THYMIC STROMAL LYMPHOPOIETIN EXPRESSION IN NASAL EPITHELIAL CELLS OF ALLERGIC ASTHMATICS

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ABSTRACT

Thymic stromal lymphopoietin (TSLP), an epithelial-derived cytokine, has a critical role in the development of allergic inflammatory responses and have been implicated in type 2 allergic disease, including asthma, allergic rhinitis, and atopic dermatitis. Genetic polymorphisms in the TSLP gene are among the most commonly cited variants associated with asthma and allergic disease, however, the functional effects of these polymorphism are not fully understood. The objective of this study was to investigate the role of a TSLP polymorphism in the Th2 inflammatory responses of the nasal epithelium, as well as in responding to nasal allergen provocation and intranasal corticosteroid treatment. We cultured nasal epithelial cells from allergic asthmatic subjects and examined cytokine and chemokine secretions and gene expression profiles in response to polyinosinic:polycytidylic acid treatment. To explore the functional consequences of the rs1837253 polymorphism we analyzed the two TSLP gene isoforms, as they have shown dichotomous effects, however, no associations were found between rs1837253 genotype and the expression of TSLP and gene isoforms. We did not find any associations of TSLP or cytokine production between genotypes, or in relation to response to nasal allergen challenge or corticosteroid treatment. Exploration of local and systemic effects of the rs1837253 SNP did not show any differences in response to INCS treatment *in vitro* or *ex vivo*. We did demonstrate that nasal epithelial cell-derived factors are capable of stimulating eosinophil/basophil colony forming units in the absence and presence of exogenous IL-3. Overall, the results indicate a role of the nasal epithelium in driving

eosinophil/basophil differentiation and highlight the complexity of gene-environment interactions and the mechanisms of asthma and allergic inflammation.

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LIST OF ABBREVIATIONS AND SYMBOLS

AHR – airway hyperresponsiveness

ANOVA – analysis of variance

AP-1 – activator protein-1

ASMC – airway smooth muscle cells

BAL – bronchoalveolar lavage

BEC – bronchial epithelial cell

BEGM – bronchial epithelial growth medium

CCL – chemokine (C-C motif) ligand

CFU – colony forming unit

COPD – chronic obstructive pulmonary disease

CXCL – chemokine (C-X-C motif) ligand

CysLT – cysteinyl leukotriene

DC – dendritic cell

dsRNA – double stranded ribonucleic acid

EAR – early asthmatic response

EGF – epidermal growth factor

Eo/B – eosinophil basophil

ERK – Extracellular signal regulated kinase

FcεRI – Fc receptors

FEV_1 – forced expiratory volume in 1 second

FVC – forced vital capacity

GADPH – glyceraldehyde 3-phosphate dehydrogenase

GATA – GATA-binding factor

GM-CSF – granulocyte-macrophage colony stimulating factor

GR – glucocorticoid receptor

GRE – glucocorticoid response element

GWAS – Genome-wide association study

HDM – house dust mite

HLA – human leukocyte antigen

hMPV – human metapneumovirus

HPC – hematopoietic progenitor cell

IFN – interferon

ICAM-1 – intracellular adhesion molecule 1

Ig – immunoglobulin

IL- interleukin

ILC – innate lymphoid cell

INCS – intranasal corticosteroid

iNKT – innate natural killer T

JAK – Janus protein tyrosine kinase

JNK – c-Jun N-terminal kinase

LAR – late asthmatic response

LD – linkage disequilibrium

lfTSLP – long TSLP isoform

LPS – lipopolysaccharide

mAb – monoclonal antibody

MAPK – mitogen activated protein kinase

mDC – myeloid dendritic cell

MDC – macrophage-derived chemokine

MHC – major histocompatibility complex

MIP-1α – macrophage inflammatory protein 1α

mRNA – messenger ribonucleic acid

NEC – nasal epithelial cell

NF-κB – nuclear factor κB

nGRE – negative glucocorticoid response element

NKT – natural killer T

OX40L – OX40 ligand

PC – provocation concentration

PC_{20} – provocation concentration that causes a 20% decrease in FEV_1

pDC – plasmacytoid dendritic cell

PI3K – phosphinositol 3 kinase

Poly (I:C) - polyinosinic:polycytidylic acid

PNIF – peak nasal inspiratory flow

qRT-PCR – quantitative reverse transcriptase polymerase chain reaction

RSV – respiratory syncytial virus

SDF-1α – stromal cell-derived factor 1α

SEM – standard error of the mean

sfTSLP – short TSLP isoform

SLP1 – secretory leukocyte protease inhibitor

SNP – single nucleotide polymorphism

STAT – signal transducer and activator of transcription

TARC – thymus and activation regulating chemokine

TBK-1 – TANK-binding kinase 1

TCR – T cell receptor

TGF-β – transforming growth factor β

Th – T helper

TLR – toll-like receptor

TNF-α – tumor necrosis factor α

TNSS – total nasal symptom score

Treg – T regulatory cells

TSLP – thymic stromal lymphopoietin

YWHAZ - tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

1. INTRODUCTION

Allergic diseases, such as anaphylaxis, allergic rhinitis, asthma and atopic dermatitis, have increased globally over recent decades. These diseases affect approximately 20% of the population worldwide, making them some of the most common chronic health conditions with a serious impact on quality of life (Kay, 2001; Zheng, 2014a). While the underlying causes for this increase in prevalence are not fully understood, genetic and environmental factors, as well as gene-environment interactions have been proposed to play roles in the development of allergic diseases (Alfven, et al., 2006). Advances in human genetics and genome mapping have provided important genetic information regarding asthma; however, the increases in prevalence of allergic disease in the past few decades have been too rapid to be attributed to genetics alone, and it is likely that they result from complex gene-environment interactions as well as epigenetic changes, in some cases (Burbank, et al., 2017).

Many have suggested the influence of significant lifestyle changes, particularly in developed countries, that have reduced microbial exposures through increased sanitation and rates of immunization. This has been proposed to alter the immune system development may affect allergic disease susceptibility: known as the "hygiene hypothesis" (Fishbein & Fuleihan, 2012), this idea postulates that increased cleanliness has deprived the early immune system of bacterial and viral induced T helper cell (Th) type 1 (Th1) responses, and skewed immune balances towards Th2 responses (Eisenbarth, et al., 2004). However, studies suggest that Th1 auto-immune and Th2-mediated allergic diseases are not mutually exclusive and that the Th1/Th2 paradigm may be oversimplified (Duffy, et

al., 1990; Sheikh, et al., 2003; Simpson, et al., 2002). Given the complexity of immune responses, it is thus likely that gene-environment interactions play a critical role in the development of allergic inflammation and atopic diseases.

ALLERGIC DISEASE AND ASTHMA

Prevalence of Asthma

Worldwide, there are over 300 million people with asthma (To, et al., 2012). While asthma affects individuals of all ages, its highest incidence occurs in childhood and teenage years (Engelkes, et al., 2015; Sears, et al., 2003; Strachan, et al., 1996), and the highest prevalence is seen in young adulthood (Hansen, et al., 2000). Rates of asthma prevalence have increased by 50% every decade (Masoli, et al., 2004), and there are approximately 250,000 premature deaths related to asthma every year (Bousquet, et al., 2006; Masoli, et al., 2004; Urbano, 2008). According to the Canadian Chronic Disease Surveillance System, in 2016 11.55% of Canadians over the age of one had been diagnosed with asthma by a health professional (Public Health Agency of Canada, 2019). Asthma is the leading cause of hospitalizations in the overall population, school absenteeism (Canadian Institute for Health Information, 2018) and hospitalizations in children (Canadian Institute for Health Information, 2018; Ismaila, et al., 2013; Tillie-Leblond, et al., 2008), and the third most common cause of lost work productivity (Theriault, et al., n.d.; To, et al., 2007).

Asthma and allergic diseases have a high socio-economic burden, with the direct and indirect costs ranking among the highest of chronic health diseases, due to significant utilization of health care resources (Bahadori, et al., 2009; Sadatsafavi, et al., 2010; Zuberbier, et al., 2014) and damaging impacts on quality of life – including professional,

physical, social, and emotional domains (Braman, 2006). In Canada, the direct costs related to asthma, such as hospitalization, healthcare professional services, and medications, as well as the indirect costs, including decreased productivity, are estimated at $2.1 billion annually (Tavakoli, et al., 2017). In 2012, the reported direct excess cost of asthma in Canada was $1028 per person per year (Tavakoli, et al., 2017), indicating a significant financial burden associated with these chronic conditions. The cost to control asthma is very high, and can be even higher when not properly controlled (Accordini, et al., 2006). With regard to quality of life, while there is limited information on the burden of the disease, studies have shown that there is a high (31%-50%) prevalence of psychological distress and diminished quality of life in individuals with asthma when compared to those without asthma (Ismaila, et al., 2013). Considering the rising prevalence and the socioeconomic impact and burden of asthma, it is critical to develop a more in depth understanding of its pathogenesis to identify potential markers and therapeutics to treat and mitigate the societal impact of this disease.

Clinical Manifestations and Diagnosis

Asthma is a heterogeneous, chronic obstructive airway disease that involves inflammation of the respiratory tract. Clinical features of asthma include recurrent wheezing, breathlessness, chest tightness and coughing. It is characterized by reversible airflow obstruction, which is associated with airway hyperresponsiveness (AHR) and inflammation (Cockcroft, et al., 1977; Hargreave, et al., 1981; Murphy & O'Byrne, 2010). AHR is defined as the predisposition of the airways of patients to narrow excessively in response to stimuli, and is characteristic of asthma (Chapman & Irvin, 2015). It can be

useful as a tool for diagnosis and has been shown to correlate with asthma severity (Sont, et al., 1999; Townley & Horiba, 2003).

Diagnosis of asthma involves physical examination, medical history, and lung function assessments using spirometry to identify variable expiratory airflow limitations (Cockcroft, et al., 1977; Hargreave, et al., 1981; Quirt, et al., 2018). Bronchoprovocation challenge testing and investigating for airway inflammation markers are also used in diagnosing asthma and are particularly important when lung function measurements are normal but symptoms of asthma continue to persist (Kaplan, et al., 2009; Lougheed, et al., 2012; Reddel, et al., 2015).

Spirometry measures airflow parameters such as forced vital capacity (FVC, the maximum volume of air that can be exhaled), and the forced expiratory volume in one second (FEV_1, the volume of air exhaled in the first second of forced expiration from full inhalation) (Miller, et al., 2005). The ratio of FEV_1 to FVC provides a measure of airflow obstruction (Cockcroft, et al., 1977; Hargreave, et al., 1981). A FEV_1/FVC ratio of less than 0.75 in adults or 0.9 in children suggests airflow limitation (Lougheed, et al., 2012; Reddel, et al., 2015). Measurement of AHR using direct airway challenges to bronchoconstrictor stimuli, such as methacholine or histamine, or indirect challenge methods, such as mannitol or exercise can be very beneficial in the diagnosis of asthma. Bronchoprovocation tests typically involve inhaling increasing concentrations of a substance (methacholine or histamine) until a specified level of bronchoconstriction is achieved, usually a 20% decrease in FEV_1 (Cockcroft, et al., 1977; Hargreave, et al., 1981). The results of this test are most often reported as the provocation concentration (PC) of the

agent that causes a 20% drop in FEV_1 (PC_{20}). A PC_{20} of less than 8 mg/mL is a threshold for a positive result indicative of AHR, and may support the diagnosis of asthma (Quirt, et al., 2018). Non-asthmatic individuals typically have a PC_{20} higher than 16 mg/mL, which suggests normal lung function (Crapo, et al., 2000). It is important to note that a positive bronchial reactivity test result is not specific to asthma and may occur in conditions like allergic rhinitis and chronic obstructive pulmonary disease (COPD). In the diagnosis of asthma, the degree of reversibility, or rapid improvements, in FEV_1 is measured at baseline and following an inhaled fast-acting bronchodilator (e.g. albuterol). An improvement of $\geq$ 12% and $\geq$ 200mL in FEV_1 following the administration of a bronchodilator is considered supportive of an asthma diagnosis (Bateman, et al., 2008; Pellegrino, et al., 2005). Other tests may be conducted to provide more information on an individual's asthma. Allergy skin prick testing (SPT) is a non-invasive method that may be used to identify possible environmental triggers of asthma and determine an individual's asthma phenotype. Additionally, inflammatory markers, such as sputum eosinophils (Gibson, et al., 1989; Hargreave, 1999) or levels of exhaled nitric oxide can provide support for asthma diagnosis. While it is not an accepted standard test in Canada, studies have shown that the measurement of exhaled nitric oxide levels can support the diagnosis of asthma and may be useful for monitoring the response to asthma therapies (Kaplan, et al., 2009).

Inhaled Allergen Bronchoprovocation Tests

For decades, standardized allergen bronchoprovocation testing has served as a proven model for mimicking the acute, as well as some chronic, features of asthma in humans, enabling investigation into the processes and characteristics of asthma in humans,

as well as the evaluation of therapeutics (Boulet, et al., 2007; Cockcroft, et al., 1987; Diamant, et al., 2013). Allergen inhalation by atopic asthmatics results in the manifestation of physiological and inflammatory features of asthma, including AHR, reversible airflow obstruction, and eosinophilic airway inflammation (Gauvreau, et al., 2015). While provocation tests that utilize direct bronchoconstrictors, such as methacholine and histamine, produce short-acting transient bronchospasm, allergen testing provides an indirect method which induces airway narrowing and prolonged bronchoconstriction through the release of inflammatory mediators and cytokines (O'Byrne, et al., 2009). Three types of allergen challenge testing in the respiratory tract include nasal challenge, segmental lung challenge, and total lung or inhaled challenge. Nasal allergen challenge has the advantage of producing a direct allergen contact in a controlled setting and helps to understand the effects of upper airway challenge on the local or systemic inflammatory response. Segmental allergen challenge involves the exposure of a small amount of allergen through the use of a bronchoscope to a well-defined area of the lung, and can be used to assess localized effects efficiently (Busse, et al., 2005). Inhaled allergen challenge includes: incremental and high-dose allergen challenges, repeated-measures low dose allergen challenges and allergen exposure rooms (Devillier, et al., 2011; Ihre & Zetterstrom, 1993; Taylor, et al., 2000), which provide information on the allergic airway response. Inhalation challenges using "provocative inhaled dose" methods offer the advantage of examining allergen-induced inflammatory responses and the changes to lung physiology (Diamant, et al., 2013). Allergen-induced bronchoprovocation tests are important research tools that offer an integral model for allergic asthma.

Genetics of Asthma

While asthma is thought to be multi-factorial and heterogenous, a genetic component has been firmly established. Heritability refers to the proportion of variation in a quantitative trait or risk of disease that can be attributed to genetic variation (Mathias, 2014). Familial studies early in the twentieth century showed evidence of familial aggregation or clustering of asthma, wherein relatives of individuals with asthma exhibited increased incidence of asthma when compared to the relatives of normal controls (Cooke & Vander Veer, Jr, 1916; Spain & Cooke, 1924). Since then, there have been numerous familial studies conducted with similar results (Aberg, 1993; Dold, et al., 1992; Gerrard, et al., 1976). Twin studies have been advantageous for studying asthma, as they enable investigations into genetic and environment causes of diseases. Numerous twin studies have shown a higher concordance rate of asthma in monozygotic twins when compared to dizygotic twins (Harris, et al., 1997; Koeppen-Schomerus, et al., 2001; Nieminen, et al., 1991). Studies have estimated that the heritability of asthma ranges between 36% and 95% (Edfors-Lubs, 1971; Laitinen, et al., 1998; Skadhauge, et al., 1999; van Beijsterveldt & Boomsma, 2007), with the largest estimates in children, compared to older individuals (Duffy, et al., 1990; Thomsen, et al., 2006, 2010; Ullemar, et al., 2016; van Beijsterveldt & Boomsma, 2007). The large spectrum of heritability encompasses the role of genetic and environmental factors, further reinforcing the postulate of important gene-environment interactions.

Although asthma has a complex genetic background, there have been many successful studies that have identified loci that contribute to disease risk. The first approach

to genome-wide genetic mapping for disease risk was through linkage analysis, which is a family-based mapping strategy used to detect genetic loci with large effect sizes that are co-localized or "linked" with disease. This type of analysis is particularly useful because it allows for novel discoveries without the biases of the disease biology. Using linkage analysis, *ADAM33* (Van Eerdewegh, et al., 2002), *DPP10* (Allen, et al., 2003), *PHF11* (Zhang, et al., 2003), *NPSR1* (Laitinen, et al., 2004), *HLA-G* (Nicolae, et al., 2005), *CYFIP2* (Noguchi, et al., 2005), were identified as genetic loci associated with asthma. While linkage analysis has been plagued with difficult replication between studies, meta-analyses have identified genomic regions that remain robust. Many of the highly-associated genes can be mapped to most replicated linkage regions (Ober & Hoffjan, 2006). The widely replicated region, 5q31-33, contains at least 14 genes that have been associated with asthma and related phenotypes, including interleukin (IL) 4 (*IL4*), *IL13*, *CD14*, and *ADRB* (Ober & Hoffjan, 2006; Yokouchi, et al., 2000, 2002).

Another approach into analyzing genetic asthma determinants is utilizing candidate gene association studies, where the hypothesis-driven gene analysis is based on either biological function, physical location within an identified linkage region, or location within a region with prior association evidence. Using a candidate gene approach, over 100 loci have been identified as genetic determinants of asthma, including genes involved in innate immunity and immunoregulation, Th2 cell differentiation and effector function, epithelial cell biology and mucosal immunity, and those involved in lung function, airway remodelling and asthma severity (Ober & Hoffjan, 2006; Ober & Yao, 2011; Vercelli, 2008). However, candidate gene analyses have shown limited reproducibility, which may

be due to sample size, differences in phenotype definition, and/or lack of appropriate case-control matches (Cardon & Bell, 2001; Rogers, et al., 2009).

An important factor in association studies to be considered is linkage disequilibrium (LD), which is the association of alleles at different sites on a chromosome (Akhabir & Sandford, 2011). When polymorphisms are further apart the LD is typically lower, although this is influenced by a number of factors, including population ancestry; in addition, the relationship between LD and gene distance is not uniform. For this reason, an identified genetic association with disease susceptibility may be in LD with multiple neighbouring variants which influence causality (Akhabir & Sandford, 2011). LD can also be beneficial as it reduces the number of variants that need to be analyzed in certain regions because one polymorphism in linkage can be utilized to assess others nearby.

With advances in genotyping arrays, decreased costs, and improved methods of analysis, GWAS have become much more feasible in the past decade, providing a non-biased approach to survey the entire genome for variants, and a very powerful tool to identify specific gene variant risk factors for disease (Vicente, et al., 2017). GWAS assess millions of single nucleotide polymorphisms (SNP), and, mostly commonly, utilize a comparison between the genotype frequencies of cases and controls. Due to the high number of comparisons that are conducted, there is a large statistical burden, so very stringent statistical corrections must be made to avoid false-positive results, requiring studies to have very large sample sizes in order to achieve genome-wide levels of significance (Akhabir & Sandford, 2011). Additionally, GWAS primarily detect common risk variants, those most commonly included in genotyping assays, since there is reduced

power to detect associations in SNPs with low frequencies. However, larger GWAS have increased power to better detect individual associations with uncommon variants (Vicente, et al., 2017).

Between 2006 and 2017 there were 25 published GWAS of asthma, with an additional 38 studies investigating an asthma-related trait (such as lung function). Across the 25 studies, there were 73 unique gene variants found, and when analysed for LD there were 39 genetic variants in low LD associated with asthma risk (Vicente, et al., 2017). The first asthma GWAS was published in 2007 by Moffatt et al. (Moffatt, et al., 2007), who identified *ORMDL3* as an asthma susceptibility locus on chromosome 17q21. Further studies showed that one of the variants was an expression quantitative locus regulating *ORMDL3* expression as well as a neighbouring gene, *GSDML* (Moffatt, et al., 2007; Verlaan, et al., 2009). Due to the high LD at this locus, it is unclear which of these genes is the culpable target; thus, this is often referred to as the "17q21 asthma locus".

Another highly replicated GWAS locus is a region associated with *IL33* on chromosome 9q24, for which large meta-analyses (European based GABRIEL Consortium, and North American asthma GWAS - EVE Consortium) have detected numerous variants upstream of *IL33* associated with asthma, consistent across ethnically diverse populations (Moffatt, et al., 2010; Torgerson, et al., 2011). *IL1RL1*, which encodes the receptor for IL-33 on chromosome 2q12.1 is another highly replicated GWAS locus that is robust to ethnicity (Moffatt, et al., 2010). Other loci that have been consistently identified as associated with asthma include *IL13* on 5q31, *HLA-DQ1/DRB1* on 6q21, *SMAD3* on 15q22, *IL1RL1/IL18R1* on 2q12, *SLC22A5* on 5q31, and *TSLP* on chromosome

5q22 (Moffatt, et al., 2010; Torgerson, et al., 2011; Vicente, et al., 2017). When looking into ethnic backgrounds, GWAS have identified SNPs specific to certain groups including: in the *PYHIN1* gene on chromosome 1q23 in African-Americans, and near *RTP2* on chromosome 3q27 in those of Latin ancestry (Torgerson, et al., 2011).

