THYMIC STROMAL LYMPHOPOIETIN POLYMORPHISM RS1837253 IN ASTHMA

THYMIC STROMAL LYMPHOPOIETIN: THE FUNCTIONAL IMPLICATIONS OF THE TSLP GENE POLYMORPHISM RS1837253 IN ALLERGIC ASTHMA

By

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ABSTRACT

Thymic stromal lymphopoietin (TSLP) is a regulator of T_h2 immune responses and is highly implicated in the pathophysiology of asthma and allergic diseases. Although robust data is present referencing the associations of polymorphisms in the TSLP gene with the development of allergy and asthma, there is very limited information on how TSLP gene variants functionally affect downstream effector pathways with regards to disease phenotypes in the clinical setting. The overall objective of this thesis is to investigate how TSLP polymorphisms are linked to the clinical phenotypic differences including alterations in TSLP secretion and subsequent downstream cellular events. We show that polyinosinic:polycytidylic acid (polyI:C) enhanced TSLP secretion from nasal epithelial cells (NEC). To explore the potential regulatory mechanisms acting on a key variant of the TSLP gene, we investigated associations between the rs1837253 TSLP variant and transcriptional regulatory factors RIG-1 and AP-1, finding no association between levels of expression of these regulators and genotype. We also investigated ex vivo production of TSLP and downstream cytokines in nasal epithelial cells (NEC) in asthmatic and non-asthmatic individuals to outline variances in expression according to disease status and genotype. We showed that NEC derived from asthmatic individuals showed significant differences in expression in asthmatic compared to non-asthmatic individuals. Furthermore, we demonstrated that there was a difference in the expression of TSLP in the asthmatic subpopulation compared to non-asthmatics when categorized by genotype, suggesting an altered regulatory mechanism of TSLP secretion for asthmatic compared to non-asthmatic individuals.

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LIST OF ABBREVIATION AND SYMBOLS

AD	Atopic dermatitis
AHR	Airway hyperresponsiveness
AR	Allergic rhinitis
AP-1	Activator protein-1
ASM	Airway smooth muscle
BALF	Bronchoalveolar lavage fluid
BEC	Bronchial epithelial cells
BEGM	Bronchial epithelial cell growth medium
CCL	Chemokine (C-C motif) ligand
CXCL	Chemokine (C-X-C motif) ligand
CFU	Colony forming units
CGRP	Calcitonin gene-related peptide
DCs	Dendritic cells
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme linked immunosorbent assay
Eo/B	Eosinophil-basophil
EOE	Eosinophilic esophagitis
EPO	Eosinophil peroxidase
ERK	Extracellular signal regulated kinase
FcεRI	Fc receptors
FEV_1	Forced expiratory volume in 1 second
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GATA	GATA-binding factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSDMB	Gasdermin B
GWAS	Genome wide association studies
HASMC	Human airway smooth muscle cells
HHP	Human hemopoietic progenitor IFNγ Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2 innate lymphoid cells
IL-7Rα	Interleukin-7 receptor-α
JAK	Janus kinase
JNK	c-Jun amino-terminal kinase
LAR	Late asthmatic response
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
mCD14	Macrophage CD14
mDCs	Myeloid dendritic cells
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MKK	MAPK kinase

MPP	Multi-potent progenitor
mRNA	Messenger ribonucleic acid
NEC	Nasal epithelial cells
NKT	Natural killer T
ORMDL3	Orosomucoid 1-like 3
OX40L	OX40 ligand
PB	Peripheral blood
PI3K	Phosphatidylinositol 3-kinase
PI(3,4,5)P3	Phosphatidylinositol 3,4,5-triphosphate
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
Poly I:C	Polyinosinic:polycytidylic acid
RIG-1	Retinoic acid-inducible gene 1
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RSV	Respiratory syncytial virus
RXR	Retinoic X receptors
SAEC	Small airway epithelial cells
sCD14	Soluble CD14
SEM	Standard error of the mean
SNPs	Single nucleotide polymorphisms
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokine
Th	T helper
TLR	Toll-like receptor

TNFα	Tumor necrosis factor-alpha
TSLP	Thymic stromal lymphopoietin
VDR	Vitamin D receptor
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta
ZPBP2	Zona pellucida binding protein 2

DECLARATION OF ACADEMIC ACHEIVMENT

Based on the work of Dr. Claudia Hui and design contributions from Dr. Helen Neighbour, Dr. Paul Keith and Dr. Gail Gauvreau, Dr. Judah Denburg and myself were responsible for the experimental study and recruitment design and the interpretation of the data. Dr. H. Neighbour, Dr. D. Tworek, Dr. D. Sommer and Dr. D. Yue medically assessed all the subjects, and performed the nasal scrape procedure for the collection of nasal epithelial samples. I was responsible for subject recruitment with assistance from T. Scime and R. Watson, who also performed methacholine challenge testing to screen subjects for asthma. Blood was drawn by D. Abraham, D. Heroux, R. Watson and T. Scime. I performed all the experiments described within the following document with assistance from a lab technician (D. Heroux) and two undergraduate students (A. Moorehead and H. Yu) whom I supervised. Drs. Jyoti Balhara and Elli Rosenberg provided valuable technical assistance with flow cytometry. Dr. Loubna Akhabir provided valuable advice on genotyping and gene expression experimental design.

CHAPTER 1: INTRODUCTION

Prevalence of immune-mediated chronic inflammatory diseases including allergy, asthma, allergic rhinitis (AR) and atopic dermatitis (AD); as well as, autoimmune diseases, such as arthritis and inflammatory bowel disease have increased globally in recent decades severely impacting the lives of those living with these conditions. The underlying causes for the increasing prevalence of allergic diseases are not well understood. Complex gene-environment interactions have been postulated to be the reason for this recent rise (Alfvén et al., 2006). The presence of atopic disorders in an individual's family history is an independent risk factor for the development of atopic disease in their children. However, the rapid increase in the incidence rates of allergic disease downplay the risk of genetic predisposition and supports the substantial role played by environmental and lifestyle changes in light of the emergence of hyper-hygienic modern industrialized populations in the development of allergic disease (Sandford et al., 1996; Steinke et al., 2008). For allergic diseases, the latter has been articulated as the hygiene hypothesis, according to which increased hygiene and cleanliness may have divested the immune system of bacterially and virally-evoked T helper (T_h) type 1 (T_h1) immune responses during early childhood, thus shifting the lifelong trajectory of the immune response towards a T_h2 bias (Eisenbarth et al., 2004). However, studies have reported that T_h1-mediated autoimmune and T_h2-mediated atopic diseases are not mutually exclusive, but associated; which may help explain, in part, the paradoxical rise in T_h1-driven autoimmune diseases (Sheikh et al., 2003; Simpson et al., 2002). Current evidence suggests that the T_h2 paradigm may be oversimplified and that gene-environment interactions may be critical in determining allergic sensitization and its clinical outcome (Duffy et al., 1990).

Recent genome wide association studies (GWAS) and meta-analyses of GWAS have identified common and distinct genes and pathways that contribute to allergic diseases. The first

GWAS on asthma by the GABRIEL consortium discovered a novel locus on chromosome 17q2, which encompasses the genes orosomucoid 1-like 3 (ORMDL3), gasdermin B (GSDMB) and zona pellucida binding protein 2 (ZPBP2), to be associated with susceptibility for early-onset childhood asthma (Moffatt et al., 2007). Two other GWAS, the phase II GABRIEL study and the EVE study, later identified associations between asthma and single nucleotide polymorphisms (SNPs) in four loci (thymic stromal lymphopoietin (TSLP), IL-33, IL1RL1, 17q21 locus) (Moffatt et al., 2010; Torgerson et al., 2011). It has been demonstrated that TSLP is upregulated in bronchial biopsies from asthmatic individuals; and its secretion, with subsequent allergic inflammatory responses, can be further induced by various stimuli such as viruses and allergens (Uller et al., 2010; Ying et al., 2005). Elevated levels of TSLP can subsequently further skew T_h1/T_h2 balance by promoting T_h2-type responses through activation of dendritic cells (DCs) as well as hemopoietic progenitor cells (Allakhverdi et al., 2009; Soumelis et al., 2002). The SNP rs1837253 in the upstream region of TSLP gene - the protein product of which is highlighted in this thesis as a key molecule which orchestrates T_h2 immune responses – was highly significantly, inversely associated with allergic asthma and related traits (He et al., 2009). Recently it has been shown that rs1837253 polymorphism may be directly involved in the regulation of TSLP secretion. This may help explain the protective association of this genetic variant with asthma and related traits. Given the critical immuno-modulatory role of TSLP in allergic inflammation, identifying functional consequences of SNPs in genes along with clinical phenotypic associations is critical in understanding and targeting allergic inflammation. The rest of this chapter will cover allergic inflammation and the pathophysiology of asthma and allergic diseases and finally, the role of TSLP in allergic diseases.

Asthma and Allergic Disease

Prevalence

Asthma is a chronic inflammatory disease which affects more than 300 million people worldwide (Urbano, 2008) and leads to an estimated 250 000 annual deaths per year (Bousquet et al., 2007). Prevalence rates for asthma increase by 50% every decade (Masoli et al., 2004). According to Statistics Canada, in 2014, 8.1% of Canadians aged 12 and older, and roughly 2.4 million people in the country, reported that they had been diagnosed with asthma by a health professional (Statistics Canada, 2014). Asthma is also the most common childhood disease affecting at least 13% of Canadian children and is a major cause of hospitalizations (Garner and Kohen, 2008), (Millar and Hill, 1998), approximately 146 000 emergency room visits annually are due to asthma exacerbations (Canadian Lung Association, 1994). Quality of life can be affected not only by asthma attacks, but also by absences from work and limitations in other activities (Chen et al., 2005). The global economic costs associated with asthma exceed those of tuberculosis and HIV/AIDS combined (Braman, 2006). The increased prevalence and economic impact of asthma emphasises the necessity for a better understanding of the mechanisms of its pathology as a means of providing potential markers and therapeutic targets to more effectively treat this healthcare burden.

Clinical Manifestations and the Diagnosis of Asthma

The clinical symptoms of asthma include recurrent episodes of wheezing, breathlessness, chest tightness and coughing. Physiologically, it is characterized by reversible airflow obstruction which is associated with inflammation of the airways and airway hyper-responsiveness (AHR) (Murphy and O'Byrne, 2010). AHR is a characteristic feature of asthma consisting of an increased sensitivity of the airways to constrictor agonists, as indicated by a smaller concentration of a

constrictor agonist needed to initiate the bronchoconstriction response, a steeper slope of the dose-response, and a greater maximal response to the agonist (O'Byrne and Inman, 2003). AHR is related to disease severity and is often used as a defining characteristic in asthma diagnosis (Townley and Horiba, 2003).

To measure AHR, a bronchoprovocation test is administered where measurements with a spirometer are made after the patient inhales incremental doses of methacholine (MCh). Spirometry is a physiological test that measures how an individual inhales or exhales volumes of air as a function of time. The primary signal measured in spirometry may be volume or flow. Spirometry is invaluable as a screening test of general respiratory health (Miller et al., 2005). The most important indices for assessing airway obstruction are forced expiratory volume in one second (FEV_1), the volume of air exhaled in the first second of a forced expiration from full inspiration, and forced vital capacity (FVC) which is the maximum volume of air that can be exhaled following full inspiration (Miller et al., 2005). A ratio of FEV_1/FVC less than 0.75 suggests airflow limitation (GINA, 2017).

The provocative concentration PC_{20} is the concentration of constrictor agonist required to cause a 20% fall in FEV_1 compared to baseline values (Cockcroft et al., 1983). Non-asthmatic subjects generally have a provocative concentration of a substance (histamine or methacholine) PC_{20} higher than 16mg/mL in the absence of corticosteroid therapy which indicates normal lung function. Asthmatic subjects generally have a provocative concentration of less than 8 mg/mL. There is, however, some overlap, and defining an exact level of airway responsiveness, which would distinguish asthmatic subjects from non-asthmatic subjects, is not possible. This is because there appears to be a continuous distribution of nonspecific airway responsiveness in the general population, with asthmatic subjects in one tail of this distribution (O'Byrne and Inman, 2003).

Thus, to diagnose asthma, FEV1/FVC is measured at a baseline and 15-20 minutes after administering a fast acting bronchodilator (such as 200μg albuterol). An improvement by more than 12% and 200mL indicates reversibility of the airflow obstruction and suggests the individual may have asthma and not irreversible obstructive diseases such as COPD (chronic obstructive pulmonary disease) (Bateman et al., 2008).

Genetics of Asthma and Allergic Disease

Heritability is the proportion of variation in a measureable trait or risk of the presence or absence of a disease that can be attributed to genetic variation (Mathias, 2014). In the early twentieth century familial aggregation, or clustering, of asthma was recognized and provided the first evidence implicating the genetic basis of its development (Sakula, 1988; Wiener et al., 1936). Further evidence of the genetic contribution to asthma can be demonstrated by twin studies where the concordance for asthma and other allergic twin was higher in monozygotic twins than dizygotic twins. However, there is an absence of complete concordance in monozygotic twins for these phenotypes, clearly indicating that environment plays a significant role in asthma development. Overall a tremendous range in the heritability of asthma is seen in family and twin studies, ranging from 36 to 95 % with higher estimates generally observed in studies implementing more objective diagnostic criteria (van Beijsterveldt and Boomsma, 2007; Duffy et al., 1990; Edfors-Lubs, 1971; Fagnani et al., 2008; Hallstrand et al., 2005; Koeppen-Schomerus et al., 2001; Nystad et al., 2005; Skadhauge et al., 1999; Thomsen et al., 2010; Willemsen et al., 2008). The degree of heritability is highly reliant on the relative contribution of genetic and environmental variability, reflective of the complex interplay of genes and environment. The multi-factorial nature of asthma along with its complex genetic background greatly complicates the search for genetic loci that cumulatively

contribute to asthma. Despite this, there has been tremendous success in identifying the genetic determinants of this disease.

Genetic studies of asthma have been conducted using family-based designs that detect the co-inheritance (linkage) of genetic variants with the phenotype. The power of this approach is that genes are detected by virtue of their chromosomal location alone and not on the basis of prior knowledge of their function. Therefore, the entire human genome can be screened in an unbiased fashion. Several novel genes such as ADAM33, DPP10, NPSR1(GPRA), PHF11 and HLA-G were identified as putative susceptibility loci using this approach. However, linkage studies are well powered to detect rare high-risk disease-causing alleles but are less successful at detecting risk alleles with modest effect sizes that may be more prevalent in the population (Risch and Merikangas, 1996). In a recent meta-analyses of 20 genome-wide linkage studies there were only two chromosomal regions (2p21–p14 and 6p21) that showed significant evidence for linkage in European families, after adjustment for multiple comparisons indicating very poor reproducibility for the available linkage study data (Bouzigon et al., 2010).

Thus hypothesis-driven association studies of asthma candidate genes using a case-control design are an alternate approach. The main advantage to this approach is the specificity of the given hypothesis in turn lacking the stringent thresholds set in place with significance testing in the more unbiased genome-wide approaches. On the other hand, the approach is limited in that it does not include novel loci that may add to the understanding of asthma biology. Each candidate gene study generally only considers a gene in contrast to the pathway from which the biological candidacy is determined and often there is lack of replication between studies because of the lack of consideration of environmental effects (Mathias, 2014). In addition the candidate gene approach is often limited through small sample sizes, differences in phenotype definition and lack of

adequate matching of study subjects for ethnic background (Cardon and Bell, 2001). A factor that must be taken into consideration in association studies is the phenomenon of linkage disequilibrium (LD), which is the association between alleles at different sites on a chromosome. LD tends to be lower for polymorphisms that are further apart (due to the effect of recombination), although there is not a simple relationship between LD and genetic distance.

In addition, linkage disequilibrium (LD) needs to be considered in association studies. LD is the association between alleles at different sites on a chromosome. LD tends to be lower for polymorphisms that are further apart (due to recombination effects), however there is no simple relationship between LD and genetic distance. The pattern of LD across the genome is not uniform and as a result a polymorphism may be in LD with several nearby variants, any one of which could be the causal locus (Akhabir and Sandford, 2011). Most recently, the association study design has been extended from the examination of a specific candidate gene to an unbiased, genome-wide approach. It has been estimated that to adequately survey the entire genome, a large number of genetic polymorphisms (250 000 to 1 million) is likely to be required. However, the number of polymorphisms will vary between studies as different populations have different levels of LD.

Most recently, the association study design has been extended from the examination of a specific candidate gene to an unbiased, genome-wide approach (Manolio, 2010). Genome-wide association studies (GWAS) utilize genotyping arrays consisting of up to millions of single nucleotide polymorphisms (SNP) as they are the most efficiently assayed type of genetic variant. However, the number of polymorphisms will vary between studies as different populations have different levels of LD (International HapMap Consortium, 2005; Kruglyak, 1999). In the most commonly used approach to GWAS the genotype frequencies at each SNP are compared between cases and controls. However, due the large number of comparisons the use of extremely stringent

statistical correction to avoid an overwhelmingly large number of false positive results is required. Therefore, very large sample sizes are needed in such studies if genome-wide statistical significance is to be achieved, for example, several thousand cases and controls. On the other hand, the large amount of genetic data generated in these studies allows efficient correction for differences in ethnic background using a subset of ancestry informative markers (polymorphisms that differ markedly in frequency between different populations) (Schork et al., 2009).

The first comprehensive GWAS in asthma was conducted by Moffatt et al. in 2007, and identified a novel locus on 17q21 locus containing a number of genes including ORMDL3 and GSDML (Moffatt et al., 2007). Subsequent GWAS utilizing genotyping arrays identified further loci consistently associated with asthma including IL33 on chromosome 9p24, HLA-DR/DQ on 6p21, IL1RL1/IL18R1 on 2q12, WDR36/TSLP on 5q22, and IL13 on 5q31 (Queiroz et al., 2017; Torgerson et al., 2011). In populations with dissimilar racial backgrounds, GWAS have uncovered evidence for loci that may be specific to particular ethnic groups, such as PHYNN1 observed in African-American asthmatics (Torgerson et al., 2011).

