

THYMIC STROMAL LYMPHOPOIETIN: EXPRESSION AND SECRETION BY AIRWAY EPITHELIUM AS A FUNCTION OF GENOTYPE

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AIRWAY EPITHELIAL-DERIVED
THYMIC STROMAL LYMPHOPOIETIN

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AND SECRETION BY AIRWAY EPITHELIUM AS A
FUNCTION OF GENOTYPE**

By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

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DOCTOR OF PHILOSOPHY (2014)

McMaster University

(Medical Sciences)

Hamilton, Ontario

TITLE: Thymic stromal lymphopoietin: expression and secretion
by airway epithelium as a function of genotype

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NUMBER OF PAGES: xvi, 142

ABSTRACT

Thymic stromal lymphopoietin (TSLP) is a pleotropic cytokine highly implicated in the pathophysiology of asthma and allergic diseases. Although there are robust data regarding the associations of TSLP polymorphisms with the development of allergy and asthma, there is very little information on how these *TSLP* variants functionally affect downstream effector pathways and disease phenotype. The overall objective of this thesis was to investigate how *TSLP* polymorphisms are linked to alterations in TSLP secretion and subsequent downstream cellular events. Initially, we investigated the influence of innate and adaptive stimuli on epithelial-derived TSLP expression and secretion, including effects on dendritic cells (DC). We show that polyinosinic:polycytidylic acid (polyI:C) and allergen-specific T cells induced enhanced TSLP secretion from asthmatic bronchial epithelial cells (BEC) compared to non-asthmatic BEC. Furthermore, activated-BEC culture supernatants induced TSLP-dependent CCL17 production from monocyte-derived DC in relation to clinical asthmatic status (Chapter 2). Next, we examined effects of TSLP on hemopoietic progenitor eosinophil-basophil (Eo/B) differentiation, demonstrating enhanced TSLP-mediated hemopoiesis *ex vivo* in relation to clinical atopic status. We further demonstrated p38MAPK-dependent autocrine signaling by TNF α in TSLP-mediated human Eo/B differentiation *ex vivo* (Chapter 3). Lastly, to explore the potential functional consequences of a key variant of the *TSLP* gene, we investigated associations between the rs1837253 *TSLP* variant and *ex vivo* production of TSLP in nasal epithelial cells (NEC). We showed that NEC derived from individuals with the “protective” minor allele have diminished TSLP secretion, which suggests that this rs1837253 polymorphism may be directly involved in the

regulation of TSLP secretion (Chapter 4). The novel work presented herein provides further evidence for TSLP regulation of distinct immunological pathways in allergic immune inflammatory airway responses initiated at the epithelial surface, and thus (by implication) of allergic disease. These observations support the concept that TSLP is potentially an important biomarker and therapeutic target for allergic diseases characterized by increased Th2 and/or eosinophilic-basophilic inflammation. Continued investigations into the functional mechanisms linking *TSLP* variants to allergic disease phenotype are of critical importance.

ACKNOWLEDGEMENTS

One year of preschool, two years of kindergarten, eight years of elementary school, four years of high school, four years of undergrad, two years of MSc, four years of PhD - 25 years later, I think I am finally done! These last four years have by far been the hardest – my intelligence, literacy, rationalism, creativity, discipline, tenacity, and sheer nerdiness have all been pushed beyond limits. But I survived. Thank you to my incredible support system.

First and foremost, I would like to thank Dr. Judah Denburg. You have been a wonderful supervisor, mentor and role model. You are an inspiration and a living example of what you teach. Through you, I learned so much about diligence, perseverance and determination – attributes that are not only crucial to a scientist, but also in life. Thank you for your continuous support and guidance and most of all, for always challenging me and having my best interest at heart. Your toughness has molded me into the person I am today.

Next, I would like to express my gratitude to my committee members for their time and commitment. To Dr. Mark Larché – there were numerous occasions when I knocked on your door and you immediately stopped typing and pretended *not* to be in your office; we all know how tremendously busy you are. Thank you for taking the time to mentor me throughout my graduate studies, your time and effort are much appreciated. To Dr. Helen Neighbour - your clinical insight and expertise made much of this work possible. Thank you for putting in the extra effort to help ease my OCD. To Dr. Gail Gauvreau - your input and feedback throughout my graduate studies were invaluable – thank you.

A special thanks to the Department of Medicine, the AllerGen Network Centre of Excellence and the Harsham Fellowship Society for their financial support.

To the students and staff of the Denburg, Larché and Cardiovascular Labs, thank you for all your assistance, from troubleshooting to agreeing to be my test subjects. To the most *awesome* lab tech and ‘housemate,’ Delia - thank you for all the early morning talks, for keeping on top of my lab orders (including cream and sugar), and most of all, for keeping

me sane when experiments failed, equipment malfunctioned and manuscripts were rejected. To my wonderful minions, Sina and Ashley - thank you for being flexible with my lab hours and coming in, or trying to, at 6 a.m. every day. Without a doubt, you have both, along with Richard, lost a few friends along the way while helping me recruit subjects. Thank you! A special thank you to Lynne, who subjected my writing to rigorous scrutiny, which vastly improved the quality of my thesis, manuscripts, grants and abstracts, and added to its readability. To the most meticulous and organized secretary, Monica - thank you for filing my expenses and holding those rare free dates in Dr. Denburg's agenda for me. My time at the lab would not have been the same without any one of you.

Next, I would like to thank my friends and family. A special thanks to all the troopers - friends (Jacky, Jen, Matty, Ray C., Ray Y. and Sonya) and family (Ada, Chole, Collins, Kelvin, Ken, Rosita, Vincent and Vico), who sacrificed their blood for science. To my wonderful friends who have been there from day one (Angie, Beta, Gaby, Jacky, Ritchie and Sonya) - thank you for your support, encouragements and inspirations, and most of all, for sticking around even when I was MIA for months at a time. To the new friends I made along the way (Christina, Helen, Henry and Jason) – thank you for all the late night Micky Dees. To my amazing boyfriend, Lloyd - thank you for your love and patience, for keeping me company while I worked on the seemingly never-ending grants and papers, for the many trips you made to work to print me my articles, for overcoming your fear of needles, for waking me up from my naps, for taming my hanger, and most of all, for putting me and my studies first – even during times when you did not want to. You are so lucky!

Finally, to my incredible parents - thank you for your continuous love, support and guidance, and for anxiously, but patiently, waiting for me to finally finish school – this thesis is dedicated to the both of you.

Words fail to express the gratitude I feel; I have truly been blessed by every single one of you. Thank you from the bottom of my heart!

<3 Claudia

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LIST OF ABBREVIATIONS

AD	Atopic dermatitis
ADEH	AD eczema herpeticum
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
ECP	Eosinophil cationic protein
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
IFN γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2 innate lymphoid cells
IL-7R α	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
TNF α	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 ACHIEVEMENT

The research documented herein is presented as a “sandwich doctoral thesis.” The three articles presented in Chapters 2-4 are three independent, but thematically related bodies of work that, as of June 2014, have been or are being peer-reviewed prior to publication. Although I was the major contributor for the work presented in this thesis, it required a collaborative effort from several individuals. As such, my contributions, along with those who assisted with the content of each article, are highlighted below.

Chapter 2: Hui CCK, Murphy DM, Neighbour H, Al-Sayegh M, O’Byrne S, Thong B, Denburg JA, Larché M. T cell-mediated induction of thymic stromal lymphopoietin in differentiated human primary bronchial epithelial cells. *Clinical and Experimental Allergy*. 2014 Jul; 44(7):953-64. doi: 10.1111/cea.12330.

This study started during my time as a Master’s student and was continued during my time as a PhD candidate due to its relevance to my thesis. Dr. M. Larché and I were responsible for the experimental design of the study as well as interpretation of the data. Drs. D.M. Murphy and H. Neighbour performed bronchoscopies and collected bronchial epithelial cell samples. I performed all the experiments with assistance provided by students whom I supervised (Dr. M. Al-Sayegh and S. O’Byrne) and a lab technician (B. Thong). With assistance from Drs. M. Larche and J.A. Denburg, I wrote and prepared the manuscript for publication. Together, we revised the paper and replied to comments from the reviewers to achieve acceptance and publication.

Chapter 3: Hui CCK, Rusta-Sallehy S, Asher I, Heroux D, Denburg JA. The effects of thymic stromal lymphopoietin and IL-3 on human eosinophil-basophil lineage commitment: relevance to atopic sensitization *Immunity, Inflammation and Disease*. 2014 Jun; 2(1):44-55. doi: 10.1002/iid3.20.

Dr. J.A. Denburg and I were responsible for the experimental design of the study as well as interpretation of the data. I performed all the experiments with assistance from an undergraduate student whom I supervised (S. Rusta-Sallehy) and a lab technician (D. Heroux). Drs. Steven Smith and Tom Mu provided valuable technical assistance with histochemical staining and flow cytometry, while L. Wiltshire was instrumental with the technical aspects of the LUMINEX assays. I wrote and prepared the manuscript for publication. L. Larocque assisted in editing of the manuscript. Together, Dr. J.A. Denburg and I revised the manuscript and the responses to comments from the reviewers.

Chapter 4: Hui CCK, Yu A, Heroux D, Akhabir L, Sandford AJ, Neighbour H, Denburg JA. Thymic stromal lymphopoietin (TSLP) secretion from human nasal epithelium is a function of TSLP genotype. Submitted in June 2014 to *Mucosal Immunology*.

I, together with Drs. H. Neighbour and J.A. Denburg, was responsible for the experimental design and interpretation of the data. Dr. H. Neighbour 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 the help of an undergraduate student I supervised, A. Yu. D. Heroux assisted with the skin prick test of the subjects and generation of the gene expression data. I conducted all the experiments while A. Yu greatly assisted with the epithelial cell cultures. L. Akhabir genotyped all the mouthwash samples. I wrote and prepared the manuscript for publication with the assistance of A. Yu. L. Akhabir and Drs. M. Larché, H. Neighbour and A.J. Sandford contributed valuable scientific input and critically appraised the manuscript, while L. Larocque, Dr. J.A. Denburg and I were involved with the editing of the manuscript.

CHAPTER 1: INTRODUCTION

In recent decades, there has been a global increase in the prevalence of immune-mediated chronic inflammatory diseases - allergic diseases, such as asthma, allergic rhinitis (AR) and atopic dermatitis (AD); and, autoimmune diseases, such as diabetes, arthritis and inflammatory bowel disease, which greatly disrupt the lives of those affected. The underlying causes for the increasing prevalence - even “epidemic” - of allergic diseases are not well understood. Many have attributed this rise to be a result of complex gene-environment interactions [1]. While a family history of atopic disorder(s) is an independent risk factor for the development of the same in the offspring [2, 3], the rapid increase in allergic incidence rates strongly argues for a substantial role of rapid lifestyle and environmental changes in industrialized countries, as opposed to genetic predisposition alone. For allergic diseases, the latter has been articulated as the “hygiene hypothesis,” according to which increased hygiene/cleanliness may have deprived the immune system of bacterially and virally-evoked T helper (Th) type 1 (Th1) immune responses during early childhood, thus altering the lifelong trajectory of the immune response towards a Th2 bias [4]. However, studies have reported that Th1-mediated autoimmune and Th2-mediated atopic diseases are not mutually exclusive, but associated [5, 6]; which may help explain, in part, the paradoxical rise in Th1-driven autoimmune diseases. Current evidence suggests that the Th1/Th2 paradigm may be oversimplified and that gene-environment interactions may be critical in determining allergic sensitization and its clinical outcome [7].

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 [8]. Two other GWAS, the phase II GABRIEL study [9] and the EVE study [10], later identified associations between asthma and single nucleotide polymorphisms (SNPs) in four loci (thymic stromal lymphopietin (TSLP), IL-33, IL1RL1, 17q21 locus). 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 Th2 immune responses – was highly significantly, *inversely* associated with allergic asthma and related traits [11]. It has been demonstrated that TSLP is upregulated in bronchial biopsies from asthmatic individuals [12]; and its secretion, with subsequent allergic inflammatory responses, can be further induced by various stimuli such as viruses and allergens [13]. Elevated levels of TSLP can subsequently further skew Th1/Th2 balance by promoting Th2-type responses through activation of dendritic cells (DCs) [14] as well as hemopoietic progenitor cells [15]. Given the critical immuno-modulatory role of TSLP in allergic inflammation, as well as TSLP effects on CD34+ progenitor cytokine and chemokine secretion, *we proposed to examine the effects of exogenous (toll-like receptor (TLR)-ligation) and host-derived (pro-inflammatory) stimuli on epithelial cell-derived TSLP secretion. Furthermore, we will examine the association of these processes with atopic sensitization and/or asthmatic status; and, the effects on CD34+ human hemopoietic progenitor (HHP) differentiation of the rs1837253 TSLP genetic variant.* 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.

1.1 Asthma and Allergic Diseases

Atopy refers to the tendency to produce immunoglobulin (Ig)-E antibodies to environmental substances known as allergens. 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 [16]. 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 [17], the prevalence of atopic sensitization in asthma have been reported to be $\leq 50\%$ [18]. Therefore, not everyone with atopy develops clinical manifestations of allergies and similarly, not everyone who has clinical manifestations of allergic-type inflammation is atopic [19].

1.1.1 Allergic Immune Responses

Allergic reactions are a result of biased Th2-immune responses and are associated with IgE synthesis, maturation and activation of mast cells, basophils and eosinophils [17]. The development of an allergic response begins with sensitization, which occurs during the body's initial exposure to an allergen. This process results in the development of unique IgE antibodies that bind to high affinity specific receptors on mast cells and basophils (Fc ϵ RI), leading to degranulation and an acute allergic reaction. Once sensitization has occurred, subsequent exposure to the same allergen is likely to give rise to the immediate, or early-phase reaction (occurs within minutes of allergen exposure). The immediate reaction is triggered when the allergen interacts with the preformed membrane bound IgE, resulting in

the release of both preformed and newly formed mediators in mast cells and basophils, such as histamine, tryptase, leukotrienes, prostaglandins, as well as an array of cytokines such as interleukin (IL)-4, IL-5, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF α). These mediators enhance mucus production, contraction of airway smooth muscle (ASM) and vasodilatation, leading to typical skin reactions such as urticaria or wheal-and-flare reactions, sneezing and nasal blockade, wheezing and bronchoconstriction or coughing – features of the early-phase reaction [17].

In some individuals, a late-phase occurs approximately 4-8 hours after the early phase, and is characterized by the recruitment of effector cells such as eosinophils, neutrophils, basophils and CD4⁺ T cells to tissues such as the airways [17]. Once at the site, antigen presenting cells such as DCs process and present allergens as peptides to naïve CD4⁺ T cells through major histocompatibility complex (MHC) class II molecules [20]. Additionally, antigen presenting cells also aid in driving the development of Th2 cells from naïve T cells, which depends on an array of factors including antigen type and dose, T-cell receptor interaction and co-stimulatory signals [21]. Subsequently, the newly differentiated Th2 cells release IL-4, IL-5, IL-9 and IL-13, which further stimulate the production of IgE and promote the infiltration and maturation of mast cells, eosinophils and neutrophils into the airways [17, 22, 23]. Eosinophilic airway inflammation is a prominent feature of the late response [24]. The newly recruited eosinophils, once activated, release a range of pro-inflammatory and cytotoxic mediators, including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), as well as leukotrienes, resulting in contraction of ASM and mucus production, leading to bronchoconstriction and airway hyperresponsiveness (AHR) (Fig. 1) [25, 26].