Additional genetic studies have investigated SNP associations with asthma-specific phenotypes. High levels of IgE are associated with allergic disorders, and are closely correlated with clinical expression and severity of asthma (Burrows, et al., 1989), given that total serum IgE levels are under strong genetic control (Palmer, et al., 2000; Weidinger, et al., 2008). Research into serum IgE levels has identified the most replicated loci associated with modulating IgE levels in the genes *FCER1A*, *STAT6*, *IL13*, *IL4*/*IL21R*, and *HLA-DRB1*. *FCERA1* encodes the alpha chain of the high affinity receptor for IgE on chromosome 1q23, and its gene variants have been shown to play an important role in the regulation of IgE (Weidinger, et al., 2008). Signal transducer and activator of transcription 6 (STAT6) is a transcription factor that is involved in gene activation for IgE synthesis; SNPs in this gene have been associated with serum IgE levels and lung function (Maier, et al., 2012; Weidinger, et al., 2008). The *FCERA1* and *STAT6* loci have solid biological bases for their influence on serum IgE levels, though the SNPs identified at *FCER1A*, *STAT6*, and *IL4R/IL21R* loci have not been associated with asthma (Moffatt, et al., 2010). SNPs in the *HLA-DQB1* and *HLA-DRB1* genes have been found to be associated with asthma and IgE serum levels (Xingnan Li, et al., 2010; Moffatt, et al., 2010). These genes are a part of family of human leukocyte antigen (HLA) class II and involved in the presentation of antigens to immune cells.

Additionally, a novel gene, *RAD50*, on chromosome 5q31, which was not previously associated with asthma or atopy, was detected as associated with IgE levels by Weidinger et al. (Weidinger, et al., 2008). *RAD50* encodes a protein involved in double strand break repair and is located near a gene cluster of cytokines (IL3, IL4, IL5, IL13, GM-CSF) that contribute to asthma inflammatory processes. The 3' end of *RAD50* also acts as a locus of control for *IL4* and *IL13* genes, and polymorphisms found in this control region were found to be associated with asthma (Xingnan Li, et al., 2010)

Another study used blood eosinophil count as a phenotype and identified polymorphisms in *SH2B3* and *GATA2*, which encode an adaptor protein involved in T-cell signaling, cytokine regulation and hematopoietic homeostasis, and a transcription factor for hematopoietic cell differentiation and eosinophil development, respectively (Gudbjartsson, et al., 2009). Polymorphisms in *IL5*, a cytokine that is highly involved in regulating eosinophils, stimulating B-cell production of IgE, and contributing to the Th2 inflammatory response characteristic of asthma, were also associated with eosinophil counts.

As mentioned above, IL-33 and its receptor IL1RL1 have been highly implicated with asthma and inflammatory disorders. Polymorphisms for these genes have been associated with the disease and asthma-related traits such as eosinophil count (Gudbjartsson, et al., 2009; Moffatt, et al., 2010). Thymic stromal lymphopoietin (TSLP), another epithelial cytokine, has many gene variants that are implicated in asthma, and is one of the most commonly cited genes involved in asthma and allergic disorders (Vicente,

et al., 2017). These associations suggest that the disruption of airway epithelium and immune responses may be an important factor in susceptibility to asthma.

While there are numerous variants that have been identified, the SNP rs1837253 in the *TSLP* gene, which has been correlated with asthma and AHR (Harada, et al., 2009; He, et al., 2009), and its functional consequences and clinical significance on asthma phenotypes, will be the focus of this thesis. Though single gene associations cannot fully account for a complex disease such as asthma, risk variants can help us understand how genetic variation can influence gene expression, cellular function, and disease pathophysiology.

IMMUNE RESPONSES AND INFLAMMATION IN ASTHMA AND ALLERGIC DISEASE

While sometimes used interchangeably, the term "atopy" – referring to the predisposition to produce specific IgE in response to environmental stimuli or allergens and the term "allergy" – referring to a hypersensitivity reaction, are both used to indicate IgE-mediated inflammatory diseases such as asthma, allergic rhinitis, atopic dermatitis, and food allergy (Bellanti & Settipane, 2017). Atopic sensitization is established by a positive SPT and/or through detectable serum levels of IgE antibodies to specific allergens; although atopic sensitization is a risk factor for allergic disease, it does not necessarily result in clinical manifestations (Zheng, 2014b). It is also important to note that not all allergic-type inflammatory conditions are atopic, since some can develop through IgE-independent mechanisms.

Allergic Asthma

Asthma is a heterogeneous condition with many phenotypes and distinctions. Clinically, it manifests as repeated episodes of breathlessness, cough, chest tightness, and wheezing due to bronchoconstriction, and mucus hypersecretion. Pathologically, asthma is typified by chronic inflammation and structural airway alterations, known as airway remodelling (Holgate, et al., 1991; Rabe, et al., 2011; Ward, et al., 2002). Allergic asthma is the most common type of asthma (Holt, et al., 1999), and is a result of prior allergic sensitization to a specific allergen, with a reaction in the airways after inhalation, causing asthmatic symptoms. IgE is a central mediator is many types of allergic disease, including allergic asthma, and plays an important role in asthma symptoms, inflammation and airway remodelling (Holgate, et al., 1991; Rabe, et al., 2011).

Allergen-Induced Airway Responses

When individuals with allergic asthma come in contact with an allergen to which they are sensitized, there is a rapid release of pro-inflammatory mediators which can trigger the contraction of airway smooth muscle, increased mucus production, and symptoms of the early asthmatic response (EAR), such as wheezing, cough, shortness of breath and chest tightness (Kay, 2001). The airway response begins when IgE binds to high-affinity IgE receptors (FcεRI) located on the surface of mast cells and basophils, and is crosslinked with the allergen, which initiates mast cell and/or basophil degranulation and release of preformed mediators, such as histamine and chemotactic factors, as well as newly formed mediators, such as cysteinyl leukotriene (CysLT), and prostaglandins. CysLTs are potent mediators of bronchoconstriction (Adelroth, et al., 1986), and studies using leukotriene receptor antagonists have shown that CysLTs account for approximately 50% of the

decrease in FEV_1 that occurs in the EAR (Hamilton, et al., 1998; Leigh, et al., 2002). CysLT are also involved in stimulating mucus secretion and inducing vascular permeability. Histamine also contributes to bronchoconstriction and the combined treatment of anti-histamines and leukotriene receptor antagonists efficiently blocks the early asthmatic response (Davis, et al., 2005). The EAR develops within 10-15 min of allergen inhalation, typically maximizes around 30 minutes and resolves around 1-3 hours (Diamant, et al., 2013). Mast cell mediators also cause an influx of pro-inflammatory cells including T cells, eosinophils, basophils and neutrophils into the airways, which in turn attract and activate inflammatory cells and release more pro-inflammatory mediators (Afshar, et al., 2008; Averbeck, et al., 2007). In approximately 60% of adults and 80% of children this results in bronchoconstriction recurrence after 3-4 hours and generally reaches a maximum between 6-12 hours, which is known as the late asthmatic response (LAR) (Diamant, et al., 2013; O'Byrne, et al., 1987). The LAR appears to be allergen-specific occurring with certain allergens, such as house dust mites (HDM) or cat allergen, in >75% and less often with others, for instance grass pollen (Gauvreau, et al., 2015; O'Byrne, et al., 1987). The LAR is associated with further release of histamine and cysLTs and is characterized by cellular airway inflammation, increased vascular permeability, and mucus secretion (Gauvreau & Evans, 2007). Mast cells are involved in the release of bronchoconstrictor mediators in the EAR, and while the origin of these are not as well established in the LAR, airway basophils and eosinophils are increased during the LAR and are able to synthesize cysLTs (Bruynzeel, et al., 1985; Holgate, 2000). Basophils are the only cell capable of producing histamine, which indicates its role in both the EAR and LAR, and although eosinophils

have been established as a major cellular source of cysLTs in seasonal allergic asthma (Seymour, et al., 2001), their role in the LAR specific is not well understood.

The immune and inflammatory processes of the LAR are complex and involve increased Th2 airway inflammation, with increased immune cell infiltration of predominantly airway eosinophils (Gauvreau, et al., 1999), but also basophils (Gauvreau, et al., 2000), and neutrophils (Gauvreau, et al., 2002). These inflammatory cells are present in the bronchoalveolar lavage (BAL), airway biopsies and induced sputum (Beasley, et al., 1988; Gauvreau, et al., 1999; Jarjour, et al., 1997). Increased airway eosinophils are associated with higher levels of circulating progenitors after inhaled allergens, which suggests that the bone marrow is stimulated after allergen inhalation to produce eosinophil/basophil progenitors (Gibson, et al., 1990, 1991; Wood, et al., 1998).

Chronic airway inflammation observed in asthmatics is theorized to cause structural remodelling of the airways (Vignola, et al., 2003). Airway smooth muscle hypertrophy and hyperplasia, goblet cell hyperplasia, epithelial shedding, sub-epithelial fibrosis and angiogenesis are commonly observed in airways that have undergone remodelling (Boulet, et al., 1997; James & Carroll, 2000; Vignola, et al., 2003). These alterations contribute to airway thickening and result in fixed reduction of airway diameter (Chiappara, et al., 2001). The underlying mechanisms of airway remodelling are not well known, but prolonged inflammation, involving longstanding exposure of the airways to a variety of environmental agents, cells and mediators, is generally considered a necessary factor. Additionally, Th2 sensitization and imbalance of Th1/Th2 responses is also thought to contribute to asthma and its pathology.

This imbalance is evidenced by a number of events involved in allergen-induced airway inflammation, including the release of Th2 pro-inflammatory cytokines, IL-3, IL-4, IL-5, and IL-6 from mast cells following IgE cross-linking on mast cells (Plaut, et al., 1989). Also, cysLTs from mast cells have been found to induce eosinophil chemotaxis in allergic asthmatics (Gauvreau, et al., 2001).

Another major contributor to airway inflammation is the dendritic cell (DC), an antigen presenting cell key to the immune response. Following inhalation, allergens encounter antigen presenting DCs lining the airway sub-epithelium which bind to the antigen (allergen) and travel to draining lymph nodes, where the antigen is presented to T cells, causing Th2 polarization and proliferation, and the overexpression of Th2 cytokines, IL-4, IL-5, IL-9, and IL-13 (Upham & Stumbles, 2003). Th2 cytokines and co-stimulatory factors promote the activation of B cells and the production of IgE. Allergen-specific IgE can then circulate throughout the blood and bind to FcεRI or FcεRII found on inflammatory cells (mast cells, basophils, lymphocytes, eosinophils) stimulating the inflammatory activity of these cells during subsequent allergen exposure (Figure 1) (Gauvreau & Evans, 2007; Lemanske & Busse, 2006). In humans, DCs have two different subsets based on cell surface antigens and functions. Myeloid DCs (mDC), derived from myeloid precursors, induce Th2-sensitization in response to inhaled allergen and promote goblet cell hyperplasia and airway eosinophilia (Lambrecht, et al., 2000). Plasmacytoid DCs (pDC), derived from lymphoid precursors, are capable of suppressing effector T-cell generation by mDCs, and the absence of pDCs can cause Th2 sensitization and features of asthma (Mo, et al., 2011).

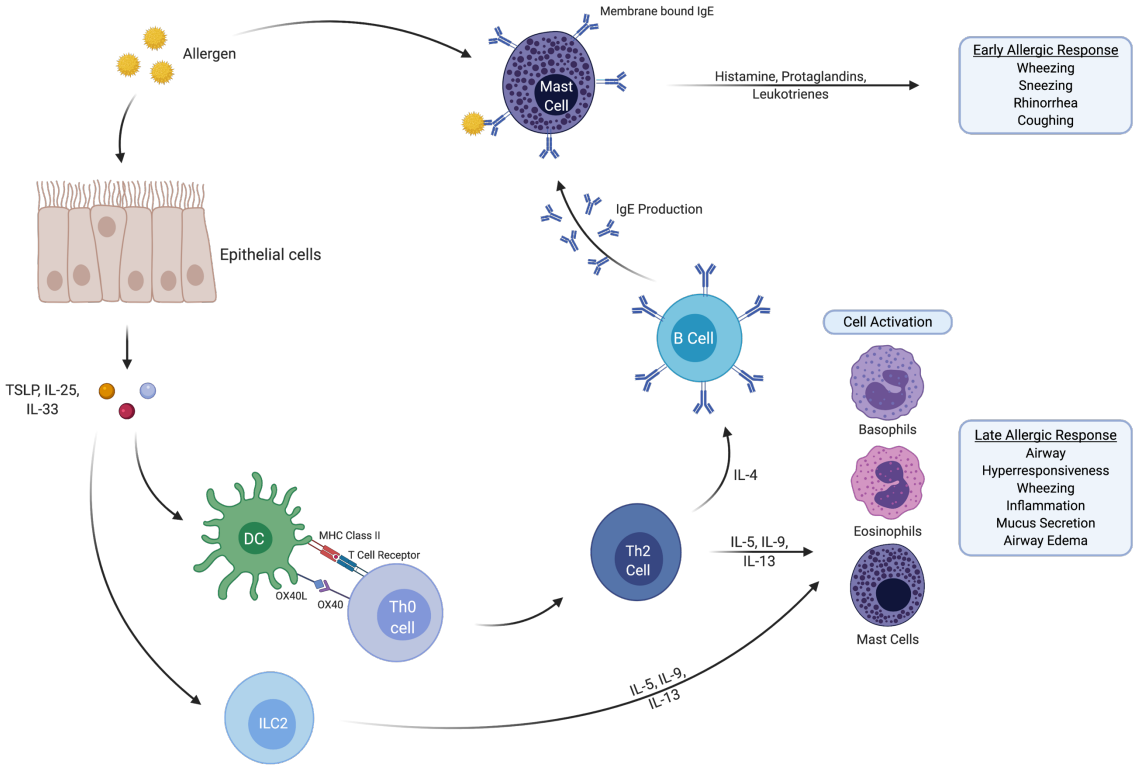

Figure 1: Pathways of early and late allergic responses. Allergens induce the release of histamine, prostaglandins and leukotrienes from mast cells, initiating the early allergic response. The late allergic response is propagated by multiple pathways and is mediated by a number of cells, including dendritic cells, Th2 cells, ILC2s, basophils, eosinophils and mast cells (adapted from Kay, 2001)

Cytokines in Allergic Inflammation and Asthma

Th2 cytokines such as IL-4, IL-5, and IL-13 are important drivers of allergic inflammation, while Th1 cytokines such as interferon-γ (IFNγ) and IL-12 antagonize the Th2 responses (Novak & Bieber, 2003). CD4+ Th2 cells produce a variety of cytokines, which promote IgE production (IL-4 and IL-13), eosinophilia (IL-5), mast cell

development (IL-9), and goblet cell hyperplasia and AHR in asthma (IL-9 and IL-13) (Kay, 2006; Kouro & Takatsu, 2009; Robinson, 2010). Studies have shown that messenger ribonucleic acid (mRNA) expression for many classical Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) is increased in bronchial biopsies from allergic and asthmatic subjects when compared to healthy controls (Hamid, et al., 1991; Humbert, et al., 1999; Ying, et al., 1997). In patients with asthma, those that expressed IL-5 in the airway showed elevated levels of activated T cells and activated eosinophils, and the mRNA in bronchial biopsies correlated with asthma severity and AHR (Humbert, et al., 1999), indicating a key role in the allergic response. IL-13 is involved in driving many features of asthma, as it promotes Ig isotype switching in B cells to produce primarily IgE, and airway mucus production through goblet cell hyperplasia (Wills-Karp, et al., 1998). In a murine model of allergic disease, blocking the IL-13 receptor alpha chain dramatically reduced AHR (Wills-Karp, et al., 1998); however, clinical trials with IL-13 receptor blocking agents have shown mixed results (Corren, et al., 2010; Wenzel, et al., 2007). Similarly to IL-13, IL-9 has also been reported to increase AHR and airway mucus production, and has other functions of enhancing Th2 cytokine production, and eosinophil and basophil differentiation, partially through the production of IL-13 (Steenwinckel, et al., 2007). Studies have shown that IL-9 enhances eosinophil survival and IL-5-mediated eosinophil and mast cell differentiation and maturation, which suggests that IL-9 may potentiate eosinophil function *in vivo* (Gounni, et al., 2000; Sitkauskiene, et al., 2005). Another cytokine produced by Th2 cells is IL-6, which is implicated in Th2 memory cell proliferation, Th2 differentiation, promotion of IL-4 production, inhibition of Th1 differentiation and, together with

transforming growth factor β (TGF-β), aids in Th17 expansion and development (Murphy & O'Byrne, 2010; Robinson, 2010). Increased levels of IL-6 have been found in the sputum of allergic asthmatic patients (Neveu, et al., 2010), and although it is thought to be independent of inflammation, the presence of IL-6 in the airways of asthmatics is believed to be the result of an "activated" state of pulmonary epithelial cells (Rincon & Irvin, 2012).

While the involvement of Th2 cytokines in asthma is well established, there are a number of other cytokines that are involved in the asthmatic inflammatory response. IL-8, also referred to as chemokine (C-X-C motif) ligand 8 (CXCL8), an important chemotactic factor, is involved in neutrophil recruitment and activation. It is secreted by a variety of structural and immune cells, including bronchial epithelial cells (BECs), smooth muscle cells, and macrophages, and studies have reported elevated IL-8 serum levels during asthma attacks (Kimata & Lindley, 1994). Increases in serum IL-8 have been shown as potential biomarkers for asthma status, with decreases indicating patient response to glucocorticoid steroids (Zhang & Bai, 2017). Additionally, IL-8 is increased in the BAL and induced sputum of asthma, which further supports its role in the pathogenesis of allergic inflammation and asthma (Chanez, et al., 1996; Nocker, et al., 1996).

Besides the classical Th2 cytokines, epithelial cell-derived cytokines, such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33, are secreted following epithelial stimulation, tissue damage, pathogen pattern recognition or allergen exposure, and are able to trigger Th2 responses at mucosal sites (Préfontaine, et al., 2010; Saenz, et al., 2010; Soumelis, et al., 2002). These epithelial-derived cytokines (termed 'alarmins') are key regulators of allergic responses: IL-33 induces TSLP production, eosinophilia, and

secretion of IgE and Th2 cytokines in mice (Saenz, et al., 2010); IL-25 drives Th2 production of cytokines (IL-4, IL-5 and IL-13), delays the apoptosis of eosinophils in humans and promotes eosinophilia in mouse models (Cheung, et al., 2006); and, TSLP acts upon DCs to polarize CD4+ T cells towards a Th2 immune response (Soumelis, et al., 2002). In murine models, overexpression of IL-25 in the airways causes Th2 inflammation and AHR, while blocking IL-25 reduces both of these responses (Yui Hsi Wang, et al., 2007). In asthmatics, IL-33 expression is increased in bronchial biopsies and airway smooth muscle cells (ASMC), and correlates with asthma severity (Préfontaine, et al., 2009). Both IL-25 and IL-33 induce TSLP production from epithelial cells. TSLP expression is increased in the airways in asthma, and blocking TSLP can reduce inflammation and AHR in murine models (Yong Jun Liu, 2007). TSLP, IL-33 and IL-25 have more recently exhibited the ability to activate a new population of lineage negative lymphoid cells (multi-potent progenitor type 2 cells), and type 2 innate lymphoid cells (ILC2s), which are capable of producing IL-5 and IL-13 and propagating a Th2 response, including mucus production and eosinophilia (Saenz, et al., 2010). Overall, there is mounting evidence that illustrates the role of these epithelial alarmins in propagating allergic inflammation in asthma. The key cytokine mediators and their actions in asthmatic inflammatory responses are listed in Table 1.

Table 1: Summary of key cytokine mediators of the asthmatic inflammatory response

Cytokine	Primary Cell Sources	Key Actions
IL-4	Th2 cells, Mast cells, Basophils, ILC2s	Immunoglobulin class switching of B cells to IgE; differentiation of Th2;
IL-5	Th2 cells, Mast cells, ILC2s	Differentiation, maturation, and survival of eosinophils
IL-9	Th2 cells, ILC2s	Eosinophil survival, mast cell and eosinophil development; AHR; mucus secretion
IL-6	DCs, Basophils, macrophages, Epithelial cells	Th2 differentiation; Th2 memory cell proliferation; Aid in Th17 expansion and development
IL-8	Epithelial cells, ASMCs, macrophages	Neutrophil recruitment and activation
IL-13	Th2 cells, Mast cells, Basophils, ILC2s	Mast cell development; Immunoglobulin class switching of B cells to IgE; eosinophilia; AHR; mucus secretion
TSLP	Epithelial cells, Basophils, Mast cells	DC and basophil activation; induction of Th2 responses
IL-33	Epithelial cells, ASMCs, DCs	Th2 differentiation; promotion of Th2 cytokine production; eosinophilia; induction of IgE secretion
IL-25	Epithelial cells, Th2 cells, Basophils, Mast cells	Th2 development and cytokine production; promotion of eosinophil survival
TNF- α	Mast cells, Macrophages, Epithelial cells, ASMCs	Induction of pro-inflammatory cytokine release from structural cells, eosinophil and neutrophil chemoattractant, T cell activation, promotion of T cell migration

TSLP, thymic stromal lymphopoietin; ASMCs, airway smooth muscle cells; DCs, dendritic cells; IgE, immunoglobulin E; AHR, airway hyperresponsiveness

The Effects of Corticosteroids on Inflammation

With the wide use of corticosteroids for treatment and management of inflammatory airway disease, it is imperative that we understand the effects that these therapeutics have at a biological level. Corticosteroids are a very effective anti-inflammatory therapy in allergic inflammation and many asthma symptoms can be managed with inhaled corticosteroids or in combination with long acting β-agonists

(Masoli, et al., 2004). They are very effective clinically as they block a number of inflammatory pathways and a wide spectrum of actions. Glucocorticoids are a class of corticosteroids that have previously shown inhibitory effects on inflammation (Erin, et al., 2005; Kato, et al., 2007).