In other genetic studies, the regulation of total IgE levels in serum has been associated with polymorphisms on the fifth chromosome, including the gene for the β chain of FcεRI and the IL-4 family of cytokines, which may contribute allergic disease development (Basehore et al., 2004; Hill and Cookson, 1996). SNP Analyses of toll-like receptor (TLR) gene variants have revealed that alteration in TLR1, TLR6 and TLR9 have protective effects on atopic asthma in children (Kormann et al., 2008). Another gene with associations to allergic diseases is CD14, which encodes for the high-affinity receptor for bacterial lipopolysaccharide (LPS). This receptor exists as a membrane molecule on monocytes and macrophages (mCD14) and as a soluble form (sCD14) in serum (Frey et al., 1992). A polymorphism in the CD14 gene has been associated with elevated

sCD14 and lower total serum IgE levels and may be involved in certain IgE- protective TLR-mediated responses. Elevated levels of sCD14 have the ability to disrupt the T_h1/T_h2 balance by antagonizing the pro-inflammatory response of mCD14 expressing cells to LPS via release of IL-12 from CD14 negative dendritic cells, favouring T_h1 responses, and thus reducing the probability of an IgE response to allergens (Baldini et al., 1999). Polymorphisms in STAT6, a member of the Signal Transducer and Activator of Transcription (STAT) family acts as a transcription factor for activating genes involved in IgE synthesis and in T_h2-associated processes, have been associated with higher serum IgE levels (Weidinger et al., 2004). Other genes such as IL-13 and vitamin D receptor (VDR) have been implicated as conferring risk for asthma and allergic disease in many genetic association studies (Ober and Hoffjan, 2006). Variants in VDR have been linked to asthma, atopy and IgE levels. SNPs in IL-13 have consistently been replicated to show associations with asthma, atopic asthma, atopy, IgE levels and AHR (Maier et al., 2006).

Recently, an SNP (rs1837253) in the TSLP gene has been shown to be highly negatively associated with asthma and AHR, while the IL-33 and IL-1R1 genes have been positively associated with asthma and eosinophil counts respectively; these latter observations suggest that the airway epithelium plays a major role in regulating susceptibility of asthma and related traits (Harada et al., 2009; Yan et al., 2017). To date, numerous loci that are linked to asthma and allergic phenotypes have been identified. In this thesis, there will be an emphasis on the rs1837253 variant SNP in the TSLP gene, its functional role in TSLP production and its clinical significance to the asthmatic phenotype. Of course, as with other complex diseases, single gene associations cannot explain fully the degree of heritability found among asthmatics, for this a multi-genetic approach that also takes into account gene-environment interactions is necessary (Moffatt et al., 2010; Yan

et al., 2017). Furthermore, to better understand the development of asthma and allergic diseases, the causal mechanism(s) behind the identified associations must be elucidated.

Inflammation in Asthma and Allergic Disease

Allergic Immune Response

The production of immunoglobulin (Ig)-E antibodies in response to environmental substances known as allergens is referred to as atopy. In humans, atopic sensitization is confirmed by a positive skin prick test and/or detectable serum levels of specific IgE antibodies to one or more known allergens (Arshad et al., 2001). In contrast, allergy refers to the hypersensitivity reaction and IgE- mediated inflammatory response to allergens, resulting in one or more allergic diseases, such as asthma, AR, AD and food allergy. Although atopy is considered a risk factor for allergic diseases (Kay, 2001), the prevalence of atopic sensitization in asthma have been reported to be ≤50% (Pearce et al., 1999). Therefore, not everyone with atopy develops clinical manifestations of allergies and similarly, not everyone who has clinical manifestations of allergic-type inflammation is atopic (Goksör et al., 2016).

Allergic Asthma

Asthma is an immune disorder of the airways involving multiple inflammatory cells and mediators (GINA, 2017). Clinically, it is typified by symptoms such as wheezing, breathlessness, chest tightness and coughing and bronchial hyper-responsiveness and pathologically by chronic inflammation and structural alterations in the airways, also referred to as remodelling. IgE is a central mediator of allergic reactions, including allergic asthma, and has a central role in asthma-related symptoms, airway inflammation and, possibly, airway remodelling (Holgate et al., 2005; Rabe et al., 2011).

In allergic asthmatic patients, contact with allergens to which they are sensitized results in the rapid release of pro-inflammatory mediators that trigger immediate contraction of airway smooth muscle (ASM) and increased mucus production and the symptoms of the early allergic/asthmatic response (EAR), that is, wheezing, shortness of breath, chest tightness and cough. The EAR results from IgE-mediated mast cell and basophil activation following allergen induced cross-linking. Mast cells and basophils release preformed mediators such as histamine, leukotrienes, and prostaglandin-D2 (PGD2) which cause bronchoconstriction and vascular permeability (Gauvreau and Evans, 2007). In addition, these mast cell derived mediators can prompt the influx of other pro-inflammatory cells, such as T-lymphocytes, eosinophils, basophils and neutrophils into the airways (Afshar et al., 2008; Averbeck et al., 2007; Platts-Mills et al., 2007).Moreover, these pro-inflammatory cells and chemokines attract and activate inflammatory cells, which release more pro-inflammatory mediators. In 50% of people this leads to a slower reaction which develops 4 to 8 hours later, and is known as the late allergic/asthmatic response (LAR). The LAR is also associated with further release of histamine and leukotrienes produced by mast cells and/or basophils (Davis et al., 2009). However, LAR is primarily characterized by inflammatory infiltration and bronchoconstriction and leads to tissue remodeling.

Remodeling of the airways is a central feature of asthma, especially severe asthma; it can lead to fixed airway obstruction and refractoriness to treatment (James, 2005; Pascual and Peters, 2005). Various elements of the airway wall are involved in the structural transformation observed during remodeling. Shedding of the epithelial, thickening of the reticular basement membrane (RBM), goblet cell hyperplasia, smooth muscle hyperplasia and hypertrophy, sub-epithelial fibrosis and angiogenesis are some of the primary findings commonly observed in remodeled airways. While we currently do not a complete understanding of the underlying mechanisms of

airway remodeling, the presence of chronic persistent inflammation, involving longstanding exposure of the airways to a variety of environmental agents, cells and mediators, is generally considered a necessary trademark of remodeling. The disruption of T_h1 and T_h2 balance is thought to drive the development of asthma pathology, progressing to the respiratory pathophysiology previously mentioned.

Helper T lymphocyte immune imbalance is readily associated with IL-4, IL-5 and IL-13 producing T_h2 cells, which can lead to increased tissue numbers of activated mast cells and/or eosinophils (Humbert et al., 1999). Following inhalation, allergens encounter antigen presenting dendritic cells lining the airway sub-epithelium. These dendritic cells bind to the antigen (allergen) and travel to draining lymph nodes, where T-cells undergo antigen presentation. This results in elevated T-helper 2 cells (T_h2 cells) proliferation which trigger the over-expression of T_h2 cytokines interleukin (IL)-4, IL-5, IL-9, and IL-13. T_h2 cytokines and co-stimulatory factors are involved in activating B-cells to produce Immunoglobulin E (IgE) antibody. Allergen-specific IgE can then circulate throughout the blood and bind to IgE receptors (FcεRI or FcεRII) found on inflammatory cells (mast cells, basophils, lymphocytes, eosinophils) stimulating the inflammatory activity of these cells during subsequent allergen exposure (Busse and Lemanske, 2001; Gauvreau and Evans, 2007).

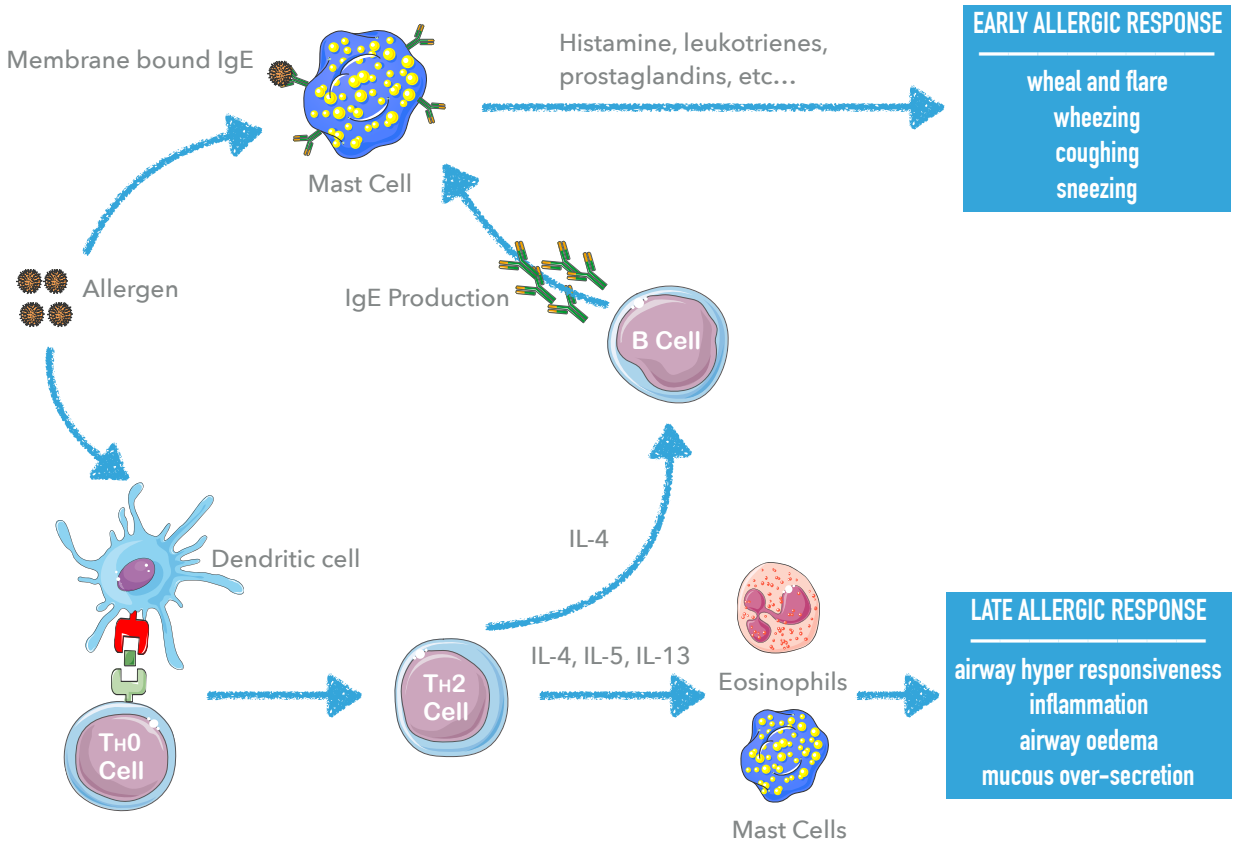


Figure 1. Pathways leading to the early and late allergic response. Antigen-induced release of histamine and lipid mediators from mast cells results in the early allergic response. The late allergic response depends on multiple pathways including infiltration of eosinophils and release of MC products (adapted from (Kay, 2001)).

T_h2 Cytokines in Asthma and Allergic Disease

T_h2 cytokines such as IL-4, IL-5 and IL-13 are primary drivers of the allergic inflammatory response while T_h1-type cytokines such as interferon gamma (IFNγ) and IL-12 antagonize T_h2 immune responses (Novak and Bieber, 2003). CD4+ T_h2 cells predominantly produce IL-4 and IL-13, which stimulate B cells to produce allergen-specific IgE antibodies, whereas IL- 5 acts to promote eosinophilia and IL-9 and IL-13 contribute to AHR in asthma (Kay, 2006; Kouro and

Takatsu, 2009; Robinson, 2010; Shimbara et al., 2000). As such, messenger ribonucleic acid (mRNA) expression for many signature T_h2 cytokines such as IL-4, IL-5, IL-13 and IL-9, is increased in bronchial biopsies from allergic and/or asthmatic individuals, compared to normal controls (Hamid et al., 1991; Humbert et al., 1999; Ying et al., 1997). In addition, asthmatic patients expressing IL-5 mRNA in the airway show a significant increase in the number of activated T cells and activated eosinophils when compared to healthy controls. IL-5 mRNA expression in bronchial biopsies also correlates with symptom severity and AHR in atopic asthma, indicating that it may play an important role in driving disease (Humbert et al., 1999). IL-13 plays a key role in the allergic response by inducing mucus hyper-secretion from goblet cells and ASM contraction, leading to the airway remodeling and subsequent reduction in airflow, characteristic of asthma (Nakajima and Takatsu, 2007; Robinson, 2010). Similar to IL-13, IL-9 has been reported to induce airway inflammation, mucus hyper-secretion, as well as AHR; elevated expression of IL-9 in allergic asthmatics has been shown to correlate with AHR (Hauber et al., 2004; Shimbara et al., 2000). Lastly, IL-9 has been shown to enhance eosinophil survival and IL-5-mediated eosinophil (and mast cell) differentiation and maturation, suggesting that IL-9 may potentiate eosinophil function *in vivo* (Gounni et al., 2000; Sitkauskiene et al., 2005).

Aside from classical T_h2 cytokines, epithelial cell–derived cytokines such as TSLP, IL-25 and IL-33, which are elevated in the airways of asthmatics, are secreted following epithelial stimulation, tissue damage, pathogen pattern recognition or allergen exposure, and have the capacity to initiate T_h2 responses at mucosal sites (Préfontaine et al., 2010; Saenz et al., 2008; Ying et al., 2005). The IL-33 and TSLP genes have emerged as two of the strongest associations for the development of asthma and related traits (He et al., 2009; Moffatt et al., 2010; Yi et al., 2017). The most established mechanism for TSLP-driven T_h2 inflammation is via the TSLP-DC

axis whereby CD4+ T cells are primed towards a T_h2-biased immune response (Soumelis et al., 2002). IL-33, which has been shown to induce TSLP production from epithelial cells, has been reported to induce eosinophilia, secretion of IgE and Th2 cytokines in mice (Saenz et al., 2008; Schmitz et al., 2005). In addition to driving T_h2 cytokine, IL-4, IL-5 and IL-13, production in T cells , IL-25 has also been shown to induce eosinophilia in murine models while delaying apoptosis of human eosinophils (Cheung et al., 2006; Kim et al., 2002; Larché et al., 2003; Pan et al., 2001). Furthermore, polymorphisms in the IL-25 receptor (IL-17RB) have been associated with asthma (Jung et al., 2009). More recently TSLP, IL-33 and IL-25 have been shown to activate a newly identified population of lineage-negative lymphoid cells (multi-potent progenitor type 2 (MPPtype2) cells, nuocytes and type 2 innate lymphoid cells (ILC2) that produce IL-5 and IL-13 and also establish a T_h2-biased immune response, including mucus production and eosinophilia (Mjösberg et al., 2011; Moro et al., 2010; Saenz et al., 2010a; Vissinga et al., 2013). Therefore, in the appropriate circumstance and genetic context, TSLP, IL-33 and IL-25 have emerged as key regulators of the allergic responses. Table 1 provides a summary of T_h2 cytokines involved in allergic responses.

Table 1. T_h2 cytokines in allergic asthma

CYTOKINE	PRIMARY CELLULAR SOURCE	MAJOR CELLULAR TARGETS	EFFECTS
IL-4	Th2 cells, Basophils, Mast cells	Th2 cells, B cells, Basophils	IgE isotype switching, Mast cell development, Eosinophil and basophil activation, mucus, secretion, induction of Th2 cytokines
IL-5	Th2 cells, Eosinophils, ILC2	Eosinophils, Basophils, CD34+ cells	Eosinophil and basophil differentiation, maturation and activation
IL-9	Th2 Cells, Th9 cells, IL-9 Eosinophils, Mast cells	Smooth muscle, Epithelium, Mast cells	Mast cell and eosinophil development, AHR, mucus secretion
IL-13	Th2 cells, ILC2	Smooth muscle, Epithelium	Mast cell development, B-cell switch to IgE production, eosinophilia, AHR, mucus hypersecretion
TSLP	Epithelium, Basophils, Mast cells	Th2 cells, DCs, Basophils, ILC2	Basophil and DC activation, induction of Th2 responses (including CD34+ cell IL-5/IL-13 production)
IL-33	Epithelium, ASM, DCs	Th2 cells, Basophils, DCs, Nuocytes,	Basophil activation, enhancing eosinophilia and IgE secretion, induction of Th2 responses
IL-25	Epithelium, Th2 cells, Eosinophils, Basophils, Mast cells	Th2 cells, Basophils, DCs, MPP, Nuocytes,	Th2 development and IL-4, IL-5 and IL-13 production

TSLP, thymic stromal lymphopoietin; ASM, airway smooth muscle; DCs, dendritic cells; ILC2, type 2 innate lymphoid cell; MPP, multi-potent progenitors; IgE, immunoglobulin E; AHR, airway hyper-responsiveness.

The role of T_h2 cells in Asthma

There is increasing evidence that airway narrowing and AHR in asthma are not mediated solely through the activation of mast cells and basophils, but that allergen-specific T cells play an important role in regulating airway tone and reactivity (Ali et al., 2004, 2007, Haselden et al., 1999, 2001). Classically, the mechanisms by which T_h2 cytokines have been thought to primarily contribute to the pathogenesis of asthma have been via IgE synthesis, maturation and activation of effector cells, such as mast cells and basophils, and IL-5-mediated eosinophil infiltration, resulting in damage to the epithelium and AHR (Kay, 2001). Recently however, it has been postulated by many that the late asthmatic response (LAR) is elicited by leukotrienes released by eosinophils,

which are recruited following the release of IL-5 from allergen-specific T cells. In a randomized, double-blinded trial, Leckie et al. reported the inability of the mAb to IL-5 to reduce the magnitude of LAR following allergen challenge; however, subsequent studies in selected patients showed benefits of IL-5 blockade (Haldar et al., 2009; Leckie et al., 2000; Nair et al., 2009)

Airway Epithelium

Airway epithelium in health and disease

The airway epithelium consists of a pseudostratified mucosal layer, with basal, ciliated, mucus secreting goblet and surfactant secreting club cells. The airway epithelium functions as a highly regulated immune barrier through the formation of tight junctions in healthy individuals (Xiao et al., 2011). Furthermore, epithelial cells undergo rapid regeneration and repair whereby basal epithelial cells differentiate and proliferate to restore the damage caused by inhaled viruses, allergen and other environmental factors (Olivera et al., 2007; Rezaee et al., 2011; Runswick et al., 2007). Finally, the airway epithelium regulates the innate and adaptive immune response by secreting inflammatory cytokines/chemokines, resulting in the recruitment of, and direct interactions with, tissue leukocytes (Holgate, 2011). In asthma, the airway epithelium shows impaired repair responses and decreased barrier function, which result from inefficient tight junction formation (Xiao et al., 2011). This leads to enhanced access of environmental stimuli, which may alter mediator production and, consequently, result in improper downstream adaptive immune responses (Holgate, 2011; Proud and Leigh, 2011). For example, compared to healthy epithelial cells, asthmatic airway epithelial cells release more pro-inflammatory mediators, including IL-13, TSLP, IL-25, IL-33, CCL17, CCL5, CCL2 and monocyte chemoattractant protein-3 (Soumelis et al., 2002). Furthermore, after *in vitro* infection of cultured asthmatic epithelial cells, there is impaired virus elimination and increased cytotoxic cell death, due to

impaired production of IFN-β and IFN-λ, cytokines responsible for viral clearance and induction of apoptosis by healthy epithelial cells (Contoli et al., 2006; Wark et al., 2005).