1.1.2 *Allergic Asthma*

Asthma may be a serious and occasionally, life-threatening respiratory disease with clinical, pathological and physiological characteristics. The main clinical features of asthma are shortness of breath, often accompanied by cough and wheezing. Allergic asthma is the most common type of asthma [27], and is a result of prior allergic sensitization to a specific allergen, with a reaction in the airways to the allergen after inhalation, leading to asthmatic symptoms [17]. An estimated 300 million individuals worldwide have been diagnosed with asthma, with an expected 100 million more by 2025 [28, 29].

Pathophysiologically, asthma is a chronic respiratory disease that is characterized by variable airway obstruction and an increase in airway responsiveness to a range of stimuli, such as allergens and methacholine, accompanied by airway inflammation and remodelling [30]. Allergic asthma is considered to be a result of disruption to the Th1 and Th2 balance, eventuating in the above respiratory pathophysiology, as it is readily associated with IL-4, IL-5 and IL-13 producing Th2 cells, which can lead to increased tissue numbers of activated mast cells and/or eosinophils [31]. The chronic inflammation seen in asthmatics has been hypothesized to result in structural remodelling of the airways [32], such as ASM hypertrophy and hyperplasia [33], subepithelial fibrosis [34], epithelial shedding, mucous gland hyperplasia, edema and infiltration of inflammatory cells [35]. Collectively, these changes contribute to thickening of the airway, and subsequently, reduction of the airway diameter [36, 37]. In both clinical and research laboratories, diagnosis of asthma can be made by measuring AHR via inhalation challenges with direct (histamine and methacholine) or indirect (allergen, cold air, exercise) airway constrictor agonists [38].

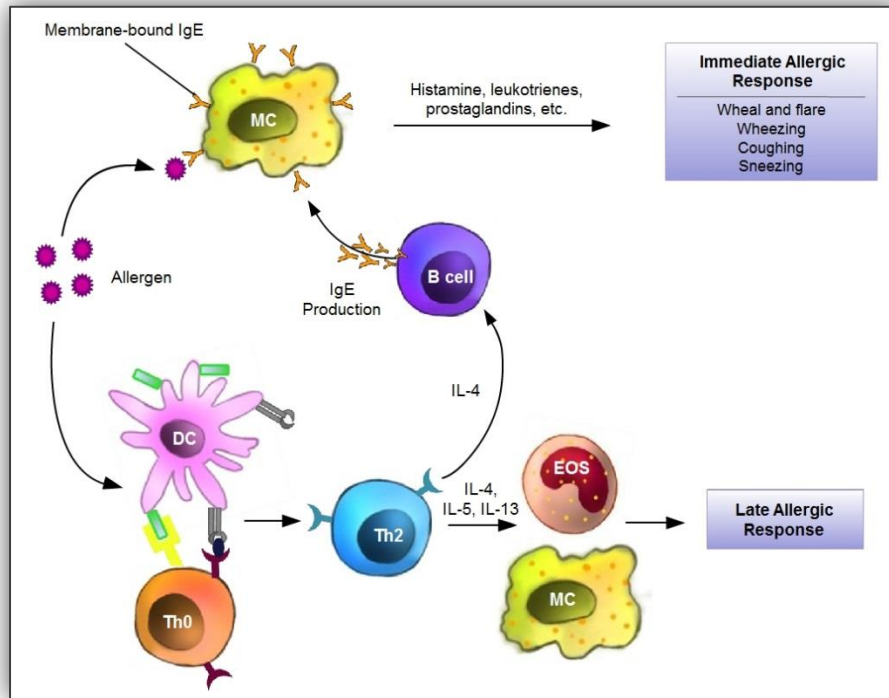


Figure 1. Pathways leading to the immediate and late allergic response. Antigen-induced release of histamine and lipid mediators from mast cells results in immediate allergic response. The late allergic response depends on multiple pathways including infiltration of eosinophils and release of MC products (adapted from [17]).

1.1.3 *Th2 Cytokines in Asthma and Allergic Diseases*

It is widely recognized that Th2 cytokines such as IL-4, IL-5 and IL-13 drive the allergic inflammatory response while Th1-type cytokines such as interferon gamma (IFN γ) and IL-12 antagonize Th2 immune responses [39]. CD4⁺ Th2 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 [40-43]. As such, messenger ribonucleic acid (mRNA) expression for many signature Th2 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 [44-46]. 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 [46]. 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 [40, 47]. Similar to IL-13, IL-9 has been reported to induce airway inflammation, mucus hyper-secretion, as well as AHR [48]; elevated expression of IL-9 in allergic asthmatics has been shown to correlate with AHR [43]. Lastly, IL-9 has been shown to enhance eosinophil survival and IL-5-mediated eosinophil (and mast cell) differentiation and maturation [49], suggesting that IL-9 may potentiate eosinophil function *in vivo* [50].

Aside from classical Th2 cytokines, epithelial cell-derived cytokines such as TSLP, IL-25 and IL-33, which are elevated in the airways of asthmatics [12, 51], are secreted following epithelial stimulation, tissue damage, pathogen pattern recognition or allergen exposure, and have the capacity to initiate Th2 responses at mucosal sites [52]. The *IL-33* and *TSLP* genes have emerged as two of the strongest associations for the development of asthma and related traits [9, 11]. The most established mechanism for TSLP-driven Th2 inflammation is via the TSLP-DC axis whereby CD4⁺ T cells are primed towards a Th2-biased immune response [14]. IL-33, which has been shown to induce TSLP production from epithelial cells [52], has been reported to induce eosinophilia, secretion of IgE and Th2 cytokines in mice [53]. In addition to driving Th2 cytokine, IL-4, IL-5 and IL-13, production in T cells [54], IL-25 has also been shown to induce eosinophilia in murine

models [55, 56] while delaying apoptosis of human eosinophils [57]. Furthermore, polymorphisms in the IL-25 receptor (IL-17RB) have been associated with asthma [58]. 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 (MPP_{type2}) cells, nuocytes and type 2 innate lymphoid cells (ILC2) that produce IL-5 and IL-13 and also establish a Th2-biased immune response, including mucus production and eosinophilia [59-62]. 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 Th2 cytokines involved in allergic responses.

Table 1. Th2 cytokines in allergic asthma

Cytokine	Major cellular source	Major cellular targets	Actions
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, 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 hyperresponsiveness.

1.1.4 Genetics of Asthma and Allergic Diseases

While expression of allergic diseases appears to be a result of both genetic and environmental factors, many allergic diseases are familial, and an increased risk can be conferred genetically [3]. Two main features of allergic disease/asthma involve the predisposition for allergen-specific IgE production and development of allergic inflammation, both of which have been highly correlated with disease severity [17]. Chromosome 5 has been implicated in the regulation of total serum IgE levels, as polymorphisms of genes on chromosome 5, including the gene for β chain of Fc ϵ RI [63] and the IL-4 family of cytokines [64], may contribute to allergic diseases. Another gene with implications to allergic diseases is *CD14*, which encodes for the high-affinity receptor for bacterial lipopolysaccharide (LPS) [65], exists as a membrane molecule on monocytes and macrophages (mCD14) and as a soluble form (sCD14) in serum [66]. A polymorphism in the *CD14* gene has been associated with elevated sCD14 and lower total serum IgE levels [67] and may be involved in certain IgE-‘protective’ TLR-mediated responses. Elevated levels of sCD14 have the ability to influence the Th1/Th2 balance by antagonizing the pro-inflammatory response of mCD14 expressing cells to LPS via IL-12 secretion from CD14⁺ DCs, favouring Th1 responses, and thus reducing the probability of an IgE response to allergens [67].

Analyses of TLR gene variants have revealed that SNPs in TLR1, TLR6 and TLR9 have protective effects on atopic asthma in children [68]. 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 Th2-associated processes [69], have been associated with higher serum IgE levels [70]. 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 [69, 71, 72]. 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 [71, 73]. Recently, a SNP (rs1837253) in the *TSLP* gene has been shown to be highly *negatively* associated with asthma and AHR [11, 74], while the *IL-33* and *IL1RI* genes have been positively associated with asthma and eosinophil counts respectively [9, 75]; these latter observations suggest that the airway epithelium plays a major role in regulating susceptibility of asthma and related traits (Table 2). To date, numerous loci that are linked to asthma and allergic phenotypes have been identified. However, in order to better understand the development of asthma and allergic diseases, the causal mechanism(s) behind the identified associations need to be elucidated. With particular emphasis on the rs1837253 variant SNP in the *TSLP* gene, some of the work of this thesis will focus on its functional role in TSLP production.

1.2 T Cell Dependence of Allergic Asthma

It is widely recognized that T cells play a significant role in asthma, in particular the involvement of Th2 cells [17]. There are reports of a possible minor contribution from CD8+ (cytotoxic) T cells in asthma [45, 76]. The overexpression of GATA-binding factor (GATA)-3 [77] and under-expression of T-bet [78], transcription factors controlling Th1 and Th2 development, respectively, in asthmatic airways, further support the Th2 hypothesis.

Table 2. Description of polymorphisms in various genes associated with allergic diseases.

Gene	Chromosome	Clinical Association(s)
<i>IL-4</i>	5	Asthma, FEV ₁ , total serum IgE [64]
<i>IL-13</i>	5	Asthma, atopic asthma, atopy, IgE levels, AHR [71, 73]
<i>CD14</i>	5	Total serum IgE [67]
<i>TLR1</i>	4	Atopic asthma [68]
<i>TLR6</i>	4	Atopic asthma [68]
<i>TLR9</i>	3	Atopic asthma [68, 79]
<i>STAT6</i>	12	Serum IgE levels [70]
<i>VDR</i>	12	Asthma, atopy, IgE levels [71, 73]
<i>FcεRIβ</i>	11	Total serum IgE [80]
<i>TSLP</i>	5	Asthma, atopic asthma, AHR [11, 74] and allergic rhinitis [81], IgE [74, 82]
<i>IL-33</i>	9	Asthma [9, 75]
<i>IL1R1</i>	2	Eosinophil counts [9, 75]
<i>IL-17RB</i>	3	Asthma [9, 75]

TLR, toll-like receptor; STAT, signal transducer and activator of transcription; VDR, vitamin D receptor; FcεRIβ, β-chain of the high-affinity receptor for IgE; TSLP, thymic stromal lymphopoietin; *IL1R1*, interleukin 1 receptor, type 1; *IL-17RB*, interleukin 17 receptor beta; FEV₁, Forced expiratory volume in 1 s; IgE, immunoglobulin E; AHR, airway hyperresponsiveness.

1.2.1 The Role of Th2 Cells in Asthma

For many years, the mechanisms by which Th2 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 [17]. However, 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 [83-86]. 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.* [87] 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 [88, 89].

1.2.2 *Peptide-Induced Late Asthmatic Reaction*

In order to investigate the role of T cells in asthma along with the downstream consequences of activating antigen-specific T cells, Haselden *et al.* [83] developed a unique *in vivo* model for T cell activation by administering synthetic peptides from a common aeroallergen protein to allergic asthmatic subjects. This group was the first to show that direct T cell activation through intradermal injection of cat allergen-derived peptides could induce isolated LAR (absence of early asthmatic response) in cat allergic asthmatic individuals, independent of IgE. These cases of isolated LAR were shown to be MHC-restricted (occurring only in individuals with MHC class II that are able to bind the injected peptides). Since then, it has been shown that inhaled peptide can also induce isolated LAR in cat allergic asthmatic individuals [86]. In a more recent study, Ali *et al.* [85] revealed significant increases in CD3⁺ T cells and CD4⁺ T cells in mucosal biopsies following inhaled peptide challenge, which was associated with increased chemokine (C-C motif) ligand (CCL)17 levels in bronchoalveolar lavage fluid (BALF) and expression in mucosal biopsies, correlating with the magnitude of peptide-induced LAR. It was also reported that inhaled peptide challenge upregulates expression of the potent vasodilator, calcitonin gene-related peptide (CGRP), in BALF and bronchial biopsies from asthmatic subjects who

develop isolated LAR. Furthermore, levels of CGRP in BALF fluid correlated with AHR [90]. Since CGRP is a potent arterial and venous vasodilator [91], it is possible that airway wall oedema may be an important component of peptide-induced late responses. Collectively, these findings support the concept that T cell activation alone is capable of triggering the LAR and related clinical manifestations.

1.3 Airway Epithelium

For many years, the airway epithelium was considered to have the simple role of a physical barrier, separating the external environment from the internal milieu of the lung; however in recent years, its new role in the regulation of immune responses has slowly emerged [92].

1.3.1 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. In health, through the formation of tight junctions, the airway epithelium functions as a highly regulated barrier [93]. Furthermore, epithelial cells undergo rapid regeneration and repair whereby basal epithelial cells differentiate and proliferate to restore the damage caused by inhaled viruses [94], allergen [95] and other environmental factors [96]. 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 [92]. In asthma, the airway epithelium shows impaired repair responses and decreased barrier function, which result from inefficient tight junction formation [93]. This leads to enhanced access of

environmental stimuli, which may alter mediator production and, consequently, result in improper downstream adaptive immune responses [92, 97]. 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 [14]. 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 [98, 99].

1.3.2 Viral Infections of the Airway Epithelium: Role of TSLP

The airway epithelium is the main target of respiratory viral infections. Viruses infect and replicate in epithelial cells, triggering the release of pro-inflammatory mediators, one of which is TSLP, initiating the inflammatory cascade [100]. Despite the fact that this inflammatory process is essential in the clearance of infection, it can augment any pre-existing inflammation in the airways of asthmatics, leading to increased airway obstruction [101]. DNA viruses synthesize double stranded (ds)RNA during replication, which are a natural source of TLR3 ligands [102], 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) [103] and primary bronchial epithelial cells (BEC) from both healthy and asthmatic individuals [13, 104], with disproportionately higher levels secreted from asthmatic epithelial cells [13]. Furthermore, dsRNA and Th2 cytokines synergistically induced TSLP production by epithelial cells in a TLR3-dependent manner [104]. Similarly, following respiratory syncytial virus (RSV) infection, Lee *et al.* [105] reported enhanced

TSLP secretion from asthmatic epithelial cells. 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 [106], which correlated with increased inflammatory responses to viral infections *in vivo* [107]. Thus, TSLP triggers DC-mediated Th2-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 [108]. Over the years, many respiratory viruses have been identified with the capacity to generate Th2-biased responses, a characteristic feature of allergic diseases. *However, the work described in this thesis will focus specifically on how (synthetic) dsRNA-activated airway epithelial cells are involved in the development of a Th2 response.*

1.4 Eosinophils and Basophils in Allergic Disorders

Eosinophils and basophils are relatively rare hemopoietically-derived myeloid leukocytes. Basophils represent the least abundant granulocyte population, constituting less than 1% of the peripheral blood population, while eosinophils make up approximately 1-3% of all circulating leukocytes [109, 110]. With particular importance to this thesis, recent studies have shown that eosinophils are pleiotropic multifunctional leukocytes with the capacity to exacerbate the inflammatory responses through the release of an array of preformed cytokines and lipid mediators [25, 26]. With respect to basophils, a role for basophils as contributors to the development and progression of allergic diseases emerged following reports of IgE-dependent IL-4 and IL-13 secretion by these cells [111].