Corticosteroids bind to cytoplasmic glucocorticoid receptors (GR) which are bound to two molecules of a 90-kilodalton heat shock protein. Binding of a corticosteroid to the GR induces a conformational change and resulting in the dissociation of the chaperone molecules (Barnes, 2001). A nuclear localization signal is exposed and the GR translocates to the nucleus, where two GR proteins bind to a glucocorticoid recognition element (GRE) as a dimer. GRE are on the 5'-upstream promoter sequence of steroid-responsive genes and increase transcription, while negative GREs (nGREs) may inhibit gene transcription (Barnes, 2001; Chandler, et al., 1983; Surjit, et al., 2011). GRs may also interact with transcription factors activating protein-1 (AP-1) and nuclear factor κB (NF-κB) to downregulate gene transcription (Schwiebert, et al., 1996).

The effects of corticosteroids are produced by activating the GRs to directly or indirectly modulate gene transcription. They may control inflammatory processes by increasing the transcription of anti-inflammatory genes while decreasing transcription of inflammatory genes. Corticosteroids increase the transcription of anti-inflammatory genes such as IL-1 receptor antagonists, secretory leukocyte protease inhibitor (SLP1), and IL-10 (Abbinante-Nissen, et al., 1995; John, et al., 1998). In asthmatics, IL-10 is decreased in macrophages, and corticosteroids may increase IL-10 to combat this. Additionally, pro-

inflammatory cytokines GM-CSF, IFNγ, and macrophage inflammatory protein 1α (MIP-1α), were all reduced following corticosteroid treatment (John, et al., 1998).

Corticosteroids reduce the number of inflammatory T cells in the airways, including T cells, eosinophils, mast cells and DCs. They markedly reduce the survival of inflammatory cells, such as eosinophils. Eosinophils survival relies on IL-5 and GM-CSF and exposure to corticosteroids reduces the effects of these cytokines and leads to apoptosis (Meagher, et al., 1996; Walsh, 1997). In asthma there is delayed eosinophilic apoptosis which is reversed by treatment with corticosteroids (Kankaanranta, et al., 2000). Conversely, peripheral neutrophil counts were increased following systemic corticosteroid treatment, which may reflect increased survival time and inhibitory action on neutrophil apoptosis (Meagher, et al., 1996).

The airway epithelium is an important target for corticosteroid treatment. Asthmatic patients have increased expression of inducible nitric oxide synthase (iNOS) in airway epithelial cells and an increased level of NO in exhaled air. These levels are reduced after administration of corticosteroids (Kharitonov, et al., 1996; Saleh, et al., 1998). Glucocorticoids inhibit the production of numerous cytokines, including IL-1, IL-6, IL-8, IL-l1, TNF-α, GM-CSF, and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) (Schwiebert, et al., 1996). The inhibition of cytokine production may explain the anti-inflammatory actions of glucocorticoids on inflammatory cell activation and recruitment, This occurs by pathways previously mentioned, such as binding to nGREs and interacting with transcriptional factors AP-1 and NF-κB. For example, the IL-6 gene contains at least 4 nGREs close to the promoter site (Ray, et al., 1990). Additionally, AP-

1 and NF-κB are involved regulating inflammatory cytokines IL-1, IL-2, IL-3, IL-6, IL-8, TNF-α, GM-CSF (Vandevyver, et al., 2013). In human airway epithelial cells, TLR-3 and Th2 cytokine dependent TSLP expression was suppressed by the treatment with glucocorticoids (Kato, et al., 2007). Additionally, glucocorticoids inhibit IL-4 and IL-5 production in the bronchoalveolar lavage in allergic asthmatics and demonstrate lower numbers of IL-4 and IL-13 expressing cells in the nasal lavage from allergic asthmatics (Ghaffar, et al., 1997; Liu, et al., 2001). Human airway epithelial cells treated with the glucocorticoid fluticasone exhibited reduced expression of the eotaxin gene, an eosinophilic chemotactic protein (Matsukura, et al., 2004). Evidence suggests that glucocorticoids may inhibit the production of Th2 cytokines from airway cells, diminishing the downstream response. In bronchial biopsies of asthmatic subjects, glucocorticoid therapy restores the continuity of the bronchial epithelium and reducing the basement membrane thickness (Laitinen, et al., 2004; Lundgren, et al., 1988; Trigg, et al., 1994). Glucocorticoids are able to reduce but not eliminate bronchial hyperresponsiveness, likely through the suppression of inflammatory events such as eosinophilic inflammation (O'Byrne & Inman, 2003; Sont, et al., 1999). Additionally, in a double-blinded, placebo control trial intranasal corticosteroids reduced bronchial responsiveness to histamine in a non-specific bronchial challenge (Agondi, et al., 2008), however, these therapeutics must be further studied to more thoroughly understand the complex mechanisms and pathways of action of intranasal corticosteroids specifically.

AIRWAY EPITHELIAL CELLS

Airway epithelium is a pseudostratified layer of cells made up of ciliated, basal columnar and secretory cells. They lie at the interface between the host and the environment and are at the frontline of mucosal immunity. Airway epithelial cells are the first line of defense against microorganisms, pathogens, gases, and allergens, and form a barrier composed of airway surface liquids, mucus, and apical junction complexes that form between neighbouring cells. Apical junction complexes are made of epithelial tight junctions and adherens junctions, and establish an impermeable barrier, cell-to-cell contact, cell polarity and regulate the movement of ions and macromolecules in healthy individuals (Georas & Rezaee, 2014). Under normal circumstances following injury, airway epithelium is able to stimulate repair pathways, mediated principally by epidermal growth factor (EGF) (Holgate, 2011). The airway epithelium is also involved in regulation of the innate and adaptive immune response through the secretion of inflammatory cytokines, resulting in the recruitment of Th2 and other inflammatory effector cells.

In asthma, the airway epithelium shows disrupted barrier integrity and dysfunctional tight junctions. Additionally, there is decreased proliferation of basal cells and the injury repair process is altered, which creates a chronic wound scenario (Perl, et al., 2011). Inhaled allergens, pollution particles and respiratory viruses can disrupt barrier integrity, allowing the penetration of the airway epithelium and promoting toxic, immune, and inflammatory responses (Georas & Rezaee, 2014). In addition, pro-inflammatory cytokines, such as IL-4, IL-13, IFN-γ, and TNF-α, can further disrupt the airway epithelial barrier, resulting in a positive feedback loop (Coyne, et al., 2002). The diminished integrity

of the airway epithelium in asthmatics is intrinsically inflammatory, with increased spontaneous epithelial cytokine secretion, including IL-6, IL-1β, IL-13, TGFβ, when compared to non-asthmatics (Freishtat, et al., 2011; Kicic, et al., 2006). A defective barrier provides access for inhaled allergens and particles into the sub-epithelial space, where they will encounter more immune cells and intra-epithelial DCs (Georas & Rezaee, 2014). Additionally, since viruses induce junction dysfunction (Coyne, et al., 2007; Rezaee, et al., 2013), this creates an opportunity for a positive feedback loop of increased susceptibility to pathogenic infection, as demonstrated in a study by Sajjan et al., where rhinovirus infection significantly increased bacterial translocation across epithelial monolayers (Sajjan, et al., 2008).

Viral Infections in the Airway

Respiratory tract infections in early life have been associated with later development of asthma (Beigelman & Bacharier, 2016). Viral infection in the airway occurs through infection and replication in epithelium, and triggers the release of pro-inflammatory mediators, including TSLP, to initiate the inflammatory cascade (Esnault, et al., 2008). The inflammatory response is an attempt to eradicate infection, but when there is damage to the epithelium, as seen in asthmatics, this reaction can contribute to airway obstruction and AHR (Gern, 2010). The common cold virus, the rhinovirus, has emerged as a key microorganism involved in asthma exacerbations, accounting for 50-80% of such attacks in children and adults (Johnston, et al., 1996; Khetsuriani, et al., 2007; Nicholson, et al., 1993). Rhinoviruses replicate primarily in airway epithelium primarily utilizing a surface glycoprotein, intercellular adhesion molecule 1 (ICAM-1), to insert the genome

into the cell (Papi & Johnston, 1999). Rhinovirus infection of BECs induces the secretion of a wide variety of inflammatory cytokines and chemokines including IL-1, IL-6, IL-8, GM-CSF, eotaxins, and RANTES (Papadopoulos, et al., 2001; Schroth, et al., 1999; Terajima, et al., 1997; Zhu, et al., 1997). Rhinoviruses have a positive, single-strand RNA genome, that form double-stranded (ds)RNA during replication; the latter are natural ligands for toll-like receptor-3 (TLR-3) and are potent inducers of *TSLP* gene expression. Previous studies have shown that *in vitro* stimulation with dsRNA induces *TSLP* expression in small airway epithelial cells and BECs, in healthy and asthmatic individuals. TSLP protein secretion is also significantly increased in epithelial cells of the asthmatic individuals (Allakhverdi, et al., 2007; Kato, et al., 2007; Schröder & Bowie, 2005; Uller, et al., 2010). Polyinosinic:polycytidylic acid (poly(I:C)), a form of dsRNA, can be utilised as a synthetic toll-like receptor-3 (TLR3) agonist, mimicking the natural TLR3 ligand, viral dsRNA; poly(I:C) has also been shown to stimulate of *TSLP* expression (Kato, et al., 2007; Uller, et al., 2010). Additionally, dsRNA and Th2 cytokines have been shown to induce TSLP expression in a TLR-3 dependent manner (Kato, et al., 2007). Another single-stranded respiratory virus, the respiratory syncytial virus (RSV), has also showed increased TSLP expression upon infection of epithelial cells from asthmatic individuals, with higher levels of pro-inflammatory cytokines (IL-6, IL-8, GM-CSF) secreted from BECs in comparison to healthy controls. These cytokine profiles also correlate with elevated inflammatory responses *ex vivo* (Hackett, et al., 2011). There are numerous studies that have demonstrated the capacity of respiratory viruses for propagating the generation of inflammatory mediators and attracting inflammatory cells to the airways, characteristic of

allergic inflammation seen in asthma. This thesis will focus on the effects of the synthetic TLR-3 agonist, poly(I:C), and the immune responses of stimulated nasal epithelial cells.

Nasal Epithelial Cells

Airway epithelial cells are some of the first sites of contact for aeroallergen, pathogens and pollution. Epithelial cells function not only as a barrier but as the first line of defense against environmental insults, and as regulators of the immune response (Sanders, et al., 2011). Immortalized cell lines can be used to model respiratory cells, but they lack the heterogeneous cell populations observed *in vivo*. To reproduce characteristics in *in vivo* respiratory epithelium, primary airway epithelial cells can be used, such as nasal epithelial cells (NECs) from human volunteers. NECs that are expanded and cultured *in vitro* generate a model of differentiated cells which mimic many of the natural features of nasal epithelium *in vivo*, providing a unique and important opportunity to study disease (Müller, et al., 2013). While culturing BECs is well documented, it is limited by availability of human lung tissue or the invasiveness associated with obtaining the requisite bronchial biopsies. Obtaining NECs is less costly, less invasive and associated with fewer side effects (Müller, et al., 2013). Further, Sridhar *et al*. showed that BECs and NECs from non-smokers have similar gene expression profiles, making NECs a good substitute for BECs (Sridhar, et al., 2008). Additionally, paired analysis of cultured BECs and NECs has demonstrated similar antiviral and pro-inflammatory responses (Roberts, et al., 2018).

THYMIC STROMAL LYMPHOPOIETIN

TSLP Biology

TSLP is a member of the four-helix-bundle cytokine family, and a distant paralog of IL-7 (Ziegler, et al., 2013), first detected in the supernatant of a mouse thymic stromal cell line, and initially studied as a factor for immature B cell and thymocyte proliferation and development (Ray, et al., 1996). A human homolog was later identified and, although the amino acid sequence shows only 43% identity between murine and human homologs, the shared functional homology between these cytokines is high (Reche, et al., 2001; Sims, et al., 2000). The TSLP receptor (TSLPR) is a complex of a unique chain, closely related to the cytokine receptor subunit γc (common γ-chain), and the IL-7 receptor α-chain (Pandey, et al., 2000; Park, et al., 2000). Like the cytokine, the murine and human receptors function similarly, despite poor amino acid sequence identity. The TSLPR is expressed on a variety of cells, including both hematopoietic cell types, such as T cells, B cells, natural killer (NK) cells, monocytes, eosinophils, basophils, DCs, as well as non-hematopoietic cells, such as epithelial cells (Ziegler, 2012).

TSLP Isoforms

The *TSLP* gene is located on 5q22.1, with 5 exons and two transcript variants in humans: a long TSLP isoform (lfTSLP), and a short TSLP isoform (sfTSLP). At the protein level, the sfTSLP is composed of the last 63 residues of lfTSLP and is expressed in all normal tissue, including lung fibroblasts (Fornasa, et al., 2015). These isoforms were originally thought to be due to alternative splicing; however, two distinct untranslated regions, indicating two open reading frames, have been discovered (Harada, et al., 2011). Recent studies suggest that the isoforms may have distinct roles in inflammatory responses.

In healthy barrier surfaces, such as human intestinal, oral mucosal and skin tissue, the main form is sfTSLP. This isoform is expressed constitutively and may have anti-inflammatory or anti-microbial effects, which are mediated by an unknown mechanism involving p38 phosphorylation (Bjerkan, et al., 2015, 2016; Fornasa, et al., 2015; Harada, et al., 2009). Pro-inflammatory signals induce expression of the long isoform (lfTSLP), which is associated with allergic inflammation, and signals through STAT5 phosphorylation.

Recently, it has been reported that RSV promotes the expression of TSLP, particularly lfTSLP, and that the transcription factor Activating Protein-2α (AP-2α) can suppress the expression of lfTSLP and sfTSLP at mRNA and protein levels in BECs following RSV infection (He, et al., 2020). In an *in vitro* mouse model of HDM-induced asthma, it has been shown that there is increased expression of lfTSLP mRNA, whereas sfTSLP mRNA is unaltered (Dong, et al., 2016). There is an upregulation of lfTSLP in a number of inflammatory conditions, including asthma, atopic dermatitis, psoriasis, and ulcerative colitis (Tsilingiri, et al., 2017). Recent data emphasize the need for analyzing the roles of the two TSLP isoforms separately in future studies (Tsilingiri, et al., 2017; Varricchi, et al., 2018).

TSLP Signalling

Based on structural homology, the TSLPR is classified as a hematopoietin receptor; with some atypical features. These include a conserved box1sequence, involved in the regulation of Janus protein tyrosine kinase (JAK) binding by other cytokine receptors, and the lack of the conserved box2 sequence (Park, et al., 2000). The TSLPR also only contains

one tyrosine residue four amino acids from its carboxy terminus, as well as a modified WSXWS motif with multiple N-linked glycosylation sites (Tonozuka, et al., 2001).

As a member of the hematopoietin receptor or type 1 cytokine receptor family, TSLPR was originally thought to activate STAT proteins utilizing JAKs; in multiple cell lines, TSLP stimulation in fact results in STAT5 phosphorylation. Despite this, TSLPR signalling can occur in the absence of JAK activation, since dominant-negative form of JAK-1 and -2 do not alter TSLP-mediated STAT5 phosphorylation (Isaksen, et al., 1999; Levin, et al., 1999). Studies have shown sustained JAK-1 and -2 activation following TSLP signalling in human DCs and human and mouse CD4+ T cells (Arima, et al., 2010). Remarkably, while IL-7Rα and γc employ JAK-1 and -3 in IL-7 signaling, the TSLPR subunit binds and uses JAK-2, together with IL-7Rα-associated JAK-1. TSLP stimulation of human peripheral blood-derived CD11c+ DCs activates STAT 1,3,4,5, and 6, as well as JAKs 1 and 2 (Arima, et al., 2010). These data indicate that TSLP is able to activate multiple STAT proteins. For example, TSLP directly activates STAT6 by binding to the promoter region of CCL17, which explains the unique ability of TSLP-mDCs to produce the Th2-attracting chemokine CCL17. Other activation pathways have been demonstrated in TSLPR signalling, such as Src kinases and phosphinositol 3 kinase (PI3K) (Isaksen, et al., 2002). Arima et al. also has demonstrated that human mDCs are capable of transmitting signals not only via STAT but also via Akt, mitogen activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and NF-κB – indicating that various signals can be initiated in a JAK-dependent manner in response to TSLP (Arima, et al., 2010).

Additionally, in humans, TSLP is able to stimulate eosinophils through p38 MAPK, ERK, and NF-κB–dependent pathways resulting in the prolonged survival, induction of adhesion molecules, and release of allergic inflammatory cytokines and chemokines, including IL-6, IL-8, CXCL1, and CC chemokine, CCL2 (Wong, et al., 2010). In BECs, TSLPR stimulation with TSLP activates STAT3 and STAT5 phosphorylation, and induces IL-13 and epithelial cell proliferation. However, BEC proliferation is lower in cells from asthmatic individuals in comparison to healthy control subjects (Semlali, et al., 2010). In ASMCs, TSLP stimulation induces pro-inflammatory cytokine and chemokine release (IL-8, eotaxin-1/CCL11, and IL-6), involving STAT3 and MAPKs (ERK1/2, p38, and JNK) signalling (Shan, et al., 2010). Together, these results suggest that TSLP has a wide and significant range of biological effects, and that its action on cells utilizes multiple complex downstream signalling pathways.

Regulation of TSLP

TSLP is secreted primarily by epithelial cells, keratinocytes, and stromal cells, but other cells are also involved in TSLP production, including DCs, mast cells, fibroblasts, ASMCs, macrophages, and endothelial cells (Moon, et al., 2011; Soumelis, et al., 2002; Ying, et al., 2005; Zhang, et al., 2007; Ziegler, 2012). TSLP plays a role in many biological functions, such as the maturation of DCs, the proliferation of naïve T cells, and the release of T cell-attracting chemokines from mast cells (Allakhverdi, et al., 2007). TSLP mRNA and protein expression are regulated by various factors, including pro-inflammatory and Th2 cytokines, inhaled allergens, trauma, mechanical injury, bacterial and viral infections, and TLR ligation (Allakhverdi, et al., 2007; Kato, et al., 2007; Oyoshi, et al., 2010). Pro-

inflammatory mediators regulate TSLP expression in BECs in a NF-κB-dependent manner (Lee & Ziegler, 2007). Additionally, pro-inflammatory cytokines such as IL-1β, TNF-α, and TGF-β, induce the expression of TSLP in human BECs and ASMCs, an effect mediated through NF-κB, ERK1/2, JAK/STAT6 and p38 MAPK signalling pathways (Kato, et al., 2007; Zhang, et al., 2007). In human ASMCs, TNF-α induced TSLP promoter activity is mediated not only by NF-κB, but also by activating protein-1 (AP-1), with mutations in NF-κB and AP-1 binding sites, abolishing the associated TSLP expression (Redhu, et al., 2011).

Rhinoviruses and RSV are known causes of asthma exacerbations and result in TSLP production (Kato, et al., 2007). Poly (I:C), a synthetic analog of dsRNA, can ligate to TLR-3 and promote TSLP production from human epithelial cells and can be upregulated by IL-4, IL-13, TNF-α, IFN-α and IFN-β or conversely suppressed by IFN-γ, TGF-β and IL-17 (Bogiatzi, et al., 2007; Kato, et al., 2007; Kinoshita, et al., 2009). In BECs, TLR-3 ligand-induced TSLP expression is dependent on NF-κB and IRF-3 signalling, and is synergistically enhanced by Th2 cytokines such as IL-4 (Kato, et al., 2007). Recently, lfTSLP, considered to have pro-inflammatory properties, has been shown to be highly inducible by poly(I:C) in both BECs and NECs (Harada, et al., 2009; Moorehead, et al., 2020). In BECs, a SNP in the TSLP promoter (rs3806933) creates a binding site in AP-1 and thus enhances binding to the regulatory element, resulting in increased poly (I:C) stimulated lfTSLP expression. Interestingly, the minor allele SNP rs1837253 has exhibited reduced expression of poly (I:C)-stimulated lfTSLP from NECs in an AP-1-independent manner (Moorehead, et al., 2020).

Apart from epithelial cells, there are a number of other structural and immune cells that are capable of producing TSLP. Mast cells are able to store TSLP intracellularly and, following the cross-linking of FcεRI in the presence of IL-4, produce TSLP (Okayama, et al., 2008). In mice, protease-activated basophils respond by producing Th2 cytokines, including IL-4 and TSLP, and are transiently recruited to the draining lymph nodes (Sokol, et al., 2008). Dendritic cells not only respond to TSLP but also produce it. Human and mouse DCs induce TSLP expression in response to TLR ligands and allergens such as HDM extract; the production of TSLP by DCs suggests a possible autocrine effect (Kashyap, et al., 2011). Additionally, DCs may serve as a source of TSLP for other responsive lineages, such as CD4+ T cells, to promote TSLP-dependent allergic inflammatory responses such as asthma and atopic dermatitis.

TSLP-Responsive Cells

TSLP has substantial effects *in vivo* and *in vitro* on a plethora of different cells during both innate and adaptive immune responses, including: DCs, mast cells, basophils, eosinophils, CD4+ and CD8+ T cells, B cells, and natural killer T (NKT) cells (Varricchi, et al., 2018). It has been established that hematopoietic progenitor cells (HPCs) and type 2 innate lymphoid cells (ILC2s) express TSLPR and respond to TSLP (Allakhverdi, et al., 2009; Halim, et al., 2012; Kabata, et al., 2013). The extensive number of cell types that respond to TSLP indicates its important role in orchestrating a wide range of immune-inflammatory responses (Figure 2).

