor symptom control

2) Frequent severe exacerbations: two or more bursts of systemic CS in the previous year

3) Serious exacerbations: at least one hospitalization, ICU stay or mechanical ventilation in the previous year

4) Airflow limitation: after appropriate bronchodilator withholds Forced Expiratory Volume in 1s, 80% predicted.

Some severe asthmatics present with neutrophilic inflammation in their airways (21). It has been suggested that pathways related to Th1, Th17 and innate immunity might be involved in the pathogenesis of severe asthma (32, 44, 45).

1.6 Role of T helper 2 Cells in Asthma

Based on initial studies in animal models and human T-cell clones (46) and bronchoscopic studies in patients with mild steroid-naive asthma (47, 48), asthma has been viewed as a disease driven by activated T helper type 2 (Th2) cells. The important role of Th2 cells in promoting airway inflammation and hyperresponsiveness was established using mouse models of allergic airways disease (49-51). This led to asthma being considered an allergic, eosinophil-dominated disease primarily mediated by Th2 cells (52-54).

Periostin, CLCA1 and SERPINB2 are the genes most strongly associated with a Type 2 asthma phenotype. These genes are also up-regulated by IL-13 in vitro (35). Serum levels of periostin have been reported to better predict sputum eosinophilia than other biomarkers (55). Elements of a Type 2 signature can be identified over a range of asthma severity levels and treatments (9, 35, 55, 56).

1.7 Role of Other Type 2 cytokine-producing Cells in Asthma

Th2-mediated airway eosinophilia can also be associated with innate immune mechanisms involving bronchial epithelial cells and innate lymphoid cells (57-59). High levels

of ILC2s have been found in eosinophilic asthmatic patients (60, 61). In mouse models of asthma induced by either Influenza A or the fungus Alternaria, both of which are characterized by airway eosinophilia, ILC2s were shown to be required to elicit eosinophilic inflammation independently of Th2 cells (62, 63). SA patients have been shown to have lower lipoxin levels in their serum and BALF. Lipoxin-4 is a potent inhibitor of ILC2 cells (64).

1.8 Available Therapies for Asthma

1.8.1 Corticosteroids

Corticosteroids have been the cornerstone of asthma therapy ever since it was reported that CS therapy could control asthma symptoms. Many of the beneficial effects of CS result from their action on Th2-associated cytokines and the control of the resulting inflammation. CSs are broad and non-specific in their activity (65-67). CSs are powerful inducers of eosinophil apoptosis (68). CSs cause eosinophil apoptosis by suppressing the synthesis of important eosinophil survival factors such as I-3, IL-5, and GM-CSF (69, 70). Studies have shown that the number of eosinophils decrease significantly in response to high doses of CSs and overall lung function improved (71, 72). CSs are most effective in asthma subjects with evidence of Th2 inflammation as manifested by sputum eosinophilia and airway periostin (34, 35, 73). Reducing eosinophils through titration of nonspecific anti-inflammatory therapy has been shown to decrease exacerbations (9, 74).

CSs are less effective in asthma phenotypes that lack an obvious Th2 involvement (7). Treatment resistant asthma is generally seen in patients with SA (75, 76).

Parsing of data from clinical trials of corticosteroid therapy has revealed that response of asthmatics to this therapy strongly relies on the presence and type of airway inflammation (7, 9, 77, 78). Some individuals show sputum neutrophilia mixed with eosinophils (30).

Corticosteroids have been shown to induce apoptotic cell death of airway epithelium in vivo and are also inefficient in attenuating epithelial cell shedding elicited by means of allergen challenge (79). Epithelial damage, as demonstrated on endobronchial biopsy, is seen in about half of subjects with mild asthma and in almost all subjects with persistent asthma (26). This suggests that CSs, in effect, could worsen the severe asthma phenotype.

CSs are effective in only about 70% of the general population with asthma (80). In severe asthma the proportion of patients with reduced responsiveness to inhaled and oral corticosteroids is higher than in mild-moderate disease. In 30% of severe adult asthma patients, oral corticosteroids (OCS) are required in addition to ICS to maintain some degree of asthma control (81-83). Moreover, CSs inhibit neutrophil apoptosis and have also been proposed to activate neutrophils suggesting that CS treatment itself could lead to development of neutrophilic inflammation (84, 85). One study has suggested that neutrophilic asthma responds well to macrolide antibiotics. This study showed a reduction in the expression of neutrophilic markers and improvement in quality of life, however, no improvement in FEV1 or asthma control was observed (86).

1.8.1.1 Mechanisms of CS Action

Mechanisms of actions of CSs can be grouped into two distinct modes – 1. Genomic mechanisms involving activation of anti-inflammatory genes and suppression of pro-inflammatory genes and 2. Non-genomic mechanisms.

1.8.1.1.1 Genomic mechanisms: anti-inflammatory gene transactivation

When glucocorticoid binds to its receptor (GR), GR dissociates from its cytoplasmic complex and is translocated into the nucleus. In the nucleus, the glucocorticoid-GR complex binds to glucocorticoid response elements (GREs), leading to increased transcription of anti-inflammatory genes, such as mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1), and glucocorticoid-inducible leucine zipper 1 (GILZ-1) (87).

1.8.1.1.2 Genomic mechanisms: proinflammatory gene suppression

The major action of corticosteroids in suppression of inflammation is to turn off the multiple genes that encode for various inflammatory mediators (88) At first, it was seen as a dominant mechanism for proinflammatory gene suppression, that is, glucocorticoid binds to DNA-bound proinflammatory transcription factors, such as NF-κB and activator protein-1 (AP-1). Another important mechanism of GR action is the recruitment of histone deacetylase 2 (HDAC2) to the activated inflammatory gene complex by ligand-bound GR with suppression of multiple activated inflammatory genes (89).

1.8.1.1.2 Non-genomic mechanisms

Glucocorticoid can increase the production of nitric oxide (NO) that displays multiple functions, including anti-inflammatory property (90) It can also induce the phosphorylation and extracellular release of annexin-1 (91). There is recent evidence for an important role of extracellular annexin-1 as an inflammation-resolving protein, acting on the formyl peptide receptors FPR1 and FPR2 (92).

1.8.1.2 Molecular mechanisms of CS resistance

1.8.1.2.1 Changes in GR

GR phosphorylation effected by several kinases can lead to reduction in GR function through altered binding, stability, nuclear translocation, and interaction with other proteins, including transcription factors and molecular chaperones (93). Exposure to cytokines, such as IL-2, IL-4, and IL-13, and subsequent activation of mitogen-activated protein kinase (MAPK) have been found to induce GR resistance in inflammatory cells through inhibition of GR ligand binding (3). In fact, a p38 MAPK inhibitor inhibits phosphorylation of serine 226 (Ser226) on GR, which is induced by IL-2 and IL-4, and this effect is mostly observed in peripheral blood mononuclear cells (PBMCs) from asthmatics (94).

MKP-1, an endogenous inhibitor of p38 MAPK signaling, is activated by corticosteroids. In alveolar macrophages obtained from severe asthmatic patients with reduced MKP-1 expression as well as murine macrophages from *MKP1* knockout mice, reduced steroid responsiveness has been found (95) In addition, the serine/threonine phosphatase protein phosphatase 2A (PP2A) is involved in the dephosphorylation of phosphorylated GR (96) Supporting this contention, PP2A expression and activity are reduced in PBMCs from patients with steroid resistance, and knockdown of PP2A or okadaic acid, an inhibitor, reduces steroid responsiveness and GR Ser226 dephosphorylation with nuclear translocation.

Microbial origin stimuli can induce steroid resistance in airway inflammatory cells. Staphylococcal enterotoxin B induces steroid resistance in human T cells *in vitro* through activation of the extracellular signal-regulated kinase (ERK) pathway linked to GR phosphorylation (97) Additionally, interferon (IFN)-γ inhibits GR nuclear translocation through activation of the TLR4/ MyD88 pathway in murine pulmonary macrophages (40).

Activation of TLR7 and TLR9, receptors for viral nucleic acids, induces steroid resistance in plasmacytoid dendritic cells (DCs) from patients with systemic lupus erythematosus (SLE) and 2 lupus-prone animal models (98).

GR can be nitrosylated by NO donors, resulting in reduced binding affinity for corticosteroids (99) SA patients produce high levels of NO, which potentially nitrosylates the GR at the HSP90 binding site, resulting in a decrease in the affinity of GR to glucocorticoid as well as HSP90 (3).

1.8.1.2.2 GR isoform identity and expression

GRα predominates in most cell types but other isoforms exist as a result of alternative splicing of GR mRNA. Responsiveness to glucocorticoids can be modulated by the relative levels of the expression of each GR isoform (100). GRβ has been shown to act as a dominant negative inhibitor through various mechanisms, including binding to GRE, formation of a heterodimer with GRα, interruption of nuclear translocation of GRα, interaction with transcriptional factors (101). Exposure to cytokines increases the expression of GRβ in airway epithelial cells and various inflammatory cells (102). Polymorphisms of GRβ have been associated with a reduced response to corticosteroids (103).

1.8.1.2.3 Activation of proinflammatory transcription factors

The transcription factors NF-κB, STAT5, and AP-1 have been implicated in the occurrence of steroid resistance. AP-1, a heterodimer of Fos and Jun protein, may be the most important transcriptional factor associated with steroid resistance of asthma given its ability to physically interact with GR, thereby preventing its binding to GREs Inflammatory cells from steroid-resistant asthmatics have been shown to possess high levels of AP-1, phosphorylated

JNK, and c-Fos (104) NF-κB activation is correlated inversely with glucocorticoid responsiveness in patients with severe asthma, and STAT5 is known to be implicated in defective GR nuclear translocation in HT-2 cells (105). It has also been suggested that activation of Type I interferon-activated IRF-1 or TNF-α may contribute to steroid resistance in airway structural cells (106).

1.8.1.2.4 Defective histone acetylation

Recruitment of HDAC2 to activated inflammatory genes is a major mechanism of inflammatory gene repression by glucocorticoids. In some diseases that are poorly responsive to steroids, decreased expression of HDAC2 is observed (107). There is strong evidence connecting decreased HDAC2 activity with steroid resistance. Low levels of HDAC2 expression have been reported in PBMCs and alveolar macrophages from refractory asthmatics and in airways of smoking asthmatics (108) Both oxidative and nitrative stresses play a crucial role in reducing HDAC2 expression, which enhances formation of peroxynite that nitrates tyrosine residues of HDAC2, thereby leading to its degradation (109).

1.8.1.2.5 Epigenetic mechanisms

Weiss and colleagues examined 31 single nucleotide polymorphisms and 14 candidate genes in the corticosteroid pathway in patients with asthma undergoing a steroid intervention trial and have identified one gene, corticotrophin-releasing hormone receptor-1 (CRHR-1), which contained a polymorphism associated with corticosteroid responsiveness in three different asthmatic populations (110).

MicroRNAs, another class of epigenetic regulatory molecules, can induce steroid resistance by promoting the degradation of mRNA and suppressing the translation of GCR proteins (111-113).

1.8.1.2.6 Immune mechanisms

Murine Th17 cells have been shown to be resistant to steroid action (44). In addition, IL-17 increases the expression of GRβ in airway epithelial cells (102). Corticosteroids stimulate secretion of IL-10, an anti-inflammatory and immune-regulatory cytokine, and the decreased

secretion of IL-10 in regulatory T cells has been reported in patients with steroid-resistant asthma (114).

1.8.1.2.7 Comorbidities

Corticosteroid insensitivity has been associated with different comorbid conditions such as obesity (115), smoking (116), low vitamin D levels (114), and non-eosinophilic (low-Th2 inflammation) mainly in adults (34).

1.8.1.2.8 ER stress

Several inhaled environmental triggers like cigarette smoke diesel exhaust, or allergens are known inducers of ER stress (117) Moreover, accumulating evidence has suggested the implications of prolonged ER stress and UPR in the development and progression of chronic lung diseases, including chronic obstructive pulmonary disorders (COPD), and bronchial asthma. ER stress is important in the pathogenesis of bronchial asthma, especially steroid-resistant bronchial asthma at least in part through modulation of NF-κB (118). Modulation of ER stress can overcome refractoriness of neutrophil-dominant asthma to steroids. Using an ER stress inhibitor, 4-phenylbutyric acid (PBA), airway hyperresponsiveness (AHR) and inflammation were significantly diminished through inhibition of nuclear translocation of NF-κB in a mouse model of neutrophil-dominant asthma, Administration of 4-PBA, an ER stress inhibitor, dramatically reduced the increased expression of IL-17, whereas it further enhances the increase in IL-10 levels, resulting in attenuation of asthmatic features (118).

1.8.1.2.9 Oxidative and Nitrative Stress

Both oxidative and nitrative stresses contribute to the development of steroid resistance in chronic airway inflammatory disorders through several mechanisms, including enhancement of proinflammatory transcriptional factors, such as NF-κB, AP-1, and HIF-1α, and a reduction in HDAC2 expression/activity (109).

1.8.1.2.10 Microbiome

The idea that the airways are sterile is rapidly changing with improvements in techniques

of sampling bronchial space. A recent study (119) has found that the airways of asthmatics whose asthma symptoms are poorly controlled by CSs, harbor members of the genus Comamonadaceae, which are known to metabolize steroidal compounds. In another study, distinct bacteria were found to be expanded in 14 subjects with CS-resistant asthma, and these were not present in subjects with CS-sensitive asthma. High level of p38 MAPK activation and reduced cellular responses to corticosteroids were observed in both peripheral blood monocytes and BAL macrophages in the presence of *H. parainfluenzae*. Activation of TAK1 by bacteria was shown to be essential for alteration of cellular responses to corticosteroids since TAK1 inhibitor–treated cells were steroid sensitive despite incubation with *H. parainfluenzae* (120).

1.8.2 Treatments targeting Th2 mediators

Treatment with molecules that target components of the Th2 pathway such as antibodies to IgE, IL-4Rα blockers and anti-IL-13 strategies, in individuals with mild-moderate asthma has confirmed the relationship of Th2 pathways in asthmatics responses (121-123). An IgE-specific antibody is the only biological treatment approved for asthma control. More specifically targeting Th2 immunity, an IL-4 variant was shown to improve physiological responses to allergen inhalation in mild corticosteroid-naïve asthma (123). Targeting GATA3, the master transcription factor controlling the differentiation of Th2 cells, reduced allergic airway disease in mouse models with preliminary evidence to support efficacy in an allergen challenge model in humans as well (50, 124-127).

Specific Th2 pathway inhibition in non-phenotyped, corticosteroid-treated individuals with chronic asthma was shown to be generally ineffective in controlling asthma symptoms (54). When a Type 2 biomarker is utilized to identify a Type 2 asthma phenotype, all recent studies of Type 2 cytokine targeted therapies have been clinically successful. When mepolizumab, a monoclonal antibody to IL-5 that had failed in previous nonselective asthma studies, was targeted to patients with moderate to severe asthma with historical sputum eosinophilia, a significant reduction in asthma exacerbations occurred over a period of a year (128). There were no effects on other phenotypic characteristics, such as symptoms or lung function. Studies targeting IL-4, alone or in combination with IL-13, although efficacious in early small trials, were not successful in larger studies (52, 129, 130). A mutant IL-4 (pitrakinra) that blocked the IL-4 receptor complex and a monoclonal antibody to IL-13 showed efficacy in inhaled allergen

challenge studies. An antibody to the IL-4 receptor was not effective in unphenotyped moderate asthma (129). Targeting IL-4/IL-13 has been beneficial in the presence of a typical Type 2 inflammatory process (130, 131).

Asthma is traditionally considered a Th2 disease, however, it is now being realized that not all asthma cases fit this traditional view (28, 132). The lack of efficacy of Th2-targeted therapies in certain asthmatic individuals (with or without corticosteroids) further bolsters the belief that a subset of asthma exists with no involvement of Th2 immunity (54, 129, 133). Apart from immune cells secreting Type 2 cytokines, other major immune inflammatory players include cells secreting IFN-γ (Th1 cells) and IL-17 (Type 17/Th17). These cells are typically elicited in response to intracellular and extracellular infections, respectively. It is increasingly being recognized that the complexity of asthma results from the superimposition of allergenic exposure along with viral or bacterial infection.

1.9 Role of Infections in Asthma Pathogenesis

Asthma patients are susceptible to airway microbial burden, and both bacterial and viral components act as pathogen-associated molecular patterns (PAMPs), which are recognized by Toll-like receptors (TLRs) (134, 135). The prevalence and effects of respiratory tract infections and activated immune pathways involved in innate host defense strongly suggest a causal role for microbes in asthma pathogenesis (21, 136-142) With the availability of gene-based detection methods most exacerbations of asthma seem to be virus related, with common cold viruses being especially important (143, 144). Respiratory viruses might also be important in the origins and persistence of asthma (145). Viral infections have been proposed to be involved in eliciting neutrophilic inflammation (146-149). Virus-induced exacerbations accompanied by lower-airway neutrophilia (149) are relatively refractory to corticosteroids, and have been linked to increased asthma mortality (150).

Certain bacteria are also associated with exacerbations of asthma, especially *Chlamydia pneumoniae* and *Mycoplasma pneumonia* (83, 151). In the study by Wark and colleagues (149) more than a third of patients with acute severe asthma showed a rise in *C pneumoniae*-specific antibodies and these patients had a more intense inflammatory response with marked sputum neutrophilia and raised serum concentrations of eosinophil cationic protein compared with people with acute asthma in whom *C pneumonia* antibodies were not detected. The presence of

these microorganisms in sputum and lung biopsies is also associated with disease severity and a poor clinical outcome. Positive *Haemophilus influenzae* and *Pseudomonas aeroginosa* cultures were reported in sputum samples of severe asthmatic patients without evidence of bronchiectasis and from those with a long duration of asthma and exacerbations in the past year (152).

There is mounting evidence that microbial infection of the airway early in life may also predispose to more severe forms of asthma. Neonatal infection of the airways with *Streptococcus pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, or combinations of these infections leads to recurrent wheeze and asthma. Furthermore, *Chlamydia* respiratory infection in childhood is increasingly linked with severe asthma (141, 142, 153-155). Infections respiratory infections are also the most frequent trigger of exacerbations of asthma, which are predominantly characterized by the activation of macrophages and neutrophils in sputum and BAL (156-158) and poor responsiveness to therapy (154, 155, 158). Patients with more severe, steroid-resistant, neutrophilic forms of asthma have chronic airway colonization with bacteria, and *H. influenzae* is one of the more commonly isolated species (159). Respiratory infection with *Chlamydia* in early life or in adulthood (142, 153, 160) or with *H. influenzae* in adulthood (137, 138) increases the severity of allergic airway disease, which is steroid-resistant in mice.

Some nontubercular *Mycobacteria* have been associated with severe airflow obstruction and greater use of corticosteroids (161). Interestingly, some *Mycobacteria* species have been shown to produce c-di-GMP for biofilm formation (162). To combat viral and bacterial infections, the host elicits Th1 and/or Th17 immune responses. IFN-γ and IL-17 are the signature cytokines of the Th1 and Th17 cell types, respectively.

Viral and bacterial infections may synergize with allergens to contribute to the induction of asthma, enhance the severity of the disease, and alter the cellular profile of the inflammatory response in lungs (163-165). Th cells of an intermediate phenotype (secreting IFN-γ and Th2 cytokines) can induce pronounced AHR in such settings (166). It has been suggested that innate host defense pathways may contribute to the pathogenesis of severe asthma, as well as to the features of disease in individuals who are refractive to anti-inflammatory therapy with glucocorticoids (8, 167). Viruses and bacteria exacerbate existing asthma possibly through type II interferon and type 17 cytokine mechanisms (168, 169).

1.10 Role Of Th17 Cells In Asthma

One of the distinguishing characteristics of these non-Type2 phenotypes, even in milder disease, is their relative CS refractoriness, perhaps attributable to lack of CS-responsive inflammatory elements (170-172). The lack of CS response with neutrophilia has suggested that the IL-17 pathway may be more important in some poorly controlled non-Type 2 molecular phenotypes (32, 44, 136). Expression of IL-17 family members has been reported to be high in severe asthma. These cytokines have also been associated with poor lung function but not necessarily with any clinical parameters (31, 173). Neutrophilic inflammation associated with SA has been attributed to Th17 lymphocytes (174, 175). IL-17A and IL-17F levels correlated with asthma severity, especially in subjects with neutrophilic, steroid-resistant disease (136). Allergens and other stimuli such as cigarette smoke and airborne particulate pollutants have been shown to induce Th17-mediated airway inflammation in human subjects. Moreover, cigarette smoking is often associated with neutrophilic inflammation of the airways, more severe asthma, and CS insensitivity (148, 176). IHC and gene expression studies in asthmatics reveal increased IL-17 in lung tissue and sputum from SA patients as compared to mild-moderate and control subjects (173, 177). On the other hand, some studies suggest that IL-17 may actually have protective effects, through important host defense mechanisms (178).

IL-17 family of cytokines can be produced by a myriad of other cell types including γδ T cells, CD8 T cells, NK cells and type 3 ILCs (ILC3). ILC3s have been detected in the VALF from patients with SA (59, 179). IL-17A and IL-17F, acting via IL-17RA and IL-17RC on epithelial cells, stimulate airway structural cells to secrete neutrophil chemoattractants such as IL-8/CxCL8 and CxCL1 (180-183). IL-17A increases airway smooth muscle proliferation and contraction suggesting it can play a role in smooth muscle hyperplasia and AHR (184, 185).

Studies of mouse models have delineated pathways by which Th17 cells could play a role in asthma pathobiology. Transfer of antigen-specific Th2 cells into mice challenged with the same antigen led to eosinophilic airway inflammation, which was corticosteroid-responsive. Whereas, transfer of Th17 cells resulted in neutrophilic inflammation that was resistant to CS treatment (44).

According to a placebo-controlled study in moderate-to-severe uncontrolled asthmatics, evaluating the effects of Brodalumab, a human IgG2 anti-IL-17RA monoclonal antibody that blocks the activity of both IL-17A and IL-17F, some clinical benefits were detected in a

subgroup of patients exhibiting a high degree of airflow limitation in response to bronchodilator inhalation (186). However, the reported decrease in symptoms was not paralleled by a significant improvement in respiratory function. Also, consistent with the role of IL-17 in imparting immunity against infectious agents (187), the use of Brodalumab increased airway respiratory infections. Other recent data also suggests that an active Type 17 immune response might actually contribute to less severe disease (188-190).

1.11 Role of Th1 cells in SA

Th1 cells have also been implicated in the causation of neutrophilic asthma (191, 192). IFN-γ and TNFα are increased in patients with SA suggesting that IFN-γ can mediate airway neutrophilia (192, 193). CD8+ T cells are known sources of IFN-γ. Tissues from fatal asthma lungs have been shown to have increased CD8+ cells compared to tissue from control or mild asthma patients with increase in IL-18, an inducer of IFN-γ (194). In a recent study, application of Bayesian Network Algorithm showed high connectivity between the asthma severity node and nodes for mast cell mediators and IFN-γ–secreting CD8+ cytotoxic T cells (195). Th1 lymphocytes and the corresponding cytokines IFN-γ are increased in SA. Tissue biopsies of airway submucosa and BAL cells from SA patients show increased IFN–γ-expressing cells, compared with that from moderate asthmatics and healthy controls (33, 196). IFN-γ has been identified in BAL fluid and serum of asthmatic patients, suggesting that Th1-like cells may in fact contribute to, rather than inhibit, the pathology in asthma (197, 198). IFN-γ has been linked to the severity of asthma (83). Elevated levels of IFN-γ are found in serum and sputum in atopic asthmatics (199, 200). IFN-γ has been implicated in airway remodeling (201). IFN-γ /LPS-induced responses are steroid-resistant (202, 203). These discoveries are supported by earlier in vitro findings, suggesting that “cross-talk” between IFN-γ and TLR4-dependent pathways can regulate inflammatory networks in macrophages (204, 205), and block the anti-inflammatory effects of glucocorticoids in human monocytes (206). Goleva et al. (139) showed that steroid-resistant asthmatic patients have increased expression of M1 and decreased expression of M2 markers on macrophages in BALF indirectly suggesting that IFN-γ might be a player in SA since M1 macrophages are activated by IFN–γ.