Eosinophilic and basophilic inflammation, with an increase in activation, degranulation and mediator release from both cell types, is a key feature of allergic diseases [112]. In the context of allergic inflammation, upon antigen exposure, eosinophils and basophils and/or their progenitors are released from the bone marrow into the circulation. Subsequently, these cells appear in the airways, in elevated numbers, where they may modulate and enhance the immune response by releasing cytotoxic cationic proteins and various inflammatory cytokines/chemokines [110, 113, 114]. Basophils are the predominant source of IL-4 following allergen- and/or IL-33-activation and have been identified in late-phase allergic responses and are found at elevated levels in bronchial biopsies of asthmatics [115]. The activation of eosinophils is significantly correlated with asthma severity, as reflected by bronchial hyperresponsiveness and asthma symptom score [109, 116]. The trafficking of eosinophils from the bone marrow to inflammatory sites is mainly promoted by IL-5, while IL-4 and IL-13 upregulate eotaxin (CCL11 and CCL24), as well other chemotactic factors such as CCL5 [117].

Eosinophils secrete an array of cytotoxic granule cationic proteins, such as MBP, ECP, EPO and eosinophil-derived neurotoxin, all of which have the capacity to induce tissue damage [114]. Activated basophils on the other hand release preformed mediators, histamine and leukotriene C4 (LTC₄), which promote inflammation and bronchoconstriction [118]. Collectively, eosinophils and basophils are highly prominent in conditions driven by allergic inflammation, and differences in activation and mediator production contribute to their unique effector functions. [26, 109, 114]. *A focus of this thesis will be on events and pathways involved in the differentiation of these cells, within the context of allergic inflammation.*

1.4.1 Hemopoiesis

Hemopoiesis refers to the generation of blood cells that occurs mainly in the bone marrow, under the influence of stromal cells, T lymphocytes and other immune cells and/or their soluble products. Hemopoietic progenitor cells are derived from pluripotent hemopoietic stem cells (HSC), which are able to self-renew, divide and differentiate into mature lineages including the erythroid, lymphoid and myeloid lineages [119] (Fig. 2). Mature cells are generated following the commitment of pluripotent progenitors into specific cell lineage, followed by terminal differentiation [119]. The CD34 antigen, an important marker of hemopoietic progenitors, is a glycoprotein whose expression appears at the highest density on early hemopoietic lineage cells, and is progressively lost on terminally differentiated cells [120, 121], with the exception of murine mast cells [122]. Therefore, CD34 is a stage-specific rather than a lineage-specific leukocyte-differentiation antigen [120]. It is not clear what function CD34 has, but it has been reported to facilitate mobilization of progenitors from the bone marrow [123] and, together with determinants such as small size and low granularity, it enables progenitor cells to be accurately enumerated in cord blood, peripheral blood, bone marrow and sputum [124], by flow cytometry.

1.4.1.1 Eosinophil-Basophil Differentiation

The differentiation of mature hemopoietic cells from the HSC is under the permissive control of a range of hemopoietic cytokines and growth factors. While stem cell factor has been shown to exert its effects very early in the hemopoietic lineage hierarchy [125], other cytokines act at later stages in the development of myeloid progenitors. The differentiation of committed progenitors towards an eosinophil-basophil (Eo/B) lineage is regulated by a

triad of cytokines found systemically and in tissue: IL-3, IL-5 and GM-CSF [126, 127], which are closely linked on human chromosome 5, and are predominantly produced by Th2 cells and mast cells [128]. These cytokines are both pleiotropic and redundant, acting in both an autocrine and paracrine fashion - signaling through a common β -receptor [129] and high-affinity receptors, which determine specific-lineage commitment and cell proliferation, differentiation and survival [130, 131], through the activation of signal transduction pathways and lineage-specific transcription factors (e.g., GATA-1, C/EBP) [126, 127].

Human eosinophils and basophils are closely linked during development and share a common progenitor as demonstrated by methylcellulose colony assays [132-134]. These assays utilise cultured cells from samples such as bone marrow, cord blood and/or peripheral blood. Cells are plated on semi-solid medium (methylcellulose) with supporting hemopoietic cytokines (IL-3, IL-5, or GM-CSF). On day 14, Eo/B colony forming units (CFU), defined as tight, granular clusters of 40 or more cells, with morphology consistent with the Eo/B precursor, are enumerated. However, the effects of IL-3, IL-5 and GM-CSF on colony formation are not uniform. The responsiveness of these progenitors to specific cytokines varies with stage of differentiation, with IL-3 and GM-CSF acting on early progenitors and IL-5 on committed progenitors, to induce specific Eo/B lineage differentiation [135]. Furthermore, IL-3 tends to promote basophil growth and differentiation, and to a lesser extent, eosinophils; while IL-5 is mainly responsible for promoting eosinophil colonies, and to a lesser extent, basophil colonies [136]. Several studies have demonstrated that IL-5 downregulates IL-5R α on the surface of mature eosinophils [137, 138], while immature eosinophils and human CD34+ progenitors acquire

IL-5R α expression and functional responses to IL-5 [139, 140], which suggest that immature eosinophils and progenitors respond better to the ongoing exposure of IL-5.

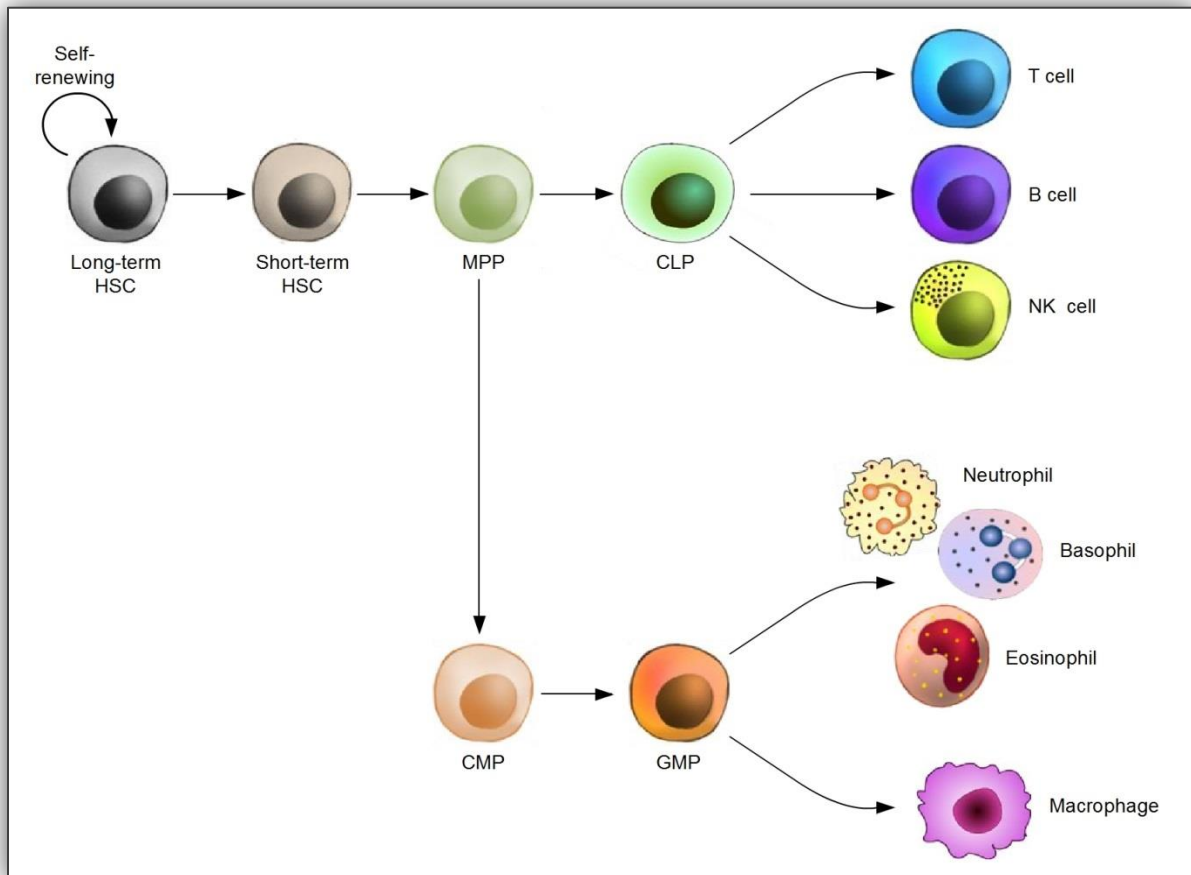


Figure 2. The hemopoietic tree. Long-term hemopoietic stem cells (HSC), which have the capacity to self-renew, give rise to all the cell types of the bone marrow and peripheral blood. Other pluripotent progenitors, short-term HSC and multipotent progenitors (MPP) have less self-renewal capacity. Together, these three cell types constitute the hemopoietic stem and progenitor cell population. MPP are thought to differentiate into the two main branches of hemopoietic development that arise from the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). Together, these progenitors give rise to mature peripheral blood cells, shown on the right. GMP, granulocyte and macrophage progenitor; NK, natural killer (adapted from [119]).

Other cytokines that have been shown to regulate Eo/B differentiation include $\text{TNF}\alpha$, which demonstrated enhancing effects on IL-3- and GM-CSF-dependent proliferation of CD34+ hemopoietic progenitors [141]. Furthermore CD34+ progenitors have been shown to express receptors for TSLP and IL-33, which suggest that they are TSLP- and IL-33-responsive [15, 142]. Recently, we (Chapter 3) [143] and others [142] have demonstrated a role for TSLP in the regulation of Eo/B differentiation. Lastly, while human eosinophils and basophils share a common progenitor, they exist as distinct cells [136, 144].

1.4.1.2 Intracellular Signaling in Eosinophil-Basophil Differentiation

The IL-3, IL-5 and GM-CSF receptors are members of the short-chain 4- α -helical bundle subset of hemopoietic cytokine receptors, with a unique α -chain that is specific for each cytokine. The shared common β c-chain has no binding capacity on its own but is recruited following the binding of IL-3, IL-5 and GM-CSF to its respective α -chain, resulting in the assembly of the heterodimeric receptors and the subsequent initiation of signal transduction [145]. Intracellular signaling can occur through three main pathways: the Janus Kinase (JAK)/STAT pathway, the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway [129] (Fig. 3).

Cytokines and growth factors signal through the JAK/STAT pathway to transmit extracellular signals through transmembrane receptors directly to promoters of target genes in the nucleus of activated genes. JAK activation leads to tyrosine phosphorylation of the β c chain, which results in the generation of various docking sites for other signaling molecules such as SRC homology 2 domains, which in turn leads to tyrosine phosphorylation of STAT

proteins followed by STAT dimerization. The dimerized STATs subsequently translocate to the where they bind to specific promoter regions and induce gene transcription [129].

IL-3, IL-5 and GM-CSF activate the MAPK pathway, which includes the extracellular signal regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 signaling pathways, to promote growth, proliferation and survival of cells [129, 146]. On cytokine stimulation, the cellular substrate Shc is phosphorylated leading to its interaction with adaptor protein Grb2, resulting in the association with a nucleotide exchange factor for Ras (SOS). The activation of Ras leads to the activation of kinases such as MEK1/2 which activates ERK [147], MAPK kinase (MKK)3/6 which activates p38 or MKK4/7 which activates JNK [148]. The activation of these signaling pathways leads to the activation of a number of transcription factors such as c-Fos and c-Jun, which contribute to cytokine-induced proliferation [147] (Fig. 3).

Finally, the PI3K pathway is involved in the regulation of proliferation, growth, cell size and apoptosis [149]. Protein kinase A is activated upon cytokine binding resulting in the phosphorylation of the β c-chain, which in turn recruits subunits of PI3K to the receptor complex [150]. Once recruited, PI3K is phosphorylated, which in turn phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and converts it to phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃), a second messenger that interacts with other signaling proteins through resulting in the amplification of the activated signal [149, 150].

1.4.2 Hemopoietic Progenitors in Atopy – Local and Systemic Regulation

While the importance of mature eosinophils and basophils in allergic inflammation has long been established, accumulating evidence supports the role of Eo/B progenitors in driving

this allergic inflammatory response [151]. Several groups have demonstrated increased numbers of CD34⁺ progenitors at sites of allergic inflammation, such as the nasal mucosa of subjects with nasal polyposis [152], and the bronchial mucosa of patients with asthma [153]. Moreover, CD34⁺ cell numbers have been shown to be inversely associated with forced expiratory volume in 1 second (FEV₁), which is consistent with the hypothesis that Eo/B progenitors may contribute to clinical symptoms in allergic individuals [153]. Indeed, there are enhanced numbers of CD34⁺ progenitors, as well as both mature and immature eosinophil cell numbers, in peripheral blood [144, 154] and bone marrow [155] of atopic individuals compared to nonatopics.

Further evidence for local airway mucosal (*in situ*) control of hemopoietic differentiation was provided by studies that showed a rise in bone marrow and circulating committed Eo/B progenitors, defined as CD34⁺/IL-5R α ⁺ cells [155, 156], in response to airway inhaled allergen exposure in atopic asthmatic subjects [155, 157-159]. Likewise, in a proof-of-concept study, Kuo *et al.* [160] demonstrated that direct stimulation of these progenitors from the marrow by IL-5, administered systemically, also enhanced CD34⁺/IL-5R α ⁺ cells. Several groups have now reported increased expression of IL-5R α on CD34⁺ cells from the bone marrow of atopic asthmatics [161]. This supports the concept that progenitor cells from atopic subjects are primed to respond to IL-5 [154], likely as a result of ambient or stimulated increased levels in bone marrow CD34⁺/IL-5R α ⁺ cells, a feature of atopic disease [162]. Along these lines, compared to asthmatics who do not develop eosinophilia, there is a higher proportion of CD34⁺/IL-5R α ⁺ cells in the bone marrow after allergen challenge in mild asthmatic subjects who develop airway eosinophilia and increased clinical symptoms to methacholine challenge [155, 158]. Taken together, these

data demonstrate eosinophil lineage skewing of CD34+ cells following allergic immune stimulation that may contribute to the subsequent development of blood and tissue eosinophilia [155].