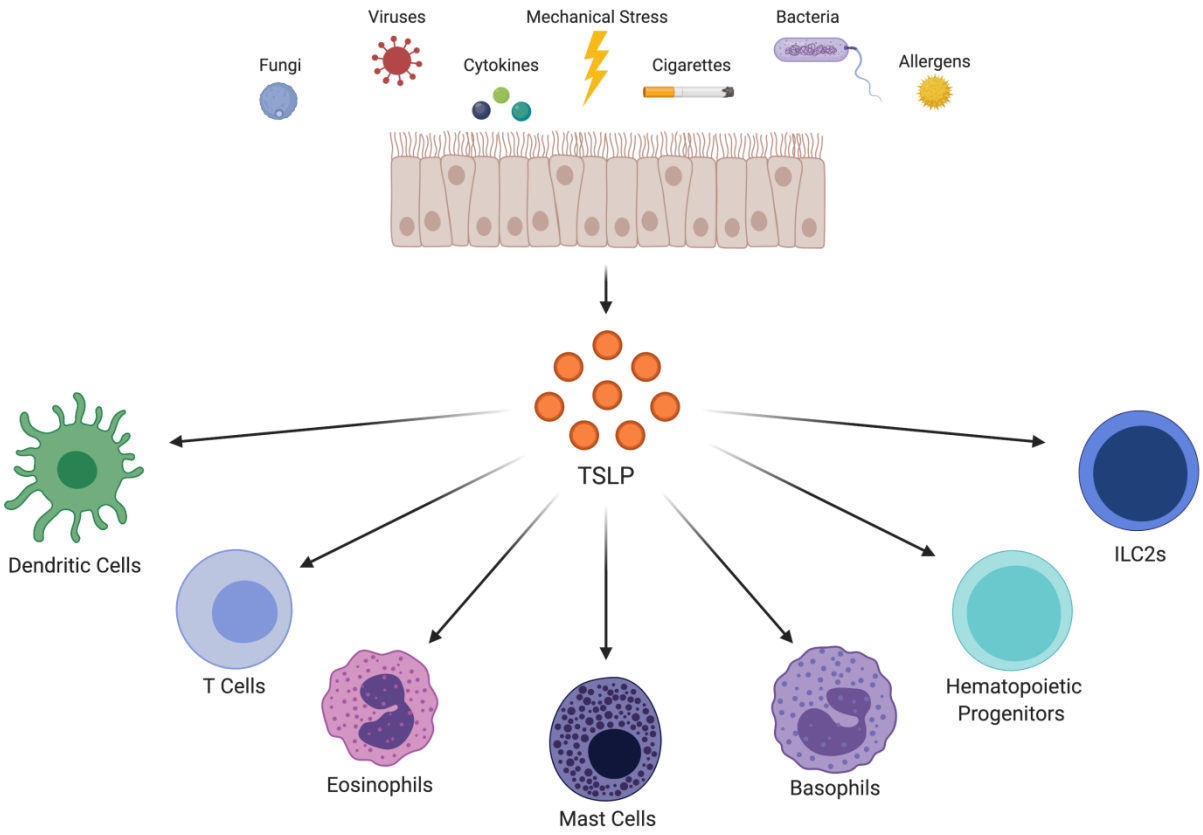

Figure 2: Cellular targets of thymic stromal lymphopoietin. Exogenous triggers, including viruses, allergens, bacteria, pro-inflammatory cytokines and fungi, can activate epithelial cells to release TSLP. Epithelial-derived TSLP can act on multiple cell types to induce differentiation, proliferation, and cytokine production (adapted from Varricchi, 2018).

Dendritic Cells

DCs are considered to be the major antigen presenting cell type in immune responses with the ability to initiate and direct immune responses and are a major target of TSLP. Human mDCs are CD11c+ and express high levels of the TSLP (Hanabuchi, et al.,

2010). Similar to stimuli that activate mDCs, including CD40L and Toll-like receptor (TLR) ligands, such as bacterial lipopolysaccharide (LPS), poly(I:C), and R848, TSLP promotes the maturation of DCs and upregulates MHC class II, and co-stimulatory molecules (CD54, CD80, CD83, CD86, and DC-lamp) (Liu, 2006; Soumelis, et al., 2002). However while mDCs typically produce IL-12, TSLP does not initiate the production of this Th1-polarizing cytokine or the pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, from mDCs (Soumelis, et al., 2002). Instead, TSLP acts on mDCs to induce IL-8, eotaxin-2, Thymus and Activation Regulating Chemokine (TARC; CCL17), Macrophage-Derived Chemokine (MDC; CCL22) and I-309/CCL1 production, which recruit Th2 cells, eosinophils and neutrophils to the airways (Liu, 2006). It has been suggested that the absence of TSLP-induced Th1 cytokine production in mDCs is an important feature in TSLP-activated DCs, creating a Th2-permissive microenvironment.

One of the most notable effects of TSLP on DCs is the upregulation of OX40 ligand (OX40L), which is critical in the polarization of naïve CD4+ T cells toward inflammatory Th2 cells. This interaction of OX40L with OX40 on naïve T cells to polarize leads to release of several Th2 cytokines, including IL-4, IL-5, IL-9 and IL-13 as well as TNF-α, amplifying the allergic inflammatory response (Soumelis, et al., 2002). CD4+ T cells that have been activated by TSLP-stimulated DCs produce decreased levels of IL-10 and IFN-γ, two cytokines known to down-regulate Th2 cell inflammation (O'Garra, 1998). OX40L-induced Th2 cell differentiation depends on the absence of IL-12, and only TSLP, but not CD40L or poly (I:C), can stimulate DCs to provide such unmitigated Th2 skewing (Ito, et al., 2005). The dominance of IL-12 over OX40L may help to explain the mechanism behind

the hygiene hypothesis which proposes that microbial and viral infections trigger Th1 responses and may decrease the subsequent development of Th2-driven atopy (Liu, 2006).

TSLP can also promote DC involvement in the maintenance and further polarization of effector CRTh2+ CD4+ Th2 memory cells (Wang, et al., 2006a). Studies have demonstrated that TSLP may promote DC induction of the differentiation of CD4+CD8-CD25- naïve thymocytes into T regulatory (Treg) cells, although it may impede the production or maintenance of FOXP3+ Tregs *in vivo* in certain diseases (Besin, et al., 2008; Duan, et al., 2010; Lei, et al., 2011; Watanabe, et al., 2005). Overall, the effect of TSLP on DCs influences innate and adaptive allergic immune responses through the production of IL-8 and eotaxin-2, which recruit neutrophils and eosinophils, the priming of the adaptive immune response by inducing chemotaxis of Th2 cells using CCL17 and CCL22, and the polarization of naïve CD4+ cells into inflammatory Th2 cells (Figure 3).

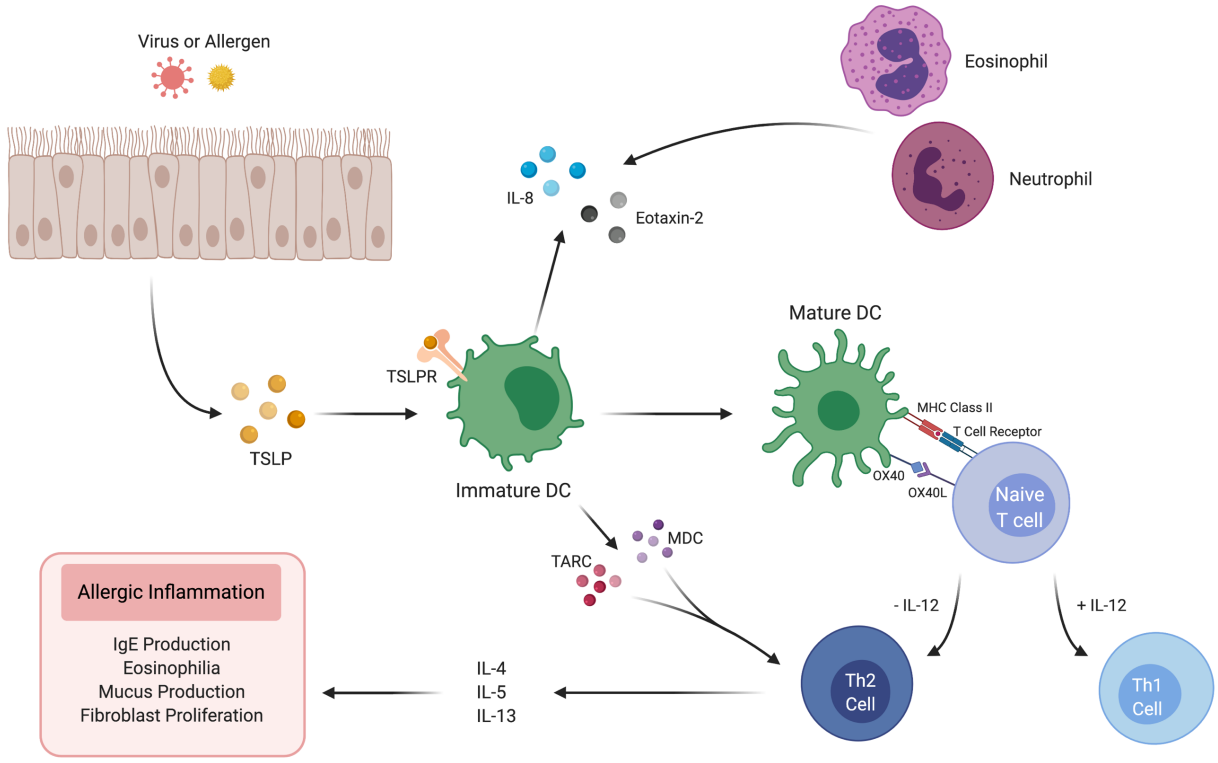

Figure 3: The role of TSLP in the pathophysiology of allergic inflammation. Allergen or viruses can trigger epithelial cells to produce TSLP, activating immature DCs. TSLP-activated DCs produce IL-8 and eotaxin-2, which attract eosinophils and neutrophils, as well as TARC and MDC, which attract Th2 cells. Additionally, TSLP-activated DCs also express OX40L, which triggers the polarization of naïve CD4+ T cells into Th2 cells in the absence of IL-12. These polarized Th2 cells migrate to the site of local inflammation and produce the Th2 cytokines, IL-4, IL-5, and IL-13, which initiate allergic inflammation by triggering IgE production, eosinophilia, and mucus production (adapted from Liu, 2006).

T Cells

While many effects of TSLP on T cells are mediated by DCs, TSLP is still able to directly activate CD4+ T cells independently of DCs. In the presence of T cell receptor (TCR) stimulation, TSLP treatment can induce proliferation and Th2 differentiation of naïve CD4+ T cells through the induction of IL-4 (Omori & Ziegler, 2007; Rochman, et al., 2007). Furthermore, IL-4 increases the expression of TSLPR on CD4+ T cells, which creates a positive feedback loop. Following TCR stimulation, TSLP initiates STAT5 activation and increases the sensitivity of CD4+ T cells to IL-2 (Rochman, et al., 2007). Recently, Ochai et al. demonstrated that culture in TSLP and Th2 conditions promotes the differentiation of naïve CD4+ T cells into IL-13+ Th2 cells, accompanied by the upregulation of IL-5 and IL-9. An *in vivo* murine model has shown that high TSLP levels promote the development of a population of IL-4^{neg}IL-13^{pos} T cells which also express *GATA3*, *IL5*, and *IL3* transcripts (Ochiai, et al., 2018). With regard to CD8+ cytotoxic T cells, both murine and human CD8+ T cells express the TSLPR complex. TSLP does not affect the proliferation of CD8+ T cells during homeostasis, but is involved in the prolonged survival of CD8+, as well as CD4+ cells, by increasing the expression of anti-apoptotic Bcl-2 in a STAT5-dependent manner (Rochman & Leonard, 2008). In the thymus, TSLP plays a positive role in the development of thymic Tregs (Watanabe, et al., 2005), but in the airways of asthmatic individuals TSLP directly impairs IL-10 production of activated pulmonary Tregs and their immunoregulatory activity (Nguyen, et al., 2010). This is consistent with studies indicating that BAL Tregs from asthmatics fail to suppress the proliferation and production of Th2 cytokines by CD4+ effector T cells (Nguyen, et al.,

2010). TSLP also has a direct effect on NKT cells: in murine NKT cells, TSLP enhances airway hyperreactivity by upregulating IL-13 production (Nagata, et al., 2007), whereas in humans TSLP-treated invariant NKT cells secrete high levels of IL-4 and IL-13, implying that TSLP may directly activate iNKT cells to secrete Th2 cytokines (Wu, et al., 2010).

Hematopoietic Progenitor Cells

In humans, hematopoietic progenitor cells (HPC) express the stage-specific antigen CD34 and are stem cells with the property of self-renewal and capacity to differentiate into all blood cell types. The majority of HPC reside in the bone marrow (AbuSamra, et al., 2017) and can travel to sites of inflammation where, under the influence of local factors, they differentiate into effector cells in the process termed "in situ hematopoiesis" (Hui, et al., 2015).

Through their differentiation into various effector cells, including basophils, eosinophils and mast cells, CD34+ HPCs are important contributors to allergic inflammation. Allakhverdi *et al*. investigated the effect of TSLP on CD34+ cells and identified the TSLP receptor and IL-7α chain subunit on circulating HPCs (Allakhverdi, et al., 2009; Hui, et al., 2014). Overnight stimulation of CD34+ cells with TSLP, in combination with IL-33, causes a dose-dependent release of Th2 cytokines and chemokines (Allakhverdi, et al., 2009). TSLP also exhibits a role in eosinophil/basophil (Eo/B) differentiation from CD34+ cells isolated from peripheral blood. Pre-exposure to TSLP and IL-33 primes the migration of HPCs towards the potent chemoattractant stromal cell-derived factor 1α (SDF-1α, also known as CXCL12), which suggests that the airway epithelial may enhance the migrational responsiveness of HPCs through the secretion of

epithelial alarmins (Smith, et al., 2015). In the presence of IL-3, TSLP significantly promotes Eo/B colony formation from HPCs and induces secretion of IL-1β, IL-6, IL-13, CXCL8, CCL2, and CCL17, as well as TNF-α, which, in conjunction with IL-3, acts to increase surface expression of TSLPR (Hui, et al., 2014). Hematopoietic progenitor cells from atopic individuals are more responsive both functionally and phenotypically to TSLP, in comparison to HPCs from non-atopic subjects. These results suggest that TSLP may underlie the "priming" effect in atopic individuals by inducing HPC differentiation into Eo/B. In addition, supernatant fluids from poly(I:C) stimulated small airway epithelial cells activates CD34+ HPCs to produce IL-5 in a TSLP-dependent manner, further reinforcing the postulation of a key role for TSLP and HPCs in the pathogenesis of allergic diseases (Allakhverdi, et al., 2009).

Group 2 Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are increasingly recognized as regulators of innate immune responses, as well as in the development of many diseases. They are classified into three major subsets: group 1 ILCs (ILC1s), ILC2s and ILC3s, all derived from common lymphoid progenitors (Scoville, et al., 2019). Although ILCs do not express B- or T-cell receptors, they do produce high levels of cytokine mediators that are characteristic of lymphocytes, via antigen-independent stimuli (Kato, 2019). ILC2s are commonly identified as $CD45^+$ lymphocytes that are Lineage-negative $CD127^+CD161^+CRTH2^+$ expressing the surface antigens ST2 and IL-17RB; however, ST2 expression can be influenced by the tissue of origin and the state of the microenvironment (Mjösberg, et al., 2011; Vivier, et al., 2018). ILC2s produce Th2 cytokines, predominantly IL-5 and IL-13,

and can mediate type 2 inflammation, which suggests functional similarities between ILC2s and Th2 cells.

ILC2s depend on the transcription factor GATA3 and, in the absence of this factor, cell development and function is dramatically hindered (Klein Wolterink, et al., 2013; Mjösberg, et al., 2012). Nasal epithelial-derived TSLP increases the expression of GATA3 and activates the phosphorylation of STAT5 in ILC2s found in blood and nasal polyps, strongly enhancing the production of IL-4, IL-5 and IL-13 (Mjösberg, et al., 2012). This is observed even without prior activation of ILC2s, in contrast to Th2 cells in which TSLP responsiveness is only seen following activation of the TCR (Kitajima, et al., 2011).

Epithelial alarmins, IL-25, IL-33, and TSLP play a role in the allergic response by acting on many immune cells, including ILC2s. TSLP is important for ILC2 survival and TSLP, together with IL-33, has a synergistic effect on cytokine production, upregulating the expression of IL-17RB and TSLPR (Camelo, et al., 2017). ILC2s respond to IL-25 and increase cytokine production, but responses are slower and less potent (Barlow, et al., 2013). The ability of ILC2s to respond to the epithelial cell-derived cytokines, and the elevated frequency of ILC2s in the sputum of asthmatics, suggest that they are involved in mediating Th2 inflammation and asthma (Chen, et al., 2017).

Eosinophils

It has been reported that TSLP activates human eosinophils through the engagement of the TSLPR complex, TSLPR and IL-7Rα (Morshed, et al., 2012; Wong, et al., 2010). TSLP delays eosinophil apoptosis and induces the production of IL-6, IL-8, CXCL1, and CCL2, with effects being concentration-dependent and specific to TSLP (Wong, et al.,

2010). TSLP induces a downregulation of L-selectin and an upregulation of the eosinophil surface expression of adhesion molecule CD18 and ICAM-1, both important for the recruitment of eosinophils to the site of inflammation. TSLP facilitates eosinophil trans-endothelial migration, and thus tissue eosinophilia, by modulating adhesion molecules (Wong, et al., 2010). Eosinophils respond directly to TSLP with degranulation and release of eosinophil-derived neurotoxin, phosphorylation of STAT5, and increased viability and survival (Cook, et al., 2012). Experiments using a blocking antibody for TSLPR show significant suppression of eosinophil degranulation. In the presence of the pro-inflammatory cytokines, IL-13 and TNF-α, eosinophils express increased levels of TSLPR mRNA and surface protein, which enhances TSLP-mediated eosinophil survival and STAT5 phosphorylation. Supporting these *in vitro* actions through an *in vivo* experiment, tezepelumab, a human IgG2 mAb anti-TSLP, inhibits the EAR and LAR and reduces blood and sputum eosinophils in patients with mild, atopic asthma (Gauvreau, et al., 2014).

Basophils

Basophils play an important role in producing Th2 cytokines and pro-inflammatory mediators; basophil development and function are thought to be largely regulated by IL-3. It has been established that TSLP can elicit an effect on murine basophils, including increasing the number of basophils in the spleen, lung, blood and bone marrow, promoting Th2 cytokine production and upregulating the surface expression of IL-33R, CD69, CD62L, CD11b, CD123 (Siracusa, et al., 2011). Further, activated basophils from human peripheral blood express TSLPR, suggesting that human basophils can respond to TSLP. Allergen inhalation in patients with allergic asthma significantly increases the number of

basophils in the airways, as well as upregulates Th2 cytokine production, and TSLPR and IL-3Rα expression on peripheral and airway basophils (Salter, et al., 2015). The increased expression of TSLPR on basophils 7 to 24 hours following allergen inhalation positively correlates with airway hyperresponsiveness and pulmonary function, suggesting that increased basophil responsiveness to TSLP may contribute to the development of the LAR. Following stimulation with cross-linking IgE antibody, basophils in the blood of atopic asthmatics upregulate the expression of TSLPR, which correlates with the level of allergen-specific serum IgE (Agrawal, et al., 2014). *In vitro* stimulation of human basophils with TSLP increases basophil responsiveness to eotaxin and induces basophil activation, both directly through TSLPR and indirectly through an IL-3-mediated basophil autocrine loop (Salter, et al., 2015). Further, basophil exposure to TSLP increases expression of IL17RB and ST2, the receptors for IL-25 and IL-33, respectively, suggesting that TSLP-induced basophils may have increased responses to other alarmins (Salter, et al., 2015). Taken together there is increasing evidence pointing to important roles for TSLP in basophil differentiative and inflammatory functions.

Mast Cells

TSLP has been found to play a role in the differentiation and proliferation of mast cells from bone marrow progenitors (Han, et al., 2014). Mast cells found in the bronchial mucosa of asthmatic patients express functional receptors for TSLP, seen at mRNA and protein levels, as well as in stained tissue sections from the bronchial mucosa. Very high levels of pro-inflammatory cytokines IL-5, IL-13, IL-6, GM-CSF, CXCL8, and CCL1 are released from mast cells after 24 hour stimulation with TSLP in the presence of IL-1β/TNF-

α (Allakhverdi, et al., 2007). TSLP also suppresses the production of TGF-β. Mast cells cultured with small airway epithelial-derived cell supernatants (unstimulated, poly(I:C) stimulated, and bacterial peptidoglycan-stimulated), with the addition of anti-TSLP mAb, show reduced production of IL-13. These results suggest that certain bacterial, viral, and inflammatory cytokine (IL-1β/TNF-α) stimuli may induce airway epithelial production of TSLP, which in turn can stimulate mast cells and propagate allergic inflammation.