Increased IFN-γ has been associated with elevations in both iNOS and Duox2, key nitrative and oxidative-stress enzymes, respectively (196). In vitro, the combination of IFN-γ and IL-13 increased the expression of both iNOS and DUOX2. A GWAS study performed as part of SARP identified 4 SNPs in Th1 pathway genes (IL12a, IL12rRB, STAT4 and IRF2) significantly associated with FEV1% predicted and asthma severity (207).

Antigen-specific Th1 cells have been shown to cause severe airway inflammation in mouse models of allergic airways disease (208). IFN-γ has also been shown to enhance mast cell responses. IFN-γ-induced chemokine CxCL10 is a known mast cell chemoattractant to smooth muscles (201, 209). It has been suggested that as the disease progresses in severity, blockade of a Th-1 cytokine in clinical asthma might be most beneficial at the severe end of the disease spectrum (210-212).

TNFα increases AHR, mucus production and expression of adhesion molecules. It may synergize with IL-17 to enhance neutrophil recruitment (213). However, a large study carried out in patients with severe asthma receiving Golimumab, a human anti-TNFα Mab, did not reveal any improvement in lung function and disease exacerbation. Also, golimumab was associated with complications ranging from sepsis to cancer (214). Etanercept, a soluble TNFα receptor, improved outcomes in patients with SA (193).

Downstream signaling for both Type II interferons and Type 17 cytokines in the lung involves airway epithelial cells and smooth muscles cells, which form the structural cell milieu of the lung.

1.12 Involvement Of Structural Cells In Severe Asthma

The complex nature of SA suggests that apart from involving immune elements, this phenotype includes changes in the structural components of the airways. Pathology of SA represents a mix of inflammatory and airway remodeling elements. Airway remodeling refers to structural changes in the airway walls and is thought to be caused by repeated cycles of injury, inflammation, and abnormal remodeling events in order to restore homeostasis. Though inflammation itself can cause structural changes, recent studies have attributed physical forces such as bronchoconstriction as a trigger for airway remodeling (215). Resident airway cells such as epithelial, fibroblast and smooth muscle cells are increasingly recognized as modulators of inflammation and remodeling. Asthmatic epithelium is phenotypically different and more

susceptible to external stimuli as compared to normal epithelium (216). Structural changes established over time may not be diminished by the dampening of inflammation alone in established disease.

Structural alterations can affect airway mechanics, while structural cells can also contribute to inflammatory processes through release of cytokines, chemokines, growth factors and extracellular matrix elements (217). First, the epithelium in severe asthma is reported to be thicker than in mild-to-moderate asthma (218), with altered proliferation, apoptosis and release of proinflammatory factors (219). Second, autopsy and biopsy studies have linked an increased amount of airway smooth muscle to asthma severity, airflow obstruction and bronchial hyperresponsiveness (220-224). Persistent AHR may be dissociated from inflammatory cell recruitment (225), implying that resident pulmonary cells may contribute to this response.

GWAS studies suggest that genes frequently associated with severe asthma are epithelium-related than allergy-related (226). It has also been suggested that certain asthma subjects have absence of airway inflammation. In such cases, the airway might be inherently more reactive and the bronchial hyperreactivity maybe independent of immune players (34, 227).

Many allergens have intrinsic protease activities that lead to their deleterious effects on the airway epithelium. Innate immune cells such as phagocytes and neutrophils produce proteases to degrade ingested foreign material and permit cell motility through the extracellular matrix (228). Lung epithelium of healthy individuals is protected from the harmful effects of neutrophil elastases by a battery of anti-proteases (229).

1.13 Role Of Anti-Proteases In Host Defense

To neutralize any excess proteases and protect host tissues, the host secretes anti-proteases. SLPI is an inhibitor of human neutrophil elastase (HNE) and Cathepsin G. SLPI has been shown to inhibit a spectrum of proteases (including HNE, cathepsin G, trypsin, chymotrypsin and chymase), its main action is likely the inhibition of elastase (230, 231)SLPI is the major protease of the upper airways and compromise of the antiprotease activity of SLPI by cleavage or oxidation due to smoking (230) has been suggested be a contributor to the pathogenesis of emphysema (232).

Human SLPI is an 11.7 kDa cationic non-glycosylated protein containing 107 amino acids, and orthologues have been demonstrated in mice, rats, pigs and sheep (233-235). It

consists of two highly similar WAP ('whey acid protein')/four-disulphide core domains. SLPI is constitutively expressed at many mucosal surfaces and is produced by a variety of epithelial cells, including respiratory, intestinal and amniotic epithelia (236-238). Production of SLPI mRNA and protein has also been noted in various inflammatory cells, including mast cells (239), neutrophils (240) and macrophages (241). SLPI production in numerous epithelial cell types has been noted in response to LPS, IL-1, TNF-α, EGF (epidermal growth factor), α-defensins and HNE (human neutrophil elastase) (236-238, 242-245). Anti-inflammatory cytokines such as IL-6 and IL-10 are capable of inducing SLPI production in macrophages (246). SLPI is cleaved and inactivated by the cysteine proteinases cathepsin B, S, and L (247).

1.13.1 SLPI As An Anti-Inflammatory Mediator

SLPI has intrinsic anti-inflammatory properties that seem to be independent of their ability to inhibit proteases (248). SLPI is thought to inhibit the breakdown of key activation regulatory proteins such as I□B, (inhibitor of NF□B), and IRAK (IL-1-receptor-associated kinase) by the ubiquitin proteasome pathway. This blocks their release from the NF□B complex, thereby preventing the entry of NFκB into the nucleus and the subsequent activation of pro-inflammatory genes. SLPI also interferes with the NFκB cascade in the nucleus by competing with p65 for the binding of the NFκB binding sites in the promoter region of pro-inflammatory genes such as IL-8 and TNFα thus inhibiting their transcription (249). SLPI has been shown to reduce eosinophil and neutrophil recruitment in models of eye and joint inflammation respectively (250, 251) SLPI has been shown to reduce histamine release from human lung, tonsil and skin mast cells (252).

SLPI binds bacterial LPS thus inhibiting the responses of macrophages to LPS, partly by blocking the transfer of LPS to CD14 and thus the uptake of LPS-CD14 complexes. Furthermore, SLPI can up-regulate macrophage production of the anti-inflammatory/repair type cytokines TGF-β and IL-10 (253). In this regard, it is notable that TGF-β can down-regulate SLPI production from cultured human bronchial epithelial cells (254) since this may represent an autoregulatory loop whereby SLPI directs production of a resolution-promoting cytokine milieu which, in turn, limits SLPI production to ensure a measured decline of inflammation. SLPI is part of the mucosal host defense of anti-microbial peptides (255-257)

1.13.2 SLPI In Lung Diseases

Low SLPI levels are found in individuals with frequently reported COPD (chronic obstructive pulmonary disease) exacerbations (258). SLPI can reduce tissue damage in several models of lung fibrosis and emphysema (259-261), and this action cannot be entirely explained by SLPI's anti-elastase activity (259). SLPI has previously been associated with suppression of asthma features in mouse models (262, 263). Higher tryptase levels in CS-insensitive patients increased numbers of tryptase-positive mast cells infiltrating the airway smooth muscle in patients with mild and severe asthma have been correlated with airway hyperresponsiveness (264, 265).

1.13.3 Therapeutic Uses Of SLPI

Aerozolized rSLPI has been evaluated in sheep and in healthy patients with CF (266, 267), where it was shown to reduce elastase activity and the levels of the neutrophil chemoattractant, IL-8. However, the limitation of aerosolized rSLPI as seen in the CF study was that rSLPI proved to be effective only in the well-ventilated areas of the lungs (268).

2.0 SPECIFIC AIMS

Severe asthma (SA) constitutes only 5-10% of all asthmatic population but accounts for nearly half of the healthcare costs and morbidity associated with the disease. SA patients remain symptomatic despite treatment with high doses of glucocorticoids (GCs), the most effective treatment for asthma. The presence of neutrophils in asthma has been correlated to refractoriness to GCs, fatal exacerbations and severity. To date, our understanding of the pathological mechanisms central to neutrophilic SA is minimal primarily because of the lack of a suitable animal model, limiting development and testing of new therapeutic options.

T cells play a causal role in asthma by secreting pro-inflammatory cytokines. T helper 2 (Th2) cytokines have been implicated in the pathogenesis of mild-moderate asthma (MMA; characterized by eosinophilic airway inflammation), whereas the role of Th17 cytokines in neutrophilic inflammation is emerging. Although, high IFN-γ expression has been associated with the severity of the disease in human asthma, its role in SA has not been addressed adequately. Studies in our lab have revealed that despite treatment with high doses of GCs, patients with SA display a high IFN-γ response accompanied by a dominant Th17 response in their airways. Based on these observations, we hypothesize that Th17 cells and IFN-γ synergistically mediate the steroid resistance in neutrophilic severe asthma. To address our hypothesis, we will –

Aim 1: Develop and characterize a stable mouse model of steroid-refractory neutrophilic airway inflammation.

Preliminary experiments from our lab show that superimposition of allergen exposure with microbial pathogen-associated molecular patterns (PAMPs) in mice increases neutrophilic airway inflammation in a temporal fashion. To mimic human SA phenotype in mice, we propose to -

a. Develop a mouse model of neutrophil-prominent airway inflammation with chronic exposure to the allergen house dust mite (HDM) with infection mimcs.

b. Study the effect of GCs on cardinal features of asthma including airway inflammation, mucus hypersecretion and airway hyperresponsiveness (AHR) in our model.

Aim 2: Characterize the Th responses in steroid resistant neutrophil-prominent SA.

Our preliminary data from human asthmatic samples shows that CD4+ T cells from the bronchoalveolar space of SA patients express relatively higher IFN-γ and IL-17 as compared to those from MMA subjects. In vitro, IFN-γ has been shown to inhibit the effects of GCs. Also, Th1 and Th17 cells have been shown to be steroid-refractory. This leads us to hypothesize that IFN-γ and IL-17 synergistically lead to steroid resistance in SA. Thus, we will -

a. Assess the expression of Th cytokines in the lungs of mice subjected to the SA model in the presence or absence of dexamethasone (Dex), a synthetic GC.

b. Study effects of cytokine deficiency (IFN-γ & IL-17A) on features of SA.

Aim 3: Study the mechanisms of CS-insensitive SA phenotype by applying bioinformatics tools to SA mouse model and human samples.

IFN-γ & IL-17A are factors involved in immune defense against intracellular and extracellular pathogens, respectively. Thus, targeting these molecules for asthma therapy could potentially make patients susceptible to microbial infections. However, targeting molecules downstream in IFN-γ and/or IL-17 signaling cascade bypasses the harmful effects of blocking IFN-γ and IL-17 directly. To achieve this, we will –

a. Identify molecules in IFN-γ and/or IL-17 signaling cascades using Ingenuity Pathway Analysis (IPA) that can be targeted for therapeutic purposes.

b. Study the expression of these molecules in the human and mouse SA.

c. Modulate the expression of these molecules in mouse SA model to study the effect on asthma features.

3.0 METHODS

Human Subjects

Male and female nonsmoking (<10 pack year and no smoking in the previous year) subjects meeting the ATS 2000 definition of severe asthma (269) and milder asthma subjects on no or lower doses of inhaled CS (Forced Expiratory Volume 1 >60% predicted with no history of frequent or severe exacerbations of asthma in previous year) of all racial/ethnic backgrounds and between the ages of 18 and 60 yrs old were recruited. Subjects underwent extensive baseline characterization, including physiologic (spirometry, diffusing capacity and PC20), allergic and clinical characterization. Subjects also underwent bronchoscopy per published protocols and consistent with procedures practiced in the severe asthma research program (270).

Human BAL fluid processing and Flow Cytometry

Red blood cells in BAL fluid were lysed using ammonium chloride solution. After washing, total cell numbers were determined. 1 million cells/mL were cultured *ex vivo* with antibody (cocktail of anti-CD3/CD28/CD2)-coated beads from a T cell activation/expansion kit (Miltenyi Biotec, Cat # 130-091-441) at a ratio of 1 bead per 50 cells. After 48 h, cell culture supernatants were collected, centrifuged and stored at -80°C until analysis. A separate group of cells was stimulated with 50 ng/mL phorbol myristate acetate (PMA, Sigma Aldrich, St. Louis, MO) plus 500 ng/mL ionomycin (Sigma Aldrich, St. Louis, MO) for 6 h with Golgi Block (BD Biosciences) added for the last 3 h. Cells were washed and processed for staining with fluorochrome-conjugated antibodies. Isotype control antibodies as well as cytokine-specific antibodies were used each time cells were used to assess cytokine production. Briefly, after cells were stained for CD4 and the relevant isotype controls, a lymphocyte gate was first applied based upon forward versus 90-degree light scatter of the whole population upon which a CD4 gate was subsequently drawn. On the selected CD4 population, the fluorescence intensity that represents the minimal cut-off/threshold was determined for each fluorescence channel using the relevant isotype control for each cytokine. Finally, this was then applied to test samples stained with antibodies against the cytokines.

Mice

BALBc/ByJ, C57BL/6J, *Ifng*-/- mice on BALB/c background (Stock # 002286), BALB/cJ (Stock # 000651; WT control for *Ifng*-/- mice) and *Stat1*-/- mice on C57BL/6J background (Stock # 012606) were purchased from The Jackson Laboratory (Bar Harbor, ME). *Il17ra-/-* on BALBc/ByJ background mice were a kind gift from Dr. Jay Kolls and were obtained through Taconic Farms. *Stat1*-/-, *Ifng*-/- and *Il17ra* -/- mouse strains were bred at the Department of Laboratory Resources (DLAR) at the University of Pittsburgh. Mice were housed under pathogen-free conditions and used between 8-10 weeks of age. Age-matched male mice were used in all experiments.

Asthma models

For the severe asthma model (SA), mice were sensitized with 25 μg House Dust Mite (HDM, low-endotoxin, Greer Laboratories, NC, Cat # XPB70D3A2.5) and 5 μg c-di-GMP (Biolog Inc., Germany, Cat # C 057-01) by the intranasal (i.n.) route on days 1,3 and 5. Mice were then rested for 5 days and then subjected to 3 challenge sets involving 3 consecutive challenges with HDM and c-di-GMP with a rest of 4 days in between each challenge set. 0.5-μg c-di-GMP was instilled with HDM on the first day of each challenge set followed by 25 μg HDM only in the next 2 challenges. The mild-moderate asthma model (MA $Th1^{lo}Th2$) followed the same sensitization and challenge scheme except that c-di-GMP was not used in this model. For the Th2 mild-moderate asthma model (MA Th2), 25 μg HDM was instilled via the i.n. route on days 1-10. Mice were then rested for 5 days and then subjected to 2 HDM challenge sets each involving 25μg HDM i.n instillation on 3 consecutive days. For all 3 models outlined above, dexamethasone (Baxter Inc.) at a concentration of 4 mg/kg was given intraperitonially (i.p.) starting on the first day of the challenge and then repeated every 3rd day. Mice were sacrificed 24 h after the last challenge.

Bronchoalveolar lavage (BAL) and differential cell counts

24 h after the final allergen challenge, mice were anesthetized with Ketamine and Xylazine given intraperitoneally. Anesthetized mice were tracheostomized and the left bronchus in each mouse

was tied off and the right lobe was lavaged with 0.7 mL of sterile PBS to obtain BAL fluid. BAL fluid was immeadiately placed on ice until determination of cell counts. Lavaged lungs were then stored in Safefix II (Fischer Scientific, Pittsburgh, PA) for 48 h for fixation and then transferred to 70% Ethanol until paraffin embedding for PAS staining. Total BAL cell numbers were determined using Trypan Blue staining and standard light microscopy. 75000 BAL fluid cells were cytospun onto clean glass slides for differential cell counts using Giemsa staining as previously described(50).

Brushing method for harvesting murine airway epithelium

Brushes were made from 60-grit sandpaper–polished polyethylene PE-10 tubing (BD). The tube was inserted into the right main and left main bronchus with gentle brushing and immediately placed in Buffer RLT (Qiagen) for RNA preparation.

RNA Isolation and cDNA preparation

Lungs were dissociated in TRIzol (Life Technologies) using a high-speed homogenizer. Debris were pelleted by centrifugation at 3000 rpm for 10 mins and the supernatant was frozen at -80 C until further use. RNA was isolated using RNeasy kit (Qiagen) and treated with RNase-free DNase (Qiagen). Integrity of the RNA was analyzed with Agilent Bioanalyzer (Santa Clara, CA). Concentration of RNA was determined using Nanodrop (Thermo Fisher Scientific, Pittsburgh, PA).

cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Life technologies) using 500 ng RNA per 50 µl reaction according to the manufacturer's instructions.

Real-time PCR

Real-time quantitative PCR was performed using validated TaqMan Gene Expression primer and probe sets (Life Technologies) according to the manufacturer's instructions. Following primer and probe sets were used: IFNG (Hs00989291_m1), SLPI (Hs00268204_m1), CCL22 (Hs01574247_m1), HPRT1 (Hs02800695_m1), Ifng (Mm01168134_m1), Il5 ((Mm00439646_m1), Il13 (Mm00434204_m1), *Il17a* (Mm00439618_m1), *hprt1* (Mm01545399_m1), Slpi (Mm00441530_g1), Il18 (Mm00434225_m1), Il27 (Mm00461162_m1), Tnf (Mm00443258_m1), Il1rn (Mm00446186_m1), Stat3

(Mm01219775_m1), Cxcl12 (Mm00445553_m1), Ccl2 (Mm00441242_m1), Ccl22 (Mm00436439_m1), Il12p35 (Mm00434165_m1) and Il12p40 (Mm00434174_m1). qRT-PCR reactions were carried out using the ABI PRISM 7700 Sequence System (Applied Biosystems) at the Genomics Research Core at the University of Pittsburgh. Results were analyzed using the SDS 2.2.2 software. mRNA expression was calculated using the $2^{-\Delta Ct}$ method using HPRT1/hprt1 as internal reference control.

Assay of secreted cytokines

Lungs were homogenized in a buffer containing 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20, and Complete Mini, EDTA-free protease inhibitor (Roche Applied Science) and debris-free supernatant was collected for cytokine measurement and stored at -80 C until further use. Secreted cytokines in human cell culture supernatants and mouse lung extracts were measured by magnetic bead-based assays (Millipore Inc., Billerica, MA and Bio-Rad Laboratories, Hercules, CA) according to the manufacturers' instructions. Data was acquired and analyzed using Luminex automated system (BioRad Laboratories, Hercules, CA). Human SLPI protein was assayed using Human SLPI Quantikine ELISA Kit (Cat # DPI00) from R & D Systems. Levels of IL-12p40 and IFN-γ protein in supernatants from bone marrow macrophages were measured using DuoSet Elisa kits (R&D Systems) according to the manufacturer's protocol.

ELISPOT

IL-13, IL-17, IL-5 and IFN-γ ELISPOT assays were performed using kits from eBioscience as per the manufacturer's specifications. Briefly, ELISPOT plates (Millipore 96-well MultiScreen HTS) were pre-coated with the appropriate primary antibody at 4 C overnight. Enriched CD4+ T cells were plated at a concentration of $1x10^5$ per well and stimulated with PMA (25ng/ml) and ionomycin (500 ng/ml) for 24 h. Biotin-conjugated secondary antibody was used to detect the secreted cytokine. The plates were developed with Avidin-HRP and peroxidase substrate (Vectastatin). The spot forming units (sfu) were quantified using an automated ELISPOT plate reader (ImmunoSpot; Cellular Technology).

Assessment of airway hyperresponsiveness (AHR)

Mice were anesthetized with 90 mg/kg pentobarbital sodium delivered i.p., tracheostomized, and

airway mechanics was assessed using the forced oscillation technique. Data were fit to the constant-phase model using FlexiVent™, a computer controlled small animal ventilator (SCIREQ, QC, Canada). Mice were mechanically ventilated at 200 breaths/min with positive end-expiratory pressure (PEEP) of 3 cm H_2O and given approximately 10 min to acclimate. AHR was assessed by a methacholine (MCh) challenge test with increasing doses of aerosolized Mch (0, 3.125, 12.5, 25 mg/ml), as previously described (271). Briefly, a standard lung volume history was established by delivering two total lung capacity maneuvers followed by two measurements of respiratory input impedance (Z_{rs}). The ascending doses of nebulized MCh were delivered for 10 sec each followed by 3 min of Z_{rs} measurements being collected every 10 sec and then repeated for each dose.

Cell isolation and sorting

Lungs of anesthetized mice were perfused with sterile PBS, removed and digested as described previously (272). Briefly, lungs were dissociated in a collagenase-DNase suspension on a gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's protocol. Single-cell suspensions were obtained by passing the dissociated tissue through a 70 μm cell strainer (BD Falcon) and washed with PBS containing 2% FBS. Cells were then stimulated with PMA (50ng/ml) and ionomycin (500 ng/ml) for 6 h with monensin added for the last 4 h. Cells were processed for staining with fluorochrome-labeled antibodies.

Antibodies and Flow Cytometry

For staining of human single cell suspensions, antibodies to CD4 (FITC; Clone RPA-T4; Cat # 555346), IL-17A (PerCpCy5.5; Clone N49-653; Cat # 560799) and IL-5 (APC; Clone TRFK5; Cat # 554396) were purchased from BD Biosciences. Anti-IFN-γ (PE; Clone 4S.B3; Cat # 502510) was purchased from Biolegend. For staining of mouse lung single cell suspensions, antibodies to CD4 (PE-TxRed; Clone RM4-5, Cat # 562314; 1:500), IFN-γ (PerCpCy5.5; Clone XMG1.2; Cat # 560660; 1:200) and IL-17A (AF488; Clone TC11-18H10; 1:200) were also purchased from BD Biosciences. Anti-IL-13 (PE; Clone ebio13A; Cat # 12-7133-81; 1:200) was purchased from e-Biosciences. Single-cell suspensions stained with combinations of the above mentioned antibodies were acquired on FACSCalibur or FACS Aria flow cytometers (BD Immunocytometry Systems) and the data were analyzed using FlowJo software (Tree Star).

Human Bronchial Epithelial Cell Culture

Primary human bronchial epithelial cells were cultured from explanted donor lungs under an Institutional Review Board approved protocol at the University of Pittsburgh. Cells were enzymatically dissociated, expanded in growth media, and seeded onto Transwell inserts at air–liquid interface. Polarized and differentiated cells were stimulated with recombinant human IFN-γ□± anti-human IFN-γ□for 8 h. Unstimulated cells were used as controls.

Plasmid Isolation and Hydrodynamic tail vein injection

pCDNA3-SLPI was a gift from Ronny Drapkin (Addgene plasmid # 18103). Endotoxin-free plasmid was used to inject into mice. 25 μg plasmid DNA in sterile saline was injected via the tail vein in mice and sterile saline was injected in the control group. Injections were performed 1 d prior to the first challenge in each challenge set.

Ingenuity Pathway Analysis (IPA)

The Disease and Functions search module of Ingenuity Knowledge Base (Ingenuity System, Redwood City, CA) was used to identify genes involved in regulating AHR. This gene set was further narrowed down by selecting for genes that are expressed in the lung tissue, epithelial and smooth muscle cells using the Build function. From this reduced set, genes that are known to be regulated by IFN-γ in lung tissue and epithelial cells were identified by IPA.