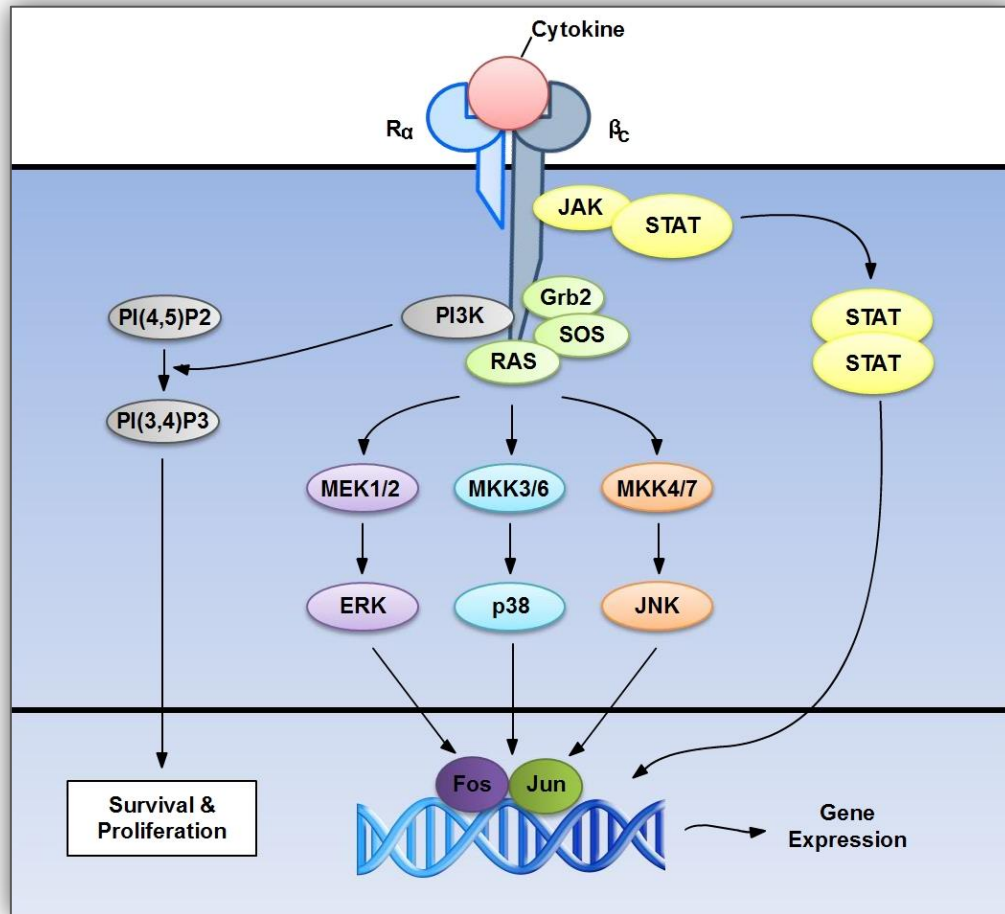


Figure 3. Schematic diagram of IL-3, IL-5 and GM-CSF intracellular signaling. Binding of hemopoietic cytokines, such as IL-3, IL-5 or GM-CSF, to their receptors activates transduction cascades leading to gene expression, cell differentiation, proliferation and survival. IL-3-, IL-5- and GM-CSF-mediated signaling begins with the high affinity binding of each of the cytokines to its respective receptor α subunit (R_{α}), subsequently, this complex binds to the common β -chain (β_c). The activation of the JAK/STAT pathway directly transmits the extracellular signals to promoters of target genes in the nucleus resulting in the expression of genes that are important in promoting cell differentiation and survival. The activation of MAPK and related pathways results in cell differentiation as well as gene expression. The activation of Ras leads to the activation of MEK1/2, MKK3/6 and MKK4/7, which target ERK, p38 and JNK to enhance transcription and cell differentiation.

In ovalbumin sensitized mice, similar increases in Eo/B CFU are observed, which coincides with increased airway eosinophils [163]. Furthermore, *in vivo* experiments demonstrate increased IL-5R α [155] and CCR3 [164] expression on bone marrow-derived CD34+ cells following allergen challenge, events which may subsequently facilitate the mobilization of these cells from the bone marrow to sites of allergic inflammation [164]. These studies confirm that progenitors in the peripheral blood and bone marrow are sensitive to allergic stimuli in the airways, and respond with enhanced systemic and local eosinophilo- and basophilopoiesis. This is supported by the close relationships that exist among increased production of Eo/B lineage-committed progenitors within the bone marrow, development of blood and tissue eosinophilia [155], and maintenance of allergic tissue inflammatory responses [154, 158].

Therapeutic interventions for allergic diseases further highlight the importance of progenitor cells in the development of allergic inflammation. Corticosteroids inhibit the development of airway eosinophilia, and are considered the most effective treatment for allergic asthma. Treatment with inhaled corticosteroids is sufficient to reduce baseline numbers of bone marrow Eo/B CFU [165]. In mild asthmatics, the gradual withdrawal of inhaled corticosteroids results in a rise in circulating progenitors, which coincides with the development of clinical symptoms [166]. Moreover, inhaled budesonide has been shown to have protective effects on allergen-mediated inflammation by inhibiting the number of allergen-induced circulating eosinophils and their progenitors grown in the presence of GM-CSF [167]. Similarly, Kim *et al.* [152] reported that intranasal steroids were associated with an increase in CD34+/CD45+ progenitors and decrease in mature eosinophils in nasal polyp mucosa, suggesting that corticosteroids block eosinophil differentiation resulting in the

accumulation of tissue (undifferentiated) CD34+ cells. Together, these studies highlight and demonstrate the direct responsiveness of hemopoietic progenitors to corticosteroids.

1.4.3 *In situ Hemopoiesis – Local Differentiation of Hemopoietic Progenitors*

A growing body of evidence suggests that progenitor cells may contribute to allergic inflammation by giving rise to mature effector cells in the bone marrow, as well as by trafficking as still-undifferentiated cells to allergic tissue sites where they differentiate into effector cells *in situ*, under the control of local inflammatory cytokines and growth factors [151]. Through this process, referred to as '*in situ hemopoiesis*,' the inflammatory response is maintained and further enhanced via the continuous production of effector cells and their soluble products [151]. The first studies to provide indirect evidence that progenitor cells may indeed traffic from the bone marrow to sites of inflammation where they undergo *in situ* hemopoiesis were performed in subjects with AR [168-170]. A series of studies reported significant elevations of Eo/B progenitors prior to seasonal allergen exposure, followed by rapid reductions in the number of circulating progenitors in subjects during the season as they developed symptoms of rhinitis, in turn followed by a rebound back to basal (high circulating) levels at the end of the season.

It has been reported that up to 50% of CD34+ cells from asthmatic subjects express intracellular IL-5 protein, with high expression of IL-5 mRNA [160]. Our group have also shown that progeny of Eo/B progenitors in colonies express GM-CSF protein and mRNA [171], and that there is an autocrine GM-CSF-dependent signaling mechanism for Eo/B differentiation mediated through TLR [172]. Furthermore, it has recently been reported that increased IL-5 and IL-13 positive CD34+ cells (a profile which can be attributed to the

effects of TSLP and IL-33—see below) are present in the sputum after allergen inhalation challenge [15]. Furthermore, Cameron *et al.* [173] demonstrated, in an explant model *ex vivo*, that IL-5 induced *in situ* differentiation of CD34+ progenitors, measured as the increase in the number of MBP immune-reactive cells, in the nasal mucosa of allergic subjects. Lastly, clinical investigational evidence supports systemic effects of IL-5 on progenitors: Stirling *et al.* [174] reported associations between systemic IL-5 given intravenously, and circulating Eo/B progenitors. Aside from a possible role in the recruitment of mature eosinophils from the circulation to the airways [174], systemic IL-5 has been implicated in the local differentiation of progenitors in the tissue [175]. Together, these studies suggest that, in addition to acting as pro-inflammatory effector cells and directly contributing to allergic inflammation, progenitors may aid in their own differentiation through autocrine production of growth factors, such as IL-5 and GM-CSF. Given that hemopoietic cells develop under the influence of the tissue milieu, the rich combination of cytokines and growth factors present in the bone marrow sinusoids, circulation or mucosal and other tissues, plays a critical role in guiding and modulating hemopoiesis. Changes in the composition of the local milieu, such as increased expression of Th2 cytokines (particularly IL-5) in individuals with atopy, may promote differentiation of lineage-committed Eo/B progenitors [176], and help explain increased eosinophils and basophils at sites of inflammation in atopic individuals.

1.5 Thymic Stromal Lymphopoietin

TSLP is a four alpha-helical bundle type I cytokine that is structurally and functionally similar to IL-7 [177]. TSLP was first identified in 1994 by Friend *et al.* [178], in the supernatants of a murine thymic stromal cell line, Z210R, and shown to support B cell development [179]. *TSLP* is located on chromosome 18 and chromosome 5q22.1 in mouse and human, respectively [180, 181]. TSLP signals through a heterodimeric receptor complex consisting of the IL-7 receptor- α (IL-7R α) chain and a TSLP binding chain referred to as TSLPR [182, 183]; it binds to the TSLPR chain with low affinity, but together with the IL-7R α chain, the affinity is greatly enhanced. Although there exists poor homology between human and mouse TSLP, with only 43% amino acid identity and 39% between human and murine TSLPR, human and mouse TSLP share very similar biological functions [181, 183]. As such, recent evidence supports a major immunomodulatory role for TSLP in the pathophysiology of allergic diseases, by acting as an important link between innate and adaptive immunity, leading to Th2 responses [14, 108, 184].

1.5.1 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 [14, 103, 104, 185, 186]. Expression of TSLP is regulated by several factors, both exogenous stimuli (e.g., injury, infection, TLR ligation) and host-derived pro-inflammatory and Th2 cytokines [103]. Pro-inflammatory cytokines IL-1 β and TNF α have been shown to induce TSLP expression in human BEC [104, 185, 187]. Likewise, Allakhverdi *et al.* [103] and Zhang *et al.* [186]

reported the ability of IL-1 β and TNF α to induce TSLP production in SAEC and human airway smooth muscle cells (HASMC) respectively. The induction of TSLP in airway epithelial cells by inflammatory Th2 cytokines has been shown to be regulated by NF κ B [185]. In HASMC, aside from NF κ B, the upregulation of TSLP by inflammatory Th2 cytokines is also regulated by the transcription factor, activating protein 1 (AP-1) [188], as well as p38 and ERK MAPK signaling pathways [186].

The first evidence for a role for pathogenic stimulation in the induction of TSLP was established by Allakhverdi *et al.* [103], who reported the ability of bacterial peptidoglycan, and polyinosinic:polycytidylic acid (polyI:C) (mimicking viral dsRNA) to induce TSLP expression in primary SAEC. The combination of TLR3 ligand (dsRNA) and Th2 cytokines, in particular, IL-4 and TNF α , has been shown to significantly enhance TSLP production in BEC in an NF κ B-dependent manner [104]. Furthermore, infection of BEC with rhinovirus or RSV, viruses known to trigger asthma exacerbations, results in the production of TSLP [104, 189]. 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 [190].

In a murine AR model, mast cells were shown to upregulate TSLP expression in nasal epithelial cells following allergen challenge [191]. Aside from regulating TSLP expression, mast cells activated by monoclonal antibodies that cross-link Fc ϵ RI have been reported to express TSLP mRNA [192]. Murine basophils, activated by protease allergens, were also reported to produce TSLP, along with other Th2 inducing cytokines; furthermore,

activated basophils in the lymph nodes have been shown to produce TSLP [193]. Although DCs are the major target of TSLP, surprisingly both human and murine DCs produce TSLP following TLR-ligation [194]. 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 RXR α and RXR β in keratinocytes resulted in enhanced TSLP expression and development of chronic skin inflammation in mice [195, 196].

1.5.2 TSLP Signaling Pathways

Despite a significant role of TSLP in driving Th2 inflammation, our knowledge of TSLP/TSLPR signaling is limited. Initial studies reported TSLP-induced phosphorylation of STAT5 in the absence of JAK activation, suggesting that STAT5 phosphorylation is mediated by an unknown kinase [179, 197]. However, two recent studies using primary human and murine CD4⁺ T cells and primary human DCs, demonstrated JAK1 and JAK2-dependent TSLP-mediated STAT phosphorylation [198, 199]. These studies shed some light on TSLP/TSLPR signaling and revealed that TSLP-induced phosphorylation of JAK precedes that of STAT.

In human myeloid dendritic cells (mDCs), aside 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 Th2-attracting chemokine [199], which helps to explain the unique ability of TSLP-activated DC to secrete CCL17. Furthermore,

TSLP-activated DCs have been shown to activate PI3K/AKT, ERK, JNK MAPK, and NFkB-dependent pathways [199].

TSLP has recently been shown to activate murine CD4⁺ T cells, resulting in STAT6 dependent Th2 differentiation [200]. In BEC, TSLP activates both STAT3 and STAT5 phosphorylation, leading to the induction of IL-13 and cell proliferation [201]. However, TSLP does not induce STAT5 phosphorylation in ASM; rather, it induces STAT3 phosphorylation and activates the MAPK (ERK1/2, p38, and JNK) signaling pathways [202]. In eosinophils, TSLPR signaling involves the ERK, p38, and NFkB signaling pathways, but not STAT3 or STAT5 [203].

1.5.3 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 hemopoietic cell types and endothelial cells [5, 38, 40]. 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 [6, 31, 32]. Recently, hemopoietic progenitors [15, 142, 143] and nuocytes [204, 205], a new type of innate effector leukocyte cells that mediate type-2 immune response, have been shown to respond to TSLP. Indeed, as reviewed below, TSLP exerts its functions on a broad range of cell types (Fig. 4).

1.5.3.1 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

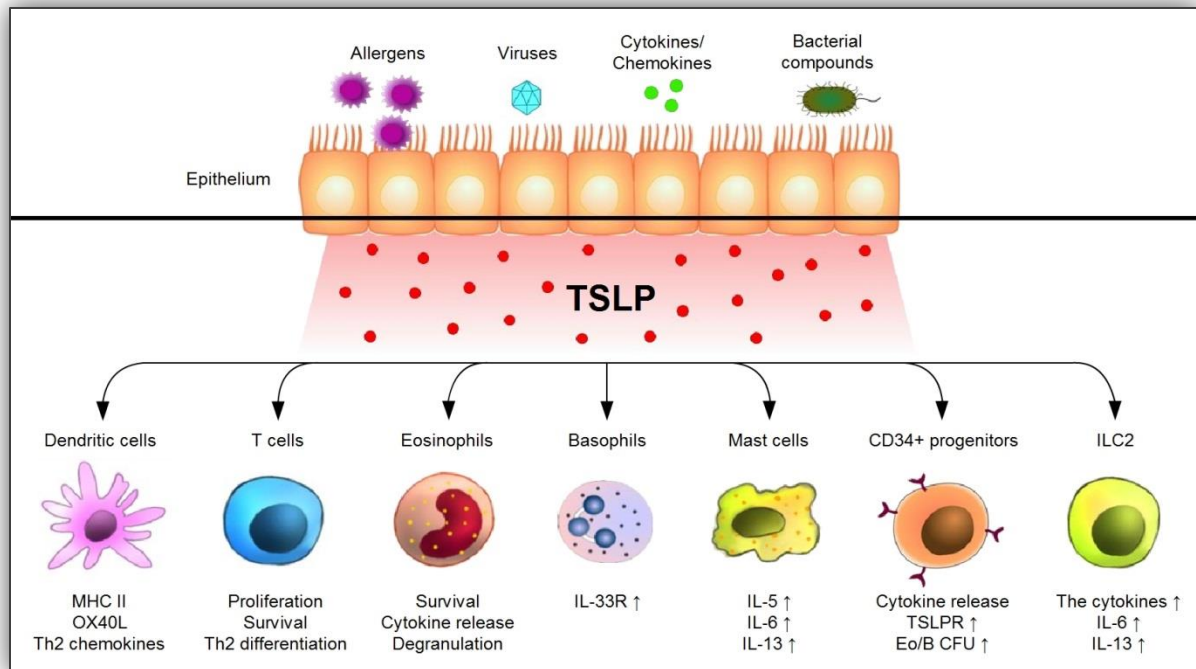


Figure 4. Cellular targets of epithelial-derived TSLP. Allergens, viruses, pro-inflammatory cytokines ($\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-4) chemokines (CCL17; TARC), and bacterial components such as peptidoglycan, lipoproteins, lipoteichoic acid, LPS are all capable of inducing TSLP production 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 [206]).

highest expression of TSLPR, which is consistent with the ability of mDCs to respond to TSLP [14, 183]. 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 Th2-polarized cells [14, 207]. 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) [14]. 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 Th2 cells that produce allergy-promoting Th2 cytokines such as IL-4, IL-5, and IL-13, as well as (uniquely) TNF α , but not IL-10 (Fig. 5) [14, 183, 208]. Gene expression analyses of TSLP-activated human mDCs shows that TSLP alone strongly induces TNF superfamily protein OX40L [208], which is critical for the induction of inflammatory Th2 cells, since blocking OX40L with a neutralizing antibody inhibits the production of Th2 cytokines and TNF α , and enhances the production of IL-10 by CD4⁺ T cells. Consistent with this finding, Liu *et al.* [209] observed that treatment of naïve CD4⁺ T cells with recombinant OX40L promoted the production of TNF, while inhibiting the production of IL-10. Moreover, *in vivo*, TSLP-induced Th2 immune responses in the lung and skin were inhibited by treating mice with OX40L-blocking antibodies [210].