TSLP in Allergic Disease

There are many studies that show the involvement of TSLP in allergic diseases. The initial link between TSLP and allergic disease was in atopic dermatitis, in which non-lesion skin did not express detectable levels of TSLP, though highly expressed by keratinocytes in acute and chronic atopic dermatitis skin lesions (Soumelis, et al., 2002). The potential role of TSLP in allergic airway disease was first established in 2002 with the finding that TSLP mRNA was present in human lung fibroblast, airway smooth muscle cells, and BECs (Soumelis, et al., 2002), with abnormal levels of TSLP being associated with respiratory disorders (Kamekura, et al., 2009; Shikotra, et al., 2012; Ying, et al., 2008, 2005; Zhang, et al., 2007). In mice, using the well-established ovalbumin/allergic airway inflammation model, TSLP protein can be detected in the BAL and the lung after ovalbumin challenges; however, TSLPR-knockout mice have been found to be protected from airway inflammation (Jang, et al., 2013). In human BECs, studies have shown increased *TSLP* mRNA in asthmatic subjects in comparison to healthy controls, associated with the degree of airway obstruction (Ying, et al., 2005). Lung epithelium from asthmatics and chronic obstructive pulmonary disease (COPD) patients had significantly higher

numbers of *TSLP* mRNA positive cells, and the BAL from asthmatic and COPD patients has increased concentrations of TSLP protein in comparison to healthy controls (Semlali, et al., 2010; Ying, et al., 2008, 2005). While the extent of TSLP expression is variable among asthmatic patients, it correlates directly with Th2 cytokine and chemokine secretion, and inversely with lung function (Shikotra, et al., 2012; Ying, et al., 2008). In patients with allergic rhinitis and nasal polyposis, TSLP is highly expressed in the nasal epithelium and associated with eosinophilic infiltration and Th2 cytokine production (Kimura, et al., 2011; Mou, et al., 2009). A clinical trial of a monoclonal antibody that targets human TSLP, tezepelumab (AMG 157), demonstrated reduction of both EAR and LAR in mild atopic asthmatics (Gauvreau, et al., 2014). AMG 157 was also able to reduce sputum eosinophil levels, the fraction of exhaled nitric oxide and circulating eosinophils, all markers of systemic and airway inflammation, which further supports the role of TSLP in the persistent airway inflammation present in asthmatics (Gauvreau, et al., 2014).

TSLP Polymorphisms

Recent GWAS and meta-analyses of GWAS have identified a number of variants that are associated with asthma and related traits. Genetic studies support the critical role of TSLP in allergic disease, with several polymorphisms at the TSLP locus found across a variety of ethnicities to be associated with increased asthma susceptibility or protection. The GABRIEL and EVE consortiums studies identified the loci IL1RL1, TSLP, IL-33, and 17q21 to be associated with asthma and suggested the role of epithelial-derived cytokines in asthma phenotypes (Moffatt, et al., 2010; Torgerson, et al., 2011).

The SNP rs1837253, located 5.7kb upstream of the transcription start site of *TSLP*, is significantly and inversely associated with asthma, atopic asthma and AHR (He, et al., 2009; Torgerson, et al., 2011). Since the minor T allele of rs1837253 is associated with reduced risk of disease and there are no other SNPs in linkage disequilibrium, 2 Mb up or downstream in the chromosomal region, this is likely a causal polymorphism with functional consequences (He, et al., 2009). The SNP rs1837253 has shown associations with reduced risk of asthma in Costa Rican boys and reduced odds of allergic rhinitis in boys with asthma (Bunyavanich, et al., 2011; Hunninghake, et al., 2010). Additionally, Hui *et al*. demonstrated that the SNP rs1837253 has functional effects on TSLP protein production, with differential TSLP protein secretion from NECs based on rs1837253 genotype (Hui, et al., 2015). When subjects are stratified by genotype, there is decreased TSLP secretion from poly (I:C)-stimulated NECs of heterozygous (CT; 1.8-fold) and homozygous minor (CC; 2.5-fold) individuals, compared to homozygous major individuals, although there are no differences based on subject atopy. Primary BECs from patients with asthma release higher levels of TSLP than those from healthy controls after dsRNA stimulation (Hui, et al., 2014; Lee, et al., 2012). Reduced TSLP secretion based on rs1837253 genotype may help explain the protective association of this SNP with asthma and AHR. Understanding the functional effect of gene variants provides important information about allergic inflammation and disease.

Furthermore, another gene variant in TSLP, rs3806933, in the promoter region of the lfTSLP has been associated with childhood and adulthood asthma, demonstrating functional effects on TSLP protein production (Harada, et al., 2011). In poly (I:C)-

stimulated BECs, rs3806833 functions to enhance the binding of AP-1, a transcription factor that mediates TLR-3-dependent cytokine expression, and increases the transcription of lfTSLP (Harada, et al., 2009). Similarly, in the NECs of asthmatics, lfTSLP is more responsive to the dsRNA stimuli poly (I:C) than sfTSLP, which suggests that there may be a dichotomy between the function of these two isoforms in inflammatory processes (Fornasa, et al., 2015). The minor T allele of the SNP rs1837253 is less inducible than the major C allele, which suggests there may be differential regulation involved in the transcription of TSLP isoforms (Moorehead, et al., 2020). Given the critical immunomodulatory role of TSLP, further investigation into TSLP and its isoforms can shed light on their role in the pathogenesis of asthma and allergic inflammation and effect on the hematopoietic milieu.

RESEARCH STUDY

Hypotheses

This project investigates NECs from allergic asthmatic in response to a dsRNA stimulus. Given that the minor T allele of the rs1837253 SNP in the TSLP gene is associated with reduced risk of allergic asthma, that there are no other SNPs in linkage disequilibrium with rs1837253, and that nasal allergen challenge models initiate Th2 responses, and that corticosteroids demonstrate the ability to reduce cytokine expression from human airway cells, the following hypotheses were made:

Hypothesis #1: NECs from individuals with the minor T allele will have a lower propensity to induce a Th2 inflammatory response and have modified gene expression and cytokine mediator profiles.

Hypothesis #2: There will be an increase in NECs *in vitro* secretion of cytokines following *in vivo* nasal allergen challenge, while after14 day treatment with an intranasal corticosteroids (INCS) cytokine profiles will be reduced.

Hypothesis #3: NEC-secreted cytokines from allergic asthmatics will stimulate Eo/B differentiation *in vitro*.

Aims

Aim #1: Analyze TSLP isoform gene expression in ex vivo TLR-induced NECs of asthmatics in relation to rs1837253 genotype.

Aim #2: Investigate the effect of nasal allergen challenge (baseline vs 24h post-challenge) and intranasal corticosteroid (INCS vs placebo treatment) on ex vivo TLR-induced NEC TSLP gene expression and secretion of cytokines.

Aim #3: Establish the influence of cytokines from TLR-induced NECs on Eo/B differentiation

Scientific Impact

The results of this study will provide insight into the role of TSLP and its gene isoforms in allergic asthma and the downstream impacts of epithelial cytokines on the hematopoietic milieu. Additionally, it will provide information on complex gene-environment interactions, and the effects of a corticosteroid treatment on allergic inflammatory processes.

2. METHODS

STUDY DESIGN

This project is part of the NACHO study being led by the principal investigator, Dr. Gail Gauvreau. In this cohort of the NACHO study, 10 subjects were enrolled and there were 10 visits for each subject. Samples for this project were collected at visits 2, 7 and 10 (Figure 4). Visit 1 consisted of subject screening. Eligible subjects were identified during the initial screening procedures with measures of spirometry, hyperresponsiveness to methacholine, complete history, physical examination, allergen skin test, and nasal allergen titration. Nasal allergen was selected based on a positive SPT response and clinical history. During visit two, subjects provided a mouthwash sample and at visits two, seven, and ten, nasal epithelial curettage were performed. The ten subjects were challenged with allergen three times. The second allergen challenge followed a placebo treatment. The third allergen challenge followed intranasal corticosteroid (INCS) treatment. The duration of study participation was 13-17 weeks.

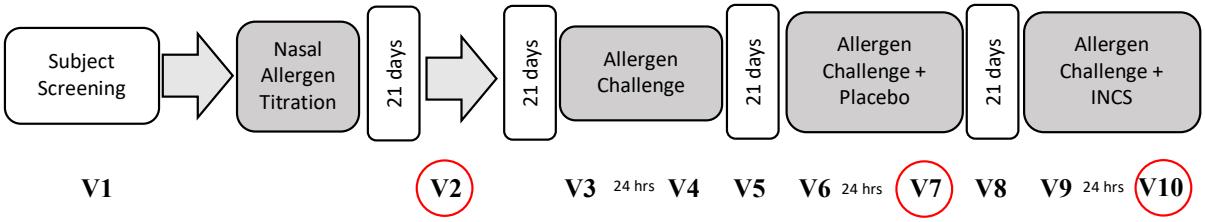

Figure 4: Study Schematic. Diagram of study design and timeline for nasal allergen challenges. Red circles indicate the visits at which NECs were obtained via nasal curettage for this study. Adapted from the NACHO protocol.

ALLERGEN AND TREATMENT INTERVENTION

Ten subjects received a placebo treatment prior to their 2nd nasal allergen challenge, and INCS (Nasacort®; triamcinolone acetonide spray) prior to the 3rd and final nasal allergen challenge. The first treatment started in the evening after visit 5 which was scheduled 7 days after visit 4 and 12 days before visit 6 allergen challenge. Treatment continued through the day of the challenge and the last dose was taken on the morning of visit 7, for a total of 14 days. The second treatment started in the evening of visit 8 which was scheduled 7 days after visit 7 and 12 days before visit 9 allergen challenge. Treatment continued through visit 9 allergen challenge and the last dose was taken on the morning of visit 10 for a total of 14 days. The subjects administered 220 mcg twice a day for a total dose of 440 mcg per day. Subjects recorded drug self-administration in a diary, and compliance was confirmed by weighing the dispenser.

STUDY SUBJECTS

For this project, 10 subjects with mild allergic asthma and allergic rhinitis completed the study. Mild asthma was defined by a methacholine $PC_{20} \leq 16$ mg/ml, with a baseline forced expiratory volume (FEV_1) $\geq 70\%$ of the predicted value. Subjects also had to have a history of nasal rhinitis and of allergic symptoms after exposure to aeroallergen, with a positive SPT to common aeroallergens. Exclusion criteria included: smoking, history of other respiratory disease, pregnancy, recent asthma exacerbations or respiratory tract infection (within 8 weeks), significant systemic disease, other lung disease, history of hypotensive episodes, history of hematologic abnormality, and use of corticosteroids within 28 days prior to initial visit.

ISOLATION OF GENOMIC DNA AND GENOTYPING

Due to the absence of significant linkage disequilibrium with other SNPs and the previous association with asthma and airway hyperresponsiveness, the SNP rs1837253 in the TSLP was selected for investigation. Mouthwash samples were collected from all subjects and DNA was extracted from the samples using QIAamp DNA Mini Kits (Qiagen) according to the manufacturer's supplementary protocol for mouthwash samples. Genotyping was then performed using a commercially available TaqMan genotyping assay for rs1837253 (Assay C__11910823_20, Life Technologies).

NASAL EPITHELIAL CELL ISOLATION AND CULTURE

Primary nasal epithelial cells (NECs) were derived from nasal curettage of the inferior nasal turbinate at baseline or 24 hours after an nasal allergen challenge using Rhino-Probes nasal cuvettes (Arlington Scientific). The NECs were cultured and expanded as described by Müller *et al* (Müller, et al., 2013). Some modifications were made to the protocol. Bronchial epithelial growth medium (BEGM) (Clonetics) comprised of bronchial epithelial basal medium (Clonetics) and supplemented with SingleQuots (Clonetics) were used to maintain NECs. Collected NECs were centrifuged and resuspended in BEGM with 10% 100X DNase 1 (Sigma Aldrich, St Louis MO) and incubated at room temperature for 20 minutes. Cells were washed and 35 mm culture dishes (Corning Costar) were coated with PureCol (Inamed), and incubated at 37°C, 5% CO_2 for 30 minutes. The PureCol were then removed, the plate was rinsed twice with PBS and the NECs were plated. Media was changed 24h after plating and replaced every second day thereafter until 80-90% confluence. Cells were passaged using trypsin (Gibco, Burlington, Ontario) and further

expanded into 75cm^2 flasks in BEGM. In this study second and third passaged cells were used for subsequent experiments .

POLY (I:C) STIMULATION

NECs were seeded into 25cm^2 flasks and allowed to grow to 80-90% confluence. BEGM was replaced with bronchial epithelial basal media (Clonetics) with 1% fetal bovine serum (R&D Systems) prior to experimental stimulation. NECs were treated with poly(I:C) (25μg/mL) or vehicle control for 3h, 6h or 24h at 37°C, 5% CO_2.

RNA ISOLATION AND REVERSE TRANSCRIPTION

RNA was extracted from NECs using RNeasy Mini-Kit (Qiagen) according to manufacturer's specifications. RNA was quantified using a NanoDrop Spectrophotometer (Thermo Fisher). The RNA in each sample time point (0, 3, 6, 24hr) from each subject were diluted with nuclease-free water based on the lowest concentration sample. The samples were reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions and cDNA was stored at -80°C.

CYTOKINE AND CHEMOKINE SECRETION

Supernatants from NEC cultures were collected after 24 hours stimulation with poly (I:C) or vehicle control and cytokine and chemokine secretion of TSLP, IL-33, IL-25, IL-5, IL-6, IL-8, IL-13, TNF-α, SDF-1α, GM-CSF, and eotaxin-3 were assessed using Multiplex assays (Meso Scale Diagnostics) according to manufacturer's instructions.

BLOOD PROCESSING AND CD34+ PROGENITOR ISOLATION

Umbilical cord blood was collected from the Labour and Delivery Unit at McMaster's Children's Hospital upon informed consent in heparinized tubes (BD Sciences). Cord blood was processed with a 1:5 ratio of 6% dextran to cord blood and incubated in a 37°C water bath for 20-30 min to separate erythrocytes through sedimentation. The mononuclear cells were isolated through density centrifugation using Lymphoprep density gradient medium. The mononuclear cell layer was extracted and diluted 1:1 with McCoys 5A media. CD34+ progenitors were enriched using MACS CD34 enrichment kit (Miltenyi Biotec) according to manufacturer's instructions.

METHYLCELLULOSE COLONY FORMING ASSAY

Enriched CD34+ progenitors were cultured (5000 cells/well) in duplicates in 0.9% methylcellulose (MethoCult H4230, StemCell Technologies) with Iscoves 2+ (modified Dulbecco's medium (Gibco) supplemented with FBS, penicillin-streptomycin, and 2-ME) in 35 mm x10 mm culture dishes. Cultures were supplemented with supernatants obtained from NEC cultures described above. NEC supernatant stimulated with poly (I:C) from visit 2 were added to the cultures (1:15), 1:150 dilution, or 1:1500 dilution, without or without optimal dose of IL-3 (1ng/mL), IL-5 (1 ng/mL), or GM-CSF (10 ng/mL). Cultures were incubated for 14 days (37°C, 5%CO_2) and Eo/B colony-forming units (CFUs) were enumerated using inverted light microscopy (colonies were defined as tight granular clusters ≥40 cells).

QUANTITATIVE REVERSE TRANSCRIPTION – POLYMERASE CHAIN REACTION

The expression of TSLP was established with SsoAdvanced Universal SYBR Green Supermix quantitative reverse transcription – polymerase chain reaction (Bio-Rad, Hercules, CA) with a MX4000 Stratagene detection system according to the manufacturer's instructions. The PrimePCR SYBR green assays for Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, (YWHAZ) were used for reference gene primers (Bio-Rad) as determined previously to be optimal reference genes by the PrimePCR Human Reference Gene Panel. All TSLP primer sets (Integrated DNA Technologies, Coralville, IA) were designed and evaluated as per the minimum information for publication of quantitative reverse transcription-PCR experiments (MIQE) guidelines. The TSLP primer sets were previously verified by Hui and colleagues using 8-point standard curves to confirm adequate amplification efficiencies for each TSLP isoform primer sets and the GAPDH/YWHAZ reference genes using SsoAdvanced SYBR green Supermix (Bio-Rad) and melt curves were used to verify expected single peak amplicons. The threshold cycle is inversely associated with the mRNA expression levels and was defined as the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence. The standard $2^{-(\Delta\Delta Ct)}$ formula was used to calculate arbitrary TSLP mRNA concentrations. The level of TSLP mRNA was normalized to the level of GAPDH/YWHAZ mRNA.

NASAL LAVAGE

Subjects were asked to blow their nose and then sit with neck flexed upwards at 45. 5mL of pre-warmed phosphate buffered saline solution was instilled into each nostril with a needless syringe with a sponge adapter, and was collected back into the syringe. Subjects were asked to refrain from breathing through their nose or swallowing. Nasal lavage fluid was filtered, centrifuged, and cytospins were made with cell suspension and analyzed for cell differentials.

PEAK NASAL INSPIRATORY FLOW (PNIF) AND TOTAL NASAL SYMPTOM SCORES (TNSS)

TNSS is a measurement of nasal symptoms experienced by study participants. They rate their nasal congestion, sneezing, nasal itching, and rhinorrhea at each time point from 0-3 and the scores are totaled. Subjects were asked to evaluate TNSS by grading each symptom (sneezing, congestion, itching, and rhinorrhea) and the score is added to reach the TNSS score. The grading levels are as follows: 0=None, no symptoms evident; 1=Mild, symptom present but easily tolerated, 2=Moderate, definite awareness of symptom, bothersome but tolerable; 3=Severe, symptom hard to tolerate, would interfere with daily activity. TNSS was determined at multiple intervals to assess onset of inflammation. PNIF is another assessment used to assess nasal patency or obstruction. Opposed to TNSS, PNIF is an objective measurement of nasal airflow. Repeating these measurements can provide important data on the nasal congestion of participants. Subjects were asked to inhale as hard and as fast through their nose as possible through the mask while keeping their mouth closed. Measurements of nasal obstruction were repeated at intervals.

WHOLE BLOOD SAMPLING

Peripheral blood was collected using standard venipuncture techniques, drawn into vacutainers, mixed thoroughly by inverting 8-10 times and used for hematology with cell differentials.

STATISTICS

Data presented within the text, figures and figure legend are expressed as mean ± SEM. Data analysis was completed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) software to perform one-way and two-way ANOVAs, and mixed model analyses. Post-hoc analyses for between groups comparisons were performed using Tukey's multiple comparisons test for one-way ANOVA and Bonferroni's test for two-way ANOVAs. Mixed model analyses were also conducted as there were some missing data points and repeated-measures one-way ANOVAs cannot accommodate for missing values. Type 1 error probability (α) was set at 0.05.

3. RESULTS

Subject characteristics

This study was approved by the Hamilton Integrated Research Ethics Board and all subjects provided written and informed consent. Ten subjects were enrolled for this part of the project. Two subjects dropped out after visit 2 but were replaced with new individuals. On visit 2, all subjects had mouthwash samples collected and were genotyped for the SNP rs1837253. The genotype distribution was 5 subjects were homozygous for the major allele (CC), 4 were heterozygous (CT), and 1 was homozygous for the minor allele (TT) (Figure 5). Nasal curettage samples were collected at visits 2, 7 and 10 and expanded *in vitro.* Of these nasal epithelial cell cultures 29 out of 30 were cultured successfully.

Poly (I:C) stimulation significantly increased chemokine and cytokine secretion from NECs of allergic asthmatics

The cytokines and chemokines TSLP, IL-33, IL-25, IL-5, IL-6, IL-8, IL-13, TNF-α, SDF- 1α, GM-CSF, and eotaxin-3 were measured from cultured NECs collected at visits 2, 7 and 10 using a Mesoplex multiplex protein assay. The cells were either treated with poly (I:C) or a vehicle control and collected at 24 hrs. IL-25 and IL-33 were not detected in any of the samples. The supernatant concentrations of TSLP, IL-5, IL-6, IL-13, GM-CSF, TNF-α, SDF-1α, and eotaxin-3 from cells that were treated with poly (I:C) for 24 hours were significantly higher than their nontreated controls (Figure 6). These levels from the poly (I:C) treated NECs were found to be significantly different for all three visits

($p<0.05$). However, when looking at the supernatant levels of IL-8, this trend was only statistically significant at visit 2.

Poly (I:C) stimulated-NEC secreted supernatant did not differ based on prior nasal allergic challenge or use of INCS

The cytokine and chemokine profiles were analyzed from poly (I:C) stimulated NECs after 24 hours. The NECs were collected at visit 2 (baseline; V2), visit 7 (24 hours post nasal allergen challenge after 14 treatment with a placebo intervention; V7), and visit 10 (24 hours post nasal allergen challenge after INCS treatment for 14 days; V10). Comparison across visits did not show any significant changes in the poly (I:C) stimulated-protein secretion of TSLP, IL-5, IL-6, IL-13, GM-CSF, TNF-α, SDF-1α, or eotaxin-3 from NECs when stratified by visit (Figure 7).

Cytokine and chemokine secretion by poly (I:C) stimulated NECs did not vary based on rs1837253 genotype

The cytokine and chemokine secretions from NECs stimulated with poly (I:C) were investigated in relation to subjects' rs1837253 genotype. Due to the limited subject number, genotype was analyzed by either the presence or absence of the minor T allele, with the heterozygotes and the homozygous minor genotypes grouped together and compared to the homozygous dominant genotype. When the results were stratified by rs1837253 genotype, there were no observed differences for any of the cytokines or chemokines of interest (Figure 8) at any visit.

Spontaneous release of cytokines and chemokines from NECs did not differ based on treatment with INCS

The spontaneous release of TSLP, IL-5, IL-6, IL-8, IL-13, GM-CSF, TNF-α, SDF-1α, and eotaxin-3 were measured from cultured NECs. The NECs were collected at visit 2 (baseline; V2), visit 7 (24 hours post nasal allergen challenge after 14 treatment with a placebo intervention; V7), and visit 10 (24 hours post nasal allergen challenge after INCS treatment for 14 days; V10). When the cytokine and chemokine profiles were compared across visits there were no statistical differences in any NEC supernatant levels when compared between the Visits 2, 7, and 10 (Figure 9).

Spontaneous release of cytokines and chemokines from NECs with the minor rs1837253 allele was selectively increased following INCS treatment

At baseline Visit 2, cells collected from subjects with the minor T allele secreted lower levels of IL-13 ($p<0.05$). However, following treatment with INCS and nasal allergen challenge, at Visit 10, NECs from subjects with the T allele had significantly higher levels of IL-13, IL-5, SDF-1α, and IL-8, when compared to their respective levels at baseline (Visit 2) and placebo treatment and nasal allergen treatment (Visit 7) other visits. These findings were not observed in the NECs from subjects with the major CC genotype (Figure 10).