Generation of bone marrow-derived macrophages (BMDMs) and transfection with c-di-GMP

Bone marrows were flushed out from femurs and tibias of WT and *Stat1-/-* mice using a 27 gauge needle. Single cell suspension of bone marrow cells was cultured for 7 d in RPMI 1640 (Life Technologies) supplemented with 10 ng/mL CSF (Peprotech Inc.), 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), 5mM Sodium Pyruvate (Life Technologies), 50 μM -βmercaptoethanol (Sigma) and Penicillin,Streptomycin and Gentamycin (Life Technologies). A media change with fresh addition of 10 ng/mL CSF was carried out on day 4 of culture. On day 7 of the culture, adherent cells were scraped off of the flask surface using a sterile cell scraper and used as BMDMs. BMDMs were transfected using Lipofectamine 2000

(Life Technologies) according to the manufacturer's protocol. C-di-GMP was mixed with LF2000 at a ratio of 1 µl LF2000/1 µg c-di-GMP, incubated at room temperature for 15 min, and added to cells at a final concentration of 5 µg/ml in Opti-MEM Reduced Serum Medium (Life Technologies). Culture supernatants were collected 24 hr later, centrifuged, and cell-free supernatants were stored at -80°C until further use.

Statistics

Results shown are mean values ± s.e.m. After testing for normal distribution of the populations, one-way ANOVA with Tukey's post hoc test was used for multiple pairwise comparisons. Student's unpaired two-tailed *t* test was used for comparisons involving two groups. Differences between groups were considered significant when $p \leq 0.05$. All statistical analyses were performed using GraphPad Prism 5 software (La Jolla, CA).

4.0 RESULTS

	Mild-Moderate Asthmatics (MMA)	Severe Asthmatics (SA)	p-value
N = 66	33	33	
Gender (Male/Female)	08/25	10/23	0.78
Race Caucasian/African-American/Other	21/9/3	30/1/1	0.01
Age (in years)	32.44 ± 2.31	46.91 ± 2.01	1.32E-05
Lung Function FEV1 % predicted	87.51 ± 3.71	55.87 ± 3.51	1.11E-09
Corticosteroid (CS) Use (No/Yes)	16/17	0/33	0.0001
CS Use (Inhaled/Systemic)	17/0	32/23	0.0007
Blood IgE (IU/mL)	284.85 ± 75.56	228.93 ± 58.77	0.55
Atopic Status (No/Yes)	01/32	06/27	0.1

Table 1. Characteristics of mild-moderate and severe asthma subjects evaluated in the study.

Subject Characterization

A total of 66 subjects, 33 each classified with mild-moderate asthma (MMA) or severe asthma (SA) were included in this study and details of patient characteristics are shown in Table 1. Of note, biological samples such as cells in bronchoalveolar lavage (BAL) fluid used for differential cell counts and cytokine expression were analyzed from a subset of these subjects based on availability as described in each Figure legend. Since the recovery of BAL fluid from different individuals is variable, especially from SA subjects, % cell type in BAL fluid is shown rather than absolute numbers. Mean percentages of lymphocytes, neutrophils and eosinophils were higher in the airways of SA as compared to MMA subjects (Figure 1A). The mean baseline FEV1% (Forced Expiratory Volume in 1s) predicted was 55.9 in SA and 87.5 in the MMA group (Figure 1B and Table 1). The dominant T cell population in cells collected by BAL was CD4+ T cells (Figure 1C).

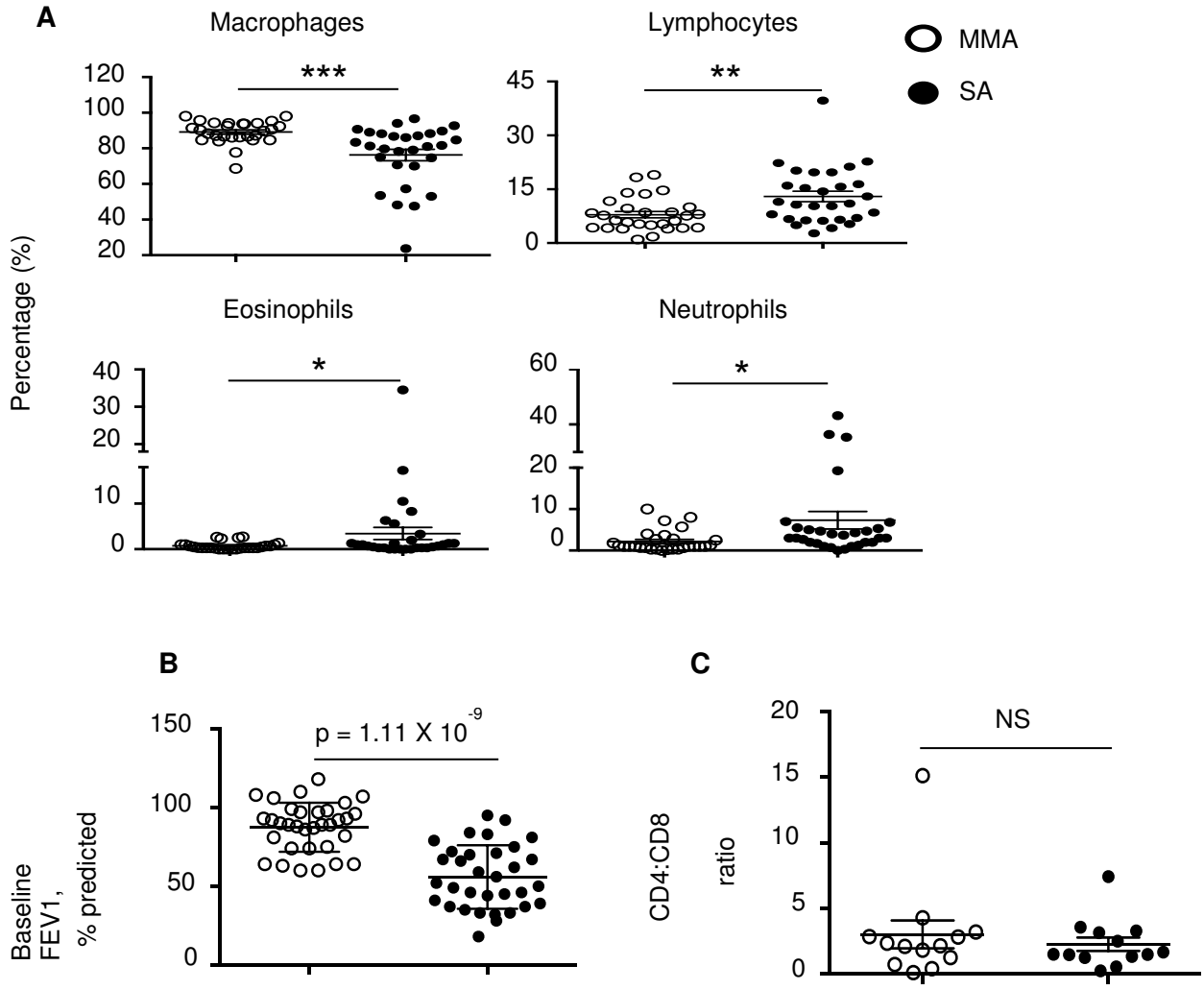


Figure 1. Cellular infiltrate in the airway lumen and FEV1 values in human asthma subjects. (**A**) Differential cell counts in BAL fluid of subjects with MMA and SA; n = 28 and 29 for MMA and SA, respectively. (**B**) Baseline FEV1% predicted for MM and SA subjects; n = 33 each for MMA and SA. (**C**) Ratio of CD4+ and CD8+ T cell percentages in the BAL fluid of MMA and SA subjects; n = 13 each for MMA and SA. For (**A-C**), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS – not significant by Mann-Whitney test.

Severe asthma subjects harbor more IFN-γ^+ $CD4^+$ T cells in their airways as compared to mild-moderate asthma subjects.

BAL cells from 9 MMA and 11 SA subjects were available for cytokine analysis by intracellular cytokine staining (ICS) and representative flow plots of cytokine$^+$ $CD4^+$ T cells in the subjects are shown in Figures 2 - 4. Asthma has historically been considered a Th2 disease. Thus, we first analyzed the production of IL-5, a Th2 cytokine, by CD4 T cells in the BAL fluid. The percentage of IL-5^+CD4^+ T cells in the BAL fluid was low, possibly due to the induction of apoptosis in Th2 cells in response to Dexamethasone therapy. More importantly, the percentages of IL-5^+CD4^+ T cells were comparable between the two cohorts (Figure 2). The percentage of IFN-γ^+ $CD4^+$ T cells (Th1 cells) was significantly greater in SA subjects averaging 20% versus 5% in MMA (Figure 3). IL-17^+ $CD4^+$ T cells was overall lower in both groups and was marginally higher in SA as compared to MMA, which reached statistical significance (Figure 4).

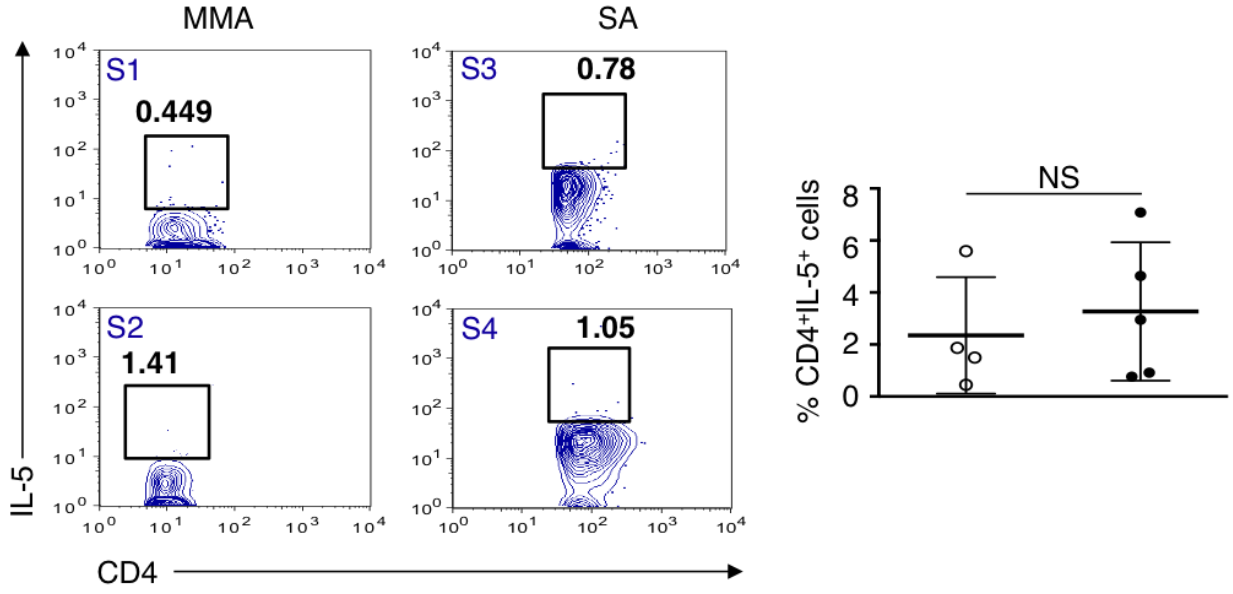


Figure 2. Comparable frequency of IL-5^+CD4^+ T cells in the BAL fluid of SA amd MMA patients. Representative flow plots (left panel) of IL-5^+ $CD4^+$ T cells in total BAL cells. Right panel is graphical representations of percentages of IL-5-expressing $CD4^+$ T cells in total BAL cells obtained from the airways of SA (n=11) and MMA (n=9) subjects studied.

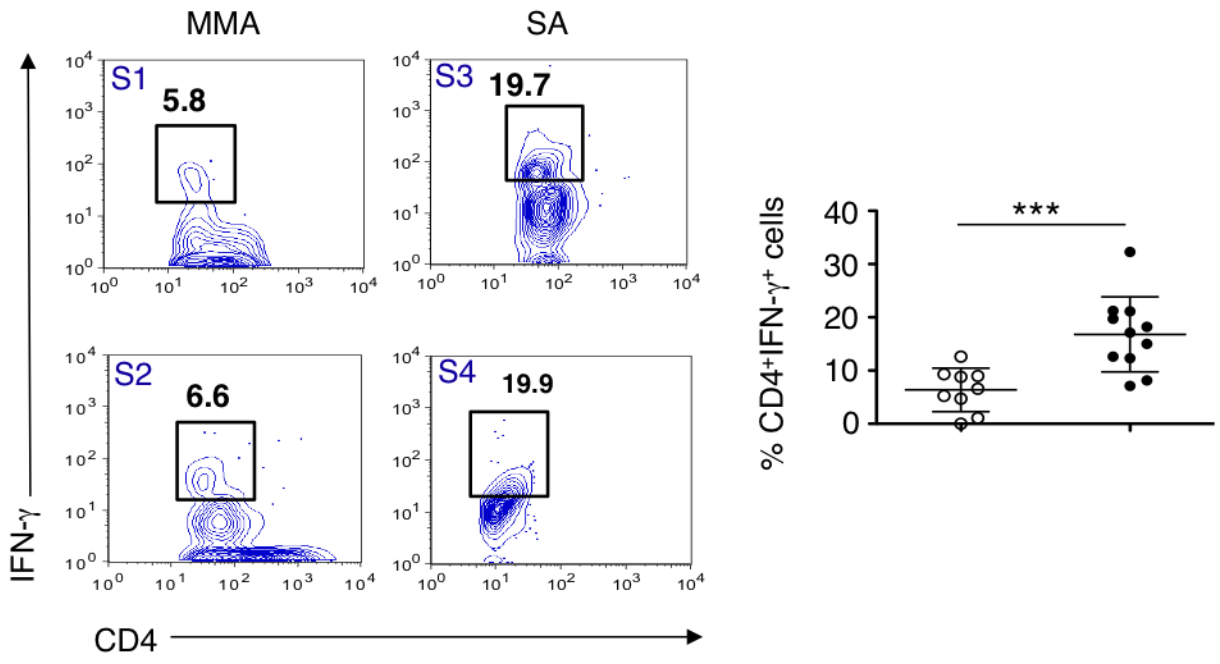


Figure 3. SA subjects harbor more IFN-γ^+ $CD4^+$ T cells in their airways as compared to MMA subjects. Representative flow plots (left panel) of IFN-γ^+ $CD4^+$ T cells in total BAL cells. Right panel is the graphical representations of percentages of IFN-γ-expressing $CD4^+$ T cells in total BAL cells obtained from the airways of SA (n=11) and MMA (n=9) subjects studied.

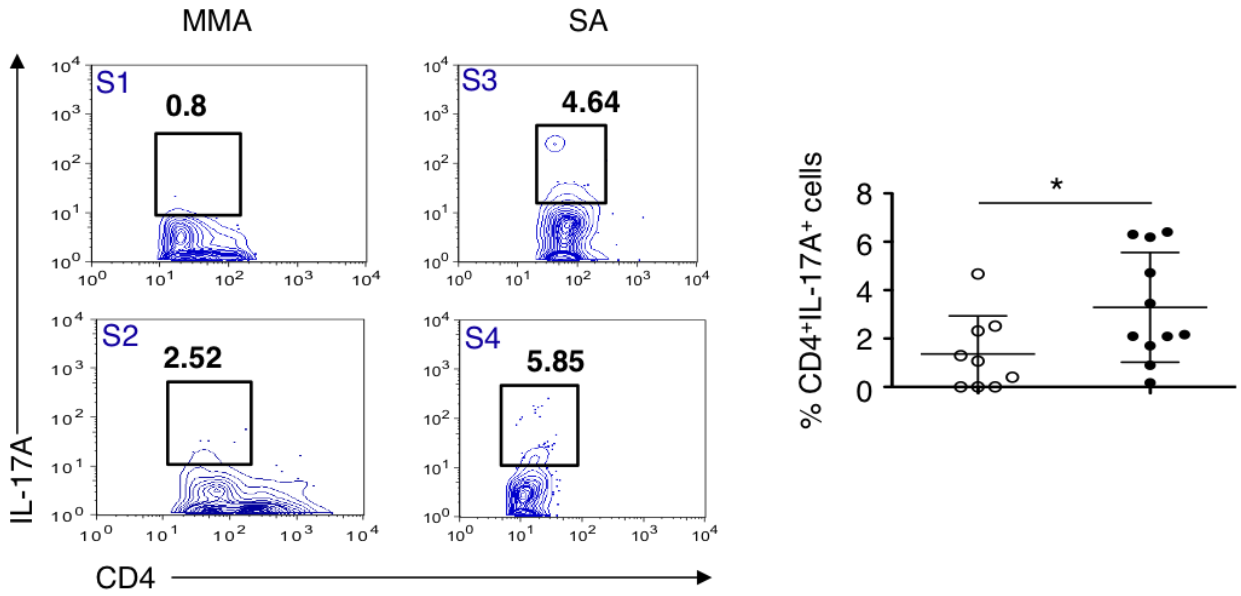


Figure 4. **SA subjects harbor more IL-17$^+$ CD4$^+$ T cells in their airways as compared to MMA subjects.** Representative flow plots (left panel) of IL17A$^+$ CD4$^+$ T cells in total BAL cells. Right panel is the graphical representations of percentages of IL-17A-expressing CD4$^+$ T cells in total BAL cells obtained from the airways of SA (n=11) and MMA (n=9) subjects studied.

Total BAL cells from 9 MMA and 14 SA subjects were cultured overnight and cytokines released were assayed by ELISA. The level of secreted IFN-γ was also higher in SA as compared to that in MMA (Figure 5). The mean levels of IL-5 and IL-17 secreted by BAL cells were comparable between the two groups although the level of secreted IL-13 was greater in the SA group (Figure 5). Secreted levels of IL-4, and IL-12p40 were also assayed. Overall, the levels of all of these cytokines were low possibly due to their rapid utilization by T cells (Figure 5). The level of IL-12p70 was below the level of detection.

It has been suggested earlier that *ex vivo* activation of cells leads to spontaneous production of IFN-γ. To address this, we analyzed the expression of mRNA transcripts of IFN-γ and IL-12, a polarizing cytokine for IFN-γ production by T cells, in freshly isolated BAL cells. Consistent with our ICS data, we observed significantly higher IFN-γ mRNA expression in SA

patients as compared to MMA patients. The mRNA transcripts for IL-12 were also higher in SA patients as compared to MMA subjects. (Figure 6).

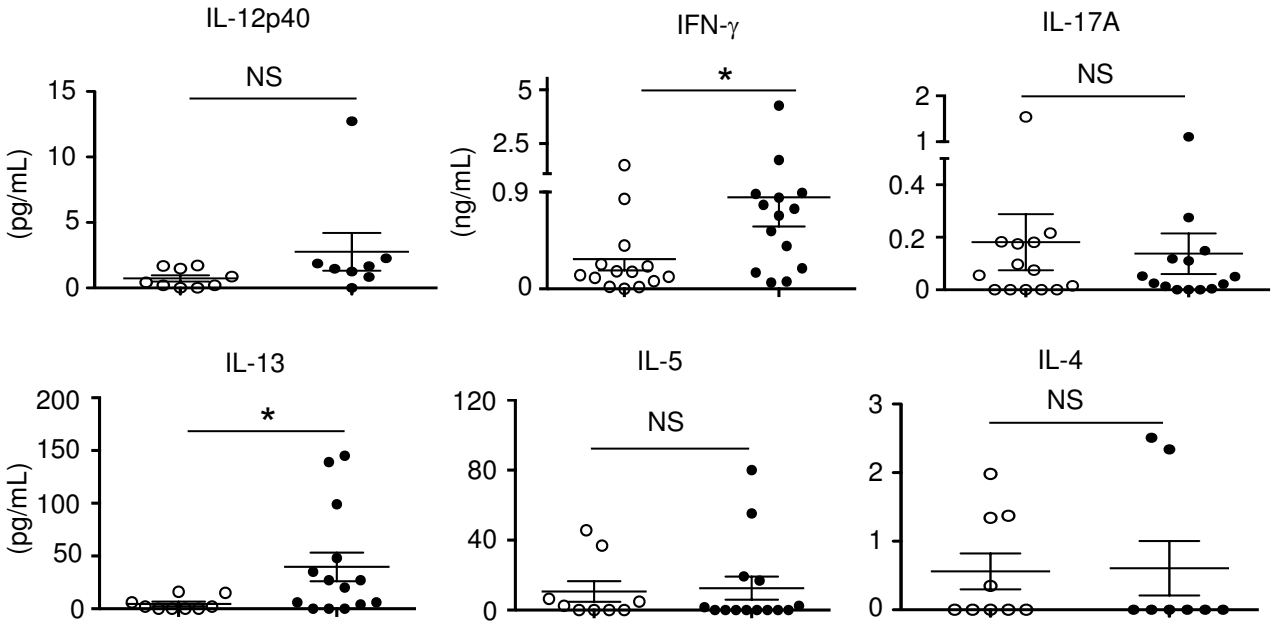


Figure 5. **Unfractionated BAL cells from SA patients secrete higher IFN-γ and IL-13 a compared to MMA subjects**. Concentration of indicated cytokines in culture supernatants of total unfractionated BAL cells from MMA and SA groups briefly cultured *ex vivo*. For IL-5 and IL-13 estimation, n = 9 and 14 for MMA and SA subjects, respectively; for IFN-γ and IL-17A estimation, n = 14 in each category. For IL-12p40, IL-4, and IL-9 estimation, n = 9 and 8 for MMA and SA subjects, respectively.

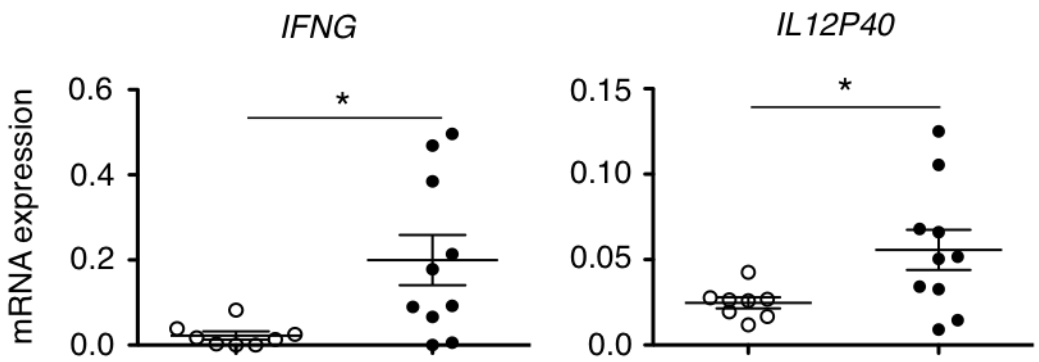


Figure 6. **Higher IFNG and IL12P40 mRNA transcripts in BAL cells of SA subjects.** *IFNG and IL12P40* mRNA expression in total BAL cells from MMA and SA subjects; n = 8 and 10 for MMA and SA, respectively.