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 Th1-polarizing cytokine IL-12 [14, 208]. In the presence of IL-12, both TSLP-activated mDCs and OX40L lose its ability to induce inflammatory Th2 differentiation [208]. To date, TSLP is the only factor that activates mDCs without the induction of IL-12 and other Th1-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 Th1-mediated immune response, which down-regulates the development of Th2-driven atopy. For these reasons, it can be suggested that TSLP plays a

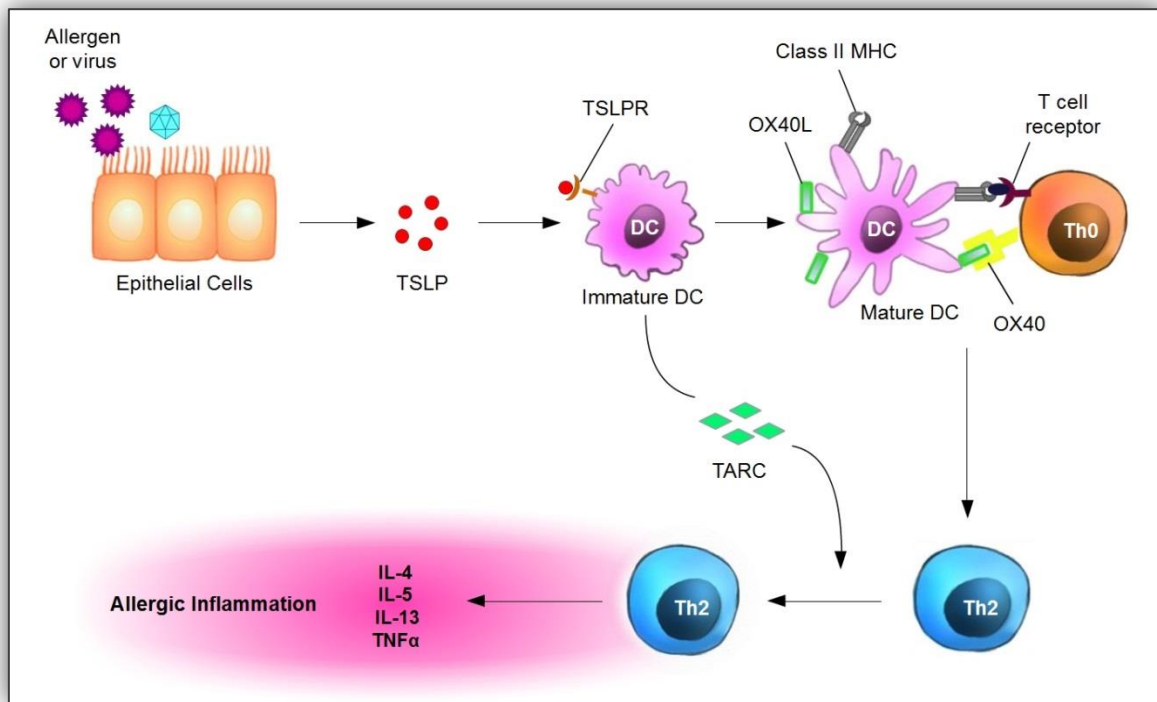


Figure 5. Pathophysiology of TSLP in allergic inflammation. Insult from allergens or viruses trigger epithelial cells to produce TSLP, which then activates immature DCs. TSLP-activated DCs express OX40L, which triggers naïve CD4⁺ T cells (Th0) to differentiate into inflammatory Th2 cells that produce IL-4, IL-5, IL-13, and TNF α . 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 [108]).

major role in classic Th2 diseases such as allergic asthma. The activation of DCs by TSLP consequently provides a permissive condition for Th2 development by upregulating the Th2-polarizing signal (OX40L), without inducing the Th1-polarizing signal (IL-12) (Fig. 6).

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 [211], which have been shown to induce eosinophilia and increased IgE production in AD, both *in vitro* and *in vivo* [212]. Moreover, TSLP-activated mDCs potently expand Th2

memory cells, without altering their central memory phenotype and Th2 commitment [213]. 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 [214]. 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 Th2 cells and cytolytic T cells.

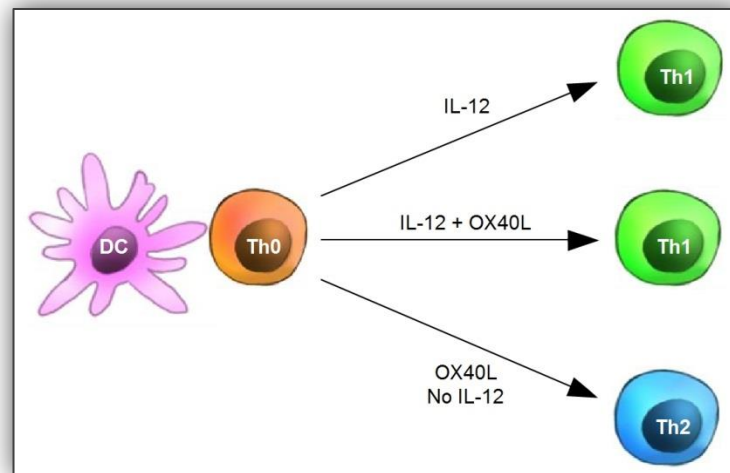


Figure 6. Schematic regulation for Th1 and Th2 differentiation. The Th1-polarizing signal (IL-12) is dominant over the Th2-polarizing signal (OX40L); therefore, Th2 differentiation will only occur in the presence of OX40L and in the absence of IL-12 (adapted from [108]).

1.5.3.2 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 [200]. Studies using TSLPR-deficient mice have shown that TSLPR expression by CD4⁺ T cells is critical for TSLP-mediated CD4⁺ T cell expansion and Th2 differentiation *in vivo* [207, 215]. In humans, Rochman *et al.* [216] 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.

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 [217]. Activated pulmonary Tregs have also been reported to express TSLPR and respond to TSLP-mediated activation of STAT5 [218]. 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 [218, 219]. Furthermore, Nagata *et al.* [220] reported on the ability of murine TSLP to activate NKT cells to preferentially produce IL-13, but not IFN γ and IL-4. Using a murine allergen-induced asthma model, this group demonstrated AHR in TSLP transgenic mice, which was almost completely eliminated in the absence of NKT cells. Furthermore, in the same asthma model, TSLP transgenic mice lacking NKT cells had significant reduction

in IL-13. Collectively, these findings highlight a unique role for TSLP in regulating the adaptive immune response.

1.5.3.3 *Eosinophils, Basophils, and Mast Cells*

Eosinophils have been shown to express both chains of the TSLPR complex (TSLPR and IL-7R α) [203, 221]. Eosinophils respond to TSLP stimulation by releasing IL-6, CXCL8, CXCL1, and CCL2 and up-regulating surface expression of the adhesion molecules CD18 and intracellular adhesion molecule-1, which suggests that TSLP may recruit eosinophils to sites of Th2 cytokine-associated inflammation [203]. Furthermore, TSLP has been shown to promote the viability and survival of eosinophils, as well as release of eosinophil-derived neurotoxin [203, 221].

Mast cells infiltrating the bronchial mucosa of asthmatic subjects have been shown to express functional TSLP receptors [103]. Allakhverdi *et al.* [103] reported that TSLP has the ability to potently activate human mast cells, in the presence of IL-1 β and TNF α , with production of high levels of IL-5, IL-13, IL-6, GM-CSF, as well as chemokines involved in allergic diseases. Furthermore, blocking native TSLP that is released by primary human SAEC completely inhibits production of IL-13 by mast cells [103].

TSLP has been shown to regulate basophil responses in mice [142]. TSLP-stimulated (as opposed to IL-3-stimulated) murine basophils show distinct attributes, such as high levels of IL-33R expression. Although the majority of human basophils express TSLPR, effects of TSLP on human basophils are still unknown. However, 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 [142]. 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.

1.5.3.4 Hemopoietic Progenitors

Hemopoietic progenitors give rise to mature eosinophils, basophils and mast cells, effector cells of allergic diseases. Recently, Allakhverdi *et al.* [222] reported that human CD34⁺ cells express receptors for TSLP and IL-33, which supports their finding that TSLP can potentially activate human CD34⁺ progenitors (and thus mast cell precursors). In the presence of IL-33, TSLP directly activates CD34⁺ progenitors, leading to the release of high levels of pro-inflammatory Th2-like cytokines and chemokines that are involved in allergic diseases [222]. Similar to its effects on mast cells [103], blocking endogenous TSLP that was released by primary human SAEC completely inhibited production of IL-5 by CD34⁺ progenitors [15].

Related to this, we recently reported the ability of peripheral blood CD34⁺ progenitors to respond to TSLP with enhanced IL-3-responsive Eo/B CFU (including both eosinophils and basophils), a process which is dependent on TSLPR-ligation [143]. Furthermore, IL-3 and TSLP-stimulated CD34⁺ progenitors secreted an array of cytokines and chemokines, key among which was TNF α which, together with IL-3, stimulated the enhanced surface expression of TSLPR, consequently increasing the sensitivity of CD34⁺ progenitors to TSLP, as reflected by Eo/B CFU (Chapter 3).

Moreover, we have recently demonstrated that CD34⁺ progenitors from atopic individuals are functionally and phenotypically more responsive to TSLP than those from

non-atopic individuals [143]. Consistent with this, Siracusa *et al.* [142] recently reported the ability of TSLP to induce basophil maturation and expansion from murine bone-marrow resident progenitors. TSLP-stimulated progenitors were predominantly CD34- and exhibited elevated levels of CD49b expression, a phenotype consistent with mature basophils [142]. *Collectively, these studies suggest that epithelial-derived TSLP may be central for regulating the differentiation of tissue-resident hemopoietic progenitors into effector cells of allergic inflammation.*

1.5.3.5 Group 2 Innate Lymphoid Cells

ILC2, part of a large family of ILCs 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 Th2 cytokines IL-5 and IL-13 [59, 62, 205, 223], suggesting functional similarities between ILC2 and Th2 cells. Mjosberg *et al.* recently reported that human ILC2, found in nasal polyps of patients with chronic rhinosinusitis, a Th2-mediated disease, express receptors for TSLP. They demonstrated STAT5 activation and increased GATA3 expression in ILC2 following stimulation by nasal polyp epithelial-derived TSLP [59]. Despite these similarities between ILC2 and Th2 cells, unlike Th2 cells, ILC2 are able to respond to TSLP with production of Th2 cytokines without prior activation, a response that is augmented by IL-33 [59]. Additionally, in combination with IL-25 and IL-33, TSLP can be shown to drive the expansion of ILC2 isolated from peripheral blood cells of healthy donors [61]. As such, ILC2 may constitute an additional population of cells that can provide an early source of Th2 cytokines, capable of initiating Th2 allergic inflammation. The

ability of ILC2 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.

1.5.4 *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, but not in uninvolved skin (normal or non-lesional) [14]. Furthermore, serum TSLP levels in children with AD are significantly higher than normal controls [224]. 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 [12]. Elevated levels of TSLP are also found in the airway epithelium and BALF of asthmatic individuals [218, 225, 226]. Experimentally, over-expression of TSLP in airway epithelial cells induces allergic airway inflammation in mice [207]. Furthermore, there is over-expression of TSLP in the nasal epithelial cells of patients with AR [227-229] and nasal polyposis [228, 230]. Recently, several GWAS have identified *TSLP* as a locus associated with asthma and allergic disease susceptibility [9, 231]. SNPs in *TSLP* have reported to be associated with several clinical outcomes and phenotypes of allergic diseases such as AR, total IgE and AHR [11, 74, 81, 82, 190, 232, 233]. 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 [234]. Evidence to date suggests a critical role for TSLP in the pathogenesis of Th2-biased allergic diseases.

1.5.5 *TSLP Polymorphisms*

Susceptibility to allergic diseases is greatly influenced by gene-environment interactions, which is multifaceted. In recent years, many genetic studies have been conducted in an attempt to elucidate the genes and pathways related to allergic diseases. GWAS use an unbiased approach to identify associations of susceptible genes and phenotype. Recent GWAS and meta-analyses of GWAS have detected a large number of loci associated with asthma and related traits.

For example, both the GABRIEL study [9] and the EVE study [10], large meta-analyses of GWAS in European populations and ethnically diverse US populations, respectively, identified SNPs in four loci (TSLP, IL-33, IL1RL1, 17q21 locus) that were associated with asthma. These findings highlight the importance of epithelial-derived cytokines in asthma. In relation to TSLP, in a recent GWAS of eosinophilic esophagitis (EoE), Rothenberg *et al.* identified a SNP rs3806932 in the TSLP gene to be significantly associated with EoE [231]. They reported that subjects with EoE had increase TSLP mRNA expression, which was significantly correlated with rs3806932 genotype. EoE subjects who were homozygous for the protective minor allele expressed less TSLP mRNA than those who were heterozygous and homozygous for the risk (ancestral) allele.

Homing in on TSLP, a recent candidate gene and genome-wide association studies identified “*protective*” associations between SNP rs1837253, found in the 5’ promoter region of *TSLP*, with asthma, atopic asthma and airway hyper-responsiveness [9, 11]. 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, have reduced association with asthma in Costa Rican boys [74] and reduced associations with AR in three independent cohorts for asthma [81]. The inverse association between rs1837253 and asthma *suggests the possibility that this TSLP variant results in diminished secretion of TSLP, consequently, modulating the Th2 inflammatory response.*

Other candidate gene association studies have demonstrated the importance of TSLP variants to the development of allergic disease phenotypes [82, 232, 233] and TSLP expression [190]. A functional SNP, rs3806933, has been identified in the regulatory element of the long form of *TSLP* [190]. 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 [232]. Furthermore, in a sex-stratified genome wide linkage analyses, Hunninghake *et al.* [82] found a 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.

AD is a common chronic inflammatory skin disease that affects 15- 20% of children in industrialized countries. AD eczema herpeticum (ADEH) is a rare but serious complication of AD. Associations between variants in *TSLP* and its receptors and risk of AD and related sub-traits have recently been reported by Gao *et al.* [233]. They found a SNP (rs1898671) in *TSLP* to be associated with a lower risk of ADEH among European

American subjects. SNP rs2416259 was also identified to be associated with risk of ADEH, as well as high total IgE concentrations, and eczema area and severity index scores. Of note, SNP rs2289276, a SNP previously associated with cockroach specific IgE levels and total IgE concentration [82], was associated with AD in their study. Table 3 provides a brief summary of TSLP polymorphisms. *Collectively, these genetic studies further highlight a critical role for TSLP in the pathogenesis of asthma and allergic diseases. This thesis will focus specifically on how the SNP rs1837253 in TSLP may be protective of the development of Th2 responses.*

Table 3. Description of polymorphisms in the *TSLP* gene associated with allergic diseases

SNP ¹	Chromosomal Position ²	Allele Change	Clinical Association(s)
rs3806932	5:110405675	A > G	Eosinophilic esophagitis [231]
rs1837253	5:110401872	C > T	Asthma, atopic asthma, AHR [11, 74] and AR [81]
rs2289276	5: 110407507	C > T	Asthma, IgE to cockroach [74, 82] and AD [233]
rs3806933	5:110406742	C > T	Childhood atopic asthma and adult asthma [232]
rs1898671	5:110408002	C > T	Lower risk of AD eczemia herpeticum [233]
rs2416259	5:110419742	C > T	AD eczemia herpeticum and total IgE [233]

¹Single nucleotide polymorphisms (SNPs) listed using the unique rs (reference SNP) number assigned by NCBI (www.ncbi.nlm.nih.gov); ²Reference sequences from NCBI Build 37.1 (chromosome: base position); AHR, airway hyperresponsiveness; AR, allergic rhinitis, AD, atopic dermatitis; IgE, immunoglobulin E.