Poly (I:C)-stimulated NEC supernatants induce Eo/B colony formation

Methylcellulose colony forming assays were conducted using 5 NEC supernatants collected at visit 2. The NECs were stimulated with 25 μg/mL of poly (I:C) and the

supernatants were taken at 24 hours post stimulation. The results show CFU formation after 14-day methylcellulose cell culture. In the absence of any additional cytokines, NEC supernatants were capable of stimulating Eo/B colony formation (Figure 11A). A dose response trend was observed at 1:15, 1:150, and 1:1500 dilutions, but only the 1:15 NEC supernatants were statistically significant when analyzed against the negative and media controls. The 1:15 NEC supernatants stimulated more CFUs when compared to negative and media controls, with significantly increased colony formation in the presence of IL-3 ($p=0.003$) as opposed to IL-3 alone (Figure 11B). There was, however, no statistically significant difference between the 1:15 NEC supernatant alone and in the presence of IL-3. Although it did not quite reach statistical significance, 1:15 supernatant in the presence of IL-5 showed a trend of increased CFU when compared to IL-5 alone ($p=0.0575$) (Figure 11C). In the conditions with GM-CSF supplementation, there were no significant differences between cultures with and without the addition of NEC supernatants (Figure 11D).

Gene expression of TSLP and isoforms of poly (I:C) stimulated NECs does not vary between visit

Analysis of total TSLP mRNA showed elevated levels after 6 hours at visits 2 and 7, with lower levels after subjects were treated for 14 days of INCS, but this difference did not reach statistical significance. The mRNA expression of sfTSLP for visits 2 and 10 was highest at 3 hours, while at visit 7 mRNA levels of sfTSLP were the highest at 6 hours. However, there were no statistically significant differences between sfTSLP in NECs at any visit for any timepoint. When the analysis of lfTSLP was conducted, a majority of the

samples did not have detectable lfTSLP mRNA at 0h. For this reason, the fold change was calculated using the mRNA levels from 3h. The results showed that lfTSLP NECs from visit 2 had elevated lfTSLP gene expression at 6 hours post poly(I:C) stimulation, none of the observed trends were not statistically significant (Figure 12).

TSLP gene expression in poly (I:C) stimulated NECs did not vary based on rs1837253 genotype

NECs collected at visit 2 were stimulated and the mRNA expression of total TSLP, sfTSLP and lfTSLP were stratified by rs1837253 genotype. Although there was higher gene expression in NECs from subjects with the homozygous dominant genotype, there were no observed significant differences in the gene expression of TSLP based on rs1837153 alleles (Figure 13).

Gene expression response to INCS in poly (I:C) stimulated NECs was not significantly different between rs1837253 genotypes

Poly (I:C) stimulated NEC gene expression was analyzed from cells collected 24 hours after nasal allergen challenge at visit 7 and visit 10, which occurred after 14 days treatment with placebo control and INCS respectively. TSLP gene expression response was analyzed in regard to rs1837253 genotypes to see if subjects had varied responses to treatment with INCS. When stratified by the presence of absence of the minor T allele, there were no significant differences in the gene expression of total TSLP, sfTSLP, or lfTSLP from NECs in response to treatment of INCS (visit 10) when compared to the placebo treated NECs (visit 7) (Figure 14).

Response to INCS treatment did not vary based on rs1837253 genotype

Nasal lavage and peripheral blood samples were collected from subjects 24 hours post allergen challenge at visits 7 and 10, following 14-day treatment with placebo and INCS respectively. These samples were analyzed to see whether the responses to INCS differed in relation to subjects' rs1837253 genotype. Eosinophils were measured in the nasal lavage and blood of subjects, and total nasal symptom score (TNSS) and peak nasal inspiratory flow (PNIF) assessments were conducted. In the nasal lavage, there was a trend of lower eosinophil % in subjects with the minor T allele overall, but there were no significant differences in these analyses between genotypes at visit 7 or 10 (Figure 15). There were no statistically significant genotype differences in eosinophil counts or percentages in the nasal lavage, eosinophil counts in the blood (Figure 15), TNSS or PNIF (Figure 16) and responses to INCS treatment did not vary when stratified by the absence or presence of the minor T allele.

FIGURES:

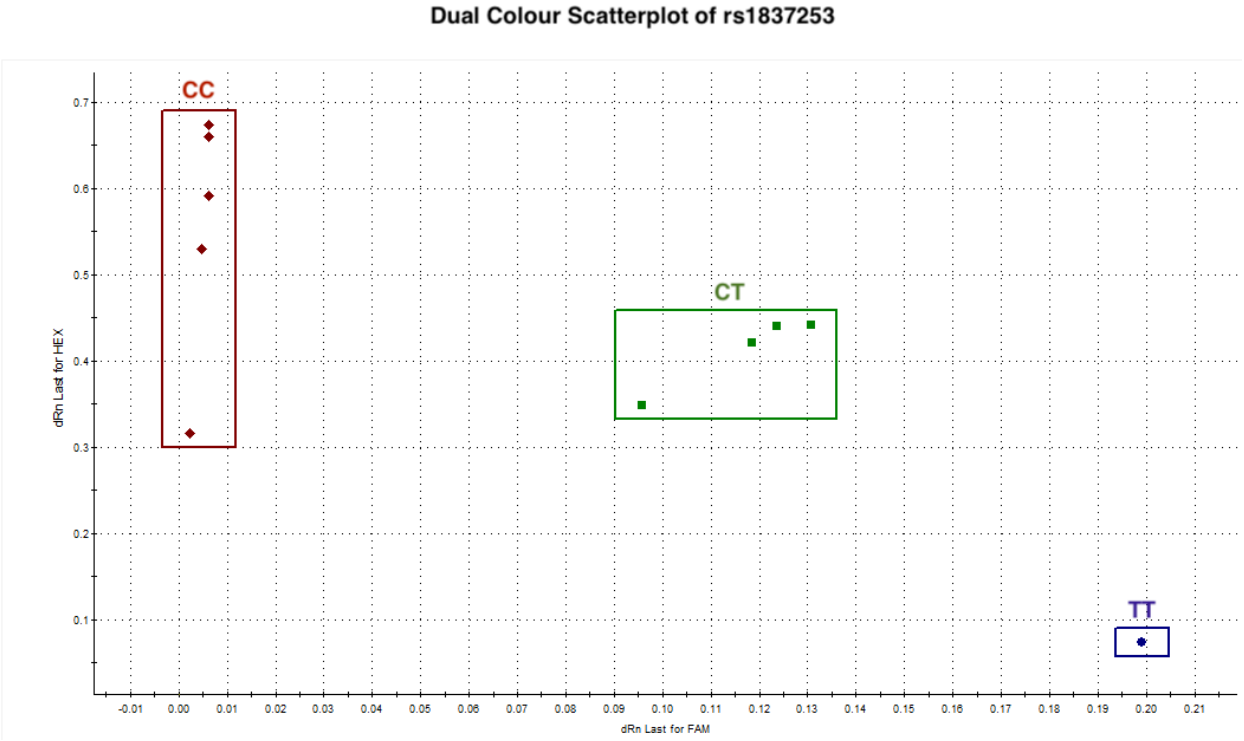

Figure 5: Allele discrimination dual scatter plot for rs1837253 TaqMan Genotyping Assay. Fluorescence for two dyes (Hex and FAM) assigned to the same wells is demonstrated in the above plot using the Stratagene Mx3000 Real-Time PCR System. Each point represents the coordinates of the fluorescence or Ct for the two dyes in a single well. Sample wells are grouped according to the amplification events indicated by either dye (i.e. homozygous for one of two alleles) or by both dyes (i.e. heterozygous for the two alleles).

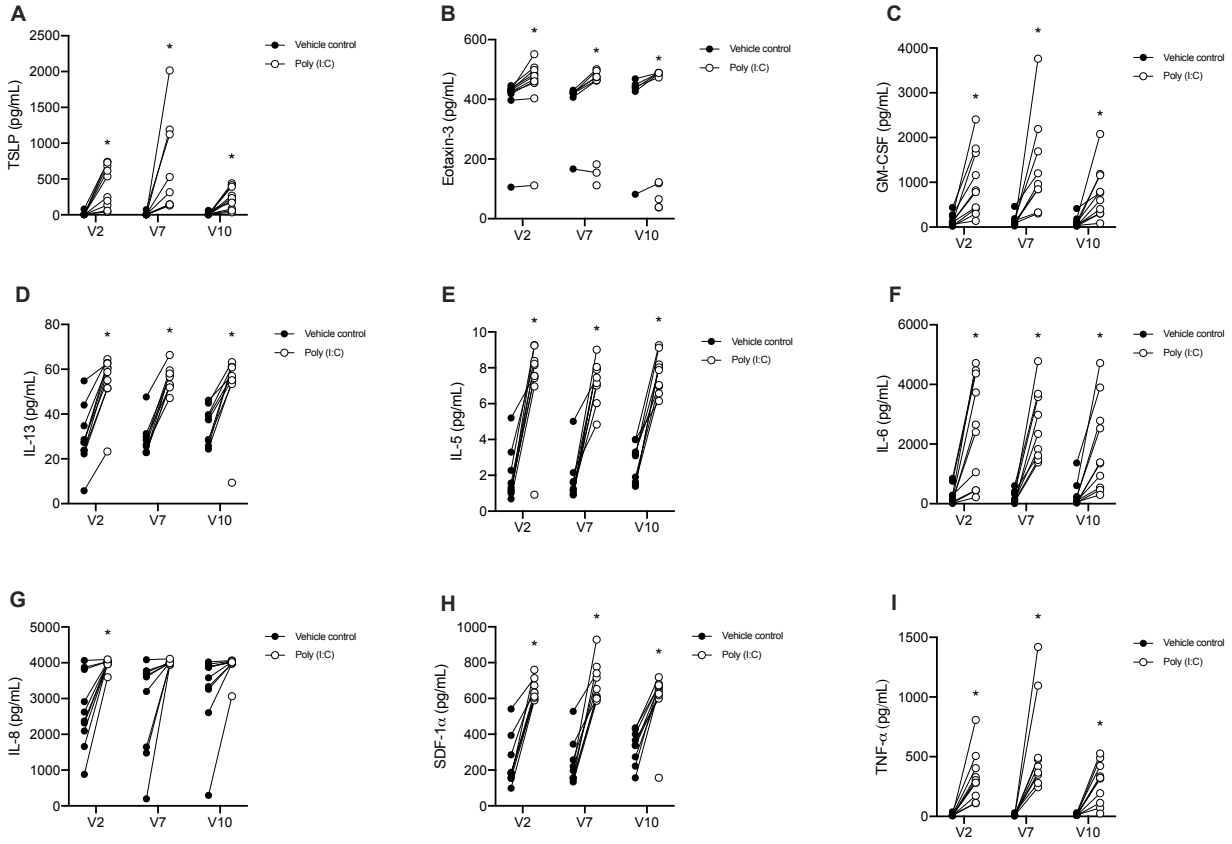

Figure 6: Poly(I:C) stimulates cytokine and chemokine expression from TLR-3 induced nasal epithelial cells. (A) thymic stromal lymphopoietin, (B) eotaxin-3, (C) GM-CSF, (D) IL-13, (E) IL-5, (F) IL-6, (G) IL-8, (H) SDF-1α, (I) TNF-α. Concentrations of secreted cytokine and chemokine protein in nasal epithelial cell culture supernatant from allergic asthmatic (n=10) individuals were measured using multiplex proteins assays following 24h stimulation with 25µg/mL of poly (I:C). One independent experiment was performed per subject. Mixed measure analyses were used to measure statistical differences. (*$p<0.05$, indicates statistical difference from vehicle control for the equivalent visit).

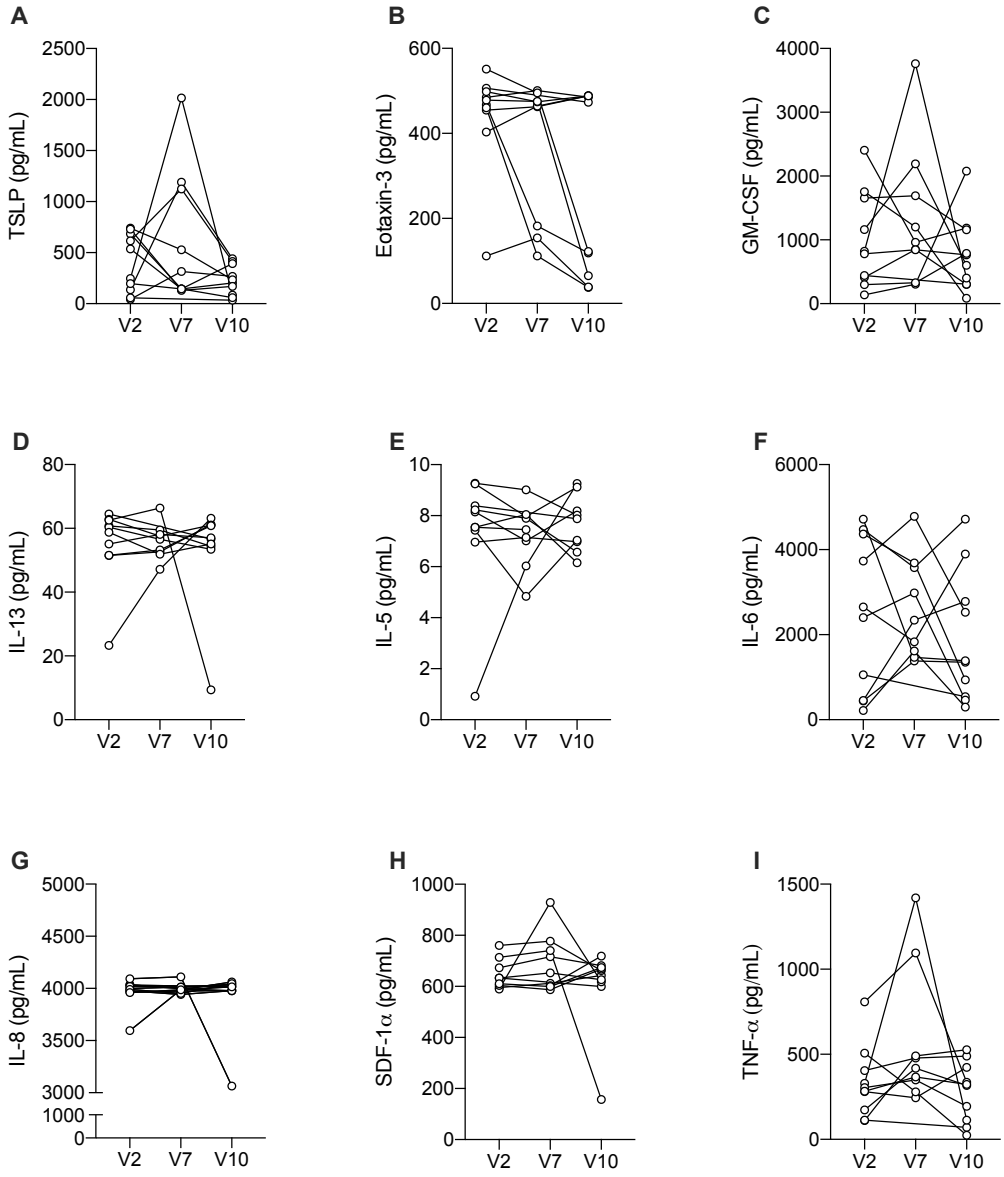

Figure 7: Relationship between visit and poly (I:C) induced nasal epithelial secretion of - (A) thymic stromal lymphopoietin, (B) eotaxin- 3, (C) GM-CSF, (D) IL-13, (E) IL-5, (F) IL-6, (G) IL-8, (H) SDF-1α, (I) TNF-α. Concentrations of secreted cytokine and chemokine proteins in nasal epithelial cell culture supernatants from allergic asthmatic (n=10) individuals were measured using multiplex proteins assays following 24h

stimulation with 25μg/mL of poly (I:C). One independent experiment was performed per subject. Mixed measure analyses were used to measure statistical differences.

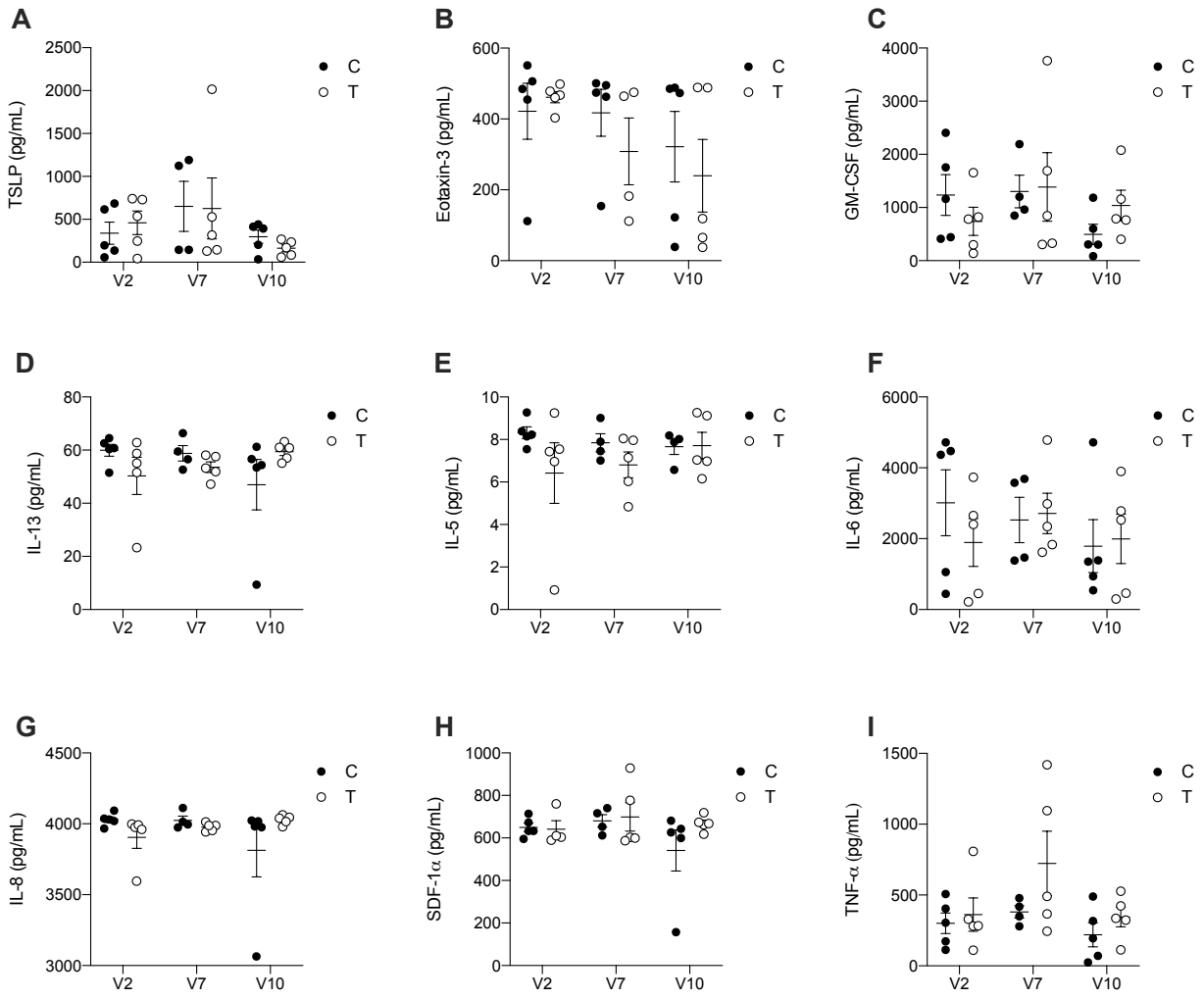

Figure 8: Relationship between rs1837253 genotype and poly(I:C) induced nasal epithelial secretion of - (A) thymic stromal lymphopoietin, (B) eotaxin-3, (C) GM-CSF, (D) IL-13, (E) IL-5, (F) IL-6, (G) IL- 8, (H) SDF-1α, (I) TNF-α. Concentrations of secreted cytokine and chemokine protein in nasal epithelial cell culture supernatant based on the absence (C; n=5) or presence (T; n=5) of the minor allele were measured using multiplex proteins assays following 24h stimulation with 25 µg/mL of poly (I:C). One independent experiment was performed per subject. Mixed measure analyses were used to measure statistical differences.