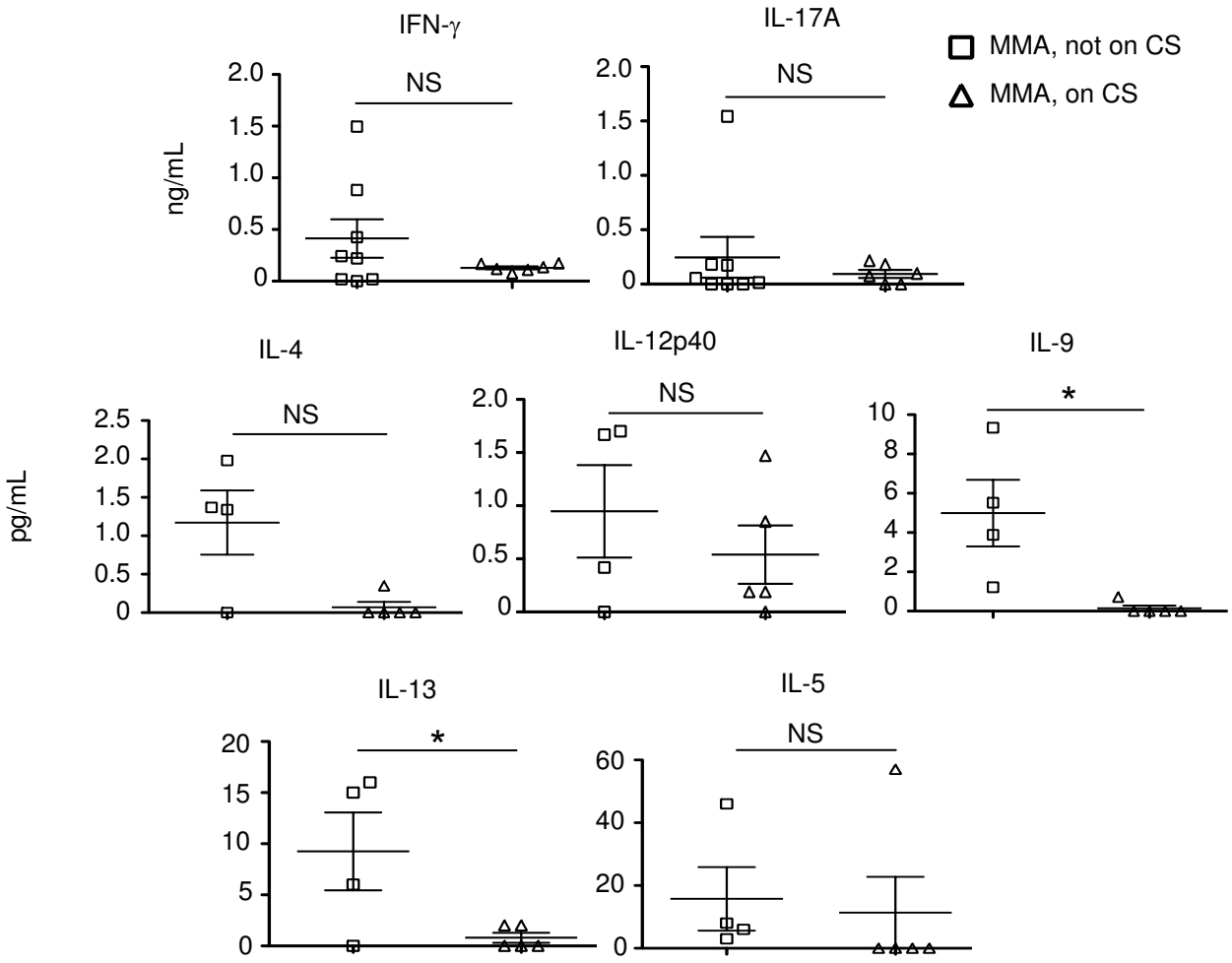


Figure 7. **Reduced cytokine levels in MMA patients on CS therapy.** Concentration of indicated cytokines in culture supernatants of BAL cells from MMA subjects treated with or without corticosteroid therapy.; n = 6 and 8 for MMA with or without CS therapy, respectively, for IFN-γ and IL-17A and n = 5 and 4 for MMA with or without CS therapy, respectively for IL-4, IL-5, IL-9, IL-12p40 and IL-13.

We further analyzed the data derived from the MMA cohort based on whether or not the subjects were on inhaled CS (ICS). Production of all cytokines assayed was lower when the subjects were on ICS, with the difference reaching statistical significance in the case of IL-9 and IL-13 (Figure 7). A comparison of cytokine levels from BAL cells of MMA subjects on ICS

versus that from SA subjects revealed higher levels of all assayed cytokines in SA with the difference in IFN-γ and IL-13 levels being statistically significant (Figure 8).

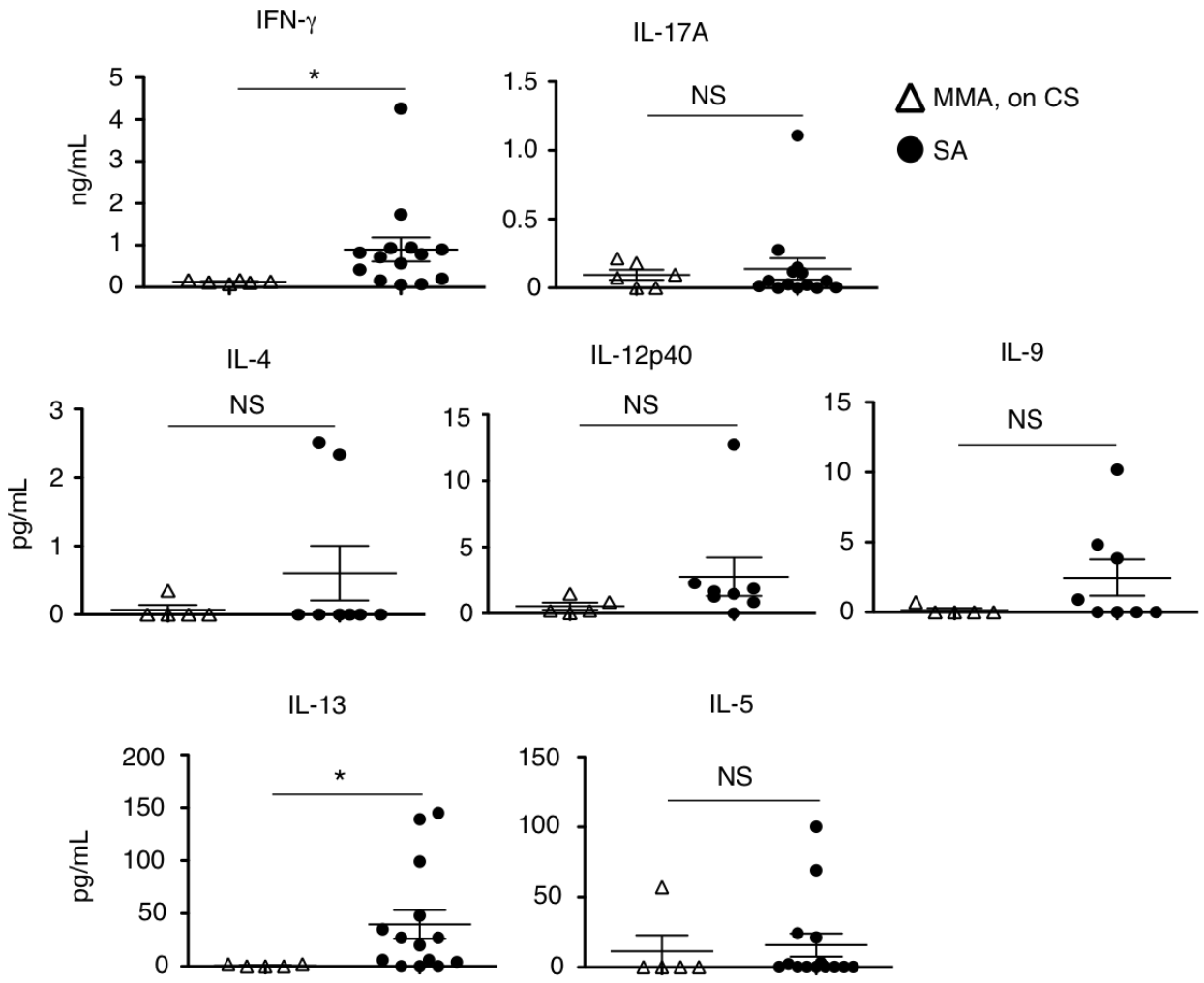


Figure 8. BAL cells from SA patients secrete more inflammatory cytokines despite being on CS therapy. Concentration of indicated cytokines in supernatants of *ex vivo* cultured BAL cells from MMA patients on CS therapy and severe asthma patients. n = 5-6 and 14 for MMA + CS and SA, respectively, for IFN-γ, IL-17A, IL-5 and IL-13 estimation, n = 5 and 8 for MMA + CS and SA, respectively, for IL-4, IL-9 and IL-12p40 estimation.

Establishment of a mouse model of severe asthma.

Our next goal was to establish a mouse model of severe asthma that would: 1) display the complex immune response identified in the airways of human severe asthma, 2) demonstrate mixed granulocytic infiltrates in the airways, and 3) demonstrate resistance to CS therapy, as observed in the SA subjects. To model this complex immune response in rodents, we took into consideration the current concept that pathogen infections play an important role in asthma pathognenesis. Given that virus infections can exacerbate asthma pathology, we used CpG, a viral nucleic acid analogue, along with HDM, to induce asthma in mice. As shown in Figure 9, mice were sensitized intranasally with either HDM alone or in combination with CpG for 10 days. During the challenge phase, mice were instilled with HDM via the airways. Lavage from the bronchoalveolar space showed that the combination of HDM and CpG recruited a mixed neutrophil-eosinophil infiltrate, with neutrophil dominance, to the airways, as seen in our human SA cohort.

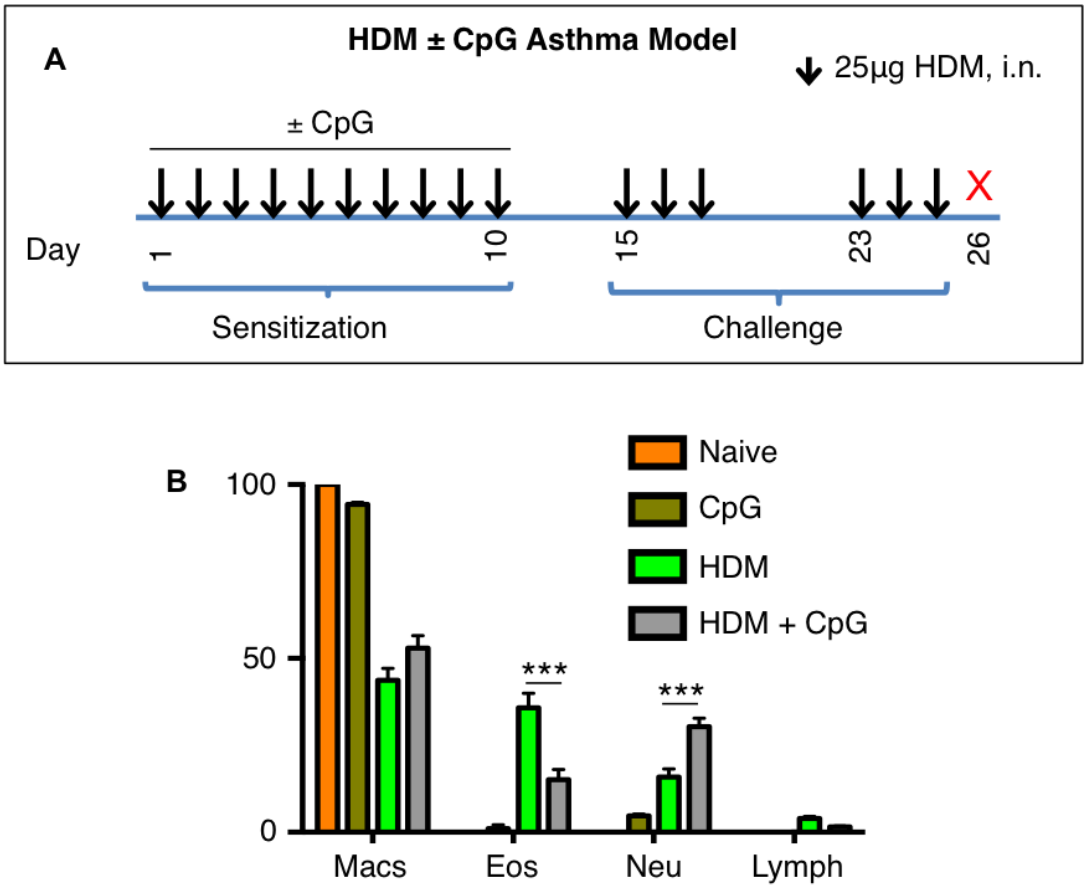


Figure 9. **Neutrophil-dominant airway inflammation in HDM + CpG asthma model.** (A) Schematic of HDM ± CpG asthma model and (B) Cellular infiltrate in the airways of mice subjected to the HDM ± CpG asthma model.

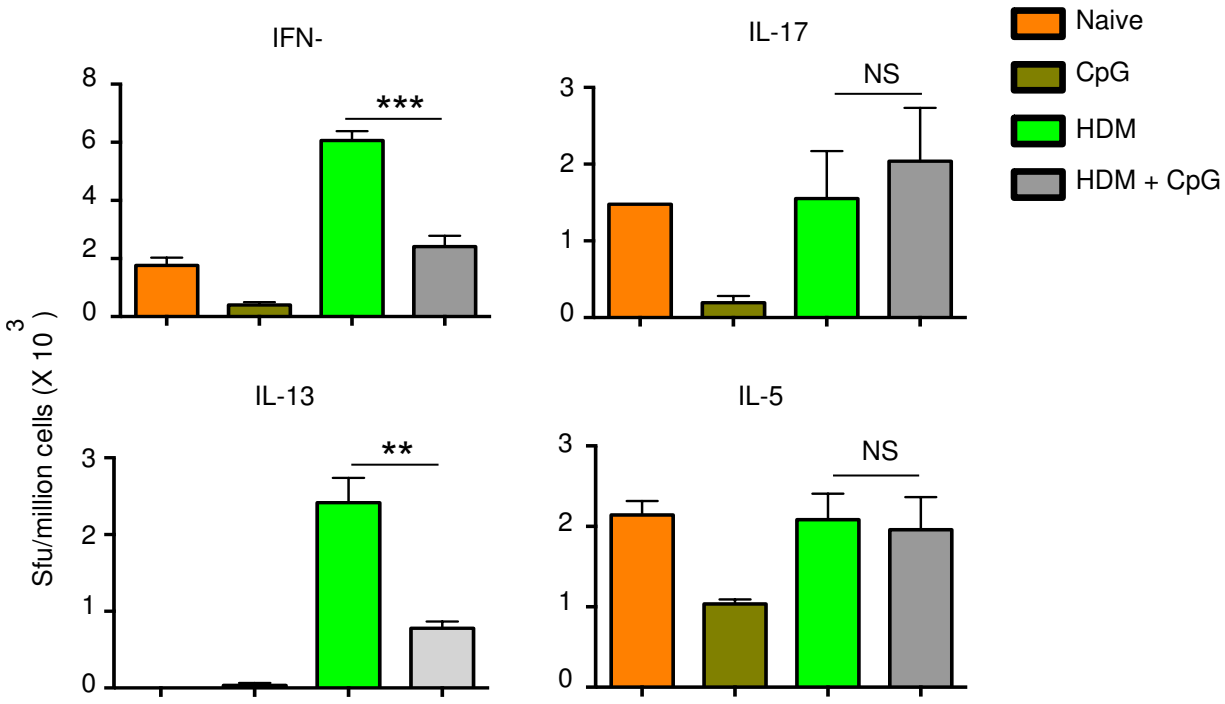


Figure 10. **Lower secretion of IFN-γ from lung CD4+ T cells of HDM + CpG-treated mice.** ELISPOT analyses of cytokine-producing CD4 T cells from lungs of mice subjected to the HDM ± CpG asthma models.

We then studied the production of Th1, Th2 and Th17 cytokines by lung CD4 T cells from mice instilled with HDM alone or in combination with CpG. ELISPOT analyses of lung CD4+ T cells showed that inclusion of CpG along with HDM reduced the ability of T cells to

produce IFN-γ and IL-13. The number of T cells making IL-17 and IL-5 remained unchanged between the two groups (Figure 10). Thus, while CpG addition mimicked the neutrophil-dominated airway inflammation as seen in the human severe asthma cohort, the cytokine profile differed between the two species.

Bacterial infections have been associated with asthma exacerbations. Most commonly associated pathogen associated molecular pattern (PAMP) with bacteria is lipopolysaccharide (LPS)/ Endotoxin. Thus, we combined HDM along with LPS to mimic asthma pathogenesis mediated by superimposition of allergen exposure with bacterial infection. Mice were sensitized via the airways for 10 days with HDM alone or in combination with LPS and then challenged with HDM after a rest period. To mimic the use of corticosteroids for asthma symptom control by subjects in our human cohorts, we also treated mice with Dexamethasone, a corticosteroid, starting during the challenge phase.(Figure 11). BAL cell analysis on day 26 revealed a predominantly neutrophilic infiltrate with a small percentage of eosinophils in the airways of mice treated with HDM plus LPS. Importantly, this infiltrate was insensitive to Dex treatment. However, the inflammatory infiltrate elicited by only HDM instillation was not only predominantly eosinophilic, but also Dex sensitive (Figure 11).

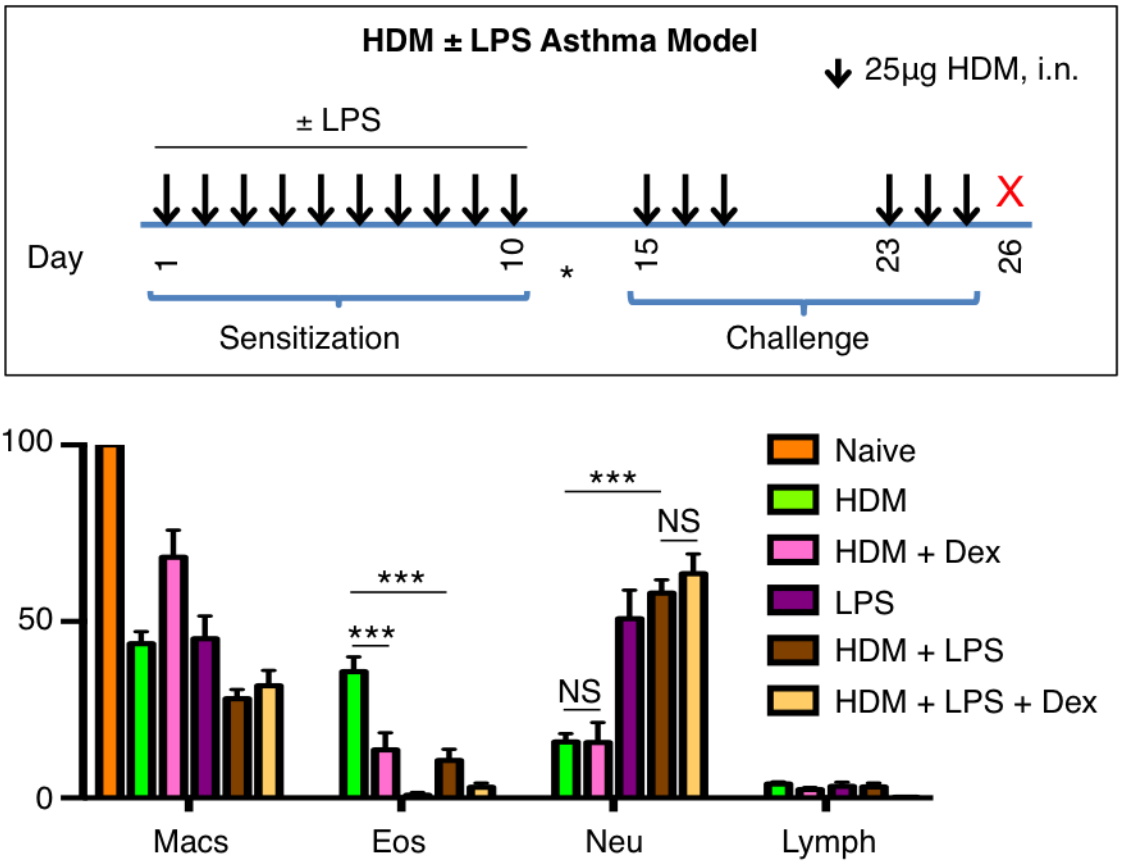


Figure 11. **Steroid-resistant neutrophil-dominant airway inflammation in HDM + LPS asthma model.** (A) Schematic of HDM ± LPS asthma model and (B) Cellular infiltrate in the airways of mice subjected to the HDM ± LPS asthma model.

ELISPOT analyses of lung CD4+ T cells showed that inclusion of LPS along with HDM enhanced the ability of T cells to produce IFN-γ and IL-17. The number of T cells producing IL-13 and IL-5 remained unchanged between the two groups. However, the production of IFN-γ by CD4 T cells was extremely sensitive to steroid treatment (Figure 12). Thus, although LPS mimicked the neutrophilic inflammation and increased IFN-γ as seen in our human asthma cohorts, the extreme sensitivity of IFN-γ to Dex treatment was a deviation from our goals of establishing a steroid-refractory severe asthma model.

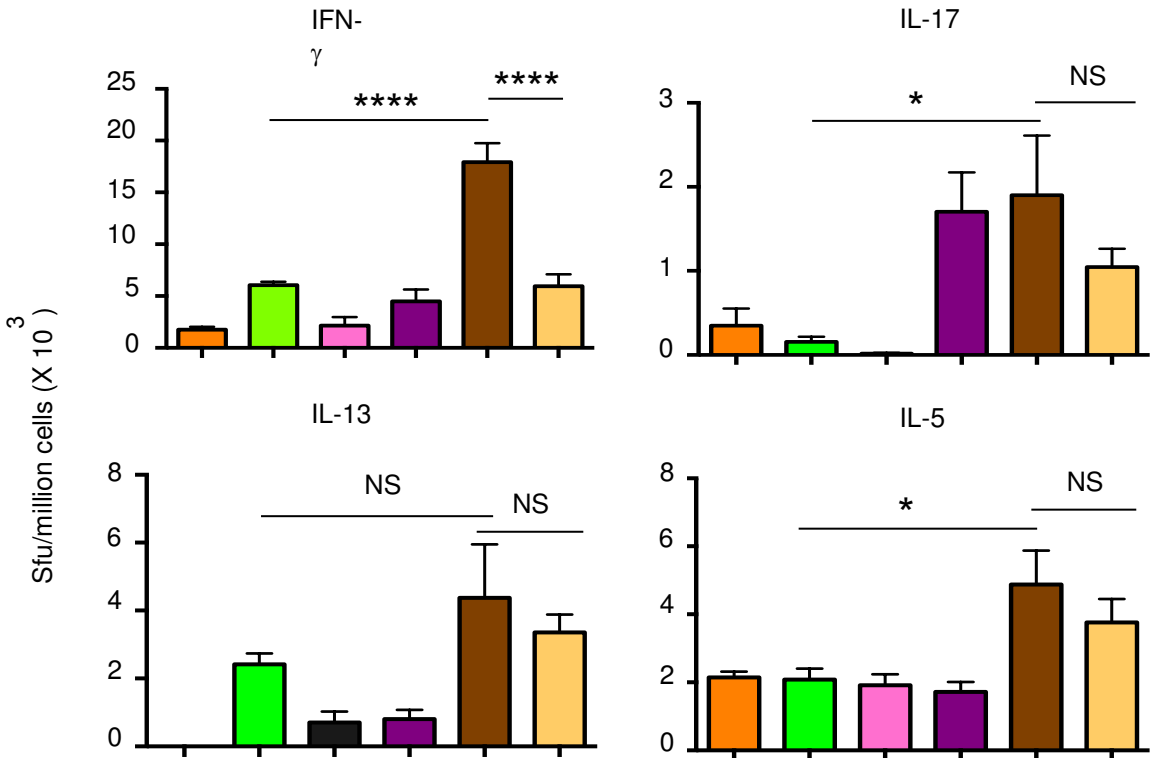


Figure 12. **Dex-induced lower secretion of IFN-γ from lung CD4+ T cells of HDM + LPS-treated mice.** ELISPOT analyses of cytokine-producing CD4 T cells from lungs of mice subjected to the HDM ± LPS asthma models.

Within the bacterial genera, intracellular bacteria such as *Chlamydia pneumoniae* have been linked to fixed airflow limitation and *Haemophilus influenzae* has been detected in the sputum of SA patients. In a more recent study, severe asthma patients were found to harbor either *Moraxella catarrhalis* or members of the *Haemophilus* or *Streptococcus genera.* All of these bacterial species are capable of intracellular growth and hence can produce the second messenger cyclic-di-GMP (c-di-GMP). c-di-GMP has been shown to be a potent mucosal adjuvant that induces a Th1-Th17 response accompanied by a low Th2 response. Thus we hypothesized that this adjuvant may be useful to model the complex immune response observed in human severe asthma. We sensitized mice with house dust mite (HDM) allergen and c-di-GMP and subsequently challenged the mice with HDM and a lower dose of c-di-GMP. A second

group of mice was sensitized and challenged with HDM alone following the same regimen (Figure 13).