Viral infections of the airway

Viral infection mainly occurs through the airway, infecting and replicating in epithelial cells, triggering the release of pro-inflammatory mediators, including TSLP, initiating the inflammatory cascade (Esnault et al., 2008). This inflammatory process is essential in the eradication of an infection, yet it can enhance any pre-existing airway inflammation in asthmatics, contributing to increased airway obstruction (Gern, 2010). DNA viruses synthesize double stranded (ds)RNA during replication, which are a natural source of TLR3 ligands, are potent inducers of TSLP mRNA. Previous studies have demonstrated that *in vitro* stimulation with dsRNA induced TSLP expression in small airway epithelial cells (SAEC) and primary bronchial epithelial cells (BEC) from both healthy and asthmatic individuals, with disproportionately higher levels secreted from asthmatic epithelial cells (Allakhverdi et al., 2007a; Kato et al., 2007; Schröder and Bowie, 2005; Uller et al., 2010). Furthermore, dsRNA and T_h2 cytokines synergistically induced TSLP production by epithelial cells in a TLR3-dependent manner (Kato et al., 2007). Similarly, following respiratory syncytial virus (RSV) infection, Lee et al. reported enhanced TSLP secretion from asthmatic epithelial cells (Lee et al., 2012). Furthermore, asthmatic BEC have been shown to produce higher levels of inflammatory cytokines (IL-6, Chemokine (C-X-C motif) ligand (CXCL)8, GM-CSF) following RSV infection, which correlated with increased inflammatory responses to viral infections *in vivo* (Contoli et al., 2005; Hackett et al., 2011). Thus, TSLP triggers DC-mediated T_h2-biased inflammatory responses with induction of IL-4, which may have paracrine effects on viral induced epithelial-derived TSLP, resulting in the perpetuation of the inflammatory response (Liu, 2006). Over the years, many respiratory viruses have been

identified with the capacity to generate T_h2-biased responses, a characteristic feature of allergic diseases. However, the work described in this thesis will focus specifically on how synthetic dsRNA-activated nasal epithelial cells are involved in the development of a T_h2 response.

Nasal Epithelial cells

Epithelial cells in the airways are among the first sites to encounter inhaled environmental stressors, such as pathogens, allergens or air pollutants. Epithelial cells are more than a structural barrier however, acting as a point of control initiating and regulating the respiratory immune response via the production of soluble mediators, and the expression of surface ligands and receptors (Borchers et al., 2006; Sanders et al., 2011; Swamy et al., 2010; Vareille et al., 2011). While immortalized cell lines can be used as *in vitro* models to study respiratory epithelial cells, they fail to differentiate into heterogeneous cell populations composed of polarized ciliated and mucus producing cells, similar to what is observed *in vivo*. To reproduce important characteristics of the respiratory epithelium observed *in vivo*, primary airway epithelial cells can be used, such as nasal epithelial cells (NECs) obtained from human volunteers. The NECs are expanded and cultured *in vitro*, yielding a cell culture model of differentiated NECs which mimics many of the features of the nasal epithelium seen *in vivo* offering a unique potential to study disease specific differences of NECs, physiological parameters associated with the airway epithelium, or the interactions between epithelial cells and other cell types, such as dendritic cell, natural killer cells or macrophages (Horvath et al., 2011; Müller et al., 2013a). Several existing methods utilize bronchial epithelial cells obtained invasively via brush biopsies during bronchoscopies, or from otherwise discarded lung tissue (Lopez-Souza et al., 2003; Turi et al., 2002; Zimmermann et al., 2009). Obtaining fresh NECs, as compared to bronchial or small airway epithelial cells, is less costly, invasive and is associated with fewer side effects. Non-invasive superficial scrape biopsies

to obtain NECs allow the a priori delineation of subject characteristics in accordance with the experimental design (Müller et al., 2013).

Thymic Stromal Lymphopoietin

Regulation of TSLP expression

A range of cells in the airway system (epithelial cells, smooth muscle cells, DCs, basophils, and mast cells) has been shown to have the ability to produce TSLP (Allakhverdi et al., 2007a; Kato et al., 2007; Lee and Ziegler, 2007; Soumelis et al., 2002; Zhang et al., 2007). Expression of TSLP is regulated by several factors, both exogenous stimuli (e.g., injury, infection, TLR ligation) and host-derived pro-inflammatory and T_h2 cytokines (Allakhverdi et al., 2007a). Pro-inflammatory cytokines IL-1β and TNFα have been shown to induce TSLP expression in human BEC (Hui et al., 2014; Kato et al., 2007; Lee and Ziegler, 2007). Similarly, IL-1β and TNFα was shown to induce TSLP production in small airway epithelial cells (SAEC) and human airway smooth muscle cells (HASMC) respectively (Allakhverdi et al., 2007a; Zhang et al., 2007). The induction of TSLP in airway epithelial cells by inflammatory T_h2 cytokines has been shown to be regulated by NFkB (Lee and Ziegler, 2007). In HASMC, aside from NFkB, the upregulation of TSLP by inflammatory T_h2 cytokines is also regulated by the transcription factor, activating protein 1 (AP-1) , as well as p38 and ERK MAPK signaling pathways (Redhu et al., 2011; Zhang et al., 2007).

The ability of bacterial peptidoglycan and polyinosinic:polycytidylic acid (polyI:C) to induce TSLP expression was first shown in SAEC (Allakhverdi et al., 2007a). The combination of TLR3 ligand (dsRNA) and T_h2 cytokines, in particular, IL-4 and TNFα, has been shown to significantly enhance TSLP production in BEC in an NFkB-dependent manner (Kato et al., 2007). Furthermore, infection of BEC with rhinovirus or RSV, viruses known to trigger asthma

exacerbations, results in the production of TSLP (Kato et al., 2007; Tu et al., 2007). Recent data suggest that the long form of TSLP is highly inducible by polyI:C in BEC. A functional SNP, rs3806933, has been identified in the regulatory element of the gene for the long form of TSLP, which creates a binding site for the transcription factor AP-1. This SNP enhances the binding of AP-1, leading to enhanced polyI:C-induced production of TSLP in BEC (Harada et al., 2009). In a murine AR model, mast cells were shown to upregulate TSLP expression in nasal epithelial cells following allergen challenge (Miyata et al., 2008).

Aside from regulating TSLP expression, mast cells activated by monoclonal antibodies that cross-link FcεRI have been reported to express TSLP mRNA (Okayama et al., 2009). Murine basophils, activated by protease allergens, were also reported to produce TSLP, along with other T_h2 inducing cytokines; furthermore, activated basophils in the lymph nodes have been shown to produce TSLP (Sokol et al., 2008). Although DCs are the major target of TSLP, surprisingly both human and murine DCs produce TSLP following TLR-ligation (Kashyap et al., 2011). Collectively, these findings suggest TSLP expression can be regulated by inflammatory stimuli produced by both innate and adaptive immune cells. Finally, in skin keratinocytes, TSLP gene expression has been reported to be regulated by both VDR and retinoic X receptors (RXR). Ablation of RXRα and RXRβ in keratinocytes resulted in enhanced TSLP expression and development of chronic skin inflammation in mice (Li et al., 2005, 2006).

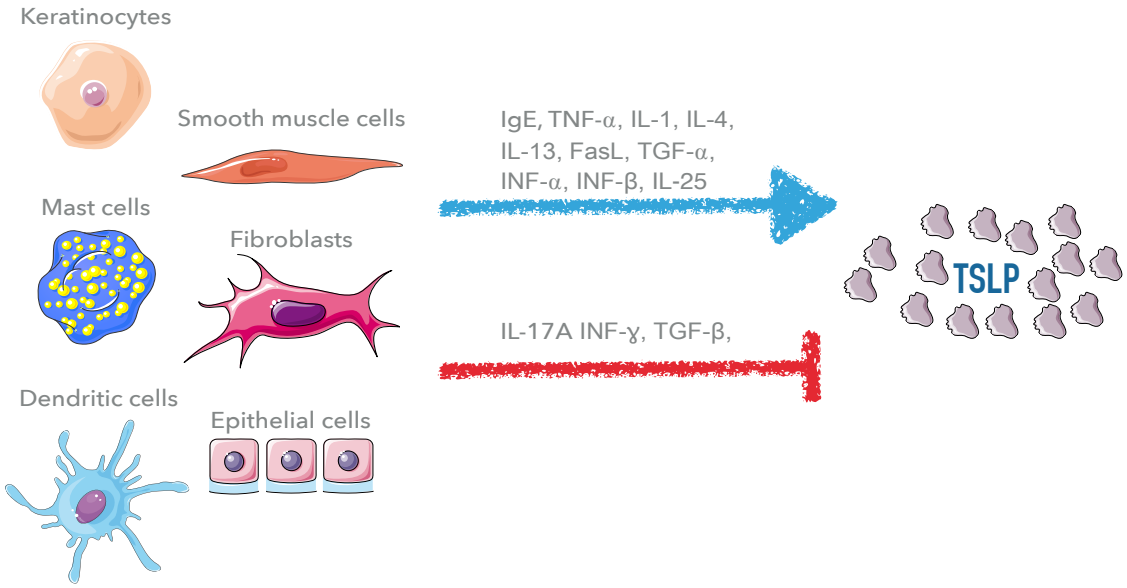


Figure 2. Regulation of TSLP expression by cytokines and IgE. Positive and negative regulation (induction of TSLP mRNA or protein expression, and enhancement or inhibition of TSLP expression by other stimuli) is shown. Positive effects of pro-inflammatory cytokines (TNF-α and IL-1) and Th2 cytokines (IL-4 and IL-13) are observed across various cell types. Cross-linking of FcεRI-bound IgE induces TSLP expression in mast cells and IgE without cross-linking induces TSLP expression in airway smooth muscle cells (ASMCs). Promotion of TSLP expression by IgE and Th2- related cytokines (IL-4, IL-13, IL-25, and IL-33) indicates amplification cycles. (adapted from (Takai, 2012))

TSLP signalling pathways

TSLP is expressed mainly by epithelial cells and keratinocytes at barrier surfaces. Its expression in the epidermis, epithelium, and submucosa in skin, airway, and ocular tissues plays a critical role in the sensitization process and exacerbation of allergic diseases. An initial analysis by real-time quantitative PCR in a panel of cDNA libraries from different cells or cell lines and a panel of purified primary cells indicated that keratinocytes, epithelial cells, smooth

muscle cells, and lung fibroblasts, cultured in growth medium and stem cell-derived mast cells activated by cross-linking of high- affinity IgE receptors (FcεRI) express TSLP mRNA in large amounts. Airway smooth muscle cells activated by IL-4, IL-13, and TNF-α and keratinocytes activated by TNF-α and IL-1β expressed higher TSLP mRNA levels than cells cultured in medium alone. Recent studies also have reported that not only keratinocytes and epithelial cells but also various other cell types can express TSLP mRNA and/or protein (Takai, 2012).

Despite the significant role of TSLP in driving T_h2 inflammation, knowledge of TSLP/TSLP receptor (TSLPR) signaling is limited. As a member of the hematopoietin receptor family, it was originally thought that the TSLPR would use JAKs to activate STAT proteins downstream of the TSLPR. It was later shown that TSLP stimulation of multiple cell lines did in fact lead to STAT-5 phosphorylation. However, initial experiments in these cell lines showed that TSLPR signaling occurred in the absence of JAK activation, and dominant-negative forms of JAK-1 and -2 did not affect TSLP-mediated STAT-5 activation (Isaksen et al., 1999; Levin et al., 1999). Several alternatives were implicated in TSLPR signaling, such as Src kinases and PI3K (Isaksen et al., 2002). However, two recent investigations have demonstrated robust and sustained activation of JAK-1 and -2 following TSLP signaling in primary human DCs and primary human and mouse CD4+ T cells (Arima et al., 2010; Rochman et al., 2010). The TSLPR subunit bound and used JAK-2 in concert with IL-7Rα-associated JAK-1. These latest findings resolve a long-standing question about the mode of TSLP signaling and show that TSLP-induced JAK activation precedes the activation of STAT proteins. Whereas multiple cell lineages express the functional TSLPR and respond to TSLP *in vivo*, most studies about TSLP-activated signaling pathways have been done in DCs and T cells (Ziegler et al., 2013). TSLP induced STAT-5 and STAT-1

phosphorylation in CD4+ T cells while activating STAT-1, -3, -4, -5, and -6 in human mDCs (Roan et al., 2012).

In human myeloid dendritic cells (mDCs), not including from STAT2, TSLP has been shown to be capable of activating all other known STATs. Noticeably, TSLP-activated STAT6 binds to the promoter region of CCL17, a T_h2-attracting chemokine, which helps to explain the unique ability of TSLP-activated DC to secrete CCL17 (Arima et al., 2010). Furthermore, TSLP-activated DCs have been shown to activate PI3K/AKT, ERK, JNK MAPK, and NFkB-dependent pathways (Semlali et al., 2010). TSLP has also recently been shown to activate murine CD4+ T cells, resulting in STAT6 dependent T_h2 differentiation. In BEC, TSLP activates both STAT3 and STAT5 phosphorylation, leading to the induction of IL-13 and cell proliferation. However, TSLP does not induce STAT5 phosphorylation in airway smooth muscle; rather, it induces STAT3 phosphorylation and activates the MAPK (ERK1/2, p38, and JNK) signaling pathways (Shan et al., 2010). In eosinophils, TSLPR signaling involves the ERK, p38, and NFkB signaling pathways, but not STAT3 or STAT5 (Wong et al., 2010).

It is important to note that there are TSLP may have dual immunoregulatory roles and that the two different isoforms of TSLP act on different signaling pathways. In a mouse model of house dust mite allergen challenge the short form of TSLP and 1α,25-Dihydroxyvitamin D3 were administered 1 h before dust mite exposure. After 8 weeks, animal lung function tests and pathological staining were performed to evaluate asthma progression. It was found that house dust mite and long form of TSLP diminished barrier function while treatment with the short form of TSLP and 1α,25-Dihydroxyvitamin D3 prevented dust mite-induced airway epithelial barrier disruption. Moreover, the short form of TSLP and 1α,25-Dihydroxyvitamin D3 treatment improved house dust mite induced asthma in mice (Dong et al., 2016).

In a chemical-induced asthma model, male BALB/c mice who were sensitized and challenged with toluene diisocyanate there was significantly increased inflammation and hyper-responsiveness of airway. This was inhibited by short TSLP treatment. Levels of mouse TSLP, high mobility group box 1 (HMGB1), and receptor for advanced glycation end products (RAGE) in airway epithelium and whole lung tissues were elevated in the toluene diisocyanate treated group compared with control mice, but decreased following short TSLP administration. Short form TSLP also inhibited STAT5(Y694) phosphorylation, whereas long form TSLP was elevated. This suggests that short TSLP prevents airway inflammation in a chemical-induced asthma model, which might be associated with the inhibitions of HMGB1-RAGE and long TSLP expression and STAT5(Y694) phosphorylation, while it is the long form of TSLP is primarily responsible for activation of T_h2 inflammatory pathways (Wang et al., 2017).

Cellular Targets of TSLP

TSLP is expressed predominantly by epithelial cells in the thymus, lung, skin, intestine, and tonsils, as well as by stromal cells and mast cells, but is not found in most hematopoietic cell types and endothelial cells and exerts its effects on a variety of different cell types (O'Byrne and Inman, 2003; Robinson, 2010; Simpson et al., 2002). In contrast, TSLPR has been found on DCs, T cells, B cells, mast cells, natural killer T (NKT) cells, and monocytes, as well as on tissues from heart, skeletal muscle, kidney, and liver (Humbert et al., 1999; Sheikh et al., 2003; Vignola et al., 1998). Recently, hematopoietic progenitors and nuocytes, a new type of innate effector leukocyte cells that mediate type-2 immune response, have been shown to respond to TSLP (Allakhverdi et al., 2009; Saenz et al., 2010b; Siracusa et al., 2011).