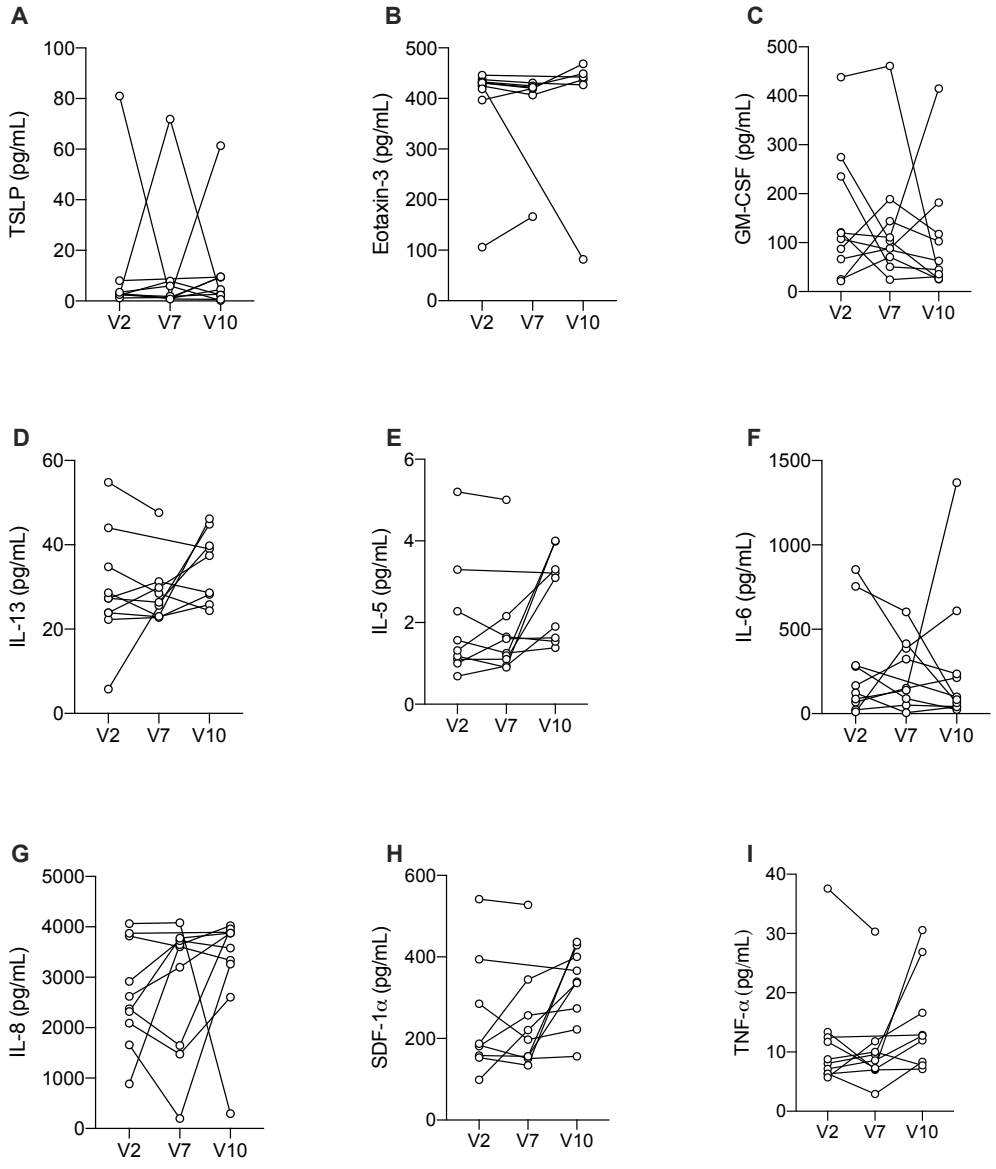

Figure 9: Relationship between visit and spontaneous nasal epithelial secretion of - (A) thymic stromal lymphopoietin, (B) eotaxin-3, (C) GM-CSF, (D) IL-13, (E) IL-5, (F) IL-6, (G) IL-8, (H) SDF-1α, (I) TNF-α. Concentrations of spontaneous release of cytokine and chemokine proteins in nasal epithelial cell culture supernatant from allergic asthmatic (n=10) individuals were measured using multiplex proteins assays. One independent

experiment was performed per subject. Mixed measure analyses were used to measure statistical differences.

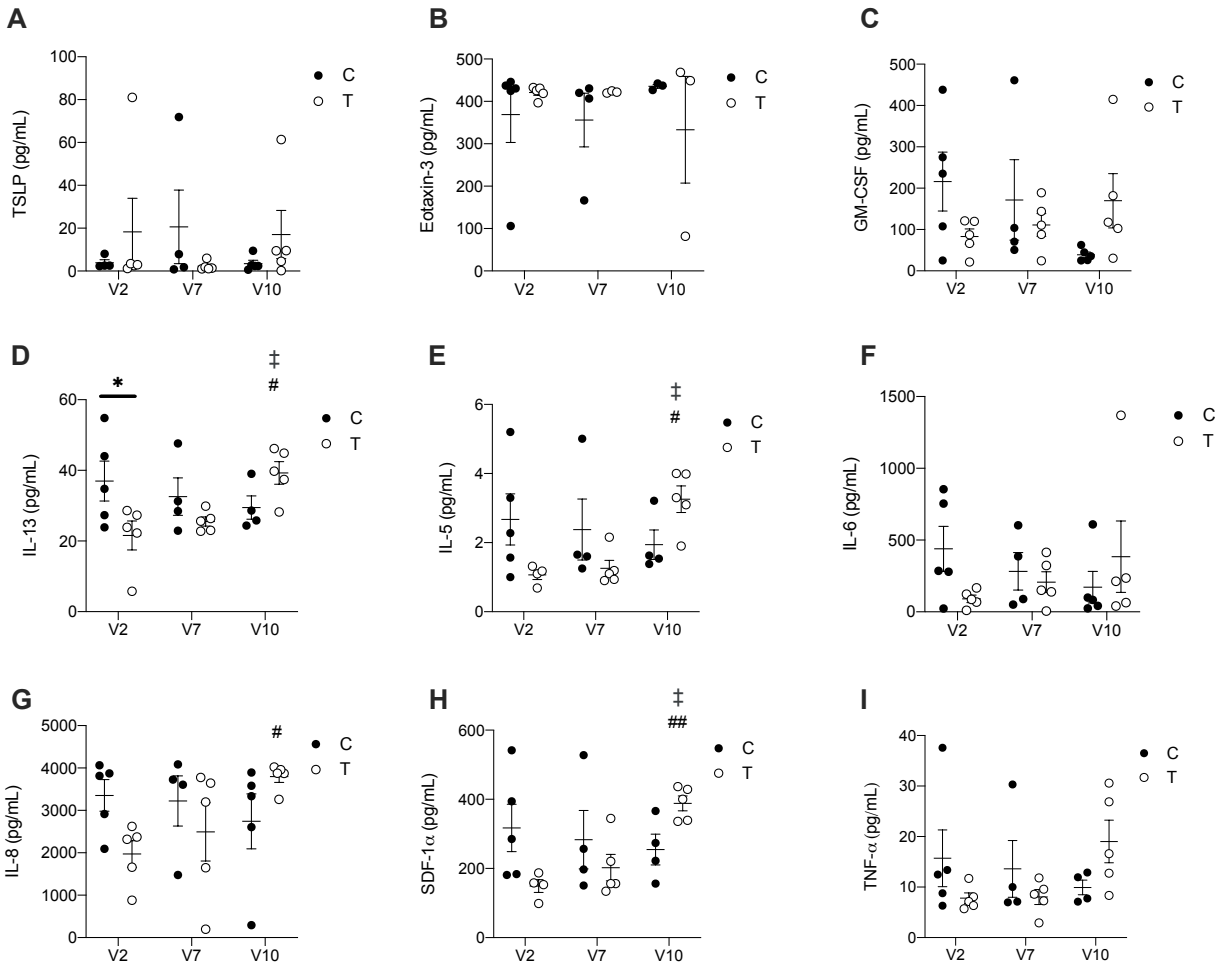

Figure 10: Relationship between rs1837253 genotype and spontaneous nasal epithelial secretion of - (A) thymic stromal lymphopoietin, (B) eotaxin-3, (C) GM-CSF, (D) IL-13, (E) IL-5, (F) IL-6, (G) IL- 8, (H) SDF-1α, (I) TNF-α. Concentrations of spontaneously released cytokine and chemokine proteins in nasal epithelial cell culture supernatant based on the absence (C; n=5) or presence (T; n=5) of the minor allele were measured using multiplex proteins assays. One independent experiment was performed per subject. Mixed measure analyses were used to measure statistical differences. * indicates a statistical difference $p<0.05$. # and ‡ indicate a significant difference ($p<0.05$) from T allele protein

concentrations at V2 and V7 respectively. ## indicates statistical difference ($p<0.001$) from T allele protein concentrations at V2.

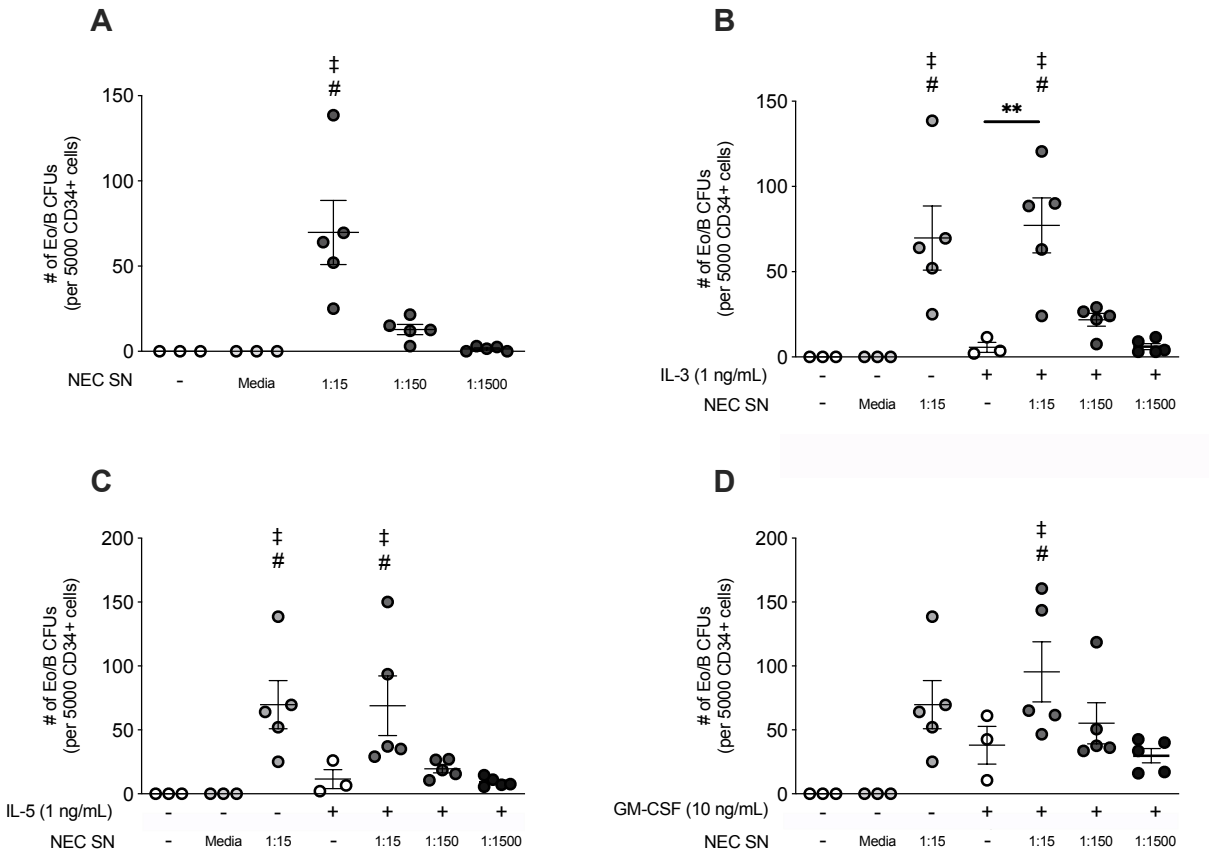

Figure 11: Nasal epithelial cell-derived supernatants capable of stimulating Eo/B colony formation. CD34+ progenitor cells were cultured in methylcellulose with TLR-3 stimulated NEC supernatants (1:15 dilution, 1:150 dilution, or 1:1500 dilution) from allergic asthmatics (n=5) in the presence or absence of exogenous supernatants. (A) no exogenous cytokines, (B) with the optimal concentration of IL-3 (1 ng/mL), (C) with the optimal concentration of IL- 5 (1 ng/mL), (D) with the optimal concentration of GM-CSF (10 ng/mL). One-way ANOVA with Tukey's post-test was used to assess differences between groups. # and ‡ refer to statistically significant differences from the negative control, and the media control respectively, $p<0.05$. ** indicates statistically significant difference from condition in the absence of NEC supernatant, $p<0.01$.

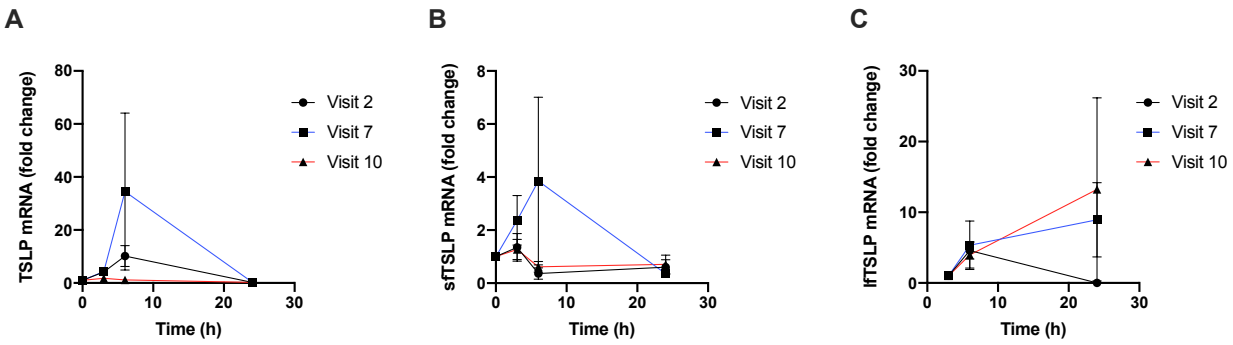

Figure 12: Polyinosinic:polycytidylic acid (polyI:C) induced thymic stromal lymphopoietin (TSLP), gene expression in human primary nasal epithelial cells (NECs). Gene expression kinetics of (A) total TSLP isoforms (B) short TSLP isoform (C) long TSLP isoform in poly (I:C) (25 μg/mL) stimulated NECs from visits 2, 7 and 10 (n=10). Results shown are expressed as fold change in the mRNA level in poly (I:C)-stimulated NECs at 3h, 6h and 24h relative to the level in unstimulated NECs (0 h) for total and sfTSLP and relative to 3h for lfTSLP. Two-way ANOVA with Bonferroni post-test was used to assess differences between groups.

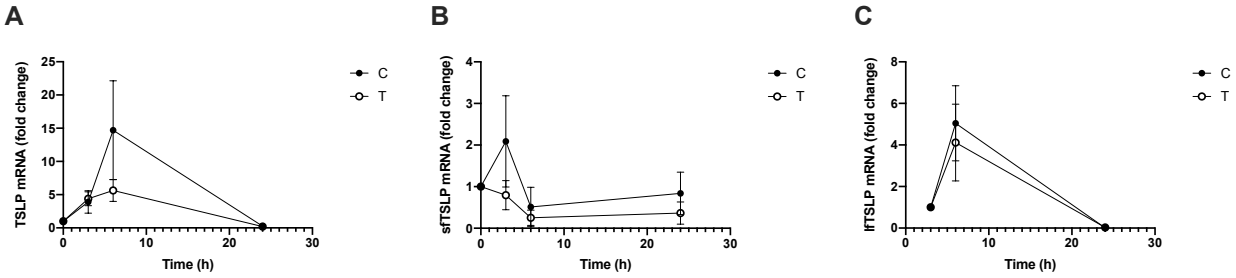

Figure 13: Polyinosinic:polycytidylic acid (polyI:C) induced thymic stromal lymphopoietin (TSLP) gene expression does not differ in human primary nasal epithelial cells (NECs) based on rs1837253 genotype. Gene expression kinetics of (A) total TSLP isoforms (B) short TSLP isoform (C) long TSLP isoform in poly (I:C) (25 μg/mL) stimulated NECs from visit 2 and are stratified based on the absence (C; n=5) or presence (T; n=5) of the rs1837253 minor allele. Results shown are expressed as fold change in the mRNA level in poly (I:C)-stimulated NECs at 3h, 6h and 24h relative to the level in unstimulated NECs (0 h) for total and short isoform TSLP and relative to 3h for long isoform TSLP. Two-way ANOVA with Bonferroni post-test was used to assess differences between groups.

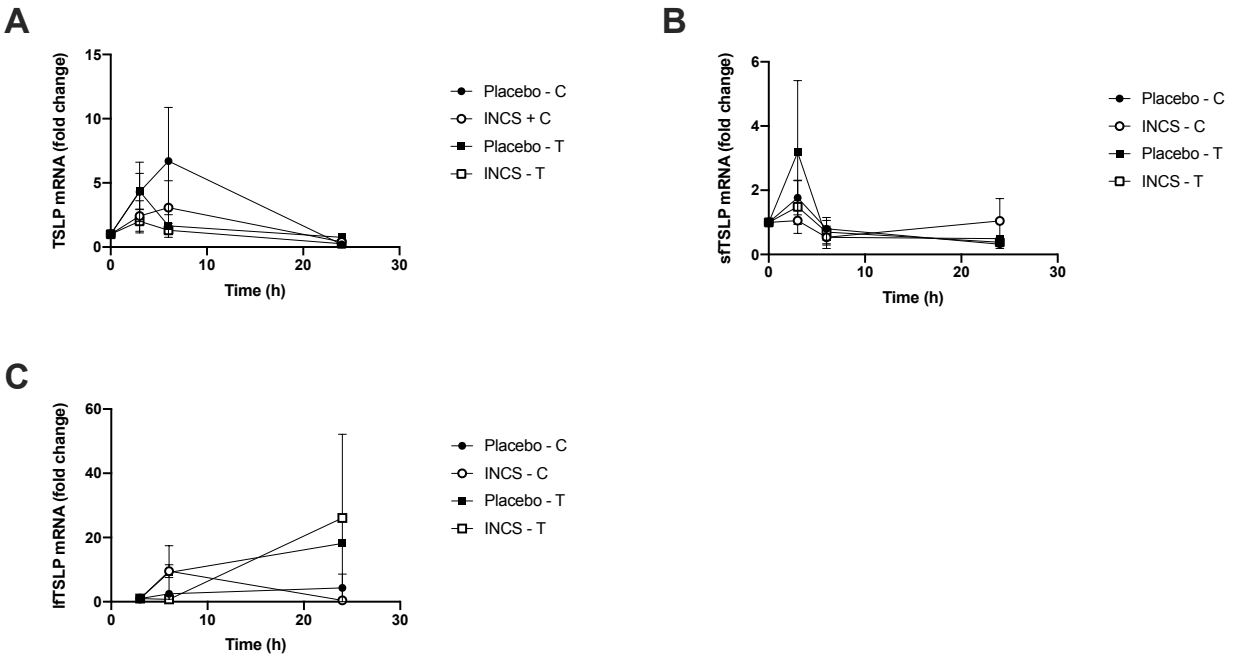

Figure 14: Polyinosinic:polycytidylic acid (polyI:C) induced thymic stromal lymphopoietin (TSLP) gene expression response to intranasal corticosteroids does not differ based on rs1837253 genotype. Gene expression kinetics of (A) total TSLP isoforms (B) short TSLP isoform (C) long TSLP isoform in poly (I:C) (25 μg/mL) stimulated NECs from visits 7 and 10 (following two week treatment with placebo and INCS treatment respectively and nasal allergen provocation) and are stratified based on the absence (C; n=5) or presence (T; n=5) of the rs1837253 minor allele. Results shown are expressed as fold change in the mRNA level in poly (I:C)-stimulated NECs at 3h, 6h and 24h relative to the level in unstimulated NECs (0 h) for total and short isoform TSLP and relative to 3h for long isoform TSLP. Two-way ANOVA with Bonferroni post-test was used to assess differences between groups.

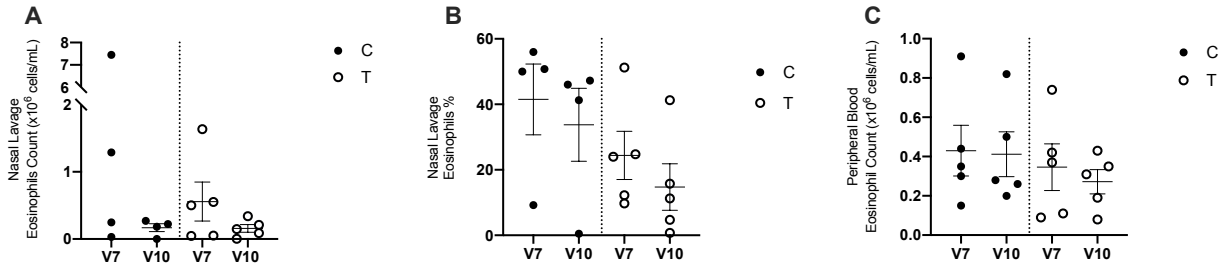

Figure 15: Nasal lavage and peripheral blood eosinophils did not differ based on the rs1837253 genotype and the response to intranasal corticosteroids was not genotype dependent. Nasal lavage samples were obtained 24 hour post nasal allergen treatment following placebo and INCS treatment (visit 7 and visit 10, respectively) and analyzed for (A) eosinophil count and (B) percentage of eosinophils. Peripheral blood was obtained and analyzed for (C) eosinophil counts. Subjects were stratified based on the absence (C; n=4) or presence (T; n=5) of the rs1837253 minor allele. Mixed measure analyses were used to measure statistical differences.