1.6 Summary

Until recently, the TSLP-DC OX40L-T cell axis was believed to be the main pathway through which TSLP mediates allergic disease [14, 187]. However, recent studies demonstrate that there may be alternate pathway(s) in which TSLP may drive Th2-biased

responses via the TSLP-progenitor axis [15, 142, 143]. To what extent these pathways are operating *in vivo* in humans in the context of allergic disease is unknown.

Recent attention has focused on the effects of TSLP polymorphisms on asthma and related phenotypes [82, 190, 232]. However, the biological effects of these polymorphisms, which ultimately leads to the manifestation of these clinical phenotypes and associations are lacking. TSLP is currently a candidate for therapeutic intervention in allergic disease but the functional consequence(s) of TSLP variants as well as the downstream effects of TSLP may be equally critical in designing novel therapeutic interventions for allergic diseases.

1.7 Research Studies

The overall objective of this thesis was to examine how TSLP drives the downstream effector pathways of allergic disease. As such, we examined two distinct TSLP-mediated pathways: a TSLP-DC axis and a TSLP-Eo/B progenitor axis. We hypothesized that variants in the *TSLP* gene are linked to alterations in TSLP secretion that then modulate downstream cellular events, such as Eo/B differentiation. Collectively, the goal of these studies was to broaden our understanding of the role that TSLP polymorphisms, and subsequently, epithelial-derived TSLP, play in the responses of downstream effector cells. *Modulation of the level of TSLP expression may ultimately influence the development of allergic diseases.*

1.7.1 *T cell-mediated induction of thymic stromal lymphopoietin in differentiated human primary BEC (Chapter 2)*

Hypothesis: Activation of allergen-specific effector T cells in the airways leads to activation of airway epithelium, resulting in the upregulation of the immunomodulatory mediators TSLP and CCL17.

Aim: Investigate the cross-talk between allergen-specific T cells and primary human BEC with respect to TSLP and CCL17 production.

1.7.2 *The effects of thymic stromal lymphopoietin and IL-3 on human eosinophil-basophil lineage commitment: relevance to atopic sensitization (Chapter 3)*

Hypothesis: Epithelial-derived TSLP mediates Eo/B lineage commitment via induction of Th2 cytokine secretion, upregulation of hemopoietic cytokine receptors, and amplification of Eo/B colony formation.

Aim: To determine whether TSLP-activated HHP differentiate into eosinophils and basophils, the mechanisms involved in this process and their relationship to atopy.

1.7.3 *Decreased nasal epithelial response to dsRNA in individuals with a thymic stromal lymphopoietin gene variant (Chapter 4)*

Hypothesis: SNP rs1837253 in the *TSLP* gene is a functional variant that alters TSLP secretion.

Aim: To evaluate the expression of TSLP in nasal epithelial cells from atopic individuals and to examine associations between rs1837253 genotype and TSLP expression.

CHAPTER 2: T cell-mediated induction of thymic stromal lymphopoietin in differentiated human primary bronchial epithelial cells

Published in *Clinical and Experimental Allergy*, 2014; 44(7):953-64

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Recent studies have demonstrated the ability of T cell epitopes from an allergen to induce airway narrowing in asthmatic subjects through the activation of allergen-specific T cells [83]. Furthermore, airway narrowing was reported to be associated with increased expression of CCL17 and increased recruitment of CD4⁺ T cells to the airways [85]. The mechanisms underlying T cell-dependent airway narrowing in asthma remain poorly understood. We undertook the current study in an attempt to better understand the link between activation of allergen-specific T cells and the induction of CCL17 in the airways. Since CCL17 is highly induced by TSLP-activated DCs, we investigated whether activated allergen-specific T cells are able to induce the expression of TSLP in primary bronchial epithelial cells from asthmatic and non-asthmatic individuals and investigated the molecular mechanisms through which this T cell-dependent TSLP expression occurs. We demonstrated that mediators from activated T cells (and individual cytokines) are able to induce TSLP and that production is higher in epithelial cells from asthmatic subjects. Furthermore, using a novel DC bioassay, we demonstrated TSLP-dependent CCL17 production and related this to asthmatic status.

T cell-mediated induction of thymic stromal lymphopoietin in differentiated human primary bronchial epithelial cells

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Clinical & Experimental Allergy

Summary

Background Inhaled peptide challenge has been shown to induce T cell-mediated, isolated late asthmatic reaction (LAR), characterized by recruitment of CD4⁺ T cells and increased levels of thymus and activation-regulated chemokine (TARC; CCL17). Epithelial-derived thymic stromal lymphopoietin (TSLP) has been shown to modulate dendritic cell function to promote T_H2 responses via CCL17 production.

Objectives To elucidate the mechanisms involved in allergen-specific T cell-induced LAR and recruitment of CD4⁺ T cells by examining the effects of T cell-derived factors on the induction of TSLP in primary bronchial epithelial cells (PBEC).

Methods PBEC grown at air–liquid interface from healthy individuals and patients with asthma were stimulated with double-stranded RNA (dsRNA) or supernatants from activated allergen-specific T cells. TSLP was measured in PBEC culture supernatants. Neutralizing antibodies and signalling inhibitors were used to examine the mechanisms responsible for the induction of epithelial-derived TSLP. The functional activity of PBEC-derived TSLP was measured using a bioassay involving the induction of CCL17 production from monocyte-derived dendritic cells (moDC).

Results Both dsRNA and allergen-specific T cells induced enhanced TSLP secretion from asthmatic PBEC compared to healthy PBEC. Activated PBEC culture supernatant induced TSLP-dependent CCL17 production from moDC in a manner related to clinical asthmatic status. IL-1 β , IL-6, and CXCL8, rather than T_H2 cytokines (IL-4/5/13), appeared to be the principle mediators of allergen-specific T cell-dependent induction of epithelial-derived TSLP, which was regulated by the MEK, MAPK, and NF κ B pathways.

Conclusion and Clinical Relevance Our data reveal a novel effect of allergen-specific T cells as a positive regulator of TSLP production by epithelial cells, suggesting T cell–airway epithelium interactions that may lead to maintenance and amplification of allergic inflammation. TSLP is currently a candidate for therapeutic intervention in asthma, but the factors that drive TSLP expression (T cell-derived factors) may be equally relevant in the treatment of allergic inflammation.

Keywords airway epithelial cells, allergens, asthma, CCL17, dendritic cells, T cells, TSLP
Submitted 26 July 2013; revised 14 April 2014; accepted 14 April 2014

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Cite this as: C. C. K. Hui, D. M. Murphy, H. Neighbour, M. Al-Sayegh, S. O'Byrne, B. Thong, J. A. Denburg and M. Larché, *Clinical & Experimental Allergy*, 2014 (44) 953–964.

Introduction

In recent years, there has been a world-wide increase in the prevalence of allergic asthma [1]. An important immunopathological hallmark of allergic asthma is the infiltration of T helper type 2 (T_H2) cells into the airways [2], a phenomenon promoted by the epithelial-cell-derived interleukin (IL)-7-like cytokine [3] thymic

stromal lymphopoietin (TSLP). TSLP is known to activate CD11c⁺ dendritic cells (DC) [4]. TSLP-activated DC express thymus and activation-regulated chemokine (TARC; CCL17), resulting in the recruitment of CD4⁺ T_H2 cells expressing the chemokine receptor CCR4 [4, 5]. TSLP-activated DC also prime T_H2 cell differentiation through expression of OX40L [4, 6], thus initiating and perpetuating the inflammatory cascade.

Recent evidence suggests that the airway epithelium is an important orchestrator in the recruitment of CD4⁺ T_H2 cells by influencing DC [4, 6, 7]. Epithelial-derived cytokines such as TSLP, IL-33, and IL-25 have been shown to play a key role in the genesis and maintenance of allergic inflammation in the airways [8, 9]. A role for TSLP in human allergic diseases is suggested because of the identified association of a single nucleotide polymorphism (rs1837253) in the upstream region of *tslp* with asthma, atopic asthma, and airway hyper-responsiveness [10]. In healthy individuals, TSLP can be elicited from human airway epithelial cells by cytokines (e.g. IL-4/TNF α) and pathogen-associated molecular patterns such as bacterial lipopolysaccharide (LPS; TLR4-agonist) and polyinosinic-polycytidylic acid (polyI:C; TLR3-agonist) [11–13]. In patients with asthma, TSLP and CCL17 are expressed in increased levels in the lungs and are linked to disease severity [14]. Studies in murine models have demonstrated reduced airway disease in TSLP receptor-deficient mice, while experimental lung-specific expression of a TSLP transgene induced experimental asthma [15].

The role of T cells in asthma, particularly T_H2 cells, has been well established [16–18]. Intradermal injection [19, 20] and inhalation [17] of allergen-derived T cell peptide epitopes have been shown to induce T cell-mediated, isolated late asthmatic reactions (LAR) in some patients with atopic asthma. Furthermore, following inhaled peptide challenge, there are significant increases in CD3⁺ and CD4⁺ T cells recruited to the airway during the LAR, which is associated with increased levels of CCL17 in the airways/lung [18]. Given that TSLP and CCL17 expression are up-regulated in bronchial epithelium of patients with asthma and correlate with asthma severity [14], T cells activated by peptide epitopes can induce isolated LAR [18], CCL17 is associated with CD4⁺ T cell accumulation during the LAR [18], and TSLP is capable of inducing CCL17 in DC [4], a potentially important yet unexplored role for allergen-specific T cells is the induction of TSLP expression in airway epithelial cells. However, to date the biological effects of T cells on epithelial-derived TSLP have not been described. In this study, we attempt to elucidate the mechanisms through which activation of allergen-specific T cells can induce airway narrowing associated with recruitment of CD4⁺ T cells [18]. We examine the effects of T cell-derived factors on the induction of TSLP in differentiated human primary bronchial epithelial cells (PBEC) grown at air–liquid interface (ALI) from healthy individuals and patients with asthma. Functional activity of PBEC-derived TSLP in the induction of CCL17 secretion by moDC was employed to create an *in vitro* model of T cell-dependent airway mucosal inflammatory responses.

Materials and methods

Airway epithelial cell isolation and culture

All studies were approved by Hamilton Health Sciences/McMaster University Research Ethics Board (approval number 07-213), and patients provided written informed consent to participate. PBEC from patients with asthma were derived from segmental bronchial brushings during bronchoscopy of asthmatic volunteers according to international guidelines [21] and as previously described [22]. Asthma was defined as either a positive methacholine provocation challenge (a methacholine concentration inducing a 20% fall in forced expiratory volume in 1 s, PC20 of <16 mg/mL) or reversibility to inhaled short-acting bronchodilator of >12% and >200 mL as per standard international guidelines [23]. Healthy PBEC were purchased from Clonetics (Lonza, Allendale, NJ, USA). PBEC were expanded and cultured as previously described with modification [24, 25]. Briefly, PBEC were maintained in bronchial epithelial growth medium (BEGM) – bronchial epithelial basal medium (Lonza) supplemented with SingleQuots (Lonza). PBEC were propagated for less than four passages from original stocks.

Culture

PBEC were seeded at a density of 8.25×10^5 cells/insert in 0.5 mL bronchial epithelial differentiating medium (BEDM) – 1 : 1 mix of Dulbecco's modified eagle medium (Sigma Aldrich, St. Louis, MO, USA) and BEGM – onto the apical surface of the polyester inserts (Corning Costar, Corning, NY, USA). One millilitre of BEDM was added to the basal compartment and cultured at 37°C, 5% CO₂. Media were replaced every second day until cells reached confluence. Thereafter, media from the apical surface were removed – establishing an ALI – and cells were differentiated for a further 21 days with the basolateral media replaced every second day.

Activation of PBEC

PBEC were stimulated with TNF α , IFN γ , IL-1 β , IL-4, IL-5, IL-6, IL-13, CXCL8, CCL5 (PeproTech, Rocky Hill, NJ, USA), activated T cell supernatants (1×10^6 cells/mL and 2×10^6 cells/mL allergen-specific T cells), and polyI:C (EMD Chemicals, Life Science Solutions, Gibbstown, NJ, USA) as indicated for 24 h at 37°C, 5% CO₂. In some experiments, cells were pretreated for 1 h with a cocktail of T_H2 neutralizing antibodies (anti-IL-4, anti-IL-5, and anti-IL-13; each at 10 μ g/mL; R&D Systems, Minneapolis, MN, USA), a cocktail of T_H1 neutralizing antibodies (anti-TNF α and anti-IFN γ ; each at 10 μ g/mL; R&D), N'-((4-Oxo-4H-chromen-3-yl)methyl-

lene) nicotinohydrizide (STAT5 inhibitor; 50 μ M; Santa Cruz Biotechnology, Dallas, TX, USA), AS1517499 (STAT6 inhibitor; 100 nM; Axon, Reston, VA, USA), PD98059 (MEK inhibitor; 10 μ M; Calbiochem, San Diego, CA, USA), SB203580 (p38MAPK inhibitor; 1 μ M; Calbiochem), SP600125 (JNK inhibitor; 50 nM; Calbiochem), and BAY 11-7082 (I κ B inhibitor; 10 μ M; Calbiochem). The concentrations of the inhibitors used in this study are in line with other *in vitro* studies [26, 27], which have shown no effects on cell survival at the selected concentrations.

Blood collection and processing

Fifty millilitre of blood was collected through direct venipuncture into heparinized vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation, washed with RPMI-1640 (Sigma), and resuspended in complete culture medium – RPMI-1640 supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin-L-glutamine (Sigma).

Cat allergen-specific T cells

Cat allergen-specific T cell lines (TCL) were generated from PBMC of patients with cat-allergic asthma. PBMC were cultured for 14 days in the presence of cat allergen (25 μ g/mL; ALK Abello; Mississauga, ON, Canada) and IL-2 (10 ng/mL; PeproTech). T cells were restimulated with autologous irradiated (3000R) PBMC and cat allergen extract for 3–6 cycles. In each 7-day restimulation cycle, IL-2 was added at days 0 and 3. Supernatant was obtained following 48-h restimulation with plate-bound anti-CD3/anti-CD28.

Monocyte-derived dendritic cells (moDC)

CD14⁺ human monocytes were isolated from PBMC by positive magnetic selection using EasySep[®] Human Buffy Coat CD14 Positive Selection kit (STEMCELL Technologies, Vancouver, BC, Canada) as per manufacturer's instructions. Monocytes were cultured (2×10^5 cells/mL) in 96-well flat-bottom plates in complete RPMI supplemented with GM-CSF and IL-4 (each at 25 ng/mL; PeproTech) at 37°C, 5% CO₂, for 6 days.

moDC activation

Mature moDC were stimulated for 48 h with supernatant from activated PBEC. Isotype control IgG₁ (eBioscience, San Diego, CA, USA) or anti-TSLP monoclonal antibody (mAb) (generous gift of Dr. G. Delespesse; University of Montreal, QC, Canada) was added to a final concentration of 10 μ g/mL.