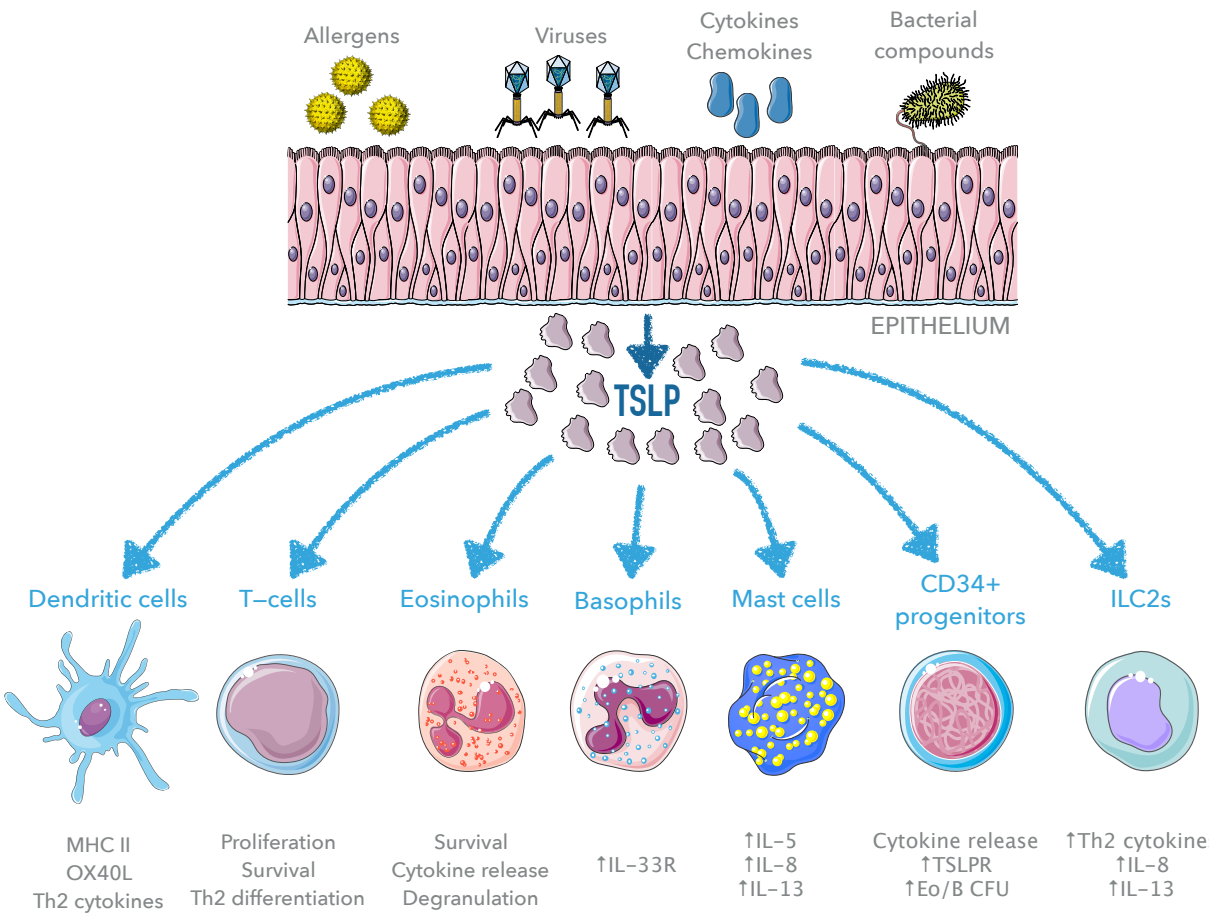


Figure 3. Cellular targets of epithelial-derived TSLP. Allergens, viruses, pro- inflammatory cytokines (TNFα, IL-1β, IL-4) chemokines (CCL17; TARC), and bacterial components such as peptidoglycan, lipoproteins, lipoteichoic acid, LPS are all capable of inducing TSLP production from airway epithelial cells. Subsequently, epithelial-derived TSLP can act on cells of multiple lineages, inducing proliferation and differentiation as well as cytokine production (adapted from (Rochman and Leonard, 2008))

Dendritic Cells

DCs are widely considered to be the major antigen presenting cell type in immune responses with the ability to initiate and direct immune responses. mDCs have been shown to have the highest expression of TSLPR, which is consistent with the ability of mDCs to respond to TSLP

(Reche et al., 2001; Soumelis et al., 2002). TSLP-activated mDCs have also been reported to produce high levels of CXCL8 and eotaxin-2, chemokines that attract neutrophils and eosinophils, in addition to producing thymus and activation regulated chemokine (TARC; CCL17) and macrophage- derived chemokine (MDC; CCL22), which functions to attract T_h2-polarized cells (Soumelis et al., 2002; Zhou et al., 2005). Furthermore, epithelial-derived TSLP enhances the maturation of mDCs by inducing expression of MHC II along with co-stimulatory molecules (CD54, CD80, CD83, and CD86) (Soumelis et al., 2002). TSLP-activation of mDCs results in the upregulation of OX40 ligand (OX40L) expression, which leads to the induction of pro-allergic inflammatory responses by triggering naïve CD4+ T cells to differentiate into inflammatory T_h2 cells that produce allergy-promoting T_h2 cytokines such as IL-4, IL-5, and IL-13, as well as (uniquely) TNF-α, but not IL-10 (Ito et al., 2005; Reche et al., 2001; Soumelis et al., 2002).

Gene expression analyses of TSLP-activated human mDCs shows that TLSP alone strongly induces TNF superfamily protein OX40L, which is critical for the induction of inflammatory T_h2 cells, since blocking OX40L with a neutralizing antibody inhibits the production of T_h2 cytokines and TNF-α, and enhances the production of IL-10 by CD4+ T cells. Consistent with this finding, Liu et al. observed that treatment of naïve CD4+ T cells with recombinant OX40L promoted the production of TNF-α, while inhibiting the production of IL-10 (Ito et al., 2005; Liu, 2007). Moreover, *in vivo*, TLSP-induced T_h2 immune responses in the lung and skin were inhibited by treating mice with OX40L- blocking antibodies (Seshasayee et al., 2007). Of interest, unlike other stimuli that activate DCs, such as CD40 ligand and TLR ligands (polyI:C and bacterial LPS), TSLP-activated DCs do not produce the T_h1-polarizing cytokine IL-12 (Ito et al., 2005; Soumelis et al., 2002). In the presence of IL-12, both TSLP-activated mDCs and OX40L lose its ability to induce inflammatory T_h2 differentiation (Ito et al., 2005). To date, TSLP is the

only factor that activates mDCs without the induction of IL-12 and other T_h1-polarizing cytokines. The molecular mechanism(s) by which TSLP activates mDCs without producing Th1-polarizing signals remains unclear. However, the dominance of IL-12 over OX40L may provide a molecular explanation for the ―hygiene hypothesis, which proposes that bacterial and viral infections elicit a T_h1-mediated immune response, which down-regulates the development of T_h2 -driven atopy. For these reasons, it can be suggested that TSLP plays a major role in classic T_h2 diseases such as allergic asthma. The activation of DCs by TSLP consequently provides a permissive condition for T_h2 development by upregulating the T_h2 polarizing signal (OX40L), without inducing the T_h1-polarizing signal (IL-12).

In addition to its differentiating effects on naïve CD4+ T cells, TSLP-activated mDCs have the capacity to robustly activate and expand naïve CD8+ T cells, in addition to inducing them to differentiation into pro-allergic IL-5 and IL-13 producing cytolytic T cells , which have been shown to induce eosinophilia and increased IgE production in AD, both *in vitro* and *in vivo* (Akdis et al., 1999; Gilliet et al., 2003). Moreover, TSLP-activated mDCs potently expand T_h2 memory cells, without altering their central memory phenotype and Th2 commitment (Wang et al., 2006). Lastly, TSLP has been shown to induce differentiation of CD4+CD8-CD25- naïve thymocytes into Foxp3+ T regulatory cells (Tregs) in a DC-dependent manner (Watanabe et al., 2005). As such, via mDCs, TSLP has the ability to regulate both the innate and adaptive phase of the allergic immune response by: (1) recruiting neutrophils and eosinophils through production of CXCL8 and CCL24, respectively; (2) preparing the local adaptive immune response by recruiting Th2 cells, through CCL17 and CCL22; and, (3) inducing the differentiation of naïve CD4+ and CD8+ T cells into inflammatory T_h2 cells and cytolytic T cells.

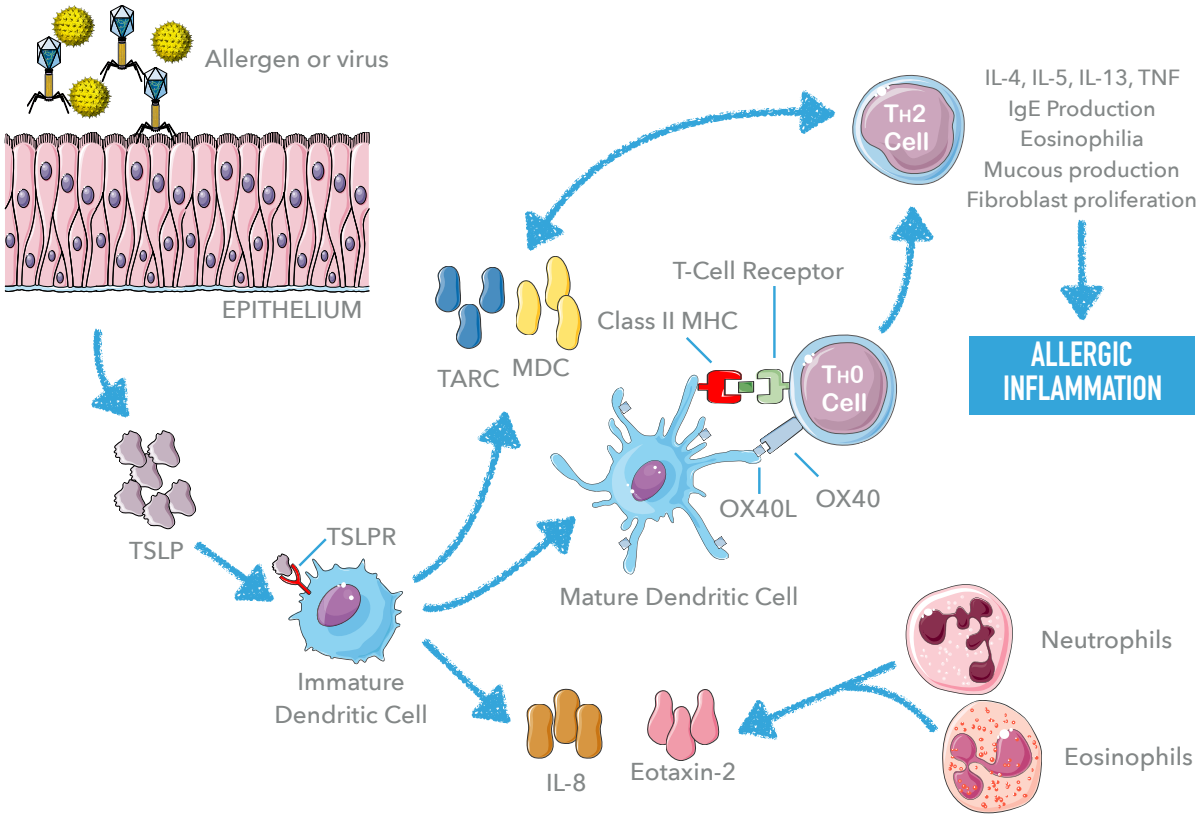


Figure 4. Pathophysiology of TSLP in allergic inflammation. Insult from allergens or viruses trigger epithelial cells to produce TSLP, which then activates immature DCs. TSLP- activated DCs express OX40L, which triggers naïve CD4+ T cells (T_h0) to differentiate into inflammatory Th2 cells that produce IL-4, IL-5, IL-13, and TNFα. Furthermore, TSLP- activated DCs produce TARC, which functions to attract helper T cells that are Th2 polarized. These differentiated Th2 cells release a profile of inflammatory cytokine, thus, initiating allergic inflammation (adapted from (Liu, 2006)).

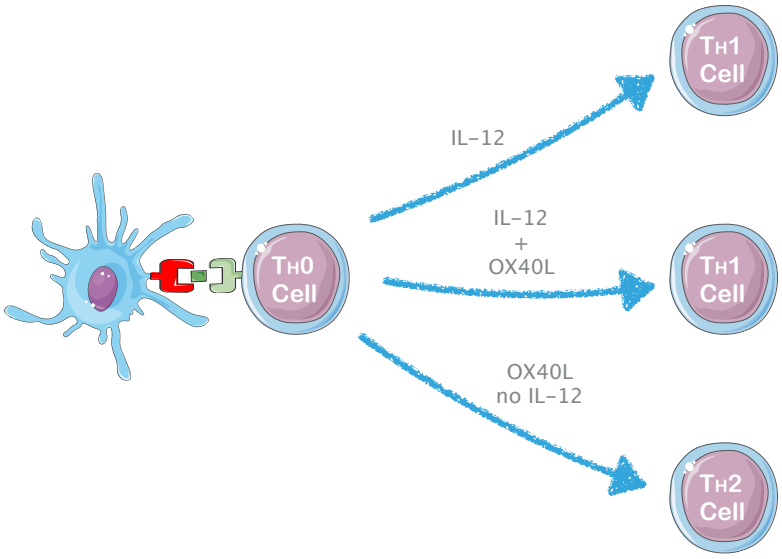


Figure 5. Regulation of T_h1 and T_h2 differentiation. The T_h1-polarizing signal (IL-12) is dominant over the T_h2-polarizing signal (OX40L); therefore, T_h2 differentiation will only occur in the presence of OX40L and in the absence of IL-12 (adapted from (Liu, 2006)).

T-cells

Although the effects of TSLP on T cells are mainly mediated by mDCs, recent studies suggest that both murine and human TSLP can directly activate CD4+ T cells, independent of mDCs. Murine TSLP appears to preferentially expand CD4+ T cells, as the addition of TSLP enhances the proliferative capacity of CD4+ T cells pre-activated through T-cell receptor stimulation. Furthermore, TSLP-treatment of murine CD4+ T cells results in IL-4 expression (Omori and Ziegler, 2007). Studies using TSLPR-deficient mice have shown that TSLPR expression by CD4+ T cells is critical for TSLP-mediated CD4+ T cell expansion and T_h2 differentiation *in vivo* (Al-Shami et al., 2004; Zhou et al., 2005). In humans, Rochman et al. demonstrated that following anti-CD3 and anti-CD28 stimulation, CD4+ T cells upregulate TSLPR expression and become responsive to TSLP, resulting in STAT5 activation and enhanced sensitivity of pre-activated CD4+ T cells to IL-2 (Rochman et al., 2007).

In addition to CD4+ T cells, both murine and human CD8+ T cells express the TSLPR complex. *In vitro*, TSLP-activated CD8+ T cells induce STAT5 and Akt activation and Bcl2 expression, which only increases survival without altering homeostatic proliferation of CD8+ T cells (Rochman and Leonard, 2008b). Activated pulmonary Tregs have also been reported to express TSLPR and respond to TSLP-mediated activation of STAT5 (Nguyen et al., 2010). TSLP-treated Tregs demonstrate impaired IL-10 production and diminished suppressive activity, which is consistent with studies that report decreased frequency and diminished suppressive activity of pulmonary Tregs in allergic asthmatics, compared to healthy controls and non-allergic asthmatics (Hartl et al., 2007; Nguyen et al., 2010). Furthermore, Nagata et al. reported on the ability of murine TSLP to activate NKT cells to preferentially produce IL-13, but not IFNγ and IL-4. Using a murine allergen-induced asthma model they demonstrated AHR in TSLP transgenic mice was almost eliminated completely in the absence of NKT cells. Furthermore, in the same asthma model, TSLP transgenic mice lacking NKT cells had significant reduction in IL-13(Nagata et al., 2007). Collectively, these findings highlight a unique role for TSLP in regulating the adaptive immune response.

Eosinophils, Basophils, and Mast cells

Following allergen challenge in patients with allergic asthma, there is a significant recruitment of hematopoietic progenitor cells to the airways. These cells express receptors for TSLP and IL-33. TSLP acts on eosinophils stimulating the release of IL-6, CXCL8, CXCL1, and CCL2. TSLP also triggers the up-regulation of the surface adhesion molecules CD18 and intracellular adhesion molecule-1 which indicates that TSLP may stimulate the recruitment of eosinophils to sites of inflammation. In addition, TSLP has been shown to increase the viability and survival of eosinophils and promote the release of eosinophil-derived neurotoxin, a protein

biomarker elevated in asthma patients (Cook et al., 2012; Kim, 2013; Wong et al., 2010). Mast cells found in asthmatic bronchial mucosa have been shown to express functional TSLP receptors. Potent activation of mast cells by TSLP has been reported in the presence of IL-1β and TNF-α by *Allakhverdi et al*. triggering the production of high levels of IL-5, IL-13, IL-6, GM-CSF, as well as chemokines associated with allergic diseases. Moreover, blocking native TSLP released in primary human SAEC greatly attenuated the production of IL-13 by mast cells (Allakhverdi et al., 2007a).

TSLP has also been shown to control basophil responses in mice. Murine basophils stimulated with TSLP show distinct characteristics, including high levels of IL-33R expression. Human basophils obtained from sites of inflammation have been shown to have significantly higher levels of IL-33R expression, indicating that TSLP may also induce basophil activation in allergic individuals (Siracusa et al., 2011). In an allergen inhalation model, allergic challenge increased basophil numbers in the airways and significantly upregulated the expression of activation markers, T_h2 intracellular cytokines, and receptors for TSLP, IL-3, and eotaxin in blood, bone marrow, and sputum basophils. Stimulation with TSLP *in vitro* primed basophil migration to eotaxin and induced basophil activation. IN that investigation basophils responded to TSLP at a magnitude and potency comparable to the well-described basophil-activating stimuli IL-3 and anti-IgE. These findings indicate that basophil activation during early- and late-phase responses to inhaled allergen might be driven at least in part by TSLP (Salter et al., 2015). Together, these studies support a role for TSLP in facilitating cross talk between epithelial cells and effector cells (mast cells, eosinophils, and basophils) of allergic inflammation.

Hematopoietic Progenitors

Hematopoietic progenitors are pluripotent stem cells with the ability to differentiate into all blood cellular types and a capacity for self-renewal. Hematopoietic progenitors are primarily identified by surface expression of the stage-specific antigen, CD34 (Szilvassy and Hoffman, 1995). Hemopoietic progenitors give rise to mature eosinophils, basophils and mast cells, effector cells of allergic diseases. Recently, Allakhverdi *et al.* reported that human CD34+ cells express receptors for TSLP and IL-33, which supports their finding that TSLP can potently activate human CD34+ progenitors. In the presence of IL-33, TSLP directly activates CD34+ progenitors, leading to the release of high levels of pro-inflammatory T_h2-like cytokines and chemokines that are involved in allergic diseases (Allakhverdi et al., 2007b). Similar to its effects on mast cells, blocking endogenous TSLP that was released by primary human SAEC completely inhibited production of IL-5 by CD34+ progenitors (Allakhverdi et al., 2009).