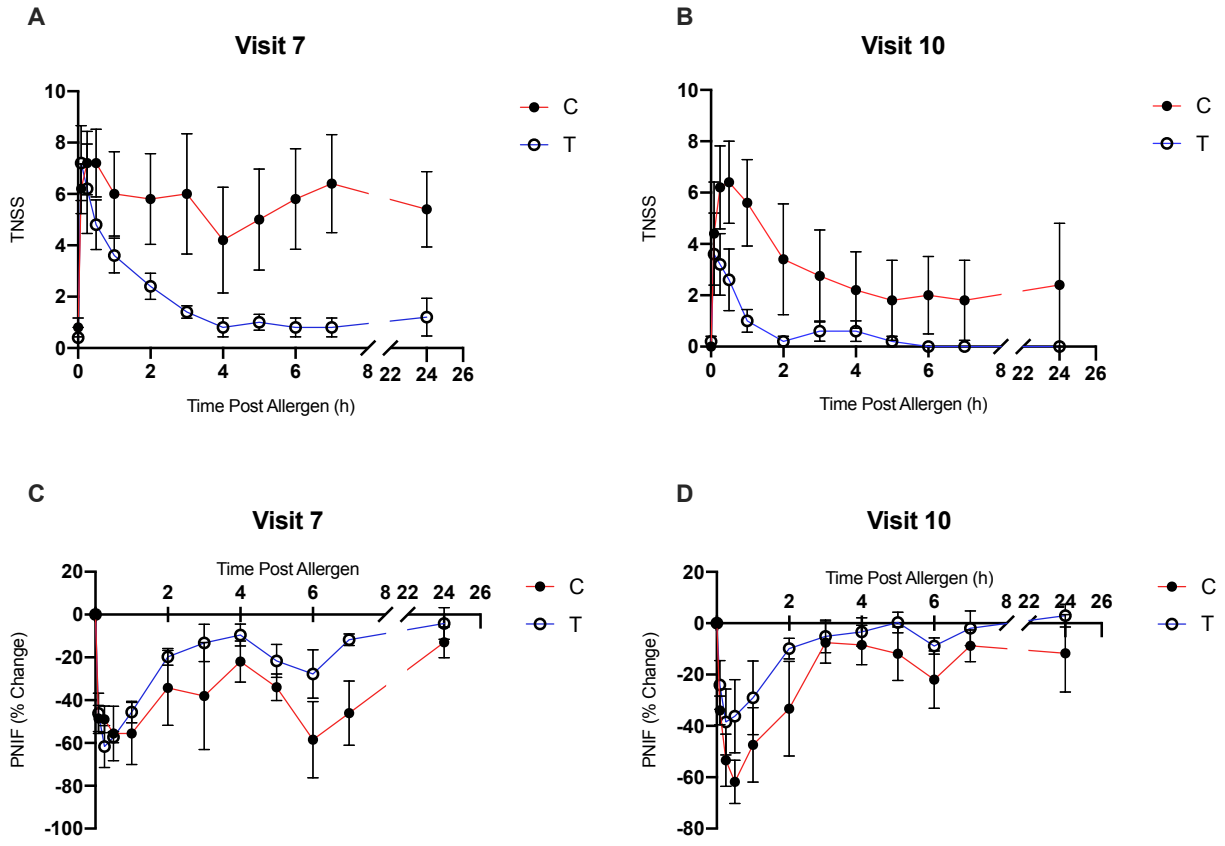

Figure 16: Total Nasal Symptoms Scores (TNSS) and Peak Nasal Inspiratory Flow (PNIF) did not differ based on rs1837253 genotype. TNSS was assessed at baseline and repeated for up to 24 hours (A) visits 7 and (B) visit 10. Subjects were asked to evaluate TNSS by grading each symptom (sneezing, congestion, itching, and rhinorrhea) and the score is added to reach the TNSS score. The grading levels are as follows: 0=None, no symptoms evident; 1=Mild, symptom present but easily tolerated, 2=Moderate, definite awareness of symptom, bothersome but tolerable; 3=Severe, symptom hard to tolerate, would interfere with daily activity. PNIF was measured as % change at baseline and repeated for up to 24 hours (C) visits 7 and (D) visit 10. Subjects were stratified based on

the absence (C; n=5) or presence (T; n=5) of the rs1837253 minor allele. Mixed measure analyses were used to measure statistical differences.

4. DISCUSSION

The role of TSLP, a crucial mediator of Th2 inflammatory responses, is well established in asthma and allergic disease and recent evidence has highlighted the influence of genetic polymorphisms in TSLP in clinical and biological phenotypes of disease. Despite the identification of numerous SNP associations with asthma, there is still limited information on the functional consequences of these polymorphisms. Hui *et al*. identified an association between the rs1837253 genotype and *ex vivo* TSLP in nasal epithelial cells (Hui et al., 2015). Additionally, there are two TSLP isoforms, sfTSLP and lfTSLP, which have exhibited distinct functions and differential expression in homeostasis and disease and it was found that in individuals with the rs1837253 minor T allele there was decreased gene expression of dsRNA-induced lfTSLP (Moorehead, et al., 2020). In this study, our aim was to investigate the nasal epithelium of asthmatics in response to *ex vivo* stimulation and evaluate the role the rs1837253 genotype in Th2 inflammatory processes, as well as in responding to nasal allergen provocation and INCS treatment.

Poly (I:C)-induced and spontaneous release of cytokines and chemokines from nasal epithelial cells

The epithelial alarmins IL-25, IL-33, and TSLP are master regulators of allergic inflammation and elicit immune cell secretion of Th2 cytokines, including IL-4, IL-5, and IL-13, which can act on mucosal barriers and key inflammatory cells, such as mast cells, eosinophils and basophils. IL-5 is involved in the recruitment, development and activation of eosinophils, promoting eosinophilia, while IL-4 induces and maintains Th2 cells. IL-4 and IL-13 regulate Th2 inflammation and are involved in activation B cell isotype

switching of to produce IgE. Additionally, IL-13 acts directly on airway epithelial cells to induce AHR and mucous production (Kuperman, et al., 2002). Another factor that is important in airway inflammation is TNF-α, which is a chemoattractant for eosinophils and neutrophils, increases cytotoxic effects of eosinophils on endothelial cells (Slungaard, et al., 1990) and is involved in the activation of T cells (Croft, 2009). It is also involved in the induction of AHR and airway eosinophilia and inflammation (Choi, et al., 2005). Anti-TNF-α therapies have had some success (Howarth, et al., 2005), however it has been dampened by safety concerns (Bongartz, et al., 2006). Like TNF-α, IL-6 is a biomarker of asthma and is increased in the BALF of asthmatics. Additionally, asthma is characterized by leukocyte infiltration, and SDF1 attracts a number of cells, including HPCs, basophils, eosinophils, T cells and monocytes.

To assess the potential role of epithelial alarmins, hematopoietic cytokines and chemokines, we analyzed TSLP, IL-25, IL-33, IL-5, IL-6, IL-8, IL-13, GM-CSF, TNF-α, SDF-1α, and eotaxin-3, from NEC epithelial cells. When the poly (I:C) stimulated cells were analyzed there were significant increases in protein secretion for all of the cytokines of interest. This aligns with current research in which poly (I:C), a dsRNA analog for TLR-3, induces inflammatory responses with increased cytokine production, elevated mucus production and decreased epithelial barrier function (Lever, et al., 2015; Ritter, et al., 2005).

In this study, we found no significant differences based on rs1837253 genotype or prior nasal allergen challenge or INCS treatment. Although other studies have demonstrated differences in TSLP protein secretion according to rs1837253 genotypes with the

homozygous minor TT allele producing the lowest levels of TSLP protein (Hui, et al., 2015), the current study may be limited because of small sample size. This limited the stratification of samples by genotypes, which is necessary in order to fully investigate the influence of the rs1837253 polymorphism but does not allow insight to be specifically made about homozygous minor genotype, TT, considered to be the most protected form of this SNP. The lack of distinctions among the cytokine profiles when analyzing genotypes and effect of INCS could reflect the potency of poly (I:C) as a stimulus of inflammatory responses, which *in vitro* may have masked potential differences. For this reason, we decided to analyze spontaneous cytokine and chemokine release from vehicle control-treated NECs. When looking at all subjects together, there were no significant differences between the cytokine and chemokine profiles of cells collected at baseline, cells with prior sensitization through nasal allergen challenge, or cells of subjects with INCS treatment followed by nasal allergen challenge. When the results were stratified by genotype, subjects with the minor T allele had decreased levels of IL-13 *ex vivo* from cells that were collected at baseline (visit 2). This suggests that individuals with this polymorphism may have a lower propensity for Th2 pathways.

IL-13 and TSLP are important mediators of allergic inflammation, with IL-13 inducing TSLP expression in the nasal epithelium (Miyata, et al., 2009). TSLP may act both upstream and downstream of IL-13. Upstream, TSLP plays a role in the polarization of Th2 cells and the activation of mast cells to produce IL-13 (Allakhverdi, et al., 2007; Liu, 2006; Ziegler & Liu, 2006); downstream, TSLP is a target of the IL-13 STAT6 pathway. IL-13-induced TSLP expression regulates Th2 cell function, maintaining memory T cells, and

producing chemokines that drive inflammation (He, et al., 2008; Wang, et al., 2006b). This may form a positive feedback loop for inflammation. The effects of these two cytokines are thus interconnected and may explain how a TSLP polymorphism influences NEC expression of IL-13.

Poly (I:C) stimulated NEC-derived factors induce Eo/B colony formation

Eosinophils are hallmark of asthma and are important players of allergic inflammation. There is evidence that *in situ* eosinophilopoietic processes can promote asthma severity and maintain tissue eosinophilia in the airways of asthmatics (Mukherjee, et al., 2014; Sehmi, et al., 2016). The findings of this study indicate that NECs can release growth factors and promote eosinophil differentiation. We tested the supernatants from asthmatic NECs which, following stimulation with poly (I:C), induced Eo/B colony formation of CD34+ HPCs. Co-culture of NEC supernatants with HPCs demonstrated a significant increase in Eo/B CFU in comparison to negative and media controls, both the presence and absence of IL-3. However, cultures with NEC supernatant in combination with exogenous IL-3 did not differ from cultures with NEC supernatant alone. This was similarly seen with exogenous IL-5, but not quite statistically significant. This finding demonstrates the role of nasal epithelium in eosinophil/basophil differentiation processes which are characteristic of allergic inflammation in asthma.

Our findings are in keeping with studies of BEC supernatants of severe asthmatics, which show increased clonogenic potential, with increased Eo/B CFU when co-cultured with bone marrow derived non-adherent mononuclear cells (Salter, et al., 2018). It has been established that TSLP/TSLPR axis plays a key role in the development of *in vitro*

eosinophilopoiesis stimulated by BEC-derived factors. Additionally, TSLP in combination with IL-3 has previously shown to induce eosinophil differentiation of CD34+ HPCs *in vitro* (Hui et al., 2014). While we did not identify specific factors in the NEC supernatant which mediated the observed differentiation, existing literature highlighting the influence of TSLP in eosinophil differentiation provides a basis for the hypothesis that TSLP may also be involved in the stimulation of eosinophilopoiesis/basophilopoiesis by NEC-derived growth factors. Concentrations of TSLP measured from the NECs in this study are within the ranges used by Salter et al. (Salter, et al., 2018), which have been shown to promote HPCs production of IL-5 and IL-13, and migration of progenitor cells towards SDF1α (Smith, et al., 2015).

Further investigation into the role of individual NEC-derived factors would provide important information on the processes and mechanisms involved in local Eo/B differentiation in the airways. It is also important to note while there are mediators, such as TSLP, that have been shown to play a major role in facilitating Eo/B differentiation, there are complex effects of many growth factors in the airways. Some factors, including IL-5, IL-3, GM-CSF and IL-9 have positive impacts and promote Eo/B differentiation, while others, such as IL-12, have a negative effect. It is likely that a complex milieu of mediators influences our observed NEC supernatant stimulated Eo/B colony formation.

Poly (I:C) induced TSLP gene expression from nasal epithelial cells

In line with previous studies on BECs, small airway epithelial cells, and NECs from healthy subjects and asthmatics, the results from this study indicate that *in vitro* exposure to dsRNA induces NEC gene expression of total TSLP, sfTSLP and lfTSLP. In current

literature, the TSLP isoforms have demonstrated dichotomous functions, with sfTSLP constitutively expressed at homeostasis, while lfTSLP is increased during inflammatory processes. lfTSLP is capable of activating DCs through binding to the receptor complex which induces STAT5 phosphorylation, however, sfTSLP does not induce STAT5 phosphorylation, and demonstrates an anti-inflammatory effect through the inhibition of cytokine production from DCs (Bjerkan, et al., 2015, 2016; Fornasa, et al., 2015). Research into the opposition of these isoforms is still relatively new and there is still much to be learned.

Previous data on human BECs and NECs indicate that the long form of TSLP is highly inducible by poly (I:C) (Harada, et al., 2011; Moorehead, et al., 2020). Similarly, human metapneumovirus (hMPV), another leading cause of respiratory tract infection in childhood, has been investigated for its role in TSLP induction in human airway epithelial cell and lung fibroblasts. hMPV strongly induced the expression of lfTSLP, which was abrogated by the silencing of TANK-binding kinase 1 (TBK1), attributed to compromised NF-kB activation (Li, et al., 2018). Unlike lfTSLP, in response to viral stimulation, sfTSLP was constitutive in this study. There is growing evidence implicating the critical role of viral infections of the airways in eliciting pro-inflammatory responses through lfTSLP, which our study further supports.

A central aim of this study was to investigate the role of the rs1837253 SNP on the asthmatic phenotype. Due to the size of this study and the lower prevalence of the minor homozygous genotype, the heterozygote subjects (CT) and homozygote minor (TT) were grouped together, as they both have the presence of at least one minor T allele. Previous

research indicates that there may be differential regulation of the two TSLP isoforms and that the minor rs1837253 T allele has protective effects by reducing expression of the pro-inflammatory lfTSLP. Although we were not able to replicate the results of decreased lfTSLP gene expression in individuals with the minor T allele, this may be due to the low ambient gene expression of lfTSLP. Levels of the long isoform were undetectable at baseline, impacting our ability to confidently analyze the effects of this isoform. Additionally, this study focused solely on individuals with asthma while our previous research included both asthmatics and non-asthmatics. Due to our small sample size and power, important underlying effects may have remained undetected. Further investigation of the functional effects of TSLP SNPs on gene expression, and the mechanisms involved, is necessary to gain a more complete understanding of how viral insult can impact the airway inflammatory processes through TSLP.

More recently, DNA methylation of the *TSLP* gene has been investigated and found associated with prenatal smoke exposure and atopic dermatitis (Wang, et al., 2013). Analysis of skin tissue exhibited *TSLP* promoter DNA hypomethylation in the inflamed skin lesion samples of children with atopic dermatitis versus normal control skin tissue samples, which was associated with increased TSLP protein secretion (Luo, et al., 2014). In the nasal epithelium of subjects with chronic rhinosinusitis with nasal polyposis there are increased methylation ratios when compared to controls, with the methylation ratio at two CpG sites, CpG3 and CpG22:23:24, positively correlating with olfactory score and unilateral nasal resistance (Li, et al., 2019). Asthma and chronic rhinosinusitis/with nasal polyps share some similarities in immunological dysfunction and the epigenetic alterations

seen in the nasal epithelium of chronic rhinosinusitis with nasal polyps suggests that investigation into the DNA methylation of the TSLP gene and promoter could provide valuable insight into the mechanisms of action in asthma. Data on the gene structure of TSLP indicate that there are two putative promoters for the two TSLP transcript variants with CpG sites that may be modified. Methylation may act as a regulatory mechanism for these transcript variants and may have implications on the functions of the isoforms.

The effect of the rs1837253 genotype on blood and nasal lavage eosinophils, PNIF, and TNSS

Inhaled corticosteroids have been a mainstay treatment for asthma, demonstrating anti-inflammatory properties. They are very effective clinically, and reduce the inflammatory pathways involved in allergic inflammation. INCS are also potent and effective therapies and are used in the treatment of allergic rhinitis, nasal polyps, chronic rhinosinusitis and upper airway inflammation. Given the relationship of allergic rhinitis and asthma, high co-morbidity of these two diseases, and evidence in support of the "united airways disease" concept (Bousquet, et al., 2008; Ciprandi, et al., 2012), investigation into the use of INCS for the treatment of asthma is necessary. This study focused on the role of the genetic polymorphism rs1837253 in influencing *in vivo* and *ex vivo* responsiveness to INCS treatment. We utilized a number of measurements to assess response to INCS treatment. We did not find any associations between rs1837253 genotype and response to INCS treatment for any of our phenotypes assessed.

In human BECs, Harada et al. investigated two TSLP polymorphisms, rs3806933 and rs2289276, which are associated with asthma disease susceptibility, and looked at the

responses of subjects with major and minor TSLP alleles to two asthma treatments, a corticosteroid and a long-acting β2 adrenergic receptor agonist. While there was response to the treatments, as seen by reduced TSLP mRNA and protein expression, the response was not associated with SNP genotypes (Harada, et al., 2011). We did find higher TNSS responses in the homozygous major genotype group, which did not decrease as quickly over time, however this was not statistically significant. Additionally, there was a trend for lower eosinophil nasal lavage percentages from subjects with the minor T allele, with the lowest levels following INCS treatment. One thing to note, when looking at the percentage of eosinophils in the nasal lavage of subjects this is a relative value based on cell proportions and is affected by other cell populations. For this reason, it may not fully encapsulate what the absolute cell numbers indicate. A larger sample size would enable more clarification on this trend. While not statistically significant, the trends seen are more localized to the nasal epithelium and not evident in the peripheral blood which may suggest that the rs1837253 exerts its effects locally, rather than systemically. Further investigation is required to elucidate this theory, and the mechanisms and pathways through which TSLP genetic polymorphisms exert their effects.

Conclusions

The role of TSLP in asthma and allergic disease is well established. Evidence suggests that TSLP polymorphisms may influence the development and manifestation of these conditions. These polymorphisms impact allergic inflammation through clinical and biological phenotypes, though research on the functional consequences of these SNPs remains limited. The TSLP SNP rs1837253 genotype has been associated with *ex vivo* TSLP production and lfTSLP mRNA expression, and we sought to further investigate this polymorphism and its responsiveness to nasal allergen provocation and intranasal corticosteroid treatment. No associations were found between rs1837253 genotype and the expression of TSLP and gene isoforms. We did not find any associations of TSLP gene expression between genotypes, or in relation to response to nasal allergen challenge or corticosteroid treatment. Exploration of local and systemic effects of the rs1837253 SNP did not show any differences in response to INCS treatment *in vitro* or *ex vivo*. Overall, the results of Eo/B colony forming assays indicate a role of the nasal epithelium in driving eosinophil/basophil differentiation and highlight the need for further investigation into the mechanisms and factors that are promoting these processes. This study highlights the complexity of gene-environment interactions and the pathways of asthma and allergic inflammation.

Limitations

This study described the effects of dsRNA on NEC TSLP gene expression, and cytokine and chemokine production, on these outcomes. There are some limitations of this study that need to be acknowledged. The study design was very beneficial as it allowed for

each subject to serve as their own control and eliminated potential variability in that regard. For the purposes of studying the rs1837253 genotype the small sample size was a factor, as well as the low frequency of the homozygous minor genotype, which is considered to be the most protective form of the rs1837253 polymorphism. While this limits the conclusions that could be made about the role of the polymorphism, the heterozygous genotype could be combined to offer some insight into the role of the minor T allele in the inflammatory processes of asthma. There were not enough samples to reach adequate power for some of the questions being explored. Although there were some nonsignificant trends that were observed, a larger sample size would provide a more robust investigation into the functional effect of the rs1837253 genotype, and a deeper understanding into the gene-environment interactions involved in asthma.

When analyzing the gene expression of lfTSLP a majority of the NEC samples did not have measurable transcripts for this isoform at baseline unstimulated conditions. This aligns with previous research where there are very low levels of lfTSLP during homeostasis and is highly increased following exposure to viruses or during inflammation (Fornasa, et al., 2015). For this reason, the change in lfTSLP expression could not be measured from baseline but rather was analyzed by the change of TSLP from 3-hour post poly (I:C) stimulation. This provides some information on the gene expression of lfTSLP, however, it may not fully capture the change from homeostatic conditions, as it had previously been seen that the greatest increase of lfTSLP mRNA expression occurs in the first three hours following exposure to poly (I:C) (Moorehead, et al., 2020).

Additionally, this study utilized nasal epithelial cells to investigate the inflammatory processes in asthma and the role of nasal allergen provocation and INCS treatment on the disease phenotypes. Nasal epithelium has been established as an appropriate surrogate for bronchial epithelial cells and have the most similar gene expression profiles (Sridhar, et al., 2008). In this study, the NECs obtained through nasal epithelial curettage were cultured to expand cell populations and establish adequate cell number to perform gene expression and protein analyses. The culture and expansion of the cells took between two and three and a half weeks to complete. This may have impacted our ability to fully explore the influence of the nasal allergen challenge and treatment with INCS. It is possible that the effects of the nasal allergen challenge and INCS treatment were not seen in the cells due to the long time period between the nasal epithelial curettage and analysis, which was required for the culture procedures. A more immediate look into the cells following the nasal epithelial swabs could provide more in depth information, although the number of cells may limit the experiments that can be performed.

Future Directions

This study demonstrated that nasal epithelial-derived factors from asthmatic individuals have clonogenic potential and are capable of inducing Eo/B differentiation. This is an important finding as it highlights the role of the epithelium in Eo/B differentiation processes that are characteristic of asthma; however, the specific factors and mediators that are contributing are still unknown. Future work into the mechanisms behind these results would provide valuable information into eosinophil differentiation in asthma and allergic inflammation. In BECs, TSLP has been found to be a key driver in *in situ*

eosinophilopoiesis (Salter, et al., 2018), which indicates a prime candidate for future investigation into the major contributors in the nasal epithelium. The use of neutralizing antibodies in the methylcellulose cultures to test for specific cytokines and factors that are involved would further expand on this study. While TSLP is a target of interest, the use of other antibodies for other potential influences, such as IL-3, IL-5, GM-CSF, and IL-33, is essential.

While there are many genetic polymorphisms that are associated with disease susceptibility, gene polymorphisms cannot fully account for an individual's predisposition for allergic disease and epigenetic factors are considered to have a role in these processes. Research into rs1837253-associated epigenetic alterations could help to explain the differences in lfTSLP gene expression that have been previously identified and found to be RIG-1 and AP-1 independent. Continued research into TSLP and pathways of asthma and allergic inflammation, will create the basis for the development of novel therapeutics to combat allergic diseases, easing the symptoms and improving the quality of life of those who experience them.

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