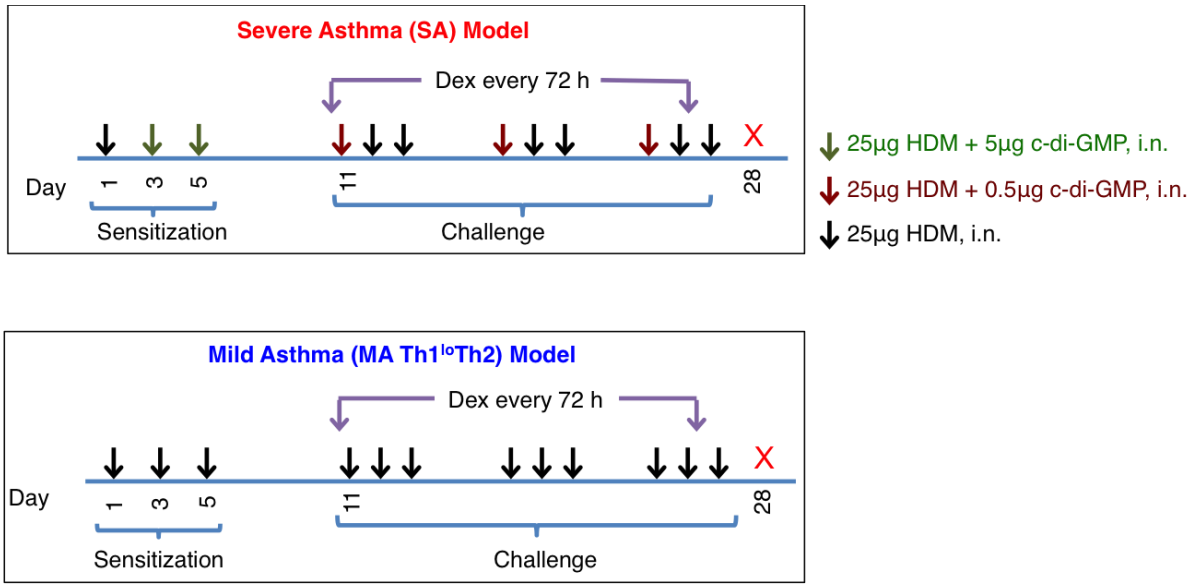


Figure 13. **Schematics of the HDM ± c-di-GMP asthma model.** Mice were sensitized and challenged as shown either with or without Dex treatment and 24 h after the last allergen challenge mice were euthanized and analyzed for different end-points.

We first tested the expression of Th1-, Th2- and Th17-polarizing cytokines in cells isolated from the lymph nodes of mice subjected to the HDM ± c-di-GMP models. mRNA transcripts for Il12p40 and Il27p28, subunits of Th-1 polarizing cytokines IL-12 and IL-27, respectively, and IL-6 and gmcsf, polarizing cytokines for Th17 cells, were significantly higher in the lymph node cells from the HDM + c-di-GMP model as compared to those from HDM alone (Figure 14). These data suggested that the ensuing T cell response in the lung would be biased towards the Th1 and Th17 lineage.

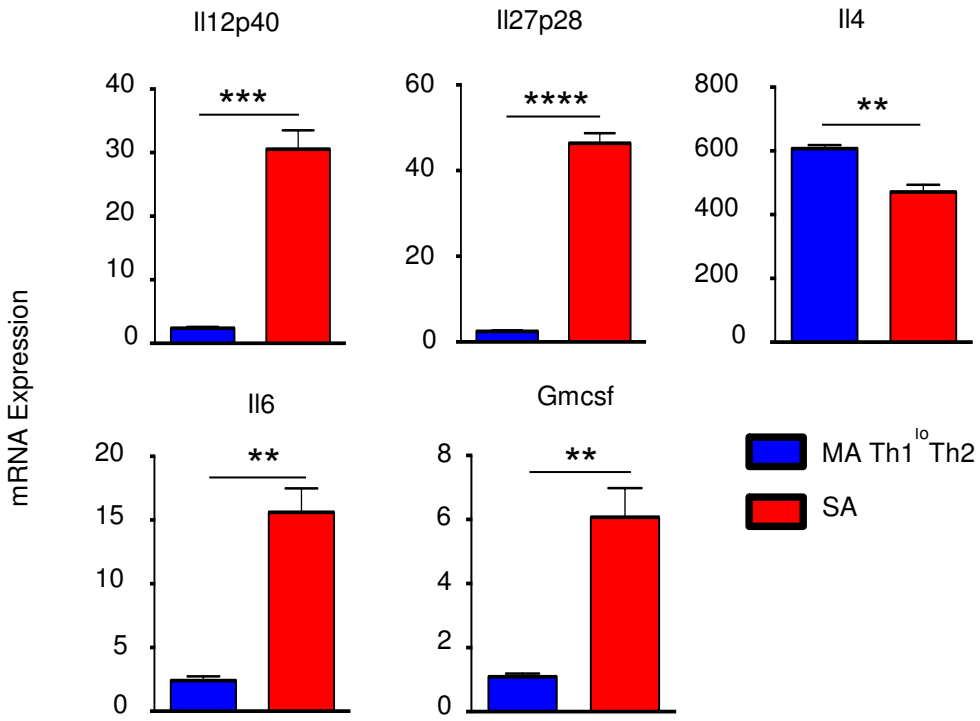


Figure 14. **c-di-GMP biases the lymph node immune response to Th1 and Th17.** Expression of indicated mRNAs in cells isolated from the lymph nodes of mice subjected to the HDM + c-di-GMP or HDM only asthma models.

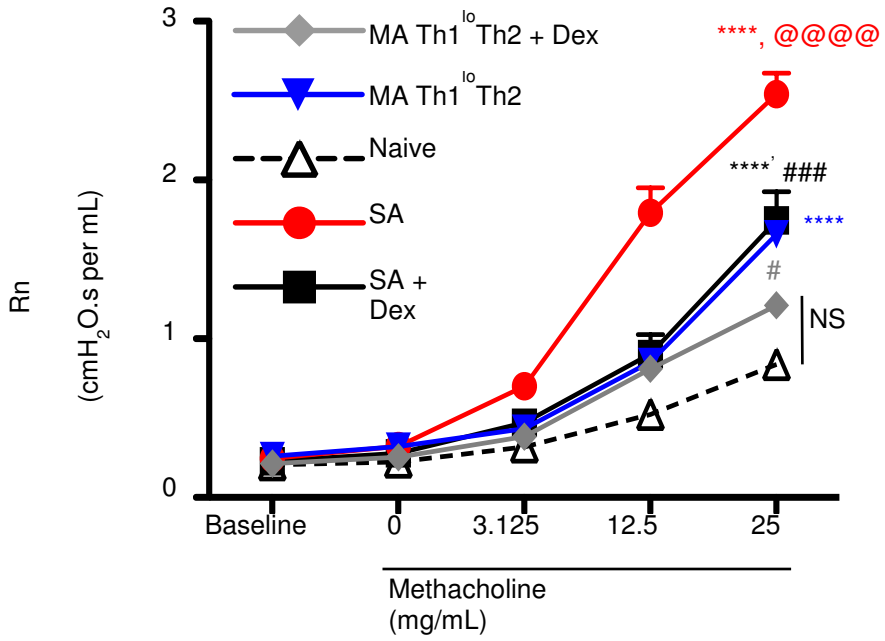


Figure 15. **AHR in HDM ± c-di-GMP asthma models.** Assessment of Central airway resistance (Newtonian resistance-Rn) as a marker of airway hyperresponsiveness (AHR) after challenge with different doses of methacholine (MCh). * significance versus naïve mice (**** $p \leq 0.0001$, @ significance of SA group versus MA Th1^{lo}Th2 group (@@@@p<0.0001), # significance of SA versus SA+Dex (### p<0.001) or MA Th1^{lo}Th2 versus MA Th1^{lo}Th2+Dex (#p<0.05) using One-way ANOVA with Tukey's post hoc test.

We next studied airway hyperresponsiveness in mice sensitized and challenged with HDM ± c-di-GMP asthma models. AHR of mice immunized with both HDM and c-di-GMP increased with increasing doses of methacholine (MCh) and was higher than that induced using HDM alone. Upon treatment with the CS dexamethasone (Dex), which was initiated during challenge and maintained until harvest, AHR in the severe model was reduced only to the response induced by HDM alone (note the almost complete overlap of the black and blue lines in Figure 15). In mice that were sensitized and challenged with HDM alone, AHR was considerably lower than that induced by HDM+c-di-GMP (Figure 2B). Also, Dex treatment reduced AHR further but not down to the level measured in MCh-challenged naïve mice (Figure 15).

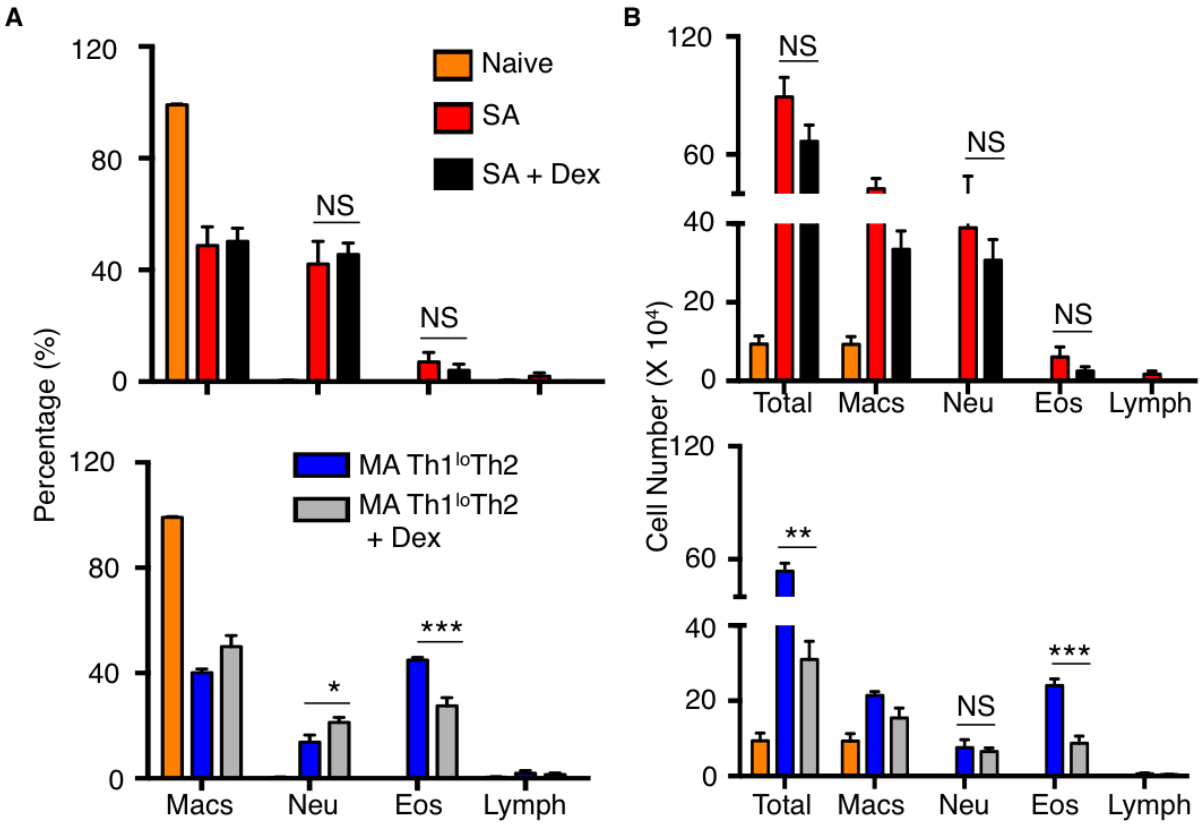


Figure 16. **Airway infiltrate in HDM ± c-di-GMP asthma models.** Differential cell counts in BALF cytospins showing total cells, eosinophils (Eos), macrophages (Macs), neutrophils (Neu) and lymphocytes (Lymph) of mice sensitized and challenged in SA and MA Th1^{lo}Th2 models ± Dex.

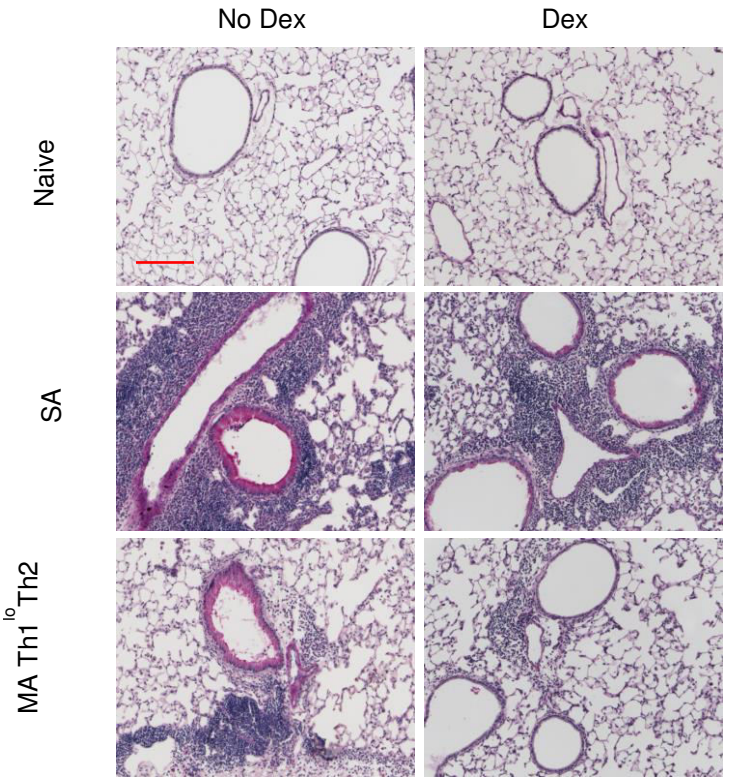


Figure 17. **Airway inflammation and mucus in HDM ± c-di-GMP asthma models**..Periodic acid-Schiff (PAS) staining of lung sections. Scale bar=200 □m

Differential cell counts in the BAL fluid recovered from mice subjected to the two models showed greater neutrophil recruitment in the SA model but more eosinophil infiltration in the HDM only model. Dex treatment substantially reduced airway inflammation in the mice that received HDM alone but was only partly effective in reducing airway inflammation in the SA mice (Figure 16). Histological examination of lung tissue sections showed that the mice subjected to the SA model harbored more intense inflammation and mucus staining compared to those subjected to the model with HDM alone (Figure 17).

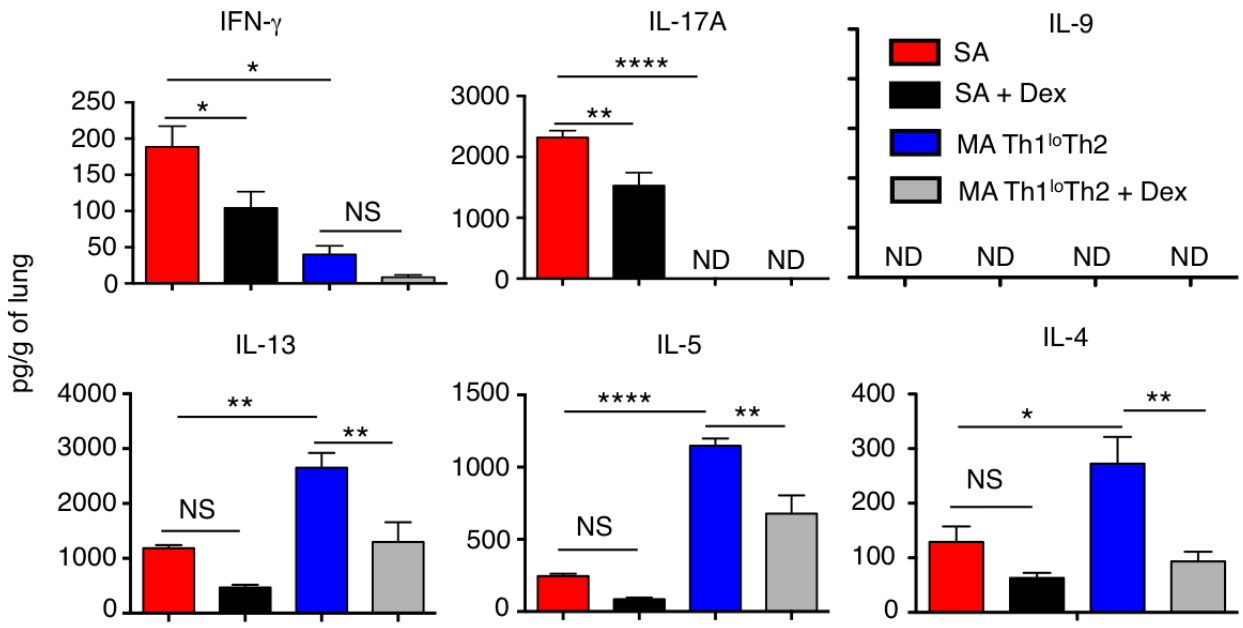


Figure 18. **Cytokine protein levels in HDM ± c-di-GMP asthma models.** The level of cytokine proteins in lung homogenates of mice subjected to the SA or MATh1^{lo}Th2 model.

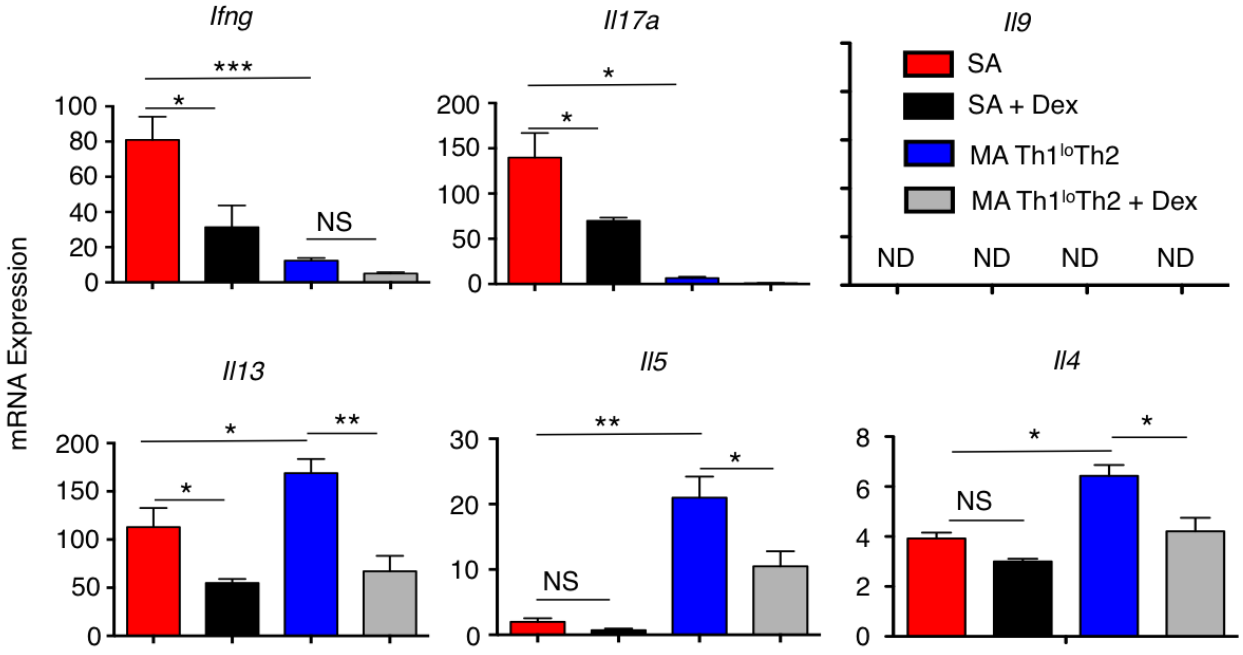


Figure 19. **Cytokine mRNA levels in HDM ± c-di-GMP asthma models.** The level of cytokine mRNAs in whole lungs of mice subjected to the SA or MATh1^{lo}Th2 model.

When assessed for cytokine levels in the models, we observed a significantly higher level of IFN-γ in the lungs of SA mice as compared to the MA mice. In contrast, the type 2 cytokines IL-4, IL-5 and IL-13 were detected at higher levels in the MA mice as compared to that in the SA mice. We have hereafter referred to the model established with HDM+c-di-GMP as SA and that in which HDM alone was instilled following the same regimen as the MA Th1^{lo}Th2 model. IL-9, another cytokine associated with a type 2 response, was not detected in either mouse model. IL-17A was detected only in the SA mice. Dex treatment only partially reduced lung IFN-γ level in the SA mice and IFN-γ was also not completely suppressed in the Dex-treated MA mice. The levels of Th2 cytokines also partially decreased upon Dex treatment in both models as did that of IL-17 in the SA model. The pattern of expression of all indicated cytokines in these two models were comparable at the mRNA and protein level (Figures 18 & 19).

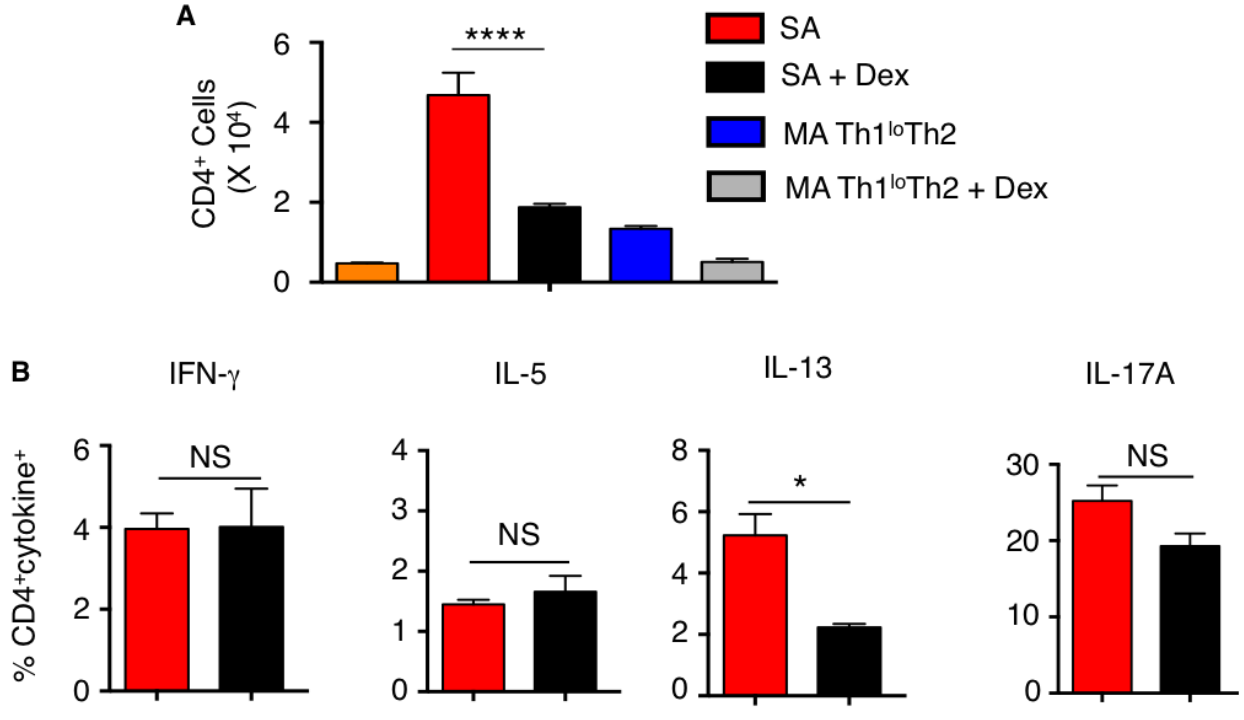
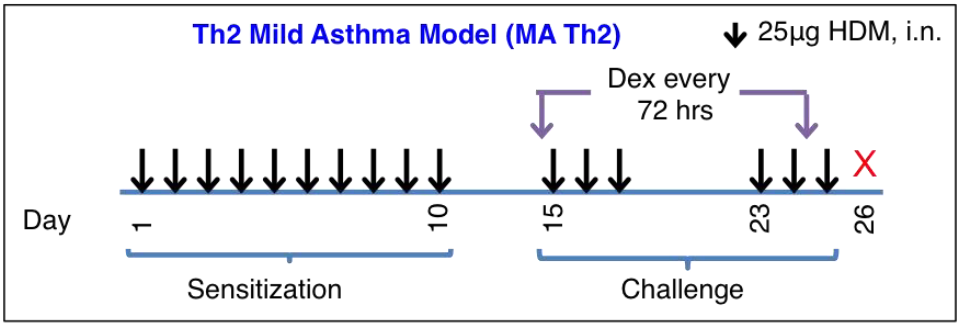
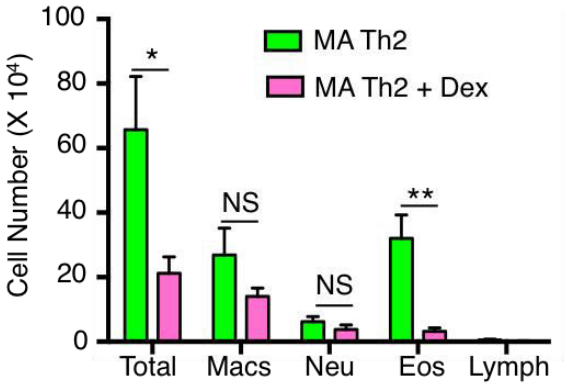


Figure 20. **CD4 T cell-associated cytokines in the mouse model of SA.** (A) Total lung $CD4^+$ T cell numbers in mice sensitized and challenged in SA and MA $Th1^{lo}Th2$ models ± Dex quantified by flow cytometry. (B). Percentages of $cytokine^+CD4^+$ T cells in the lungs of mice subjected to the SA model.