Hui et al. demonstrated a direct role for TSLP in eosinophil-basophil differentiation from human peripheral blood CD34+ progenitor cells. In the presence of IL-3, TSLP significantly promoted the formation of eosinophil and basophil colony forming units (CFU) TSLP–TSLPR interaction dependent manner. IL-3/TSLP-stimulated progenitors actively secreted an array of cytokines/chemokines, key among which was TNFα, which, together with IL-3, enhanced surface expression of TSLPR. In progenitots isolated from atopic individuals, the stimulated cells were functionally and phenotypically more responsive to TSLP than those from non-atopic individuals. This suggests that hematopoiesis can be enhanced through TSLP and may be a major driver for TSLP-driven allergic inflammation initiated at the epithelial surface.

Group 2 Innate Lymphoid Cells

ILCs are a newly characterized population of innate immune effector cells. Although they have

lymphoid morphology and secrete what have been regarded as mediators characteristic of lymphocytes, they have no recombinase-dependent B or T cell receptors nor do they express myeloid or lymphoid cell-surface markers . ILC2s, part of a larger family of ILCs expressing the ST2, IL-17RB and CD127 cell surface antigens, in a recently discovered group of innate immune cells are found in the gut and airway mucosa of mice and humans. These ILC2 have the capacity to produce T_h2 cytokines IL-5 and IL-13, suggesting functional similarities between ILC2 and T_h2 cells (Aron and Akbari, 2017; Kumar et al., 2014). Mjosberg *et al.* recently demonstrated the expression of TSLP receptor on human ILC2s found in nasal polyps of patients with chronic rhinosinusitis. They also demonstrated STAT5 activation and increased GATA3 expression in ILC2s following stimulation by nasal polyp epithelial-derived TSLP. Despite these similarities between ILC2 and T_h2 cells, unlike T_h2 cells, ILC2 are able to respond to TSLP with production of T_h2 cytokines without prior activation, a response that is augmented by IL-33 (Mjösberg et al., 2011). Additionally, stimulation with IL-25, IL-33, and TSLP was demostrated to drive the growth of ILC2s isolated from peripheral blood cells of healthy donors. ILC2s may comprise an additional cell population providing an early source of T_h2 cytokines, capable of initiating T_h2 allergic inflammation. The ability of ILC2s to respond to epithelial cell-derived cytokines (i.e. TSLP, IL-33, and IL-25) suggests that these cells may interact closely with the epithelium (Vannella et al., 2016).

TSLP in Allergic Diseases

Recent studies show that TSLP plays a pivotal role in allergic diseases. In AD, TSLP is highly expressed by keratinocytes in acute and chronic skin lesions (Soumelis et al., 2002). Furthermore, serum TSLP levels in children with AD are significantly higher than normal controls (Lee et al., 2010). The first evidence for a link between TSLP and human asthma was provided by

Ying et al. Through in situ hybridization they demonstrated elevated levels of TSLP mRNA expressing cells, which correlated with disease severity, within the bronchial epithelium submucosa in asthmatics (Ying et al., 2005). Elevated levels of TSLP are also found in the airway epithelium and BALF of asthmatic individuals (Nguyen et al., 2010; Shikotra et al., 2012; Ying et al., 2008). Over-expression of TSLP in airway epithelial cells induces allergic airway inflammation in mice (Zhou et al., 2005). Furthermore, there is over-expression of TSLP in the nasal epithelial cells of patients with AR and nasal polyposis (Kimura et al., 2011; Liu et al., 2011). Recently, several GWAS have identified TSLP as a locus associated with asthma and allergic disease susceptibility (Moffatt et al., 2010; Rothenberg et al., 2010). SNPs in TSLP have reported to be associated with several clinical outcomes and phenotypes of allergic diseases such as AR, total IgE and AHR (Bunyavanich et al., 2011; Gao et al., 2010; Harada et al., 2009, 2011; He et al., 2009; Hunninghake et al., 2008, 2010). In a recent clinical trial, a human TSLP mAb (AMG 157) was demonstrated to reduce both the early and LAR following an allergen challenge in mild atopic asthmatics. Moreover, AMG 157 was able to reduce markers of systemic and airway inflammation measured as fraction of exhaled nitric oxide, sputum eosinophil levels and circulating eosinophils, which further supports TSLP‘s role in persistent airway inflammation in asthmatics (Gauvreau et al., 2014). Evidence to date suggests a critical role for TSLP in the pathogenesis of T_h2-baised allergic diseases.

TSLP Polymorphisms

Susceptibility to allergic diseases is greatly influenced by gene-environment interactions (Herbert et al., 2017). In recent years, there has been a concerted effort to elucidate the genes and pathways related to asthma and other allergic disease through genetic analyses. Recent GWAS and meta-analyses of GWAS have detected numerous loci associated with asthma and related traits.

Large meta analyses such as the GABRIEL study and the EVE study identified SNPs in four loci (TSLP, IL-33, IL1RL1, 17q21 locus) that were associated with asthma (Moffatt et al., 2010; Torgerson et al., 2011). These findings highlighted the importance of epithelial-derived cytokines in determining the asthmatic phenotype. Regarding TSLP, in a recent GWAS of eosinophilic esophagitis (EoE), Rothenberg et al. identified the SNP rs3806932 in the TSLP gene to be significantly associated with EoE. They reported that subjects with EoE had elevated TSLP mRNA expression, which was significantly correlated with the rs3806932 genotype. EoE subjects who were homozygous for the protective minor allele expressed significantly less TSLP mRNA than those who were heterozygous and homozygous for the risk (ancestral) allele (Rothenberg et al., 2010).

Recent candidate gene and genome-wide association studies identified "protective" associations between the SNP rs1837253, found in the 5' promoter region of TSLP, with asthma, atopic asthma and airway hyper-responsiveness (He et al., 2009; Moffatt et al., 2010). The absence of linkage disequilibrium of rs1837253 with other SNPs in the region suggests that it is likely the causal polymorphism for the associations, and/or has functional consequences on TSLP expression. Other studies have reported that this same SNP, rs1837253, has reduced association with asthma in Costa Rican boys and reduced associations with allergic rhinitis in three independent cohorts for asthma (Bunyavanich et al., 2011; Hunninghake et al., 2010). The inverse association between rs1837253 and asthma suggests the possibility that this TSLP variant results in diminished secretion of TSLP, consequently, modifying the T_h2 inflammatory response. Hui et al. found that although atopic sensitization does not affect the secretion of TSLP from NECs, there was decreased TSLP secretion in NECs obtained from heterozygous (CT; 1.8-fold) and homozygous minor allele (TT; 2.5-fold) individuals, as compared with NECs from homozygous

major allele individuals (CC; $P<0.05$), after double-stranded RNA (dsRNA) stimulation (50 μg ml^{-1}) (Hui et al., 2015). These results imply that the rs1837253 polymorphism may be directly involved in the regulation of TSLP secretion. This may help explain the protective association of this genetic variant with asthma and related traits. Identifying functional consequences of SNPs in genes with previously reported clinical associations is critical in understanding and targeting allergic inflammation.

Other candidate gene association studies have illustrated the significance of TSLP variants to the development of allergic disease phenotypes and TSLP expression. A functional SNP, rs3806933, has been identified in the regulatory element of the long form of TSLP (Hunninghake et al., 2008). This variant enhances the binding of AP-1, a transcription factor that is critical for TLR3-dependent cytokine expression, resulting in enhanced transcriptional efficiency of the long form of TSLP in response to dsRNA stimulation in primary BEC. This same SNP, rs3806933, was later reported to be associated with childhood atopic and adult asthma (Harada et al., 2011). Furthermore, in a sex-stratified genome wide linkage analyses, Hunninghake et al. found an SNP in TSLP, rs2289276, to be inversely associated with cockroach-specific IgE in Costa Rican girls and total IgE in Costa Rican girls with asthma (Hunninghake et al., 2008). Collectively, these genetic studies further highlight a critical role for TSLP in the pathogenesis of asthma and allergic diseases.

Research Study

Hypothesis

Given that the minor T allele of the rs1837253 SNP in the TSLP gene is associated with reduced risk of allergic asthma, and as there are no other SNPs in linkage disequilibrium with rs1837253, we hypothesize that individuals with the minor allele would have a decreased

propensity to induce a T_h2 inflammatory response related to an altered cytokine mediator profile, leading to protection from allergic asthma. We therefore evaluated the double-stranded RNA (dsRNA)-induced secretion of TSLP from primary nasal epithelial cells (NECs) from non-asthmatic and asthmatic individuals, examining associations among rs1837253 genotype, atopy, and T_h2 cytokine secretion.

Aims

Aim 1: Investigate differences in TSLP production between asthmatic patients and subjects possessing the protective minor T allele in TSLP gene SNP rs1837253.

Aim 2: Outline variances in phenotype between asthmatic populations and subjects possessing the protective minor T allele in TSLP gene SNP rs1837253 by investigating altered protein and gene expression of key immune proteins and immune receptors.

Aim 3: Investigate functional differences associated with SNP rs1837253 in order to suggest a mechanism by which the genotype exerts its intermediary effects in the process of asthma development.

Scientific Impact

The results of this study will provide insight how an individual's genetic makeup may lessen or worsen the development of asthma. It will also further our understanding of TSLP and its function within the context of allergic asthma and its effect on the hematopoietic milieu. Moreover, this investigation will help us better understand the complex gene-environment interactions and the methods of control that regulate the development of not only asthma but other allergic diseases as well.

CHAPTER 2: METHODS

Study subjects

Subjects were non-smoking men and women, aged 18 to 65 years of age. Asthmatic subjects with atopy, non-asthmatic subjects with atopy, and healthy controls all between 18 and 65 years of age were recruited to participate in the study. No subjects required medication for treatment of asthma or allergies. All asthmatic subjects with allergy were atopic with asthma and a methacholine $PC_{20} \leq 16$ mg/mL. Subjects were excluded if they had asthmatic exacerbations or respiratory tract infections within 4 weeks of the study start date or underlying diseases other than asthma. Participating subjects underwent a screening procedure to determine allergic status by skin prick test and lung function by spirometry. All subjects provided written informed consent. Subjects treated with nasal steroids, antihistamines and anti-leukotrienes unable to withdrawn these medications for 2 weeks before collection of nasal scrapings were excluded from the study. Sample size was based on power calculations based on observed differences in TSLP expression in work completed by *Hui et al.* The targeted sample size was 100 subjects, in order to obtain approximately 15 subjects possessing the homozygous minor alleles.

Isolation of genomic DNA and genotyping

The SNP rs1837253 in the TSLP gene was selected based on previous associations with asthma and related traits as well as the absence of significant linkage disequilibrium with any other SNP. DNA will be extracted from mouthwash samples using QIAamp DNA Blood Mini Kits (Qiagen, Toronto, ON, Canada) according to the manufacturer's supplementary protocol for mouthwash samples. Genotyping was then be performed using a commercially available TaqMan genotyping assay for rs1837253 (Assay C__11910823_20, Life Technologies, Burlington, ON, Canada). The genotyping data was be verified for Hardy–Weinberg equilibrium by the X^2 test.

Nasal epithelial cell isolation and culture

Primary nasal epithelial cells (NECs) were derived from the inferior nasal turbinate using Rhino-Probe nasal curettes (Arlington Scientific, Springville, UT). NECs were expanded and cultured as previously described by Meuller *et al.* with some modification to the procedure (Müller et al., 2013b). NECs were maintained in bronchial epithelial growth medium (BEGM; Clonetics, Allendale, NJ) supplemented with SingleQuots (Clonetics). The SingleQuots comprise of 2 ml bovine pituitary extract (52 μg ml^{-1}) and 0.5 ml each of insulin (μg ml^{-1}), human epidermal growth factor (0.5 ng ml^{-1}), hydrocortisone (0.5 μg ml-1), epinephrine (0.5 μg ml^{-1}), transferrin (10 μg ml-1), retinoic acid (0. ng ml^{-1}), triiodo-L-thyronine (6.5 ng ml^{-1}), and Gentamicin/Amphotericin-B (GA1000; μg ml^{-1}). Collected cells were centrifuged and resuspended in BEGM with 10% 100x DNase 1 (Sigma Aldrich, St Louis, MO; at room temperature for 20 min). Cells were washed twice and plated on 35 mm culture dishes (Corning Costar, Corning, NY), coated with Purecol (Inamed, Fremont, CA), and incubated at 37°C, 5% CO2. Media was changed 24h after plating; after which, media was replaced every second day until cells reach 80–90% confluency. In this study, second and third passaged cells were used.

Poly(I:C) stimulation

NEC were seeded onto 100mm dishes (Corning) and allowed to grow to 80–90% confluence. Thereafter, BEGM was replaced with bronchial epithelial basal media (Clonetics) and 1% fetal bovine serum (R&D Systems, Minneapolis, MN) before experimental stimulation. NECs were treated with poly(I:C) (EMD Chemicals, Gibbstown, NJ) as indicated or vehicle control for 3h, 6h or 24 h at 37°C, 5% CO2.

RNA isolation and reverse transcription

RNA was extracted from a number of different human nasal epithelial samples using the RNeasy Mini-kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA in each sample was quantified using a NanoDrop Spectrophotometer (Thermo Fisher, Wilmington, DE). The RNA in each sample time point (0, 3, and 6h) from each participant was diluted based on the lowest concentration sample using RNase-free water. The samples were reverse-transcribed using the Quantitect Reverse Transcription kit with genomic DNA wipeout buffer (Qiagen) and complementary DNA were aliquoted and stored at -80°C.

Quantitative reverse transcription-PCR

The expression of TSLP was determined by quantitative reverse transcription- PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) with a MX4000 Stratagene detection system according to the manufacturer's instructions. The PrimePCR Human Reference Gene Panel (Bio-Rad) was used to determine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, (YWHAZ) to be the optimal reference genes. The following primer set for quantitative reverse transcription-PCR was used: (NM_033035.4 and NM_138551.4), 50 – CTAAGGCTGCCTTAGCTATC-30 and 50–AAGCGACGCCACAATCCTTG-30 as previously described. PrimePCR SYBR green assay for Human GAPDH and YWHAZ were used for reference gene primers (Bio-Rad). 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. Pooled complementary DNA was used to optimize annealing temperature (found to be 60°C for all primer sets). Primer sets were previously verified by Hui and colleagues using 8-point standard curves to confirm

adequate amplification efficiency for each TSLP isoform primer sets and the GAPDH/YWHAZ reference genes using Sso Advanced SYBR green Supermix (Bio-Rad). Melt curves were used to verify expected single peak amplicons. The threshold cycle (Ct) is inversely correlated with the target mRNA expression level and was defined as the cycle number at which the reporter fluorescence emission exceeded the midpoint along the amplification curve. 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.

Cytokine and Chemokine Secretion

TSLP, GM-CSF, IFNy, TNF-α, IL-8 and IL-5 were assessed using Bio-Plex assays (Bio-Rad, Hercules, CA, USA) according to manufacturer's recommendation. The detection limits for these cytokines were TSLP (3.1 pg/mL), GMCSF (9.5pg/mL), IFNy (0.1 pg/mL), TNF-α (0.1 pg/mL), IL-8 (0.2 pg/mL) and IL-5 (0.1 pg/mL).

Flow Cytometry

Flow cytometric analysis of type 2 cytokine and toll-like receptors was conducted using mononuclear cells (which contains lymphocytes including T cells, B cells, NK cells as well as monocytes) isolated from donated peripheral blood using Lymphoprep^{TM} density gradient medium. Peripheral blood was centrifuged at 1500rpm for 20 minutes. Mononuclear cell fraction was isolated using a bulb pipette. Four-color immunofluorescent staining was performed. The mononuclear cells were resuspended in 1 mL of FACS buffer (PBS containing 0.1% sodium azide) and placed in aliquots in individual tubes at a concentration of 1 3 105 cells per tube. Cells were resuspended in murine block (PBS containing 0.2% sodium azide, 2.5% murine serum, and 2.5% human serum) and incubated in the refrigerator for 30 minutes. Optimal amounts (1uL) of PerCP, FITC, or PE-labeled anti–TSLPRa, IL-3Ra, IL-5Ra, IL-13a, IL-17RBa, ST2a, GM-CSFRa, TLR-

2, TLR-4, or their respective isotype controls were added to the tubes, and incubated for 30 minutes in the refrigerator. Cells were then washed with FACS buffer and fixed in 250 mL of 1% paraformaldehyde. Compensation tubes were likewise prepared, staining cells individually with anti-CD45–fluorescein isothiocyanate, anti-CD45–PE, anti-CD45–allophycocyanin, and anti-CD45–PerCP. For intracellular analysis of TLR-9, IL-5, and GMCSF cells were resuspended in a fixing solution (Caltag Laboratories, Carlsbad, Calif) for 20 minutes at room temperature, washed with FACS buffer, and resuspended in permeabilization buffer (Caltag Laboratories). Antibodies for anti-human TLR-9, IL-5, and GMCSF and rat isotype control were added to the permeabilization solution and incubated for 30 minutes at room temperature. Cells were washed and resuspended in 250 mL of 1% paraformaldehyde. All samples were covered with aluminum foil and refrigerated until ready for acquisition. Acquisition was performed with an LSR II flow cytometer (BD Bioscience) and data analysis was conducted using Flow Jo software (Tree Star, Inc, Ashland, Ore).

Statistical Analysis

Data presented within the text, figures and figure legends are presented as mean ± SEM. Data analysis was completed using Graphpad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) software to perform students, t-test and ANOVAs. 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. Type 1 error probability (α) was set at 0.05.

CHAPTER 3: RESULTS

Subject Characteristics

This study was approved by the Hamilton Integrated Research Ethics Board and all subjects provided written informed consent. From December 1st 2015, to June 1st 2017, 45 subjects have been enrolled to participate in the study. Of the 45 enrolled, 14 are atopic asthmatic and 1 is non-atopic asthmatic and 30 are non-asthmatic (methacholine challenge results were negative). There were 23 atopic non-asthmatics and 7 subjects were non-atopic non-asthmatic (Table 2). The homozygous minor T allele (TT) was found in 8 of the subjects, 18 subjects possessed the homozygous major C alleles (CC) and 19 subjects were heterozygous (CT) for both forms of the allele (Figure 1).