Cytokine and chemokine measurement

TSLP and CCL17 in cell-free supernatant were measured using DuoSet ELISA Development kit (R&D) as per manufacturer's instructions. Cat allergen-specific T cells (2×10^6 cells/well) were stimulated for 48 h, and cell-free supernatant was harvested, and IL-1 β , IL-4, IL-6, IL-9, IL-10, IL-13, GM-CSF, IFN γ , TNF α , CXCL8, CCL11, CCL2, and CCL5 were assessed using Bio-Plex assays (Bio-Rad, Hercules, CA, USA) according to manufacturer's recommendation. The detection limits for these cytokines were 3.2 (IL-1 β), 12 (IL-3), 2.2 (IL-4), 3.1 (IL-5), 2.3 (IL-6), 2.1 (IL-9), 2.2 (IL-10), 3.7 (IL-13), 2.2 (GM-CSF), 92.6 (IFN γ), 5.8 (TNF α), 1.0 (CXCL8), 40.9 (CCL11), 2.1 (CCL2), and 2.2 pg/mL (CCL5).

Statistical analysis

All data are expressed as the means \pm SEM. Significance was assumed at $P < 0.05$. All analyses were performed with PASW Statistics (version 18) (SPSS, Chicago, IL, USA) using nonparametric tests. Differences within groups were assessed by Friedman test with Dunn's post hoc test. Differences between groups (healthy individuals vs. patients with asthma) were assessed by Mann-Whitney U-test.

Results

Clinical characteristics of patients with asthma

All subjects had either a positive methacholine challenge or significant airways reversibility to bronchodilator [23]. Additionally, all subjects were taking regular

Table 1. Clinical characteristics of patients with asthma

Characteristics	Asthmatics (n = 6)
Age (years)	52 (24–64)
Ratio M/F	2:4
BMI (kg/m ²)	32.28 (21.1–48.1)
FEV ₁ , L (prebronchodilator)	2.39 (1.47–4.5)
FEV ₁ ,% predicted (prebronchodilator)	79.5 (50–97)
FEV ₁ , L (post-bronchodilator)	2.53 (1.67–4.51)
FEV ₁ ,% predicted (post-bronchodilator)	86 (64–103)
Δ FEV ₁ bronchodilator (%)	5 (1–14)
PC ₂₀ methacholine (mg/mL)	12.5 (1.33–33)
Eosinophil (absolute)	0.25 (0.2–0.5)
Total IgE	63 (24–344)
ICS/LABA (n)	6
Inhaled corticosteroids (μ g)*	400 (100–1000)

FEV₁, forced expiratory volume in 1 s; PC₂₀ methacholine, concentration of methacholine provoking a fall in FEV₁ of 20% or more; ICS/LABA, inhaled corticosteroid/long-acting β -agonists.

Values shown are numbers of median (minimum–maximum).

*Inhaled equivalent.

inhaled steroids ($\geq 400 \mu\text{g}$ beclomethasone/day). Further subject characteristics are shown in Table 1.

Pro-inflammatory cytokines induce TSLP expression in PBEC

Pro-inflammatory cytokines have been shown to induce TSLP production (mRNA or protein) from human airway epithelial cells [11, 12, 28]. To investigate whether airway epithelial cells grown at ALI behave similarly, healthy PBEC were stimulated with cytokines (1, 10, 100 ng/mL) or medium alone and culture supernatants assayed for TSLP. Dose-dependent release of TSLP was observed in response to $\text{TNF}\alpha$ ($P < 0.05$). IL-1 β , IL-13, and IL-4 induced a substantial amount of TSLP although no dose response was observed within the range examined (Fig. 1).

TLR3 ligand induces TSLP expression in PBEC

To further validate our ALI model, we examined the effects of polyI:C on the induction of TSLP from PBEC

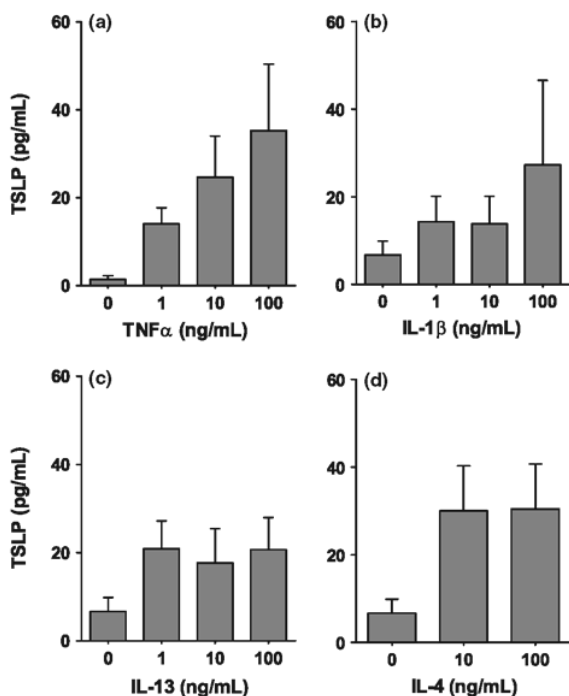


Fig. 1. Pro-inflammatory cytokines induce TSLP in healthy human PBEC. Healthy human PBEC were incubated for 24 h with varying concentrations (0–100 ng/mL) of $\text{TNF}\alpha$ (a), IL-1 β (b), IL-13 (c), and IL-4 (d). Concentrations of TSLP protein in the culture supernatant were measured by ELISA. One independent experiment performed per subject. Results shown are mean \pm SEM in duplicate ($n = 5$). Significant difference from untreated cells ($*P < 0.05$)

obtained from healthy individuals and patients with asthma grown at ALI. Stimulation with polyI:C induced a dose-dependent release of TSLP in both groups, with significantly higher expression of TSLP following 50 $\mu\text{g}/\text{mL}$ of polyI:C compared to medium alone (healthy controls: $97.91 \pm 17.67 \text{ pg}/\text{mL}$ vs. $0.45 \pm 0.45 \text{ pg}/\text{mL}$, $P < 0.001$; and patients with asthma: $184.74 \pm 33.37 \text{ pg}/\text{mL}$ vs. $19.82 \pm 14.67 \text{ pg}/\text{mL}$, $P < 0.01$, respectively; Fig. 2). PBEC derived from patients with asthma expressed higher levels of TSLP than those from healthy controls across all concentrations ($184.738 \pm 33.37 \text{ pg}/\text{mL}$ vs. $97.91 \pm 17.67 \text{ pg}/\text{mL}$ at 50 $\mu\text{g}/\text{mL}$ poly I:C; $P < 0.05$; Fig. 2).

Allergen-specific T cell-soluble products elicit greater TSLP production in PBEC from patients with asthma

We investigated whether mediators secreted by allergen-specific T cells could activate airway epithelial cells to produce TSLP. Supernatants from activated cat allergen-specific T cells cultured at 2×10^6 cells/mL induced significantly more TSLP secretion from ALI-differentiated PBEC from both clinical groups, compared to medium alone (healthy subjects: $8.78 \pm 2.44 \text{ pg}/\text{mL}$ vs. $3.62 \pm 1.19 \text{ pg}/\text{mL}$, $P < 0.05$; patients with and asthma: $22.57 \pm 5.05 \text{ pg}/\text{mL}$ vs. $9.14 \pm 1.71 \text{ pg}/\text{mL}$, $P < 0.05$; Fig. 3). PBEC from patients with asthma expressed higher levels of TSLP than PBEC obtained from healthy controls (2.6-fold; $P < 0.05$; Fig. 3).

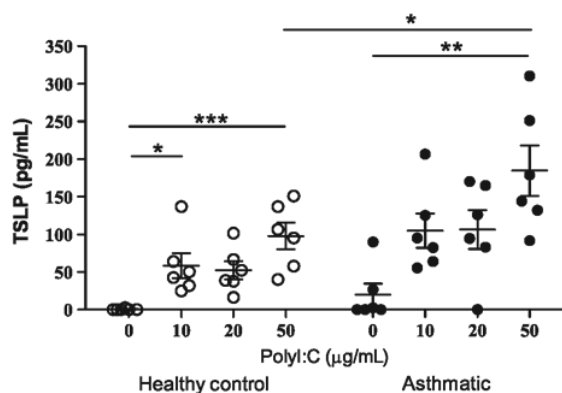


Fig. 2. Triggering of TLR3 induces TSLP production in human PBEC grown at ALI. Healthy (open circles) and asthmatic (closed circles) PBEC grown at ALI were incubated for 24 h with increasing concentrations of poly I:C (0–50 ng/mL). Concentrations of TSLP protein in the culture supernatant were measured using ELISA. Results shown are mean \pm SEM in duplicate ($n = 6$). One independent experiment performed per subject. Significant difference within group from untreated cells ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$) and between groups ($*P < 0.05$).

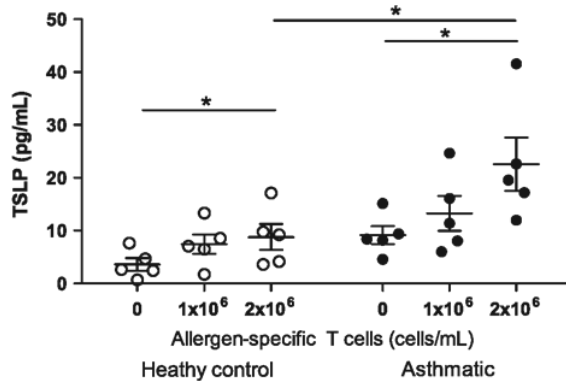


Fig. 3. Allergen-specific T cell-soluble products elicit greater TSLP production from asthmatic PBEC. Both healthy (open circles) and asthmatic (closed circles) PBEC were stimulated for 24 h with supernatants from purified allergen-specific T cells (1×10^6 and 2×10^6 cells/mL). Concentrations of TSLP protein in the culture supernatant were measured using ELISA. Results shown are mean \pm SEM in duplicate ($n = 5$). One independent experiment performed per subject. Significant difference within group from untreated cells ($^*P < 0.05$) and between groups ($^*P < 0.05$).

Inhibition of MEK, MAPK, and NF κ B pathways reduces allergen-specific T cell-induced TSLP

To elucidate the responsible mediators in the supernatant of activated allergen-specific T cells, PBEC were stimulated with allergen-specific T cell supernatants in the presence or absence of neutralizing antibodies against a cocktail of T_H2 cytokines (IL-4, IL-5, and IL-13) or a cocktail of T_H1 cytokines (TNF α and IFN γ) and the supernatants were collected and analysed for TSLP levels. We demonstrated that neutralizing a pool of T_H2 or T_H1 cytokines did not effectively inhibit TSLP production (Fig 4a). Next, pharmacological inhibitors were used to elucidate the signalling pathway(s) responsible for mediating allergen-specific T cell-induced TSLP expression. Preincubation with STAT5/6 inhibitors or LY294002 (phosphatidylinositol 3-kinase (PI3K) inhibitor) had no effect on allergen-specific T cell induction of TSLP expression. In contrast, SP600125 (c-Jun N-terminal kinase (JNK) inhibitor), PD98059 (MEK inhibitor), SB203580 (p38), or BAY 11-7082 (I κ B inhibitor) significantly inhibited the production of TSLP ($P < 0.05$; Fig. 4b).

IL-1 β , IL-6, and CXCL8 secreted by allergen-specific T cells induce TSLP production in PBEC

To further characterize the responsible mediators involved in the induction of TSLP, supernatants from activated cat allergen-specific T cells cultured at 2×10^6 cells/mL were analysed for cytokine/chemokine secretion using Luminex. IL-1 β , IL-5, IL-6, IL-13, CCL2,

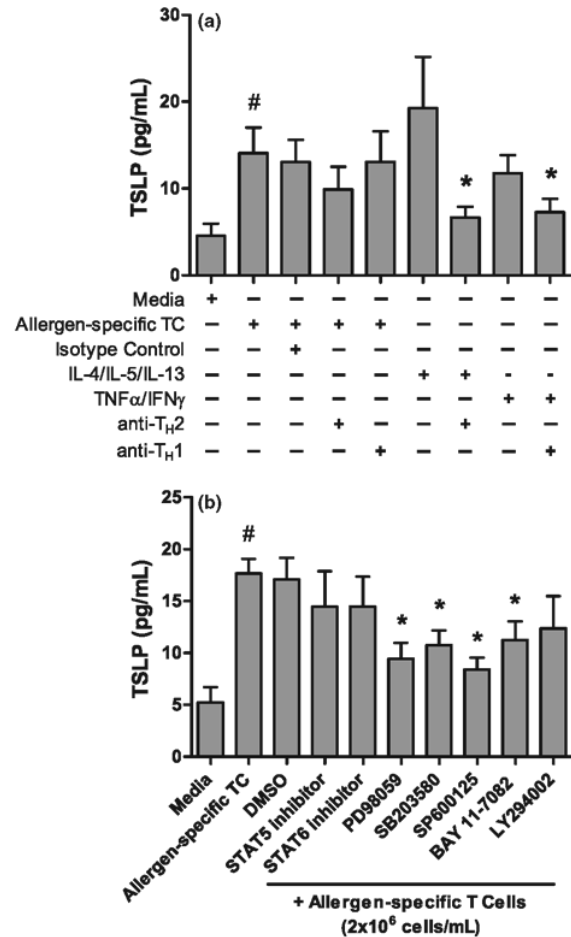


Fig. 4. Allergen-specific T cell-induced TSLP is dependent on MEK, MAPK, and NF κ B pathways. Healthy PBEC were preincubated with (a) a cocktail of T_H2 neutralizing antibodies (anti-IL-4, anti-IL-5, and anti-IL-13; each at 10 μ g/mL) or a cocktail of T_H1 neutralizing antibodies (anti-TNF α and anti-IFN γ ; each at 10 μ g/mL) before stimulating with supernatant from activated allergen-specific T cells, or a cocktail of T_H2 cytokines (IL-4, IL-5, and IL-13; each at 10 ng/mL) or T_H1 cytokines (TNF α and IFN γ ; each at 10 ng/mL). (b) STAT5 inhibitor (50 μ M), STAT6 inhibitor (100 nM), PD98059 (MEK inhibitor; 10 μ M), SB203580 (p38MAPK inhibitor; 1 μ M), SP600125 (JNK inhibitor; 50 nM), BAY 11-7082 (I κ B inhibitor; 10 μ M), and LY294002 (PI3K inhibitor; 50 μ M) for 1 h before stimulating with supernatant from activated allergen-specific T cells. Results shown are mean \pm SEM in duplicate ($n = 6$). One independent experiment performed per subject. Significant difference from media ($^{\#}P < 0.05$) and allergen-specific T cells ($^*P < 0.05$).