The cytokines detected at the whole lung level in SA mice could be detected in $CD4^+$ T cells by ICS. Also, Dex treatment reduced the frequency of IL-13^+ $CD4^+$ T cells. However, since CS treatment inhibits the expression of a wide range of molecules such as cell adhesion molecules, Dex treatment also led to lower tissue T cell accumulation (Figure 20A). Thus, based on the reduced $CD4^+$ T cell numbers in Dex-treated mice, the total numbers of each of the cytokine-expressing T cells was lower upon Dex treatment at the whole lung level (Figure 20B).

Airway inflammation and AHR in a Th2-dominant mild asthma model, MA Th2





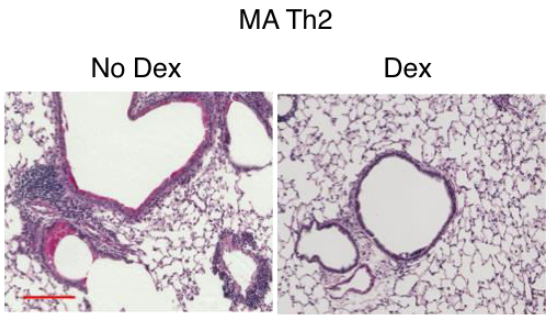


Figure 21. **Airway inflammation in the MA Th2 model.** (A) Schematic of the Th2 Mild Asthma model (MA Th2). (B). Differential cell counts in BALF cytospins showing total cells, Eos, Macs, Neu and Lymph.. (C). PAS staining of lung sections. Scale bar=200 μm.

Dex treatment of the MA Th1^{lo}Th2 mice significantly attenuated airway inflammation, mucus production and AHR (Figures 15-17 & 20). However, AHR in these mice was still not completely reduced to that induced in naïve MCh-challenged mice (Figure 15). We next examined another mouse model in which Th2 cytokines were exclusively induced in response to HDM (referred here on as MA Th2). This was achieved by instilling HDM on a daily basis initially for 10 days and subsequently challenging the mice with HDM after a period of rest (Figure 21A). Repetitive HDM instillation induced a Th2 response and eosinophilic airway inflammation (Figure 21B) with barely detectable IFN-γ or IL-17 induction and low neutrophil infiltration in the airways (Figures 21B and 23). Levels of Th2/type 2 cytokines produced in the lungs were comparable between the MA Th1^{lo}Th2 and MA Th2 models (Figures 18-19 and 23). The key difference in cytokine response between the MA Th2 model and the MA Th1^{lo}Th2 model was that IFN-γ was induced only in the latter, albeit at levels below that induced in the SA model. No IL-17 was detected in either model. Dex treatment completely ablated airway inflammation and mucus production in this model and completely suppressed AHR induced by MCh challenge (Figure 21C and 22). The level of AHR induced in this Th2-driven model was lower than that observed in the SA model but was equivalent to that induced in the MA Th1^{lo}Th2 model (Figures 15 and 22).

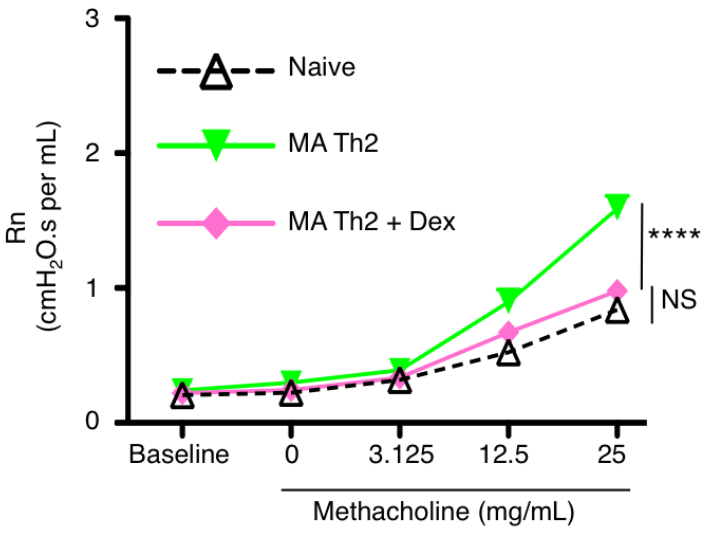


Figure 22. **AHR in MA Th2 model.** Airway hyperresponsiveness (AHR) assessment using forced oscillation technique.

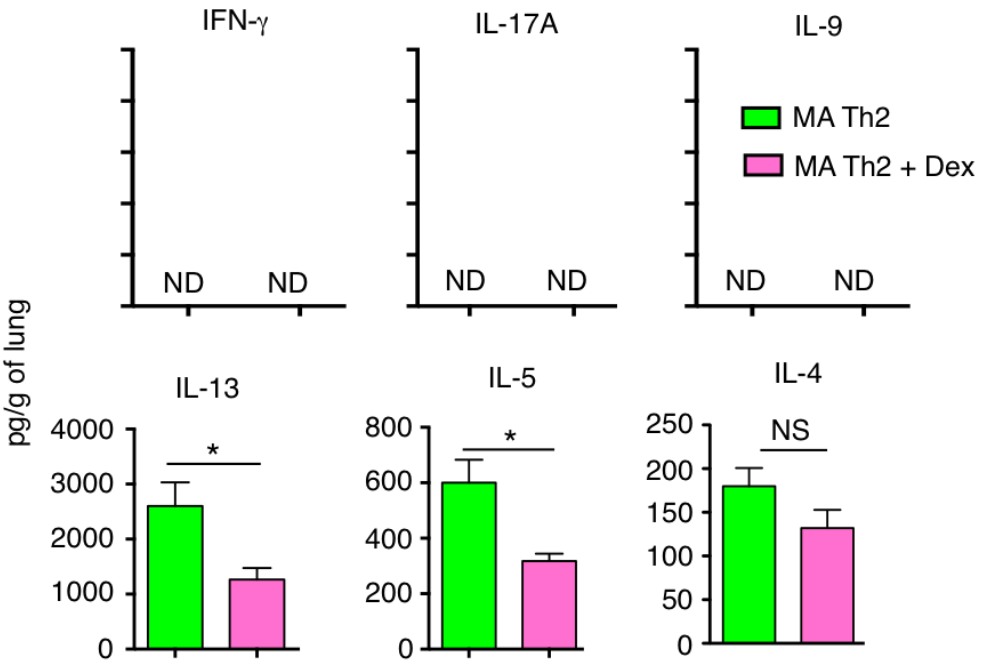


Figure 23. **Cytokine protein levels in MA Th2 model.** Cytokine proteins in whole lungs of mice subjected to the MA Th2 model.

IL-17 controls neutrophilic influx, but not AHR, in the SA mouse model.

Given the increase in IL-17+CD4+ T cells in human severe asthmatics and the increased mRNA and protein expression of IL-17A in the mouse SA model, we tested the importance of IL-17A signaling in the pathogenesis of SA. To this end, we subjected *Il17ra* -/- mice to the SA model. Loss of *Il17ra* results in loss of signaling by two members of the IL-17 family of cytokines, namely IL-17A and IL-17F. Surprisingly, AHR in *Il17ra*-/- mice subjected to the SA model remained similar to that measured in WT mice (Figure 24). However, as expected, neutrophil recruitment to the airways and tissue inflammation was lower in the *Il17ra*-/- mice as compared to that in their WT counterparts. (Figure 25A,B). Addition of Dex did not mitigate the inflammation. These results suggested that neutrophils and IL-17A do not contribute to AHR in this model of allergic airway disease. At the whole lung level, the levels of secreted Th1/2/17 cytokines were largely comparable between WT and *Il17ra*-/- mice subjected to the SA model (Figure 26A,B). Cytokine mRNA expression was similar between WT and *Il17ra*-/- mice except for a reduction in IL-17A in the *Il17ra*-/- mice (Figure 26B).

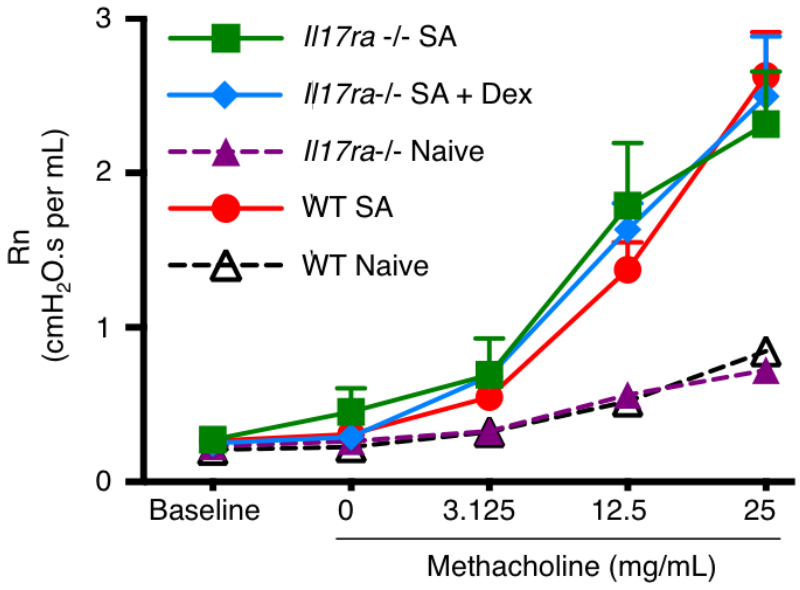


Figure 24. **Comparable AHR in WT and *Il17ra*-/-.** AHR in WT and *Il17ra*-/- mice subjected to the SA model after MCh challenge.

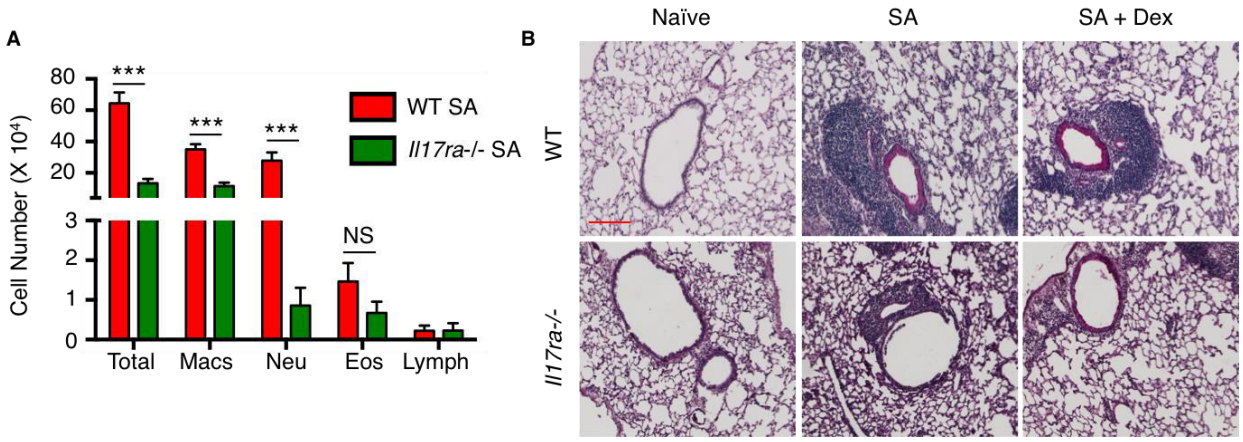


Figure 25. **Reduced airway inflammation in *Il17ra* -/- mice subjected to the SA model.** WT and *Il17ra*-/- mice were subjected to the SA model and the following end-points were analyzed 24 h after the final allergen challenge - (A) Differential cell counts in BALF cytospins showing total cells, Eos, Macs, Neu and Lymph. (B) PAS staining of lung sections.

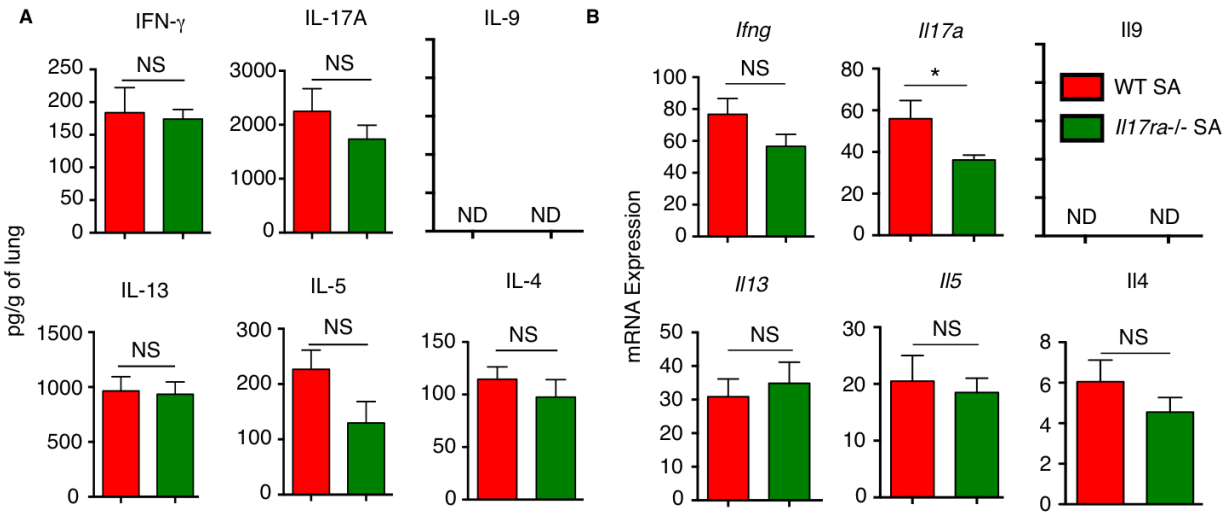


Figure 26. **Cytokine levels in WT and *Il17ra* -/- mice subjected to the SA model.** Cytokine protein (A) and mRNA (B) expression in whole lungs of WT and *Il17ra*-/- mice subjected to the SA model.

IFN-γ controls AHR in the SA mouse model.

We next focused on the contribution of IFN-γ to AHR and airway inflammation in the SA model. While the WT mice showed increased AHR, the *Ifng*-/- mice failed to develop AHR. Again, Dex treatment only partially reduced AHR in the WT mice (Figure 27). Despite the inability of *Ifng*-/- mice to mount appreciable AHR upon MCh challenge, airway inflammation was unabated in these mice with or without Dex treatment. The *Ifng*-/- mice showed slightly greater neutrophil and eosinophil infiltration in their airways (Figure 28). These mice also displayed a significantly higher level of IL-17A protein and mRNA in the lungs as compared to the WT mice and yet failed to mount AHR (Figure 29). The increase in IL-17 levels in the absence of IFN-γ was expected because of cross-regulation between IFN-γ and IL-17. Taken together, these data showed that IFN-γ, and not IL-17, contributes to AHR in this model of asthma. Also, these data show that airway inflammation and hyperresponsiveness can be independent of each other and do not always complement each other.

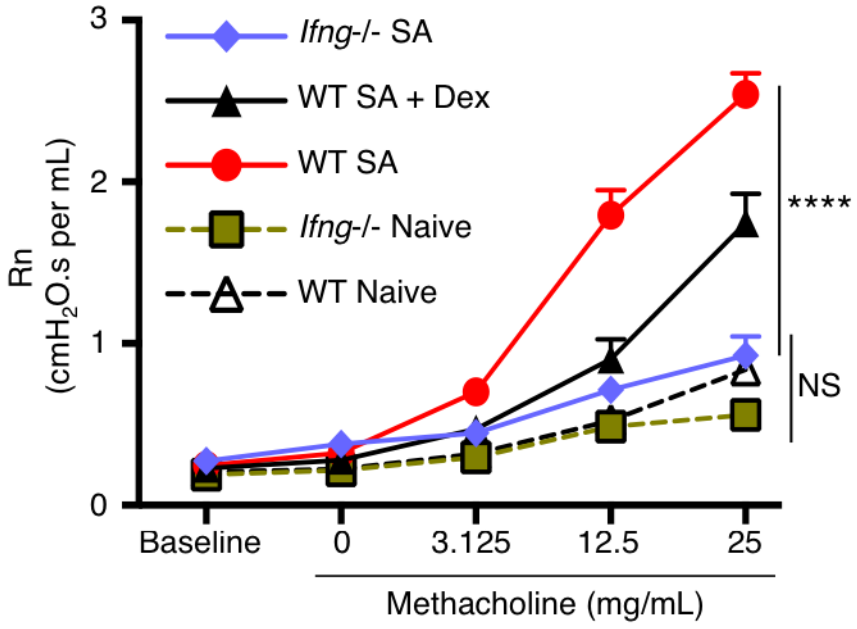


Figure 27. ***Ifng*-/- mice fail to develop AHR after being subjected to SA model.** AHR in WT and *Ifng*-/- mice subjected to the SA model after MCh challenge.

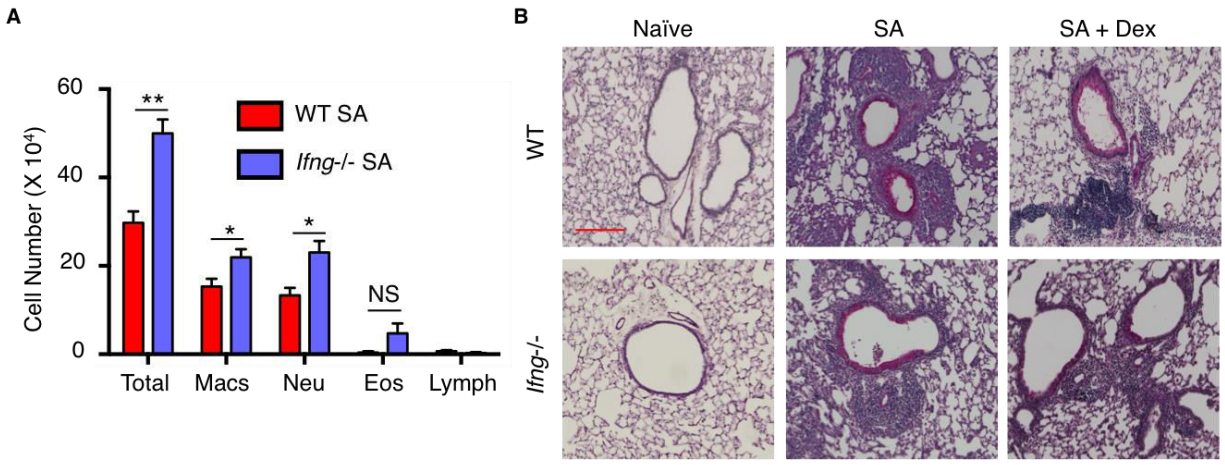


Figure 28. **Increased airway inflammation in *Ifng*-/- mice subjected to the SA model.** WT and *Ifng*-/- mice were subjected to the SA model and the following end-points were analyzed 24 h after the final allergen challenge - (A) Differential cell counts in BALF cytospins showing total cells, Eos, Macs, Neu and Lymph. (B) PAS staining of lung sections.

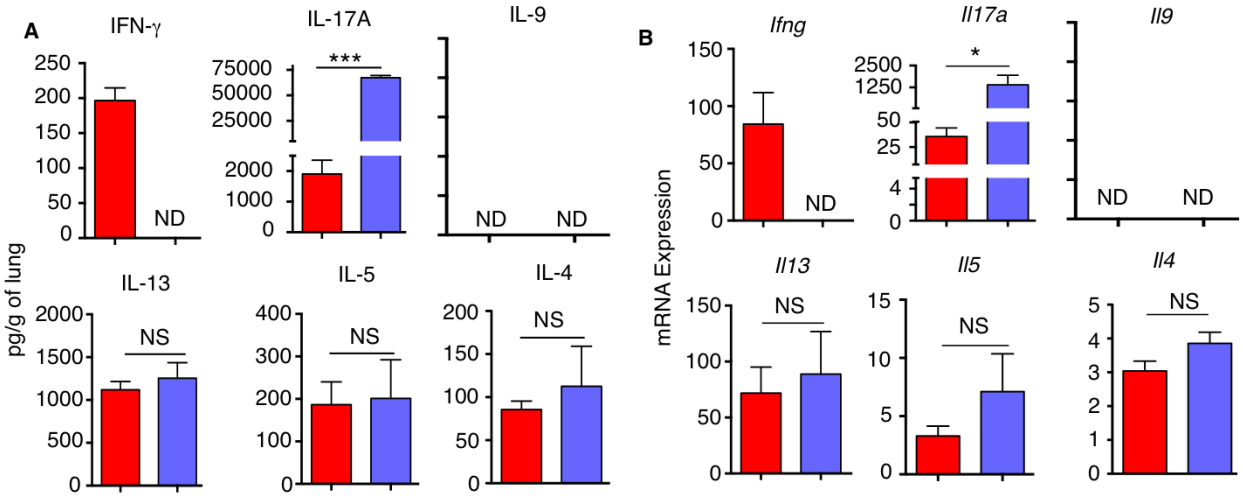


Figure 29. **Cytokine levels in WT and *Ifng* -/- mice subjected to the SA model.** Cytokine protein (A) and mRNA (B) expression in whole lungs of WT and *Ifng*-/- mice subjected to the SA model.

Mechanism of AHR control by IFN-γ

We next asked how IFN-γ might associate with increased AHR. Using Ingenuity Knowledge Base (IKB), we identified multiple genes that have been associated with AHR in humans or mice (Table 2). Since, persistent AHR is a feature of severe asthma (53) and it has been suggested that structural changes in the airways contribute to persistent AHR (270). Thus, we focused on molecules that are modulated by IFN-γ and also expressed in either structural cells of the lung, namely epithelial cells or smooth muscle cells (Figure 30).