Gene expression differs in a genotype-dependent manner following PolyI:C stimulation

Polyinosinic:polycytidylic acid (polyI:C) has been previously reported to induce TSLP production (messenger RNA (mRNA) or protein) in human airway epithelial cells. There have been reported differences in the immune response between the BEC of healthy and asthmatic individuals, with enhanced TSLP secretion in BEC from asthmatics compared to their healthy counterparts following viral exposure. The higher capacity of asthmatic BEC to sense and respond to viral infections and thus, to release elevated levels of TSLP was reported to be due, in part, to increased expression of the retinoic acid-inducible gene 1 (RIG-1) in asthmatic airway epithelial cells following viral infections. A recent study found that TNF-α upregulates TSLP mRNA and induces high levels of TSLP protein release in primary human airway smooth muscle cells. TNF-αa induced the TSLP promoter activity which was activator protein-1 (AP-1) binding sites (Redhu et al., 2011). To investigate whether primary cultured nasal epithelial cells behave in a similar manner, mRNA expression of RIG-1 and AP-1 was detected following PolyI:C ($25\mu gmL^{-1}$)

stimulation for 0, 3, and 6 hours. The expression of the common isoform of TSLP was elevated in a time dependent manner following polyI:C (25μg/mL) stimulation for 3h ($p<0.01$) and 6h ($p<0.001$) compared to the unstimulated control (figure 2a). This time dependent increase in expression was found in the long form (figure 2b) but only at the 6h time point for the short form of TSLP (figure 2c). RIG-1 expression was also elevated in a time dependent manner following polyI:C (25μg/mL) stimulation for 3h ($p<0.001$) and 6h ($p<0.001$)(figure 2d). AP-1 expression was reduced in a time dependent manner following polyI:C (25μg/mL) stimulation for 6h ($p<0.05$)(figure 2e). When comparing the gene expression of key regulatory proteins across different genotypes. The common isoform of TSLP was significantly increased at the 6h time point across genotypes (figure 3a), where the homozygous major and heterozygous major alleles were higher compared the homozygous minor alleles. When stratified according to the short and long isoforms, the long isoform was elevated for subjects possessing the homozygous major allele and heterozygous alleles compared to the homozygous minor after 6h of polyI:C stimulation (figure 3b-c). The short isoform was not differentially expressed according to genotype however (figure 3b). RIG-1 and AP-1 expression did not change significantly between genotypes (figure 3d-e) at any time point. When comparing the gene expression of key regulatory proteins in atopic and non-atopic individuals no associations were found in the common isoform, the long and the short isoform of TSLP (figure 4a-c), RIG-1 (figure 4d) and AP-1 (figure 4e).

Using a two-way ANOVA and a Bonferroni post test the contribution of asthmatic status to differences in shared isoform TSLP expression was found to be significantly different. The contribution of genotype was not significant ($p = 0.0853$). When comparing the gene expression of the long and short isoform of TSLP in asthmatic individuals, expression was not found to be significantly different in the asthmatics groups or when stratified by those carrying the major (C)

and minor (T) allele (figure 9a-c). Interaction between asthma and genotype was found to be non-significant.

TSLP protein expression in subjects stratified by rs1837253 genotype following 24h stimulation with polyI:C is significantly different.

Nasal epithelial cell cultures were stimulated with 25µg/mL polyI:C for 24h and subsequent differences in the protein levels from isolated supernatant were analyzed using a multiplex cytokine and chemokine assay. TSLP significantly elevated protein levels in individuals with the homozygous major allele compared to those possessing the homozygous minor allele (figure 5a) ($p<0.05$). No differences were identified in the expression of IL-8, IL-5, GMCSF, INF-y and TNF-α (figure 5b-f) when stratified by genotype. Only TSLP had significantly elevated protein levels in asthmatic individuals (figure 6a). No differences were identified in the expression of IL-8, IL-5, GMCSF, INF-y and TNF-α (figure 6b-f). ($p<0.05$)

Cell surface protein expression in asthmatic individuals shows no differences compared to non-asthmatic subjects

Peripheral blood mononuclear cells were stained with antibodies specific to several key immune cytokine receptors and intracellular immune proteins (TLR-2 , IL-5 receptor , intracellular GMCSF , IL-3 receptor , intracellular IL-13 , GMCSF receptor , IL-17 receptor B ,TSLP receptor , TLR-9 , ST2 receptor & intracellular IL-5). No significant differences were found among any targets in the panel (figure 7 a-l) when the mean fluorescent intensity was compared in subjects with allergic asthma to those without asthma. No significant differences were found among any targets in the panel (figure 8 a-l) when the mean fluorescent intensity was compared across different genotypes of rs1837253.

Figures:

Table 2: Subject Characteristics

Characteristics	Asthmatic	Non-Asthmatic
Sample Size, n	15	30
Sex, male:female	7:8	16:14
Age, years (mean)	39	29.2
Atopy, n	14	23
Ethnicity		
Asian	2	7
Caucasian	10	13
Other	3	10
Genotype		
Homozygous major (CC)	7	12
Heterozygous (CT)	7	11
Homozygous minor (TT)	1	7

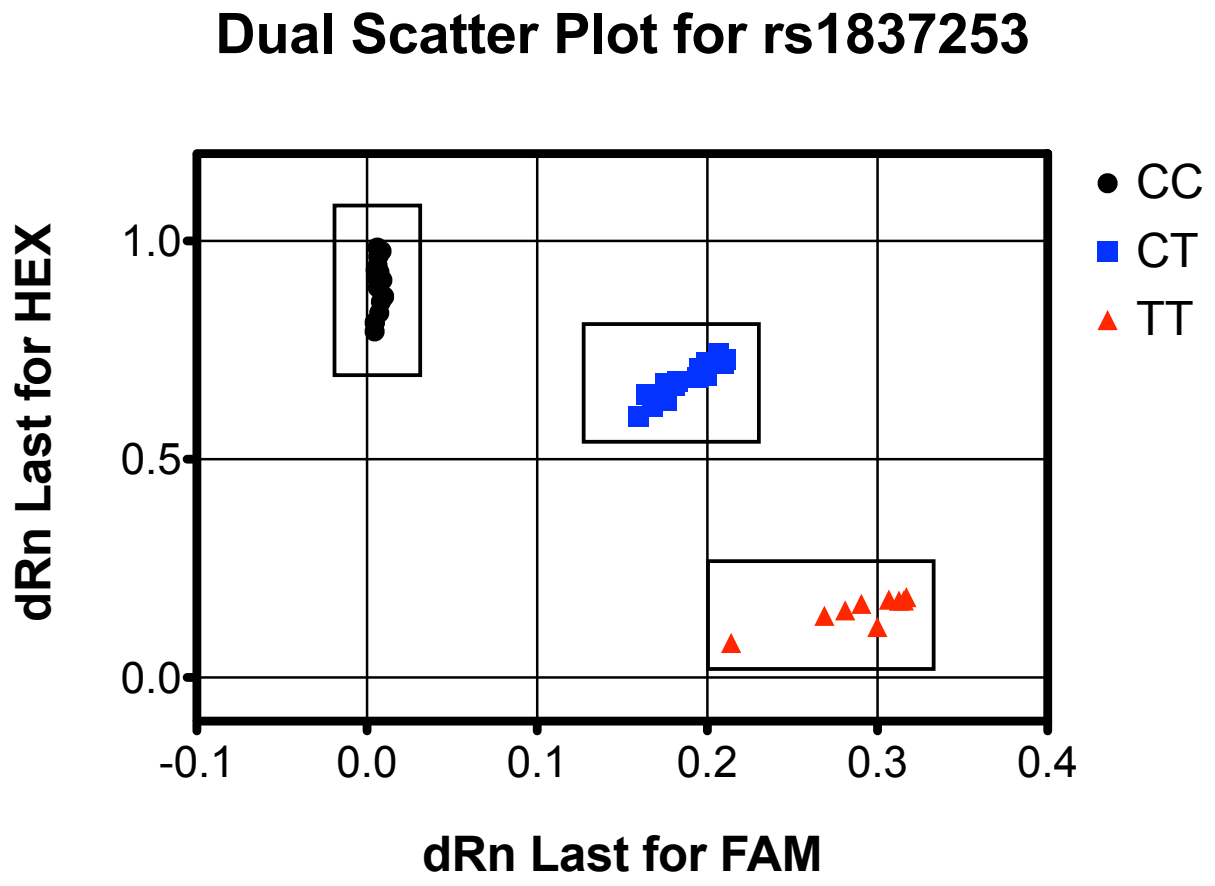


Figure 1: Allele Discrimination Dual Scatter Plot for rs187253 using Taqman Probes. Fluorescence for two dyes (Hex and FAM) assigned to the same wells is demonstrated in the above plot using the Mx3000PTM Real-Time PCR System. Each point represents the coordinates of the fluorescence or Ct for the two 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).

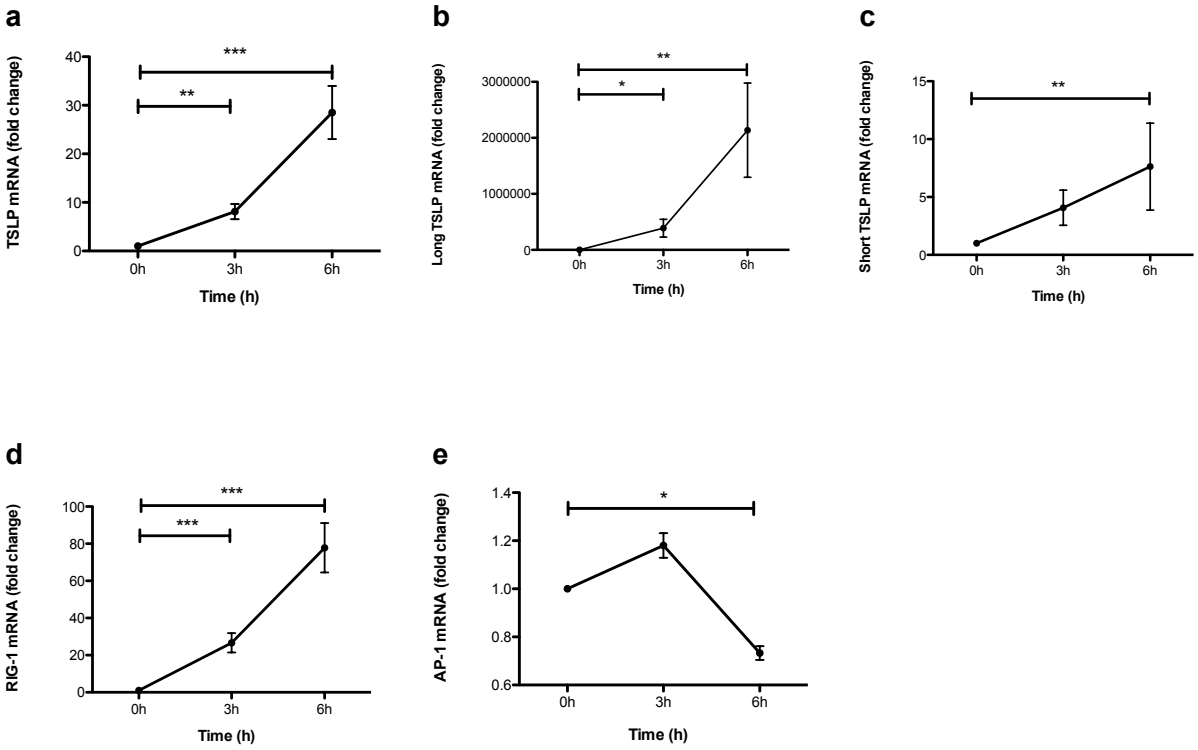


Figure 2: Polyinosinic:polycytidylic acid (polyI:C) induces thymic stromal lymphopoietin (TSLP), RIG-1 and inhibits AP-1 gene expression in human primary nasal epithelial cells (NECs). Gene expression kinetics of the (a) common TSLP isoform (b) long TSLP isoform (c) short TSLP isoform (d) RIG-1 (e) AP-1 in polyI:C (25 mg mL^{-1}) stimulated NECs (n = 40). Results shown are expressed as fold change in the mRNA level in polyI:C-stimulated NECs at 3 and 6 h relative to the level in unstimulated NECs (0 h). One-way ANOVA with Tukey's post-test was used to assess differences between groups. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

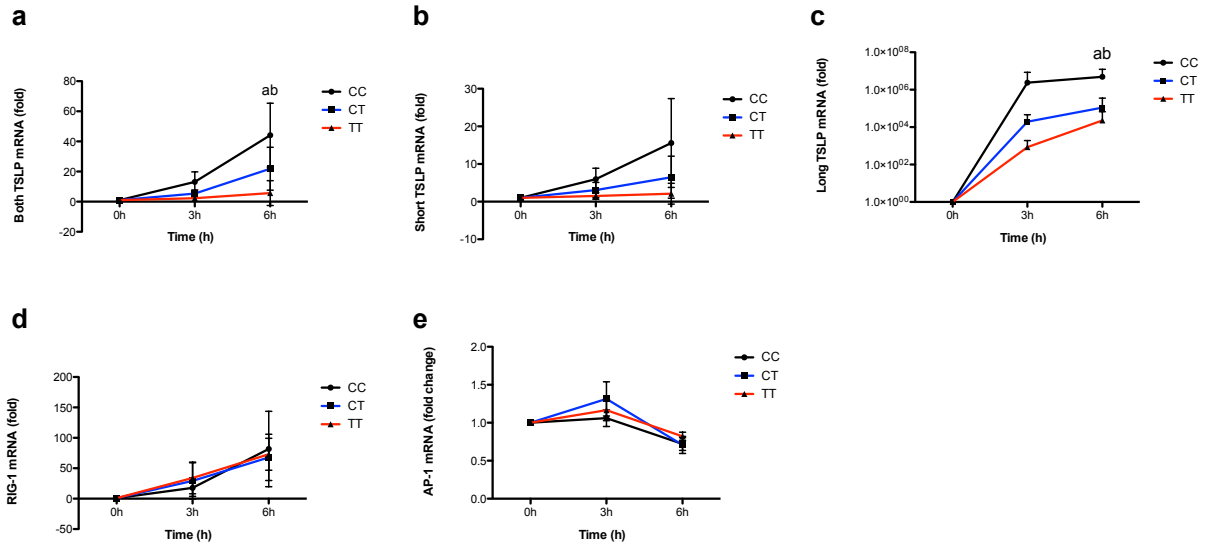


Figure 3: Polyinosinic:polycytidylic acid (polyI:C) induces the expression of the long isoform of thymic stromal lymphopoietin (TSLP) differently according to genotype of SNP rs1837253. Gene expression kinetics of the (a) common TSLP isoform (b) long TSLP isoform (c) short TSLP isoform (d) RIG-1 (e) AP-1 in polyI:C (25 mg mL^{-1}) stimulated NECs (n = 40). Results shown are expressed as fold change in the mRNA level in polyI:C-stimulated NECs at 3 and 6 h relative to the level in unstimulated NECs (0 h) and are stratified by genotype. Two-way ANOVA with Bonferroni post-test was used to assess differences between groups (**a** indicates significant difference between homozygous major and homozygous minor allele groups, **b** indicates significant difference between heterozygous and homozygous minor allele groups; **a, b** indicates $p<0.05$).

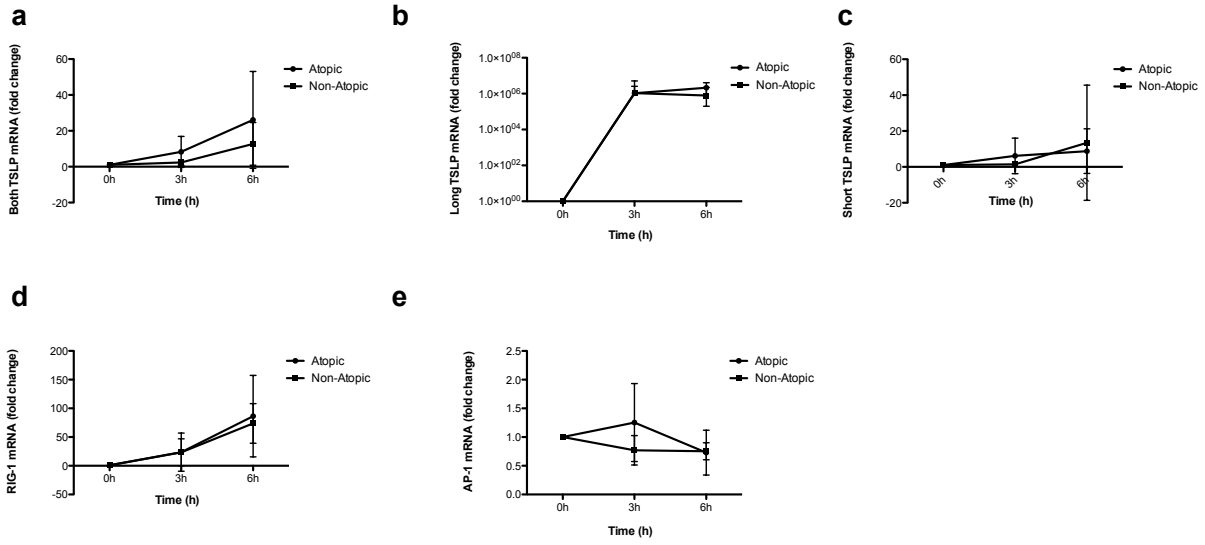


Figure 4: Polyinosinic:polycytidylic acid (polyI:C) does not alter the expression of thymic stromal lymphopoietin (TSLP), RIG-1 or AP-1 differently according to atopic status. Gene expression kinetics of the (a) common TSLP isoform (b) long TSLP isoform (c) short TSLP isoform (d) RIG-1 (e) AP-1 in polyI:C (25 mg mL^{-1}) stimulated NECs (n = 40). Results shown are expressed as fold change in the mRNA level in polyI:C-stimulated NECs at 3 and 6 h relative to the level in unstimulated NECs (0 h) and are stratified by genotype. Two-way ANOVA with Bonferroni post-test was used to assess differences between groups. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

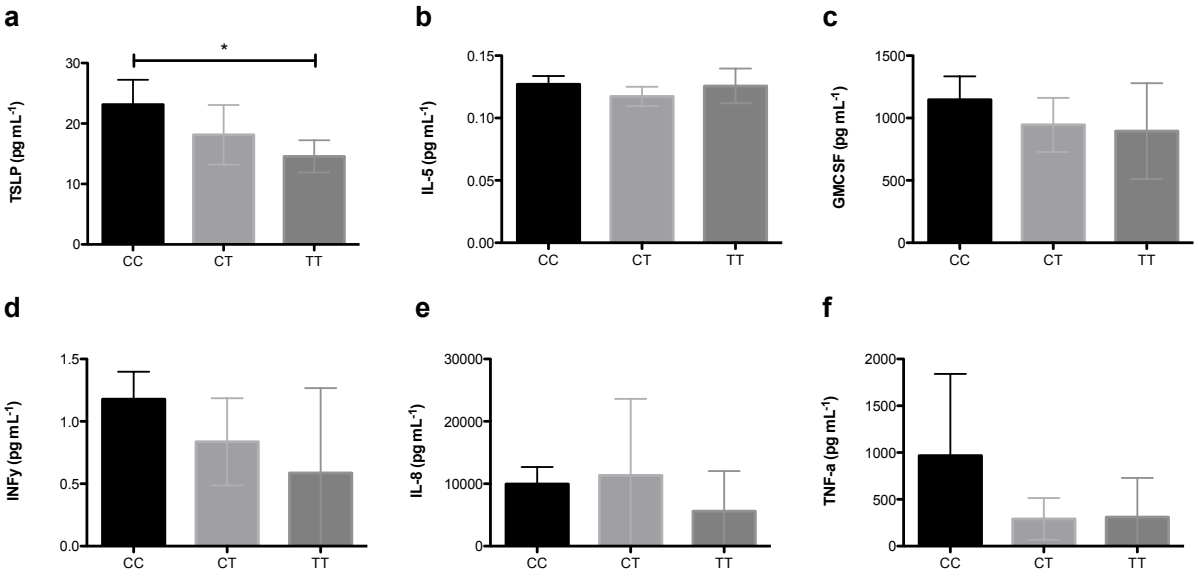


Figure 5: Relationship between TSLP rs1837253 genotype and polyinosinic:polycytidylic acid (polyI:C)-induced secretion of (a) thymic stromal lymphopoietin (TSLP) (b) IL-5 (c) GM-CSF (d) INF-ɣ (e) IL-8 (f) TNF-α in nasal epithelial cells. Concentrations of secreted cytokine and chemokine protein in nasal epithelial cell culture supernatant from rs1837253 homozygous major allele (CC; n=17), heterozygous (CT; n=14), and homozygous minor allele (TT; n=7) individuals were measured using Biorad Bioplex assays following 24h stimulation with 25µg mL^{-1} of polyI:C. One independent experiment was performed per subject. One-way ANOVA with Tukey's post test was used to assess differences between groups. (*$p<0.05$)

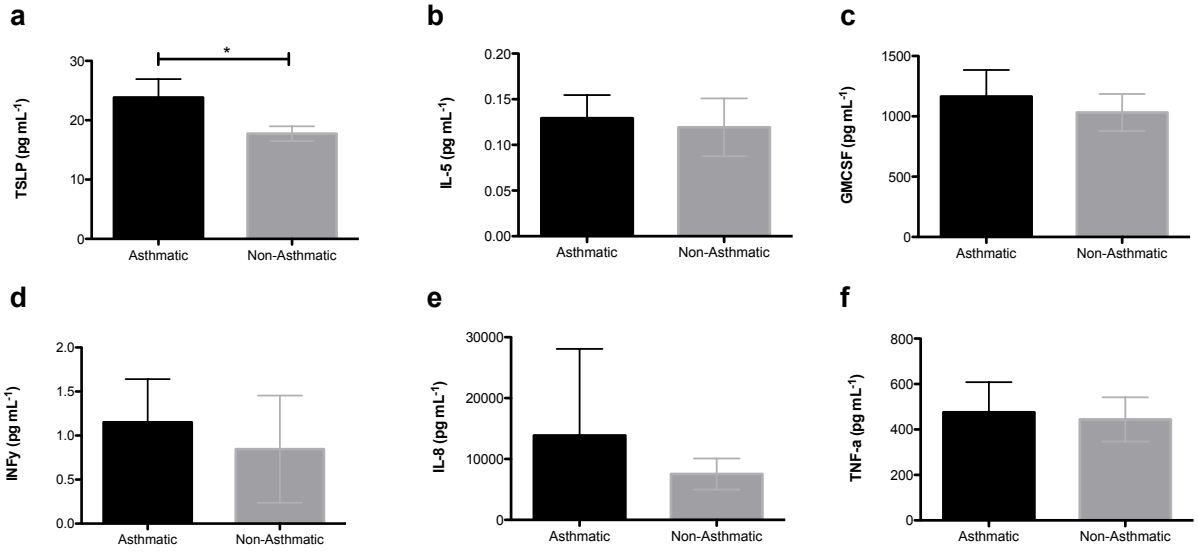
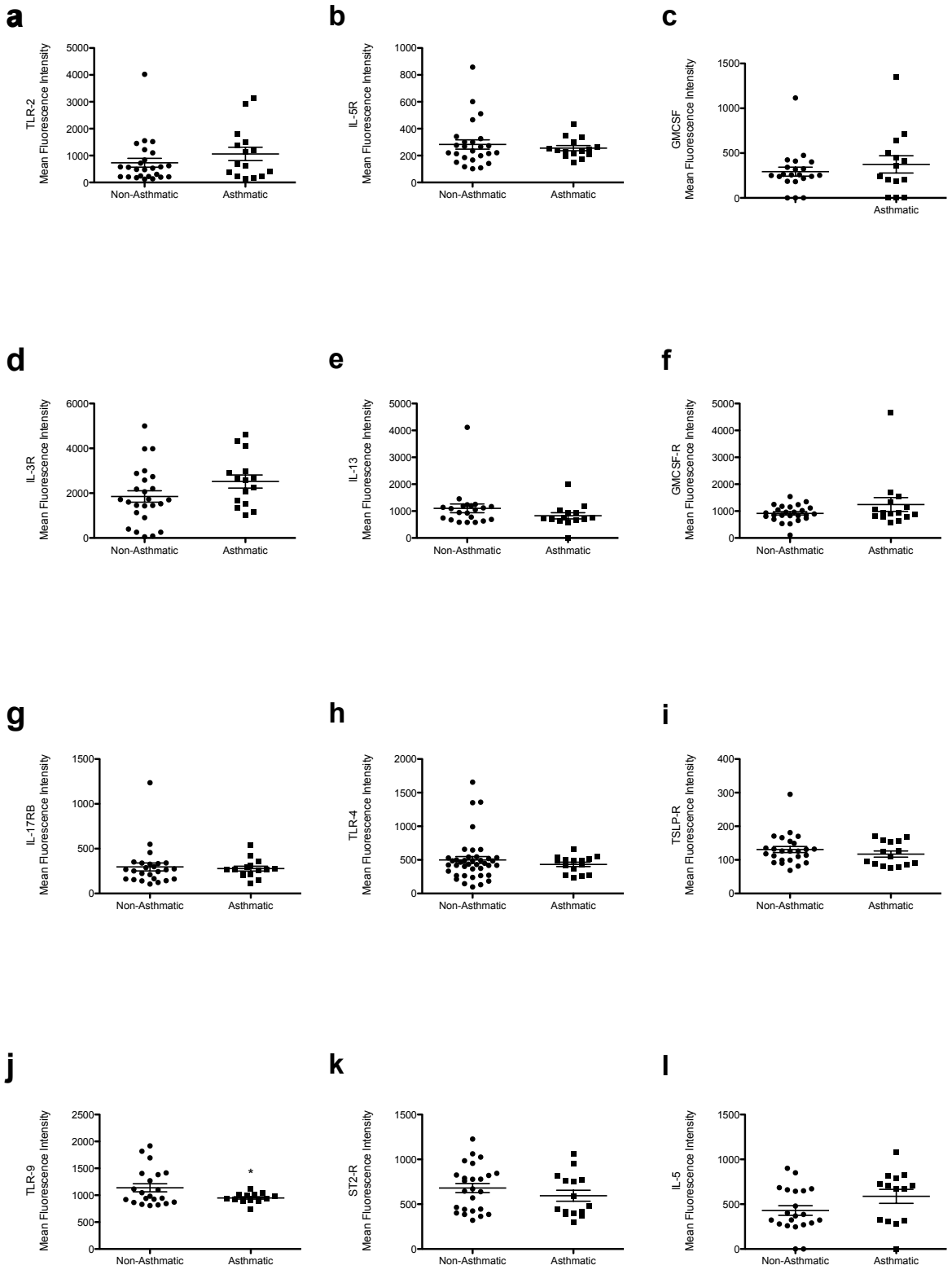


Figure 6: Relationship between asthmatic status and polyinosinic:polycytidylic acid (polyI:C)-induced secretion of (a) thymic stromal lymphopoietin (TSLP) (b) IL-5 (c) GM-CSF (d) INF-y (e) IL-8 (f) TNF-α in nasal epithelial cells. Concentrations of secreted cytokine and chemokine protein in nasal epithelial cell culture supernatant from asthmatic (n=13) and non-asthmatic (n=25) individuals were measured using Biorad Bioplex assays following 24h stimulation with 25µg mL-1 of polyI:C. One independent experiment was performed per subject. Students t-test was used to assess differences between groups. (*$p<0.05$)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Non-Asthmatic
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IL-5R
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Asthmatic
c
GMCSF
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IL-3R
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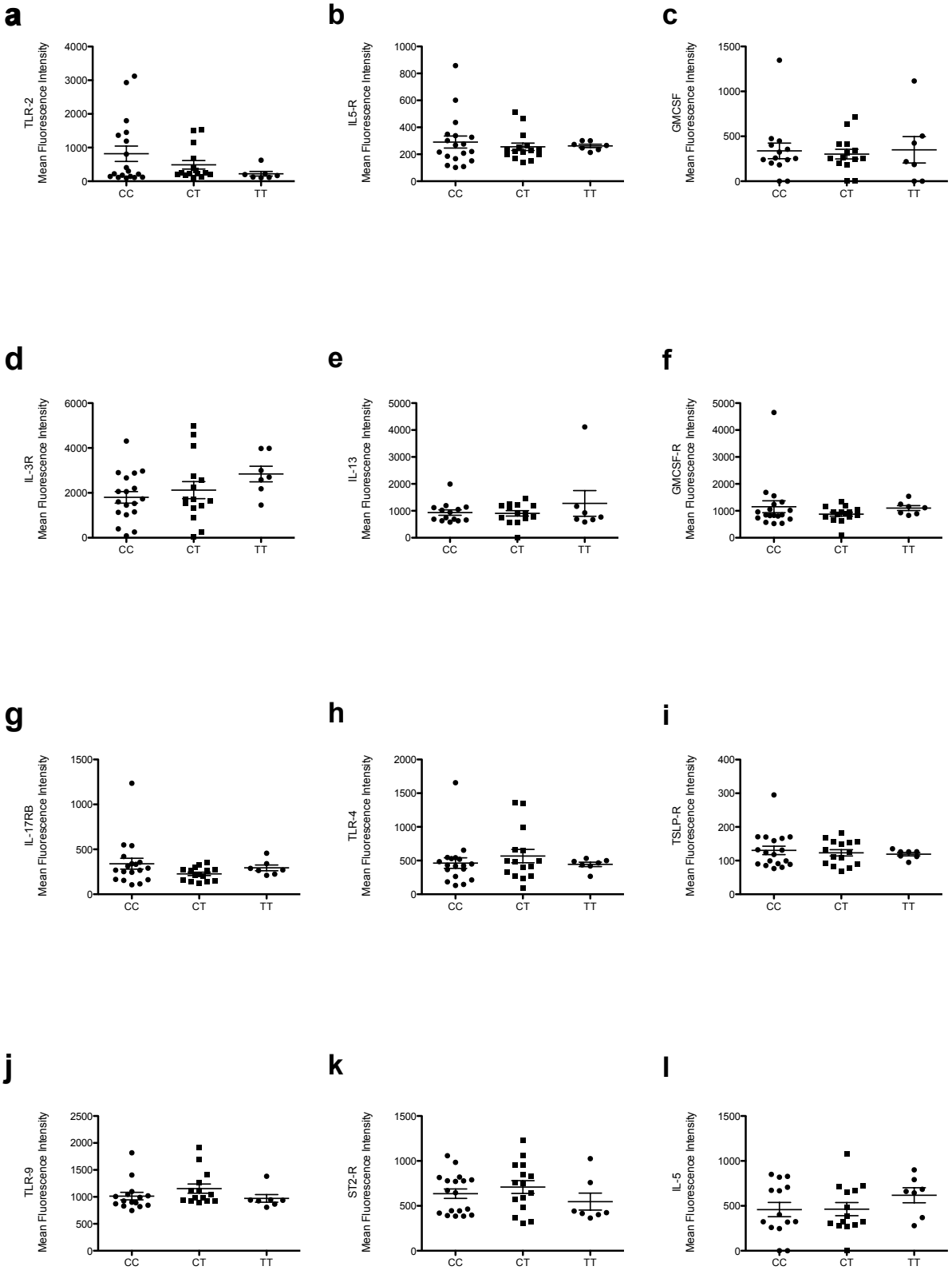
Figure 7: Peripheral blood mononuclear cell surface and intracellular cytokine expression in asthmatics (n=15) and non-asthmatics (n=25) using flow cytometry. Peripheral blood mononuclear cells were stained with antibodies for cytokines (a) TLR-2 (b) IL-5 receptor (c) intracellular GMCSF (d) IL-3 receptor (e) intracellular IL-13 (f) GMCSF receptor (g) IL-17 receptor B (h)TSLP receptor (i) TLR-9 (k) ST-2 receptor (l) intracellular IL-5. Students t-test was used to assess differences between groups.


a
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Mean Fluorescence Intensity
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IL5-R
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IL-5
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Figure 8: Peripheral blood mononuclear cell surface and intracellular cytokine expression according to TSLP rs1837253 genotype (CC=17), (CT=15), (TT=8) using flow cytometry. Peripheral blood mononuclear cells were stained with antibodies for cytokines (a) TLR-2 (b) IL-5 receptor (c) intracellular GMCSF (d) IL-3 receptor (e) intracellular IL-13 (f) GMCSF receptor (g) IL-17 receptor B (h)TSLP receptor (i) TLR-9 (k) ST2 receptor (l) intracellular IL-5. One-Way ANOVA with Tukey's post-test was used to assess differences between groups.

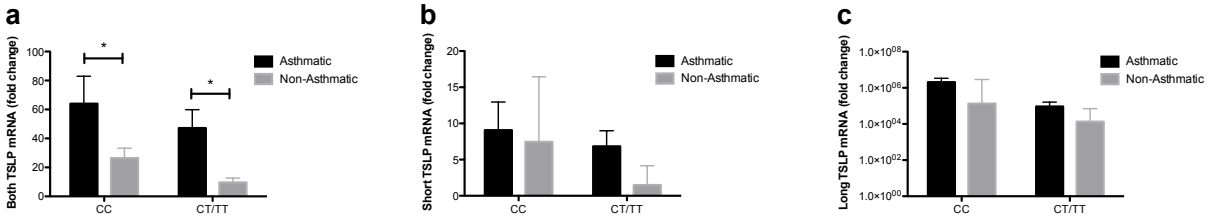

Figure 9: Relationship between genotype and asthmatic status in polyinosinic:polycytidylic acid (polyI:C)-induced secretion of thymic stromal lymphopoietin (TSLP). Gene expression analysis comparing the common isoform, the short and the long isoform of TSLP using RT-qPCR given the presence (CT/TT) or absence of the minor T allele (CC) in asthmatic subjects compared to non-ashtmatic subjects. Two-way ANOVA with Bonferroni post-test was used to assess differences between groups. (*$p<0.05$).

CHAPTER 4: DISCUSSION

Recent evidence suggests that genetic polymorphisms in the TSLP pathway may play a major role in the development of allergic disease and asthma. Genetic polymorphisms influence disease traits which manifest through various intermediary biological or clinical phenotypes. Information on these transitional stages is limited at the moment. Hui et al recently showed an association between rs1837253 genotype and the ex vivo production of TSLP, a crucial cytokine for the induction of T_h2 inflammatory responses in nasal epithelial cells. No association was found between atopic status and TSLP secretion (Hui et al., 2015). However, within the clinical context, the functional role played by rs1837253 SNP in TSLP has not been previously detailed. In this study, our aim was to examine TSLP secretion from NECs in response to ex vivo stimulation and identify differences in asthmatic TSLP production to those possessing the protective alleles—which could shed insight into the underlying involvement of rs1837253 in the pathogenesis of asthma and allergic disease.

Gene expression analysis of nasal epithelial cell cultures following polyI:C stimulation

Consistent with previous studies on small airway epithelial cells, NECs, and BECs from healthy and asthmatic individuals the present results indicate that exposure to dsRNA *in vitro* induces a time-dependent expression of the common, short and long isoform of TSLP mRNA in NECs (Hui et al., 2015). In this study, dsRNA induced differential TSLP secretion in NECs obtained from non-asthmatics compared with asthmatic individuals. To identify a potential method of TSLP regulation we investigated the expression of a key transcription factor, retinoic acid-inducible gene 1 (RIG-1) involved in regulating the viral response. A recent study found that in conjunctival epithelial cells, TLR-3 activation through polyI:C stimulation elicits elevated RIG-1 expression. Elevated RIG-1 following viral infection may play a role in the higher capacity of

asthmatic BECs to sense and respond to viral infections and thus to secrete more TSLP. Furthermore, the role that is played in a TLR3 stimulated TSLP asthma model has not been explored. Whether these regulators affect one another's expression is an interesting question, however the present data do not indicate an association between RIG-1 and the rs1837253 genotype (figure 3); this suggests other mechanisms underlying its effect (Gleich, 2000).

Previous data demonstrate that the long form of TSLP is highly inducible by polyI:C in BECs. A functional SNP, rs3806933, has been identified in the regulatory region of the TSLP gene that enhances the binding of AP-1, a prominent transcription factor in airway diseases that regulates the expression of multiple inflammatory proteins. Furthermore, the rs3806933 SNP influences AP-1-driven promoter activity of the long form of TSLP in BECs. AP-1 expression is enhanced in the asthmatic airway, consistent with reports that SNP rs3806933 is associated with adult asthma and childhood atopic asthma. Interestingly in our dataset, following polyI:C stimulation AP-1 expression was reduced (figure 2). To better understand this, other downstream regulatory factors need to be examined.