Table 2: Airway hyperresponsiveness-related genes (IPA output)

2210013O21Rik	ICOS	PIK3R1
ADAM12	**IFNG**	PLAT
ADIPOQ	IgG	platelet activating factor
ALOX5	IGHG1	PRG2
AMBP	IL4	PRMT2
arginase	IL5	PTAFR
C3	IL6	PTEN
C5	IL9	RAC1
C3AR1	IL10	Ras
C5AR1	IL13	RIPK2
C5AR2	IL18	RORC
CARD11	IL22	RUNX3
CAT	IL25	**SLPI**
CCL2	IL27	SMAD3
HRAS	IL13RA2	SOCS3

CCL11	IL15RA	SPI1
CCL17	IL17A	SPON2
CCL22	IL17B	SPRED1
CCL28	IL17RA	STAT3
CCL3L3	IL1RL1	STAT4
CCR3	IL1RN	STAT6
CD44	IL27RA	TACR1
CD86	IL2RB	TBX21
CD1D	IL4R	Tgf beta
CD300LF	IL6R	TICAM1
CDH13	ITK	TLR4
CHD4	KIT	TNF
CLCA1	LAMA2	TNFAIP6
CMA1	LGALS3	TNFRSF4
CX3CR1	LTA	TNFRSF9
CXCL12	LTB4R	TNFRSF1A
CXCR4	LTC4S	TPSG1
DLL4	MAPK3	TRAF3IP2
F2RL1	MGAT5	Traj18
FLT3LG	mir-1	TSLP
GATA3	MYD88	U0126

GGT5	NFE2L2	VDR
HAS2	Nos	VEGFA
HGF	NRTN	PIK3CD

Table 2. List of AHR-related genes identified using Ingenuity Knowledge Base (IKB) Diseases and Functions search bar from the IPA software.

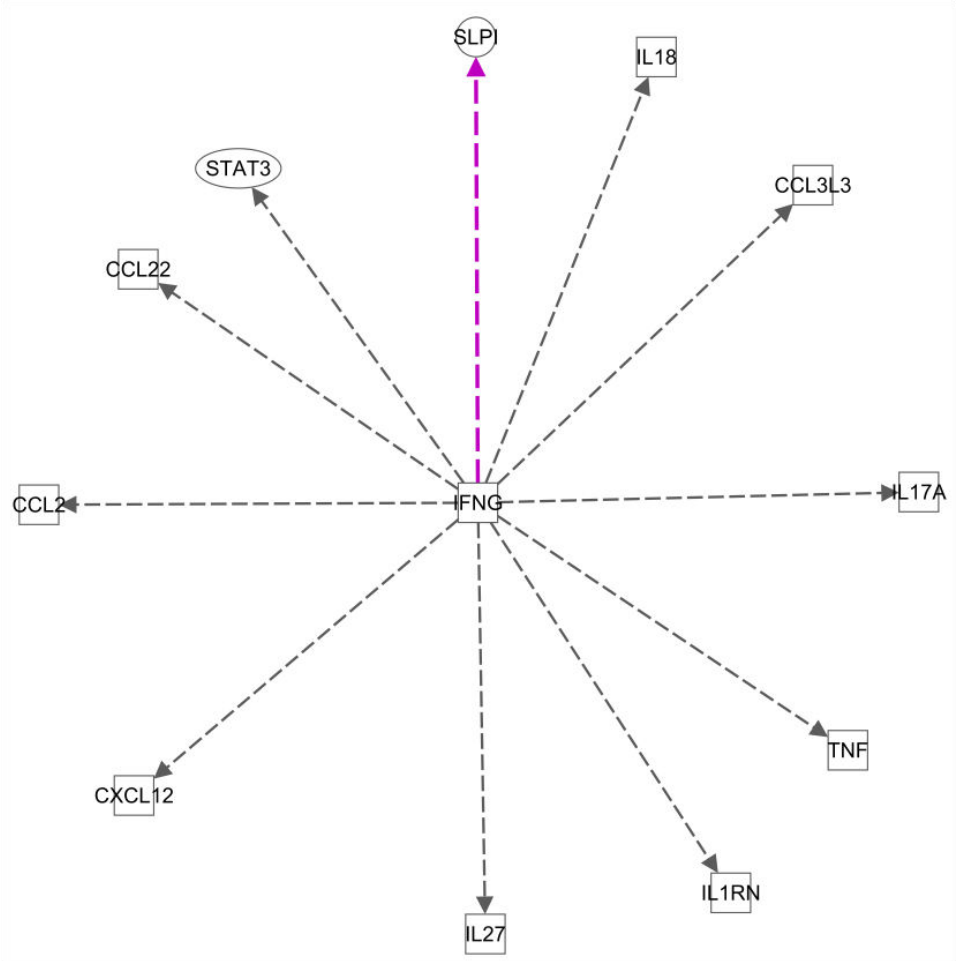


Figure 30. **IFN-γ-regulated AHR-related genes expressed in structural cells of the lung identified using IKB.** Using IPA's Build function, AHR-related genes shown in Table 2 were specifically selected for genes expressed in the lung, epithelial cells and smooth muscle cells.

Genes from this reduced set were further tested for regulation by IFN-γ in the lung using Path Explorer function.

The expression of all of these genes except for CCL3L3 was analyzed by qRT-PCR in the lung epithelium of WT and *Ifng*-/- mice subjected to the SA model (Figure 32). qPCR analysis of 2 epithelial-restricted genes and 2 non-epithelial genes confirmed the enrichment of airway epithelium using the brush technique (Figure 31). As already discussed, levels of IL-17A mRNA and protein were increased several fold in the lungs of *Ifng*-/- mice as compared to that in WT mice and yet the mice did not develop AHR which ruled out a role for IL-17 in promotion of AHR in the SA model. The expression of two other differentially expressed genes, *slpi* (secretory leukocyte protease inhibitor) and *Ccl22*, was of interest based on available information on their role in asthma.

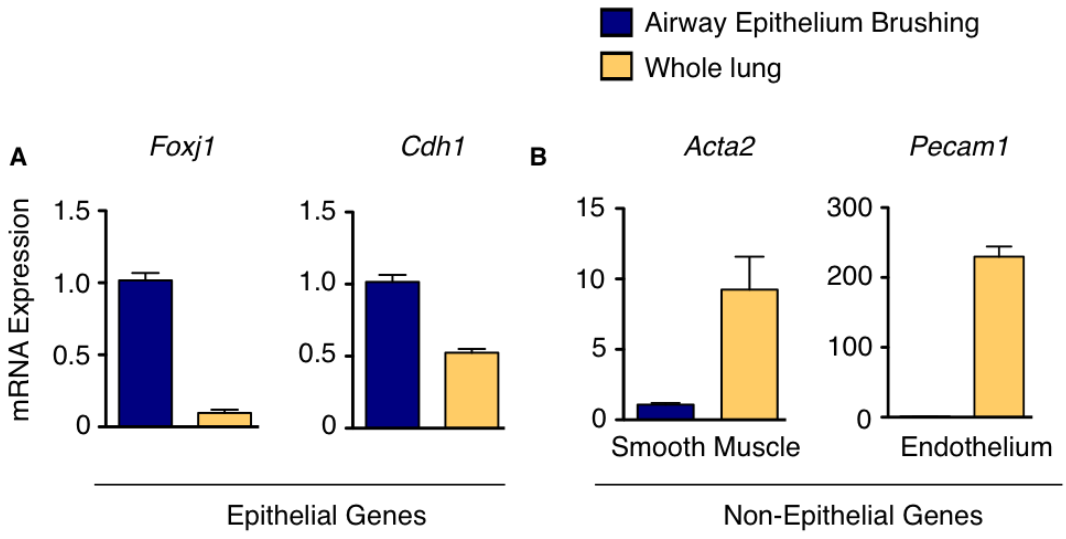


Figure 31. **Characterization of mouse airway epithelial cell brushings.** qRT-PCR analysis of expression of (A) Epithelial-specific genes and (B) non-epithelial genes in whole lung and brush-harvested samples.

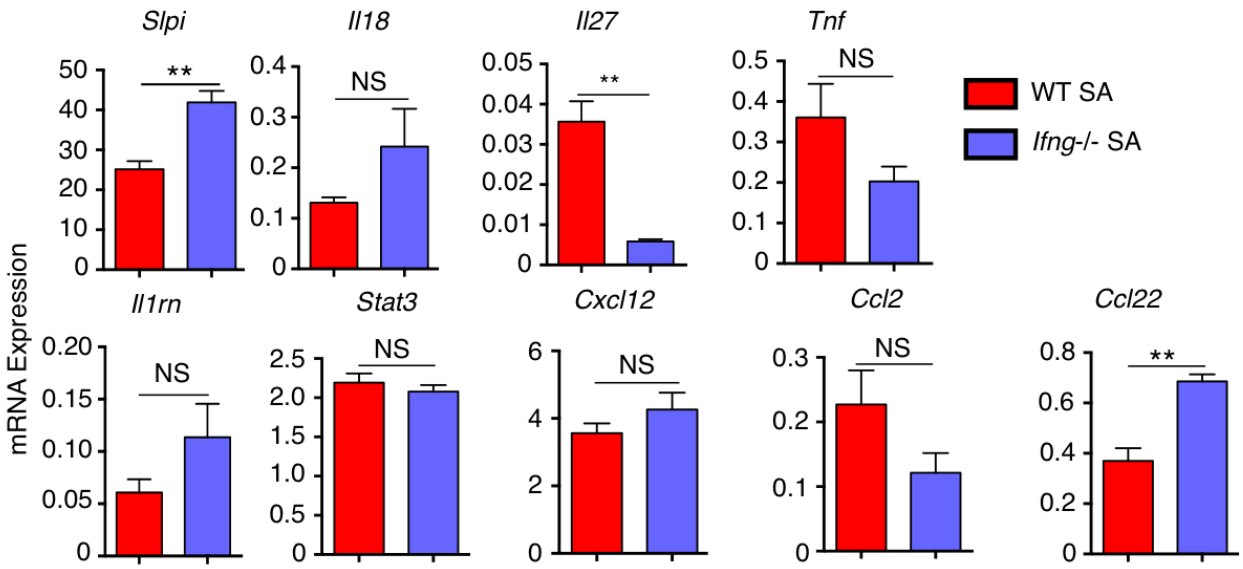


Figure 32. Comparison of AHR-linked IFN-γ-regulated genes in WT and *Ifng*-/- mice subjected to the SA model. qRT-PCR analysis of the indicated genes in WT and *Ifng*-/- mice subjected to the severe asthma model.

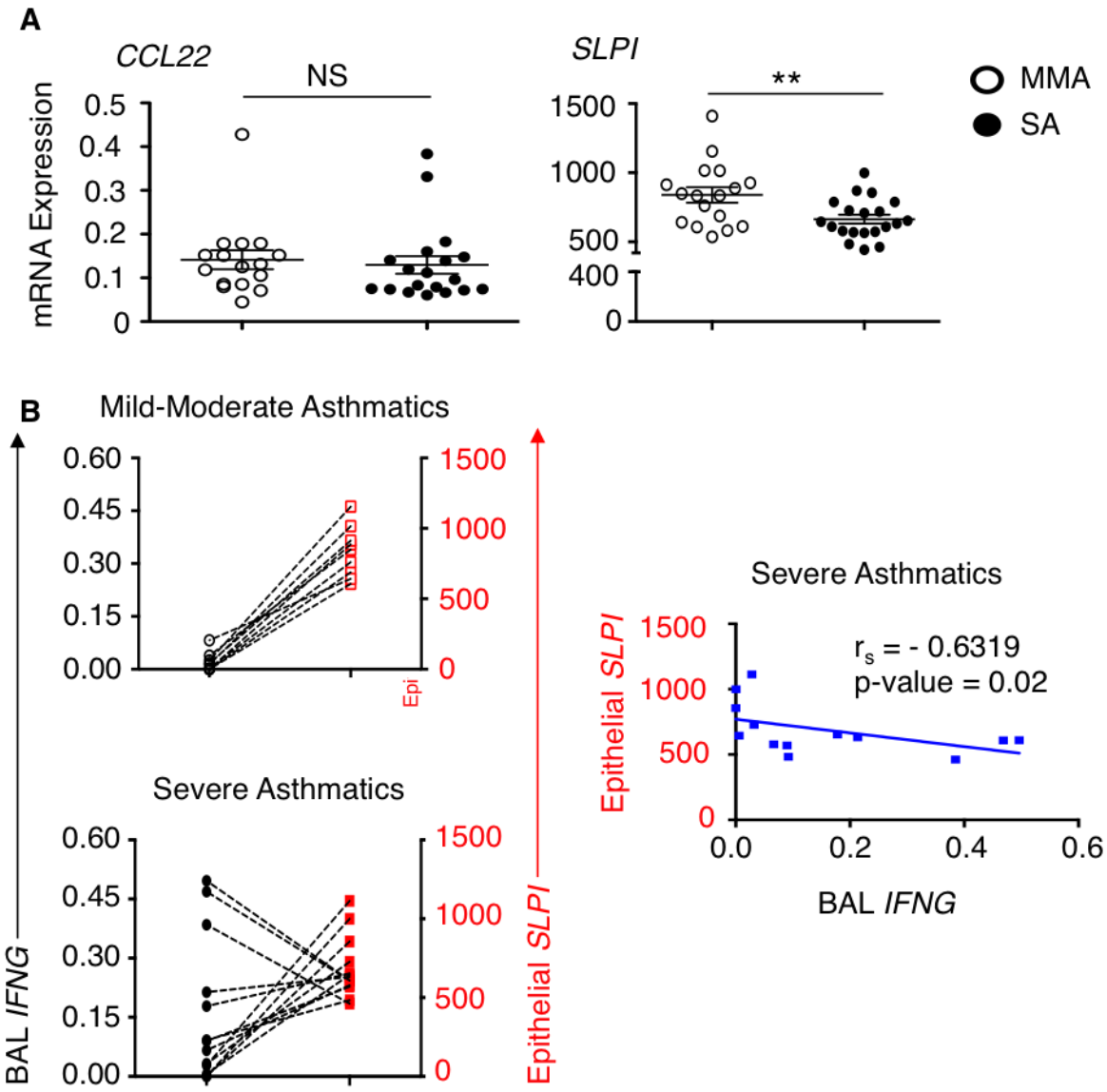


Figure 33. **Inverse correlation between IFN-γ and SLPI expression in human severe asthma.** (A) CCL22 and *SLPI* mRNA expression analyzed by qRT-PCR in epithelial brushings of MMA and SA subjects; n = 17 and 20 for MMA and SA, respectively. ** $p \leq 0.01$, Student's unpaired t-test. (**B**) Correlation analysis between BAL cell *IFNG* and airway epithelial cell-expressed *SLPI* mRNA in MMA (top) and SA (bottom) subjects. Spearman's Rank Correlation Test was used to calculate the correlation coefficient (r_s) using GraphPad Prism Software. Regression line for the SA cohorts is shown in the right panel; n = 9 and 13 for MMA and SA, respectively.

Inverse correlation between IFN-γ and SLPI expression in human severe asthma.

To assess the importance of SLPI and CCL22 in our human asthma samples, we their mRNA expression in bronchial brushings of MMA and SA subjects. *CCL22* mRNA expression was comparable between the two cohorts (Figure 33A, left panel). However, *SLPI* mRNA expression was lower in the bronchial brushings of SA subjects as compared to that in the MMA subjects (Figure 33A, right panel).

We next explored the relationship between epithelial *SLPI* expression and *IFNG* expression in BAL cells in the same subjects. All MMA subjects and most SA subjects (except for 2) used for this analysis were new subjects (not included in Figure 1 but included in Table 1). The MMA subjects displayed high epithelial *SLPI* mRNA expression but again low *IFNG* mRNA expression in their BAL cells. In the majority of SA subjects, the opposite was true and epithelial cell-expressed *SLPI* showed a significant negative correlation with *IFNG* expressed by the corresponding BAL cells (Figure 33B).

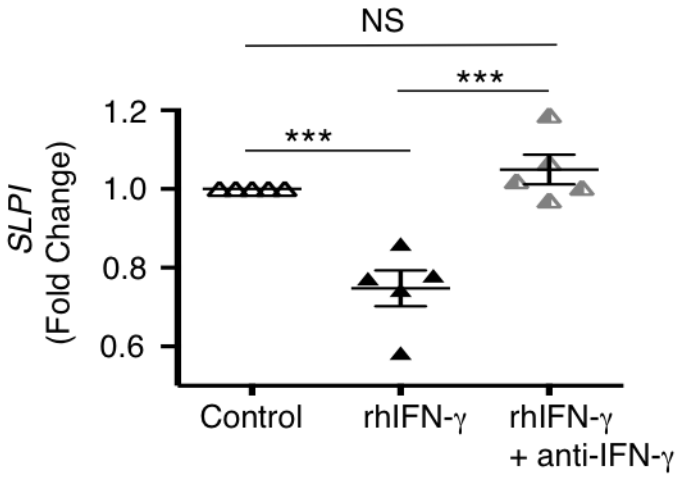


Figure 34. **Recombinant IFN-γ decreases SLPI mRNA expression.** Primary airway (bronchial) epithelial cells from non-asthmatic human subjects were stimulated with rhIFN-γ ± anti-IFN-γ or left untreated for 8 h. *SLPI* mRNA expression was analyzed by qRT-PCR and fold change over untreated was calculated using *HPRT* as internal reference control; n = 5.

To further confirm the negative regulation of SLPI by IFN-γ, primary human bronchial epithelial cells were treated with recombinant interferon-gamma with or without a neutralizing anti-IFN-γ antibody. Recombinant IFN-γ decreased *SLPI* mRNA levels which was reversed with the neutralizing anti-IFN-γ antibody (Figure 34).

We also tested the expression of SLPI in the airway epithelium of mice subjected to the three asthma models used in this study. Both mild asthma models (MA Th1^{lo}Th2 and MA Th2) displayed higher *Slpi* mRNA levels as compared to the SA model (Figure 35A). Also, *Slpi* mRNA expression was similar in lungs of WT and *Il17ra*-/- mice, suggesting that IL-17 signaling is not involved in *Slpi* regulation (Figure 35B).

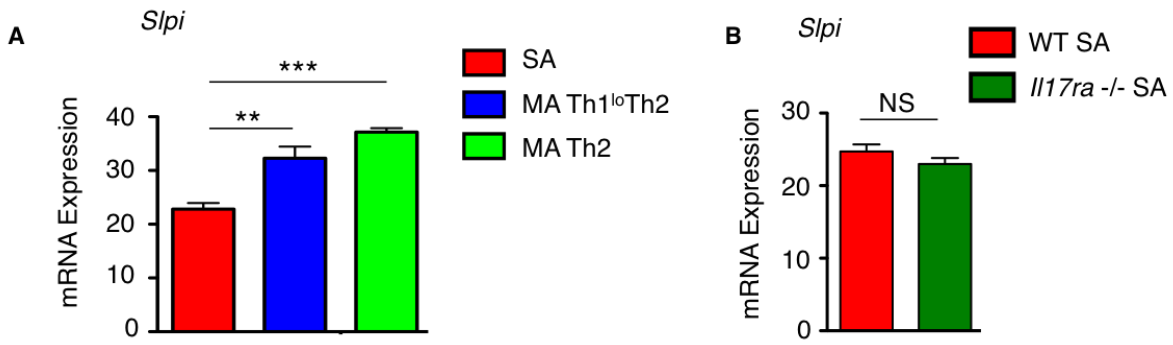


Figure 35. **SLPI mRNA expression in different models of asthma used in the study**. (A). SLPI mRNA expression in airway epithelial brushings from mice subjected to the SA, MA Th1^{lo}Th2 and MA Th2 asthma models. (B) SLPI mRNA expression in airway epithelial brushings of WT and *Il17ra*-.- mice subjected to the severe asthma model.

Forced expression of SLPI attenuates AHR in the SA model

Given that the *Ifng* -/- mice had higher SLPI expression and no AHR induction after being subjected to the SA model, we tested whether overexpression of SLPI in WT mice subjected to the SA model will impact AHR. An expression plasmid for human SLPI (hSLPI), which was

shown to be functional in mice, was introduced by hydrodynamic tail vein injection into mice one day before each challenge set (Figure 36A). hSLPI was detectable in the sera of mice 4 days after the last tail vein injection of hSLPI expression plasmid, with/without Dex treatment, but not in control mice which received PBS only (Figure 36B). As compared to control mice, those that received the hSLPI expression plasmid (but no Dex treatment) mounted lower AHR.

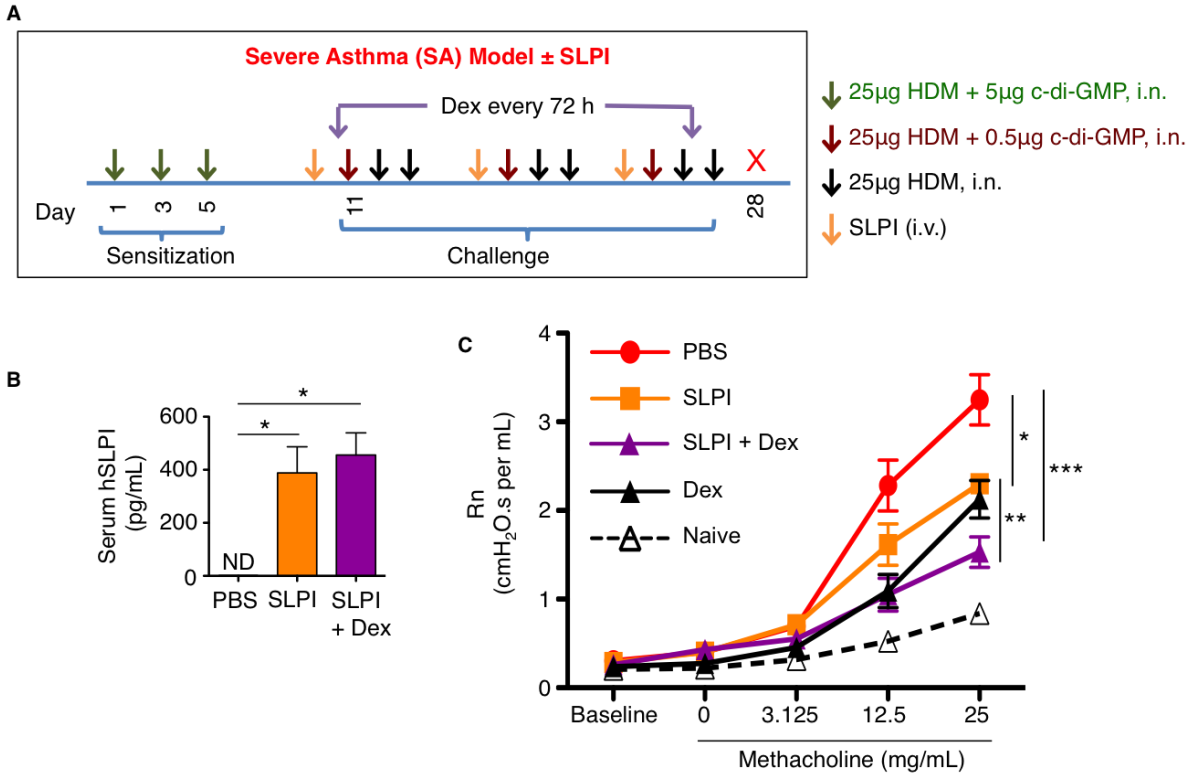


Figure 36. Forced SLPI expression attenuates AHR in WT mice subjected to the SA model. (A) Schematic of the SA model with orange arrows indicating the days when hSLPI-expressing plasmid was administered via tail vein injection. (B) Detection of hSLPI protein in sera of mice that received hSLPI expression plasmid by tail vein injection. (C) AHR measurement in mice subjected to the SA model with or without human SLPI introduced in an expression plasmid via hydrodynamic tail vein injection.