The central question addressed in this study is whether there is a difference among TSLP genetic polymorphisms on secretion of poly I:C-induced TSLP by asthmatic- vs. non-asthmatic-derived NEC. We addressed this by comparing non-asthmatic with asthmatic subjects possessing the different alleles of rs1837253 SNP. Due to the lower prevalence of the heterozygous minor allele within the population, we combined the heterozygous (CT) and homozygous (TT) minor allele data into one group, and then compared TSLP secretion ex vivo to the homozygous major (CC) allele group. Using two-way ANOVA, we analyzed the contribution of zygosity/genotype to the amount of TLSP secreted by asthmatic and non-asthmatic NEC (figure 9). Following polyI:C stimulation asthmatics secreted more TSLP protein, regardless of genotype, though considerably

less when comparing CT/TT to CC. There was a strong trend (p=0.0853) showing a difference in TSLP secretion by asthmatic vs. non-asthmatic status for subjects with the minor allele (CT/TT), compared with the difference between asthmatics and non-asthmatics who possess the major allele (CC), however due to the lower prevalence of the minor alleles in the asthmatic population it would be warranted to increase our sample size and power to further investigate this trend.

Interestingly, TSLP protein expression was elevated regardless of genotype, suggesting other driving factors linking TSLP expression and the development of asthma. These results do not preclude the validity of GWAS findings which indicate a protective effect for those carrying the minor (T) allele. Our findings indicate that there may still be a benefit through the reduction in total TSLP expression, yet it does put into question the degree of influence that TSLP rs1837253 genotype has on asthma development. Our results also disagreed with *cis*-eQTL analyses of 34 asthma genes performed by *Li et al.* in cells from human bronchial epithelial biopsy (BEC, n =107) and from bronchial alveolar lavage. In both bronchial epithelial cells biopsies and bronchial alveolar lavage from both asthmatic and non-asthmatic subjects the group found no differences in TSLP expression according to rs1837253 genotype (Li et al., 2015). Conversely, GSDMB expression based on an ORMDL3 SNP was significantly correlated in bronchial epithelial cells and cells derived from bronchial alveolar lavage. *Li et al.* also found multiple SNPs (including rs3806932, rs3806933, and rs2289276) in the TSLP-WDR36 region which were significantly correlated with the expression levels of TSLP (Li et al., 2015). Our study, in contrast, did show differences in nasal epithelial production of TSLP according to rs1837253 genotype, which highlights that the nasal airways contribute to the development in asthma as well through modulation of cytokine expression (figure 3). It should also be noted that *Li et al.* did not use a

dsRNA virally induced model which may also suggest that there may be differences in the regulatory mechanism that this SNP impacts, contributing to an altered viral response pathway.

Genome-wide expression analysis of baseline and rhinovirus-infected BECs from normal and asthmatic donors have identified a unique transcriptomic pattern at baseline and during rhinovirus infection (Bochkov et al., 2010). Complementing these genome-based observations, analysis of asthmatic BECs have also identified dysregulated innate immune responses and abnormal repair mechanisms that stimulate airway remodeling, both of which are considered critical drivers of the asthmatic phenotype (Freishtat et al., 2011). In characterizing the contribution of genetic components of allergic disease one may be able to better delineate the reason for the variety of phenotypes and endotypes typified by diseases such as asthma. Through patient categorization based on observable clinical characteristics, it may be easier to link the underlying molecular mechanisms of asthma to the present endotype. To date, primarily two endotypes of asthma are described, T_h2-high and T_h2-low. Patients with T_h2-high asthma have increased eosinophils in their sputum and airways, whereas T_h2-low asthma patients have either an increase in neutrophils or a paucigranulocytic (minimal inflammatory cells) profile in their sputum and airways. TSLP contributes to the T_h2-high endotype.

Further elucidation into rs1837253 involve understanding differences in function between the long and short form of TSLP. In a recent investigation by Wang et al. explored the differential roles of the TSLP isoforms performed in mice airway epithelium and human lung tissue. Results suggest that short TSLP prevents airway inflammation in a chemical-induced asthma model through the inhibitions of HMGB1-RAGE and long TSLP expression as well as through inhibition of STAT5(Y694) phosphorylation. In contrast, long TSLP is thought to be the pro-inflammatory agent. In this study, solely the long form seemed to have increased expression with presence of the

CC alleles of rs1837253. We subcategorized our subjects into an asthmatic group to identify the strength of the contribution of genotype to asthma when compared to non-asthmatic individuals. In our present study, we found significant differences between our asthmatic and non-asthmatic subgroup. A trend was found associating genotypic contribution to shared isoform TSLP expression, however our sample size was still small. When the protective short isoform and T_h2 driving long isoform were assessed no significant differences were found in expression. Due to the differential roles of the long and short form of TSLP it will be necessary to investigate the outcome of this increase in total TSLP expression as differences may have been undetected due to low power in our present study design, and thus further investigation would be merited. Finally, the question of whether this genotype contributes to asthma in a genotype dependent manner was assessed. No interaction was found however in our analysis putting into question the role that rs1837253 plays in asthma.

Protein expression levels derived from supernatants of polyI:C stimulated nasal epithelial cells.

The epithelial master regulators of allergic inflammation IL-33, IL-25, and thymic stromal lymphopoietin (TLSP) stimulate the innate or adaptive immune system to secrete cytokines IL-4, IL-5, and IL-13 (T_h2 cytokines). The accumulation of these type 2 cytokines stimulates key inflammatory cells, such as eosinophils, mast cells, and basophils. Both IL-4 and IL-13, through the activation of the transcription factor GATA3, regulate T_h2 inflammation. IL-13 and IL-4 are both involved in activation of B-cell isotope switching to produce immunoglobulin E (IgE). IL-5 is central for eosinophilic development, survival, and chemotaxis. Innate lymphoid type 2 cells (ILC2) are also involved in the production of IL-5 and IL-13. Type 2 cytokines also contribute to mucous cell hyperplasia and fibrosis leading to airway remodeling (Tabatabaian et al., 2017).

Furthermore, higher numbers of eosinophil/basophil colony-forming units are observed in the blood of atopic asthmatics following allergen-inhalation challenge. It has been shown previously that mature basophils and eosinophils synthesize cytokines important to allergic inflammation (Gauvreau et al., 1998). Tumor necrosis factor-α (TNF-α) is an important cytokine in the innate immune response that directly attacks tumor cells but has no toxic effect on normal cells, which incites an inflammatory response. Previous experiments have found that TNF-α plays a critical regulatory function in activating the airway inflammation associated with asthma. Likewise, anti-TNF-α therapy has had some success, although limited due to side effects as a biologic used to treat asthma. Very similar to TNF-α, interleukin-8 (IL-8) is an anti-inflammatory cytokine which has a regulatory effect on the airway inflammation caused by asthma (Jiang et al., 2017; Tan et al., 2016). To assess the potential role of growth factors and regulatory cytokines in allergic disease we examined the expression of the hematopoietic cytokines, GMCSF, IL-5, IL-8, and TNF-α to determine whether nasal epithelial cells are contributing to eosinophil or basophil formation or recruitment. In our analysis, we found no difference in protein expression of these key hematopoietic and regulatory molecules from NECs when comparing asthmatics to non-asthmatics and when comparing different genotypes of rs1837253 (figures 5,6). This suggests that the epithelium does not affect the differentiation of circulating progenitors through altered production of hematopoietic cytokines due to differences among rs1837253 alleles. In our analysis of TSLP we found differences in the time dependent expression of TSLP protein and the genotype dependent expression of TSLP protein (figures 5,6). This correlates with our transcript level findings. Our assay was not equipped to detect the different isoforms of TSLP, hence validation of gene expression results highlighting differences between the short and long form of TSLP remains an important question to be investigated.

Surface receptor expression levels derived from peripheral blood mononuclear cells using flow cytometry.

Peripheral blood conditioned with TSLP skews the adaptive immune responses to a pathogenic and pro-allergic T_h2 type. CD8+ T cells expanded by TSLP-conditioned DCs generate the hallmark T_h2 cytokines IL-5 and IL-13. Danger signals produced by the epithelium and secreted toward underlying tissues alert many cell subsets to the presence of pathogens/allergens. Thus DCs are not the only population of innate immune cells that responds to TSLP, type 2 innate lymphoid cells also robustly upregulate T_h2 cytokines IL-4, IL-5, and IL-13 in response to TSLP alone or in synergy with other cytokines. Thus, to better understand whether altered secretion of TSLP changes a snapshot of the adaptive immune response, flow cytometric analysis on key peripheral blood mononuclear cell markers was performed.

Although levels of expression of toll-like receptors, cytokine receptors and intracellular cytokines were investigated using flow cytometry. No significant differences were found between asthmatic and non-asthmatic subjects or between subjects carrying different genotypes of rs1837253 (figure 7,8). We investigated a variety of peripheral blood mononuclear cell surface receptor markers and intracellular markers. This suggests rs1837253 acts local to the epithelium and does not alter circulating cell surface expression levels in the blood. The effects on circulating progenitors may be acute following viral infection. Thus, it may be necessary to stimulate the isolated peripheral blood mononuclear cell population with polyI:C to elicit an effect that may bring genotypic differences in immune regulation to the surface. When looking at airway remodelling genes, Wieczfinska and Pawliczak found a significant increase in the expression of TGF-β1 and arginase 1 on the mRNA and protein levels following viral stimulation. This effect was inhibited by apocynin, though only on the mRNA level. TIMP-1 expression was not

influenced by human rhinovirus; however, apocynin caused a significant increase of TIMP-1 mRNA expression. TSLP increased the expression of TGF-β1 and arginase 1 mRNA in fibroblasts, but not in peripheral blood mononuclear cells, thus it may have been necessary to introduce a viral insult in our experiments to observe a response *in vitro* (Wieczfinska and Pawliczak, 2017). Alternatively, a co-culture model exposing peripheral blood mononuclear cells to polyI:C treated airway cells may better simulate the *in vivo* environment and elicit any differences which are not native to the hematopoetic milieu of individuals with asthma (Horvath et al., 2011).

Conclusions

There is increasing evidence connecting TSLP polymorphisms to the development of asthma. Inverse associations between the T allele of rs1837253 and asthma have been previously reported. Hui et al recently showed an association between rs1837253 genotype and the ex vivo production of TSLP, a key cytokine for the provocation of T_h2 inflammatory responses in nasal epithelial cells. No association was found between atopic status and TSLP secretion (Hui et al., 2015). However, within the clinical context of asthma, the role played by rs1837253 SNP in TSLP has not been previously detailed. We demonstrated that asthmatic nasal epithelium produce more TSLP than controls. There was also a trend showing differential TSLP mRNA production by genotype in asthmatic subjects compared to controls. We also demonstrated increased time dependent expression of TSLP protein following polyI:C stimulation and an increase in TSLP secretion in asthmatic individuals irrespective of genotype. No differences were identified in the immune modulatory cytokine expression (GMCSF, IL-5, IL-8, and TNF-α) and no differences were found in cell surface receptor expression (TLR-2,4,9, GM-CSFR, IL-3R, IL-5R, & ST-2R) of our subjects. Thus, in nasal epithelial cells, we demonstrated that:

1) TSLP is over-expressed following TSLP expression in a time-dependent manner.

2) The minor rs1837253-T allele was associated with decreased TSLP expression, for the long isoform but not the short isoform, consistent with an asthma-protective effect.
3) TSLP expression was higher in asthmatics regardless of genotype, however there was a trend showing a difference in TSLP secretion by asthmatic vs. non-asthmatic status for subjects with the minor allele (CT/TT), compared with the difference between asthmatics and non-asthmatics who possess the major allele (CC).
4) There was no interaction between rs1837253 genotype and asthmatic status suggesting independent mechanisms driving TSLP associated asthma development.

Our study suggests that rs1837253 acts in an additive manner influencing TSLP production according to genotype contributing to the T_h2 biased phenotype in asthmatic nasal epithelial cells. This highlights the complexity of gene environment interactions in allergic disease and points to alternate directions for further investigation into the mechanism of control of TSLP secretion and the subsequent development of asthma and associated allergic diseases.

Limitations

Here we described the effects of dsRNA on NEC-derived TSLP production and associated levels of cytokine expression in asthmatic and non-asthmatic subjects. There were several limitations faced in the study design. Due to the low frequency of the minor alleles within the asthmatic subject population gathered in a limited time frame, differences in the phenotype of asthmatics carrying the most protective form of the rs1837253 polymorphism were characterized in a limited capacity as the study did not reach power. This limits the conclusions we can draw on the role of TSLP in asthma, yet still offers valuable insights into the degree to

which TSLP SNPs influence the expression of TSLP. It also offers insight into the circumstances under which these differences become apparent which may bear significant relevance in the clinical setting as it helps better define the root causes of disease and perhaps uncover a deeper understanding of the interplay between gene environment interactions.

Due to the ease of acquisition and manipulation of nasal epithelial cells, bronchial epithelial cells were not used. While the use of bronchial epithelial cells would offer a more accurate representation of the asthmatic disease model, using a smoking model Sridhar *et al*. showed that bronchial and nasal epithelium from non-smokers were most similar in gene expression when compared to other epithelial and non-epithelial tissues, with several antioxidant, detoxification, and structural genes being highly expressed in both the bronchus and nose (Sridhar et al., 2008). Furthermore the link existing between the upper and lower airways has been observed repeatedly in the past bolstering the united airway concept airway (Bellanti and Settipane, 2014; Håkansson et al., 2015; Papadopoulou et al., 2014). This high degree of similarity and relatedness in asthma pathology makes the nasal epithelium the ideal surrogate for bronchial epithelial cells in our asthma model.

Future Directions

In our study, we demonstrated diminished TSLP secretion in NEC derived from individuals who are carriers of the protective rs1837253 T allele in an RIG-1 and AP-1 independent manner; however, the mechanism for reduced TSLP expression remains unclear. We also outlined that asthmatic nasal epithelium produces TSLP in different amounts. There was also a trend showing differential TSLP mRNA production by genotype in asthmatic subjects. There was no significant difference in the amount of TSLP expression by genotype in asthma compared to controls. Perhaps this indicates that other SNPs in the TSLP gene contributed to the enhanced TSLP phenotype and

that rs1837253 acts in an additive manner contributing to the T_h2 biased phenotype. Understanding the regulatory mechanisms of TSLP secretion is the next major hurdle.

TSLP has recently been shown to be a methylation-sensitive gene (Luo et al., 2014; Wang et al., 2013). The methylation status of TSLP was significantly associated with prenatal tobacco smoke exposure and was further associated with childhood AD. Moreover, TSLP 5‘-CpG island methylation was inversely related with its downstream TSLP protein secretion (Wang et al., 2013). In a more recent study, Luo et al. reported DNA hypo-methylation of the TSLP promoter region, which was accompanied by overexpression of TSLP in skin lesions from patients with AD compared with controls (Luo et al., 2014). Further investigation into SNP rs1837253-associated epigenetic alterations in airway epithelial cells will be critical in understanding the mechanisms underlying the involvement of rs1837253 in regulating TSLP secretion. Furthermore, given that SNP rs1837253 showed the greatest association with asthma, continued research on the effects of rs1837253 genotype on TSLP secretion in different phenotypes of asthma, such as asthma severity, will help unravel the association. Although the antagonist of TSLP seems promising as a therapy for allergic airway inflammation (Gauvreau et al., 2014), reaching a greater understanding of the molecular pathogenesis of asthma and allergic diseases will eventually pave the way for novel therapies targeting the source of inflammation rather than life-long therapies aimed at dampening inflammation and easing symptoms.

Due to the similarity of immunological dysregulation between asthma and chronic rhinosinusitis/nasal polyposis, another interesting investigation would be to identify a common connection in altered TSLP expression in different TSLP gene polymorphisms. It was previously reported that epithelial cells derived from nasal polyps of patients with CRSwNP release TSLP and IL-25 when specifically stimulated by poly(I:C). Eosinophilic and non-eosinophilic CRSwNP

also have distinct epithelial endotypes regarding TSLP/TSLPR/IL-7Ra and ST2 expression (Liao et al., 2015).. The positive feedback loop between TSLP, IL-33 and their receptors, and T_h2 responses can facilitate T_h2-skewed eosinophilic inflammation in CRSwNP. The authors found that the increased expression of IL-25 they observed in nasal polyp tissues of patients with CRSwNP correlated with worse computerized tomography scores and blood eosinophilia (Boita et al., 2016). Poly(I:C)-induces expression of TSLP in primary nasal epithelial cells from polyposis patients only and that a deregulation of the expression of the DUSP-1 transcription factor may play a role in this process. No other TLR agonists nor primary healthy epithelial cells show the induction of this T_h2 skewing mediator. Similar to the observations made in our present asthma study, perhaps investigation into rs1837253 in the context of CRSwNP may lead to observations that open up a way to explore the involvement of viral infections as a causative or contributing factor to the T_h2-skewed inflammatory environment in polyps or to the pathogenesis of nasal polyposis (Golebski et al., 2016).

Thymic stromal lymphopoietin (TSLP) may have dual immune-regulatory roles. In inflammatory disorders of the bowel, the long isoform of TSLP promotes inflammation while the short isoform inhibits inflammation (Dong et al., 2016). In our study our gene expression analysis was underpowered to detect differences in long and short form expression, however trends were evident of a bias towards long for expression in our genotype of interest. We were also unable to distinguish between the long and short form in our protein expression analysis. Delineating the precise effects of rs1837253 on the isoforms of TSLP is essential in outlining what functional implications stem from altered TSLP expression due to differences in genotype.

Allergic diseases are characterized by tissue eosinophilic and basophilic inflammation. There is substantial evidence that this particular inflammatory profile results from the migration

to tissues of a common eosinophil/basophil (Eo/B) progenitor which then undergoes a differentiative process regulated by local cytokines. Investigating effects on CD34+ differentiation through the use of ALI tissue culture inserts in a co-culture model to determine the effects on eosinophil and basophil colony formation. If asthmatics have an increased propensity for progenitor differentiation in the presence of nasal epithelium of asthmatics with the C allele, it may offer unique insight into the effects of TSLP on hematopoietic progenitor cells.

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