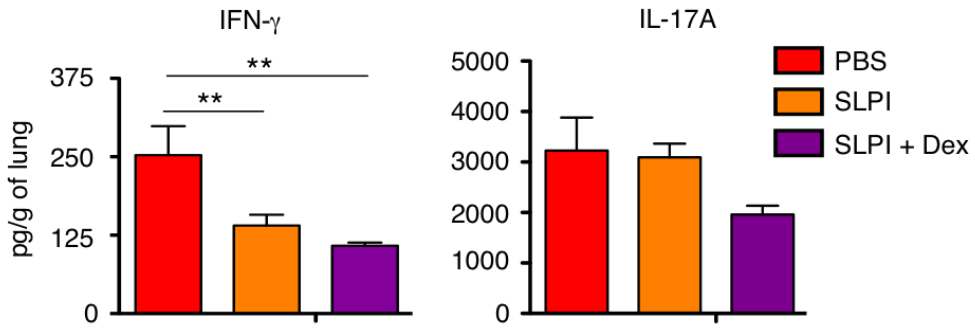


Figure 37. **Forced SLPI expression reduces IFN-γ protein in lung homogenates.** Cytokine concentrations in whole lungs of mice mice subjected to the SA model with or without human SLPI introduced in an expression plasmid via hydrodynamic tail vein injection.

Mice that received the hSLPI expression plasmid along with Dex had even lower AHR at the highest dose of MCh than those that received the expression plasmid alone (Figure 36C). IFN-γ concentration in lung homogenates of mice that received the hSLPI expression plasmid was lower than in the mice that received PBS suggesting a feedback regulation between SLPI and IFN-γ. SLPI overexpression did not alter the level of IL-17A (Figure 37).

Regulation of IFN-γ production by c-di-GMP via IL-12 induction is dependent on STAT1

STAT1 is a well-studied molecule involved in IFN-γ signaling. To study the effect of c-di-GMP signaling on IFN-γ induction, bone marrow-derived macrophages (BMDMs) were used since macrophages can express both IL-12 and IFN-γ and IFN-γ can upregulate its own expression. Also, c-di-GMP, via cytosolic recognition, has been shown to induce inflammatory cytokine production in BMDMs. BMDMs generated from WT and *Stat1*-/- mice were transfected with c-di-GMP. C-di-GMP-induced IL-12p40 production was significantly lower in *Stat1*-/- BMDMs as compared to that in WT BMDMs (Figure 38A). C-di-GMP transfection did not induce IFN-γ production in either WT or *Stat1*-/- BMDMs suggesting that c-di-GMP-stimulated induction of IFN-γ occurs via IL-12 and is dependent on STAT1 (Figure 38B).

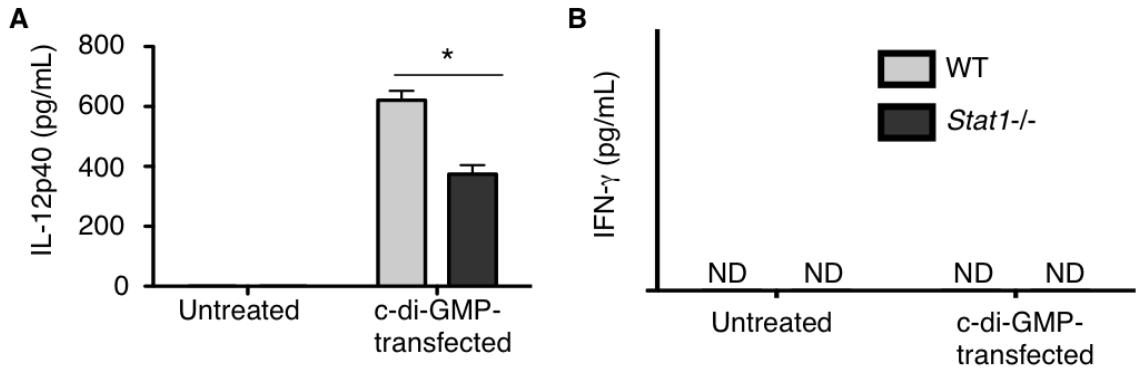


Figure 38. **c-di-GMP-mediated IL-12p40 induction is STAT1-dependent.** (A) IL-12p40 and (B) IFN-γ concentrations in culture supernatants of WT and Stat1-/- BMDMs transfected with c-di-GMP.

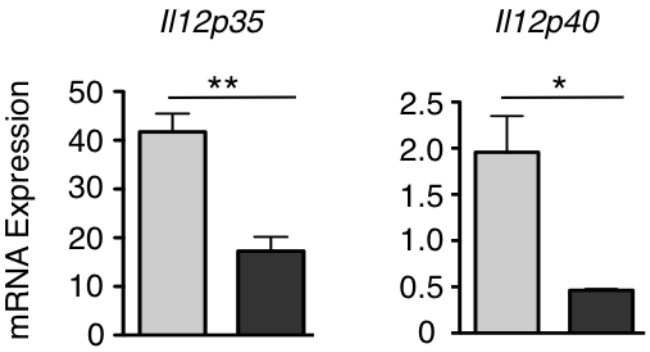


Figure 39. **Stat1 deficiency leads to decreased expression of Th1 polarizing cytokines in lung draining lymph nodes.** *Il12p35* and *Il12p40* mRNA expression in lymph nodes of WT and *Stat1-/-* sensitized in the severe asthma model.

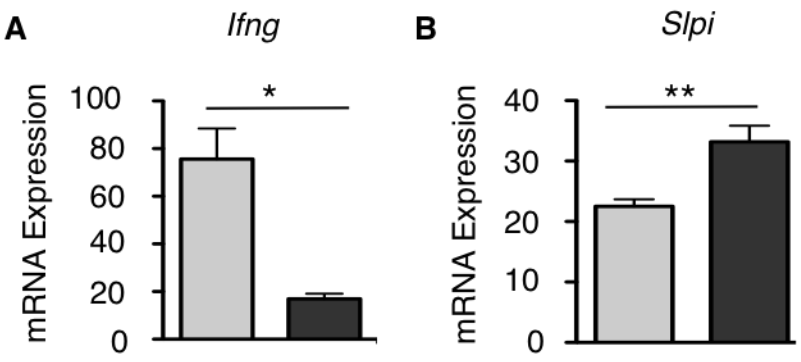


Figure 40. **Reduced lung *Ifng* and increased epithelium Slpi mRNA transcripts in Stat1 -/- mice.** (A) *Ifng* mRNA expression in whole lungs of WT and *Stat1*-/- mice sensitized and challenged in the severe asthma model. (B) *Slpi* mRNA expression in airway epithelial brushings from WT and *Stat1*-/- mice sensitized and challenged in the severe asthma model.

We also observed reduced expression of *Il12p35* and *Il12p40* mRNA expression in the lung-draining lymph nodes of sensitized *Stat1*-/- mice as compared to that in WT mice in the SA model (Figure 39). *Ifng* mRNA expression in the lungs of *Stat1*-/- mice subjected to the SA model was significantly lower as compared to that in WT mice (Figure 40A) which resulted in higher *Slpi* mRNA expression in the airway epithelium (Figure 40B).

5.0 DISCUSSION

Our study, using both human asthma samples and a novel mouse model of severe asthma, has revealed a dysregulated Th1/IFN-γ immune response in SA. The combined human-animal data in the present study also suggests a role for lower SLPI levels in the context of high IFN-γ levels contributing to increased AHR in SA subjects. While a relationship between IL-17 levels and neutrophil recruitment in the airways is evident in both humans and mice (44, 177, 273, 274), neutrophilic inflammation *per se* may not portend poor lung function. That airway inflammation and AHR may not be always linked is being increasingly appreciated (39, 275). Using the animal model of SA we show that neither neutrophils nor IL-17 signaling is required for AHR induction. This focuses our attention on other effector functions of the T cell cytokines in asthma pathophysiology.

Based on research in the 1990s and early 2000s, asthma was largely considered to be an eosinophil-dominant Th2 disease that could be well controlled with CS therapy. However, based on clinical observations and research studies, it is becoming increasingly apparent that not all asthmatics present with eosinophilia and/or a sole Th2 response in their airways. Furthermore, a small proportion of asthmatics respond poorly to CS therapy as well as antibodies that inhibit cytokines/cytokine receptors involved in the Th2 arm of adaptive immunity. This suggests involvement of non-Th2 pathways in the pathology of CS-insensitive asthma. Studies have implicated multiple factors like IL-33, TNF-α, GM-CSF, IFN-γ, etc in CS-insensitive asthma pathology (276, 277). Some of these reagents have either been found to be minimally effective in clinical trials or only been validated in mouse studies till date(278-280). Due to the unavailability of efficient non-steroidal therapies for severe asthma, such patients are prescribed higher doses of CS which provides minimal relief and also endangers them to potential side effects of CSs. Studies on human samples allow only morphological assessment and *in vitro* experimentation. To date, our understanding of the pathological mechanisms central to steroid-resistant neutrophilic SA is minimal primarily because of the lack of a suitable animal model, limiting development and testing of alternative to CS therapy.

We have recently shown synergistic interactions between IFN-γ and low levels of IL-13 in induction of nitro-oxidative stress in primary airway epithelial cells (196). Despite use of high dose of oral CS, IL-13 was still detectable at low levels in the BAL cell culture supernatants of

SA subjects in our study. Using *in vivo* and *in vitro* models, it has been shown that dexamethasone action can be inhibited in an IFN-γ rich environment(16, 202, 203, 281). Transgenic mice overexpressing IFN-γ in the lung develop local tissue inflammation, increased lung volumes and alveolar enlargement thus indicating a role for IFN-γ in lung function decline(282). It will be interesting to determine whether in addition to CD4+ T cells, the recently identified IL-25-induced IL-17RB^{+} cells in the blood of severe asthmatics and associated with steroid-resistant asthma (283) is a source of increased IL-13 from BAL cells isolated from severe asthmatics. In the mouse model as well, despite Dex treatment, a low level of IL-13 along with IFN-γ and IL-17 was maintained in the lungs of the mice. Whether the combination of different levels of T helper cytokines causes different degrees of disease severity will be important to investigate in future studies with large patient cohorts. However, our analysis has shown that among the three T helper subsets, Th1 is dominant in ~70% of SA subjects.

Viral and bacterial infections are associated with severe asthma exacerbations and severe asthmatics are also more prone to such infections(141, 163, 284, 285). While combining HDM with CpG or LPS did not completely recapitulate the pathology as seen in our human asthma cohorts, using c-di-GMP as a surrogate for infection(s), along with HDM, we show that the airway inflammation and AHR are poorly responsive to steroids. Observations from BAL analyses and tissue staining clearly suggest that the inflammation in the severe asthma model is poorly responsive to treatment with steroids, as seen in severe asthma patients(21). Also, the poor response of AHR in severe asthmatic mice to Dex mirrors the persistent AHR seen in severe asthma patients (83). Apart from Th1, Th17 cell induced airway inflammation has been shown to be steroid-resistant (44). Also, IL-17 induces chemotaxis of neutrophils by stimulating production of neutrophil-attracting chemokines and cytokines.

Although a previous study showed the ability of IL-17 to cause airway smooth muscle (ASM) contraction using tracheal rings from humans and mice (185), a recent study failed to associate an IL-17 sputum signature with worse lung function in SA subjects (178). The animal model in the former study employed intraperitoneal (i.p.) sensitization with ovalbumin (OVA) and alum followed by intranasal (i.n.) challenge with OVA and thus the primary site of T cell priming was not the lung. When mice with CD11c+ cell-specific deletion of $\alpha_v\beta_8$ were subjected to this model, the partial reduction in AHR was attributed to a deficiency of Th17 cells

all of the animal models used in our study, mice were sensitized and challenged i.n. to induce T cell priming in the lung-draining lymph nodes. In our SA model, in which both IFN-γ and IL-17 were induced, failure to respond to IL-17 in *Il17ra*-/- mice did not reduce AHR while lack of IFN-γ in *Ifng*-/- prevented AHR. It is not known if any IFN-γ was induced in the OVA model, since Th17 cells can also produce IFN-γ (286). In our analysis of cytokine production in human asthmatics, IFN-γ was the dominant cytokine detectable in appreciable amounts in severe asthmatics despite use of high dose of CS and the amount of IL-17 secreted by BAL cells from severe or milder asthma subjects was much lower and essentially similar. Our cytokine analysis of BAL cell culture supernatants revealed a mean concentration of 0.29 ng/ml of IFN-γ in cultures established from milder subjects. Among SA subjects, BAL cells from 10/14 (70%) produced more than this level while only 3/14 (20%) of MMA subjects were found to produce at a level higher than average. In the 3 animal models used by us, in two models (SA and MA $Th1^{lo}Th2$) in which IFN-γ was induced, AHR was only incompletely suppressed by Dex. In contrast, in the MA Th2 model, which did not produce any detectable IFN-γ protein, AHR was completely suppressed by Dex. Most remarkably, AHR in the *Il17ra*-/- mice was almost identical to that induced in WT mice although inflammation was substantially reduced. It is important to note that among the 4 SA subjects whose BAL cells generated below average IFN-γ, IL-17 concentrations were <0.050 ng/ml and IL-13 levels were below the mean level detected in the SA group (~40 pg/ml). Thus, in some subjects, other mechanisms, which may involve tissue-resident cells such as ILC2s (64), may drive the SA phenotype. Collectively, these data show that in the context of a complex immune response, as observed in 70% of human severe asthma and also modeled in mice, IFN-γ plays an important role in AHR while IL-17 promotes neutrophilic inflammation in the airways.

An inverse relationship between IFN-γ expression and SLPI, as observed in the SA subjects and in the mouse SA model, has been also observed previously in entirely different contexts. For example, overexpression of IFN-γ in the airways of transgenic mice was shown to selectively inhibit expression of SLPI but not of other protease inhibitors (282). How might low SLPI contribute to the SA disease state? After its cloning in 1986 and description of its presence in various body fluids including nasal and bronchial mucosal secretions, SLPI was described as an epithelial product. Later, it was also shown to be a product of macrophages hyporesponsive to

LPS (287). IFN-γ was shown to inhibit SLPI expression restoring LPS responsiveness to macrophages (287). Most interestingly, relevant to SA, overexpression of SLPI in macrophages inhibited nitric oxide production (287). It is important to note that in humans, airway epithelial cells rather than alveolar macrophages express iNOS and reduced SLPI expression in these cells in severe asthmatics, as observed in our study, may contribute to increased exhaled nitric oxide (FeNO) production. In our recent cluster analysis, subjects in cluster 6 with mixed granulocytic airway inflammation, who required the highest health care utilization and were on high dose of oral CS also expressed the highest level of FeNO (11). It is possible therefore, that lower SLPI expression in SA due to high IFN-γ expression contributes to increased FeNO levels.

SLPI broadly inhibits multiple leukocyte serine proteases including chymase and tryptase produced by mast cells. Tryptase was shown to promote AHR by activating protease activated receptor 2 (PAR2) rendering PAR-2 responsive to peptide agonists (288). Stimulation of activated PAR-2 caused AHR by release of neurokinins from afferent neurons in the bronchial tissue (288). It is interesting that in another OVA-based model of chronic asthma in which mice were sensitized i.p. with OVA (without alum) and then challenged i.n. with OVA for 9 weeks, a mast cell-dependent role for IFN-γ on airway remodeling, AHR and airway inflammation was found (201). It is possible that in this chronic model as well, IFN-γ-mediated decrease in SLPI expression in airway epithelial cells with derepression of mast cell protease activity is involved in increased AHR in the mice. Although in the chronic OVA-induced model, a role for IFN-γ in airway inflammation was also observed, in our SA model, a role for IFN-γ in airway inflammation was not evident but instead IL-17 was involved in promoting airway inflammation. In a study of wound healing in skin, *Slpi*-deficient mice were found to generate active TGF-b, which played a role in wound healing (289). Since TGF-b is a central mediator of airway remodeling (290), SLPI deficiency may also contribute to airway remodeling in asthma, another hallmark of severe and chronic asthma, which is believed to be responsible for persistent AHR (291). It is interesting to note that multiple allergens have protease activities as do different cell types such as neutrophils and mast cells. Thus, it is possible that SLPI may play a fundamental role in inhibiting both allergen- and cell-associated proteases such that its downregulation promotes the SA phenotype via effects on AHR, FeNO levels and airway remodeling. It will be useful to develop an inducible airway-specific SLPI transgenic mouse system to study effects of

SLPI expression on allergic airway disease - this approach may inhibit AHR to a greater degree due to local overexpression in airway epithelial cells where SLPI is normally expressed.

Observations from our study and emerging literature highlight an urgent need for non-steroidal anti-inflammatory drugs for the treatment of severe asthma. The limited success of anti-cytokine therapies suggests too selective an approach by targeting one moiety for a disease as complex and heterogeneous as asthma. Thus, there is an unmet clinical need to find alternatives to steroid therapy, preferably targeting multiple factors, to control inflammation and AHR so as to improve the health-related quality of life for SA patients. Besides targeting the obvious inflammatory players involved in asthma pathology, it would also be fruitful to target downstream molecules, especially molecules like SLPI that are expressed in airway structural cells, to achieve maximal symptom control.

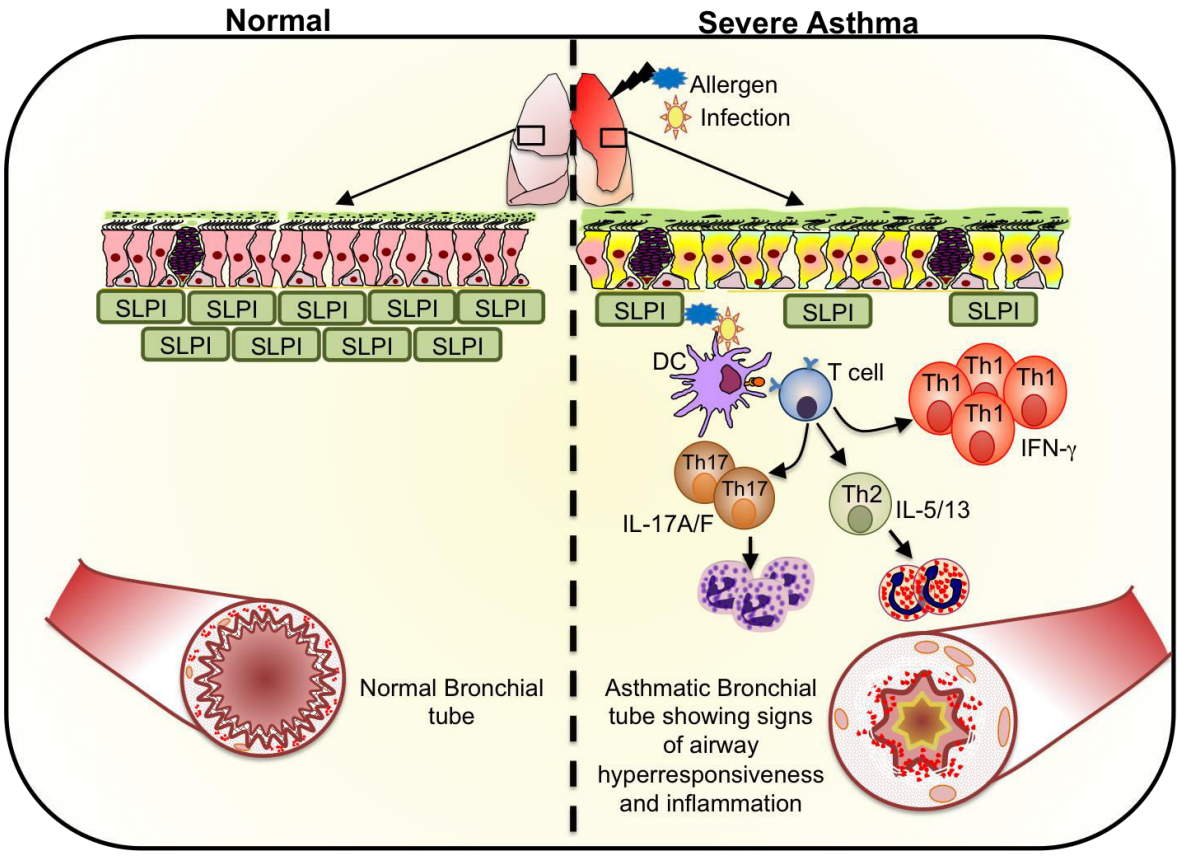


Figure 41. **Outline depicting the important findings of the project.**

The concept of asthma has advanced over time from a singular disease to a complex of various phenotypes, with varied natural histories, physiologies and responses to treatment (292). Further research is needed to clarify additional clinical and molecular phenotypes, identify and validate biomarkers and find new molecules for possible intervention(s). Well-characterized asthma populations of diverse etiology and backgrounds with longitudinal follow-up are likely to add new information on molecular networks.

6.0 Future Directions

Asthma heterogeneity has led to challenges in treatment, especially in patients poorly responsive to current therapies(293). Corticosteroids, which are the gold standard therapy for asthma, do not work effectively in all asthmatics. Few effective alternatives to CS therapy are currently available for treating severe asthma patients.

Using human samples and a novel mouse model of severe asthma, in this study we showed that the immune response in severe asthma is distinctly different from that seen in mild-moderate asthma. While asthma has traditionally been considered a Th2 disease, our study shows a distinct IFN-γ signature in severe asthmatics. Using a novel animal model of severe asthma established in our laboratory, we prove that IFN-γ is central to controlling AHR in this model of severe asthma. On the other hand, IL-17A controls neutrophilic influx into the airways but has no role in controlling AHR in this model. Recent clinical trials using Brodalumab, a monoclonal antibody against IL-17RA, also proved ineffective in controlling asthma symptoms. Using biological network analysis tools, we were able to show an inverse correlation between BAL cell-expressed IFN-γ and epithelium-expressed SLPI in both human samples and the mouse model of severe asthma. Forced expression of SLPI in the mouse model of SA attenuated AHR which decreased further when combined with Dexamethasone.

It has been suggested that anti-protease supplements should be administered to reduce deleterious effects of chronic inflammation by neutrophil serine proteases (229). Inhaling inhibitors directly into the lungs to target extracellular proteases is very attractive since it limits the treatment to the site of the disease. It thus provides high concentrations of active molecules where they are needed, minimizing systemic side effects. Inhaled α1-PI has been shown to reduce the HNE-mediated destruction of lung tissue in mice exposed to cigarette smoke(294) and in patients with CF(295, 296). Accessibility of pulmonary airways to aerosols of drugs makes aerosol therapy with anti-proteases a powerful way of treating lung diseases. However, depositing aerosols in the lungs requires particles of appropriate sizes and the assurance that the drug reaches poorly ventilated areas of the lung. Since SLPI can be cleaved and inactivated by cysteine cathepsins (247), encapsulating SLPI within liposomes can bypass the unwanted effects of proteolysis. Encapsulated SLPI was recently shown to be protected from degradation by Cathepsin L (297). Excess mucus in the airways of patient with severe asthma presents another challenge to the deposition of the aerosols containing SLPI.

Systemic corticosteroid use has been associated with an increased risk of fracture and cataracts (298, 299) while high doses of ICS are associated with an increased risk of adrenal suppression and growth retardation in children (298, 300, 301). Systemic corticosteroid-related weight gain may further impact negatively on asthma control (302). Thus, steroid sparing or lowering therapies, such as those with small molecule inhibitors, are needed to reduce the side-effects of CSs and overall health benefit for asthma patients.

To better meet the therapeutic needs of severe asthmatics, identifying molecules using high throughput sequencing of clinical samples is the need of the hour. These molecules then need to be tested rigorously in animal and cell-culture models to assess their functionality and specificity to an asthma phenotype. Asthma treatment needs to be personalized in the sense that it should be matched to an individual's genetic and environmental susceptibility. Proof of concept for endotyping asthma for better understanding of its mechanisms and more focused drug development can lead to better and more personalized therapeutic options that will far outweigh the current 'one-size-fits-all' approach to asthma care.

7.0 PUBLICATIONS

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