CCL5, and CXCL8 were the predominant mediators secreted by allergen-specific T cells (Fig 5a). Allergen-specific T cells failed to secrete detectable levels of CCL11. We next examined the effects of human recombinant IL-1 β , IL-5, IL-6, CCL5, and CXCL8 on TSLP production in PBEC. When combined, IL-1 β , IL-6,

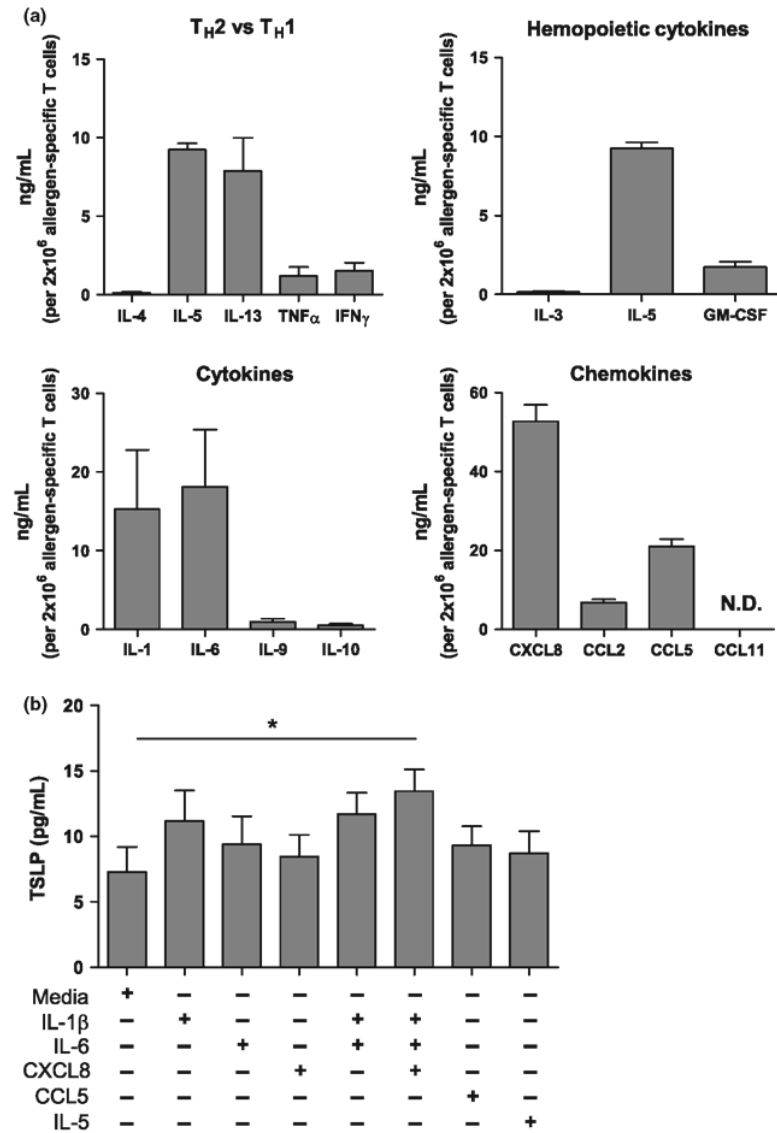


Fig. 5. Allergen-specific T cells induce TSLP production in PBEC via IL-1 β , IL-6, and CXCL8. (a) Cat allergen-specific T cells (2×10^6 cells/well) were stimulated for 48 h, and cell-free supernatant was harvested and measured for cytokine/chemokine secretion (pg/mL) using Luminex. (b) Healthy PBEC were incubated for 24 h with IL-1 β (15 ng/mL), IL-6 (20 ng/mL), CXCL8 (50 ng/mL), CCL5 (20 ng/mL), and IL-5 (10 ng/mL). Concentrations of TSLP protein in the culture supernatant were measured by ELISA. One independent experiment performed per subject. Results shown are mean \pm SEM in duplicate ($n = 5$). Significant difference from untreated cells ($*P < 0.05$). N.D., not detected.

and CXCL8 together significantly enhanced TSLP expression from PBEC (Fig. 5b).

TSLP-dependent production of CCL17

To assess the biological activity of TSLP in the supernatants of activated airway epithelial cells, moDC were stimulated with supernatants (SN) from [1] polyI:C-

exposed PBEC from healthy individuals (healthy polyI:C-SN) and patients with asthma (asthmatic polyI:C-SN) and [2] cat allergen-specific TCL supernatant-stimulated PBEC from healthy subjects (healthy TCL-SN) and patients with asthma (asthmatic TCL-SN). Healthy and asthmatic polyI:C-SN induced CCL17 production from moDC that was significantly inhibited by anti-TSLP mAb ($P < 0.001$) (Fig. 6a,b). moDC cultured with asthmatic

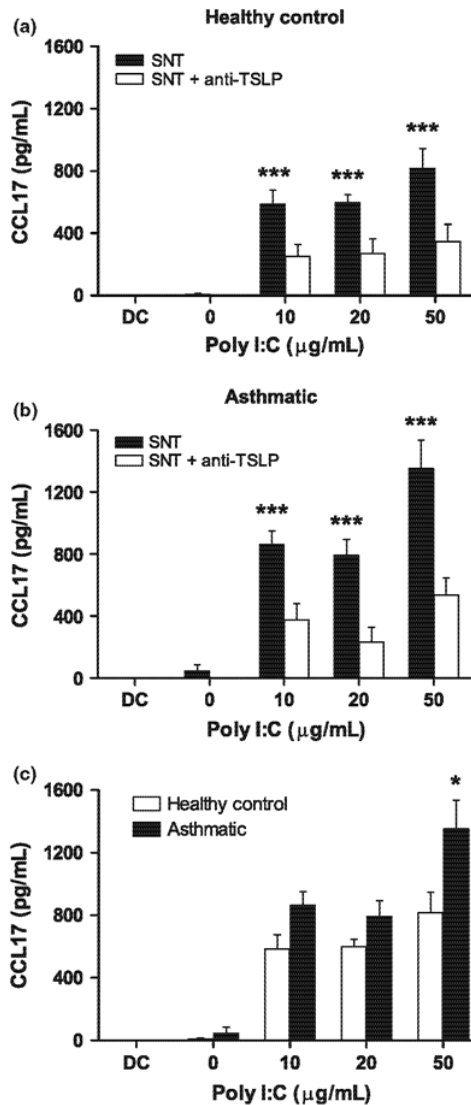


Fig. 6. TSLP-dependent production of CCL17 by moDC following culture with PBEC polyI:C-SN. Fully differentiated healthy and asthmatic PBEC grown at ALI were stimulated with polyI:C-SN for 24 h and supernatant collected for activation of moDC. moDC were stimulated for 48 h with healthy (a) and asthmatic (b) polyI:C-SN in the presence or absence of neutralizing anti-TSLP and supernatant collected and measured for CCL17 using ELISA. (c) CCL17 secretion by moDC following healthy (white bars) and asthmatic (black bars) polyI:C-SN. Results shown are mean \pm SEM in duplicate ($n = 5$). One independent experiment performed per subject. Significant difference within group from SNT + anti-TSLP ($***P < 0.001$) and between groups ($*P < 0.05$).

polyI:C-SN produced significantly higher levels of CCL17 than that cultured with healthy polyI:C-SN (1.7-fold at 50 $\mu\text{g/mL}$ poly I:C; $P < 0.05$; Fig. 6c). Likewise, healthy and asthmatic TC-SN induced CCL17 produc-

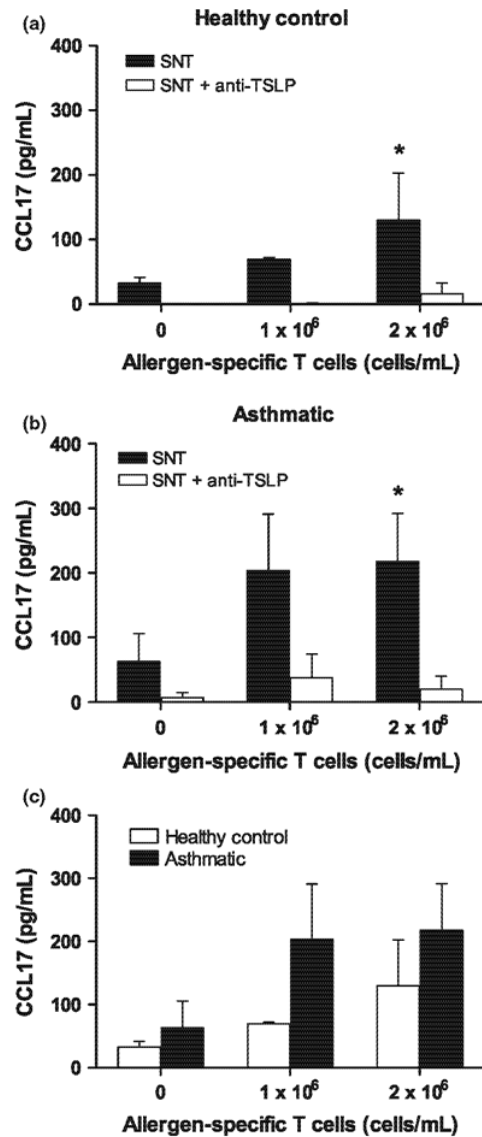


Fig. 7. TSLP-dependent production of CCL17 by moDC following culture with PBEC TC-SN. Fully differentiated healthy and asthmatic PBEC grown at ALI were stimulated with TC-SN for 24 h and supernatant collected for activation of moDC. moDC were stimulated for 48 h with healthy (a) and asthmatic (b) TC-SN in the presence or absence of neutralizing anti-TSLP and supernatant collected and measured for CCL17 using ELISA. (c) CCL17 secretion by moDC following healthy (white bars) and asthmatic (black bars) TC-SN. Results shown are mean \pm SEM in duplicate ($n = 3$). One independent experiment performed per subject. Significant difference within group from SNT + anti-TSLP ($*P < 0.05$).

tion from moDC that was significantly inhibited by the addition of anti-TSLP mAb ($P < 0.05$; Fig. 7a,b). In agreement with data from polyI:C-SN, asthmatic TC-SN

induced higher levels of CCL17 from moDC than healthy TCL-SN, although the difference between groups did not achieve statistical significance (Fig. 7c).

Discussion

The active role of the epithelium in the initiation and regulation of mucosal inflammation has become increasingly clear [8, 9]. The crosstalk between epithelial cells and DC, mediated by allergen-specific T cell-induced TSLP, may be an important mechanism for the allergic immune cascade. In a recent study [18], bronchoscopic analysis of T cell recruitment to the airway revealed significant increases in CD3⁺ and CD4⁺ T cells following inhaled peptide challenge (in individuals who developed LAR), which was associated with increased levels of CCL17 in the airways/lung. Levels of CCL17 correlated with the magnitude of the LAR, suggesting an association between CCL17 and CD4⁺ T cell accumulation, in the induction of the LAR. Together, these observations support our finding of enhanced allergen-specific T cell-mediated CCL17 by DC *in vitro* in relation to clinical asthmatic status – a process that we demonstrate to be dependent on TSLP. The capacity of TSLP to participate in allergen-specific T cell-driven allergic response points to the potential importance of T cell–epithelium interactions in the development of allergic inflammation.

Airway epithelial cells produce an array of cytokines and chemokines that have the capacity to orchestrate allergic inflammation in the lung [15]. We demonstrate herein the production of TSLP by both healthy and asthmatic PBEC grown at ALI. To date, the ability of individual T_H2 cytokines to stimulate TSLP protein expression in the airway epithelium remains controversial. Kato *et al.* [12] did not find detectable levels of TSLP following stimulation with TNF α , IL-4, or IL-13. Similarly, Lee *et al.* [28] found that IL-4 and IL-13 had no effect on TSLP expression, but demonstrated increased TSLP production with IL-1 or TNF α . Allakverdi *et al.* [11] reported the combined effects of IL-1 β and TNF α on TSLP production in small airways epithelial cells (SAEC). In the present study, we found a dose-dependent release of TSLP in healthy PBEC grown at ALI following stimulation with TNF α or IL-1 β (Fig. 1). Recently, TNF α or IL-1 β alone was shown to induce TSLP production from human airway smooth muscle cells [29]. *In vivo*, a combination of ciliated, non-ciliated, and mucous-secreting cells constitutes the airway epithelium [30]. PBEC grown in ALI cultures are well differentiated, with a mixed mucociliary phenotype, compared to the poorly differentiated PBEC grown in submerged monolayer cultures [31, 32]. Epithelial cells in ALI cultures have been reported to secrete tonic levels of inflammatory cytokines including TNF α [33]. There-

fore, the ability to detect TSLP in our *in vitro* cultures could result from the synergistic effects of autocrine TNF α and our selected cytokines of interest. This is in line with the reports that showed that a combination of TNF α with IL-4, IL-13, or IL-1 β up-regulates TSLP expression in healthy PBEC [12] and SAEC [11].

Previous studies have demonstrated that exposure to dsRNA *in vitro* induces TSLP expression in SAEC [11] and PBEC from healthy individuals and patients with asthma [12, 13]. We demonstrate herein that PBEC from patients with asthma have a higher response to stimuli, resulting in the release of more TSLP than PBEC from healthy controls (Fig. 2), which is consistent with previous reports [13, 34, 35]. Our data support the reproducibility of dsRNA-dependent TSLP production in human PBEC and extend these findings by showing it in PBEC grown in ALI conditions, which allow cells to differentiate to a mixed mucociliary phenotype more representative of *in vivo* conditions than liquid submerged cultures [32]. Of note, Lee *et al.* [34] failed to induce TSLP from ALI cultures from healthy children using respiratory syncytial virus (RSV). This discrepancy may be a result of intrinsic and functional differences between children- and adult-derived PBEC. However, consistent with our data, Uller *et al.* [13] were able to demonstrate TSLP secretion from healthy bronchial epithelial cells following stimulation with polyI:C. RSV represents only one of many strains of viruses; other strains of viruses have been successful in inducing TSLP from airway epithelial cells [12]. Furthermore, polyI:C is a highly accepted dsRNA surrogate commonly used in research to simulate virus infection, as it mimics many aspects of rhinovirus infection in PBEC [13], allowing for the yield of robust and reproducible data.

Little is known about the details of interaction between activated allergen-specific T cells and the epithelium. Our study provides the first evidence that exposure of PBEC to soluble products from allergen-specific T cells induces disproportionate production of TSLP in PBEC from patients with asthma compared to healthy controls. Given that allergen-specific T cells are largely associated as being T_H2 in nature, we speculate IL-4 and IL-13 [11, 12, 28] to at least partially account for the activity of the T cell supernatants. To our surprise, we found that neither neutralizing a pool of T_H2 nor T_H1 cytokines effectively inhibited TSLP production (Fig 4a). Of note, although we blocked a cocktail of T_H2 cytokines (IL-4, IL-5, and IL-13), we did not specifically block the combination of IL-1 β and IL-13 in the T cell supernatant-dependent TSLP production in PBEC. As allergen-specific T cell supernatants contained high levels of IL-13 (~8 ng/mL) and IL-1 β (~15 ng/mL; Fig. 5a) and given the modest induction of TSLP in PBEC by IL-13 (10 ng/mL) and IL-1 β (10 ng/mL; Fig. 1), there may very well be additive effect of IL-1 β and IL-13.

We further demonstrate that blocking STAT5, STAT6, or PI3K has no effect on inhibiting T cell-induced TSLP expression (Fig. 4b). In contrast, we demonstrate that the regulation of TSLP expression by allergen-specific T cells involves the MEK, mitogen-activated protein kinases (MAPK) – p38 and JNK – and nuclear factor kappa beta (NF κ B) pathways. SP600125 (JNK inhibitor) inhibited allergen-specific T cell-induced TSLP expression by more than 50%, while PD98059 (MEK inhibitor), SB203580 (p38 inhibitor), and BAY 11-7082 (I κ B inhibitor) inhibited the production of TSLP to a lesser extent (46.7%, 39.3%, and 36.4%, respectively; Fig. 4b). Of note, when the *P*-values were corrected for multiple analyses, the inhibitors no longer achieved a statistical significance, which is likely due to small sample size. Nonetheless, our results are in agreement with the findings that MAPK and NF κ B signalling regulates TSLP induction in airway epithelial cells [28, 36, 37]. Upon further characterizing the responsible mediators in driving TSLP expression, we detected high levels of IL-1 β , IL-5, IL-6, IL-13, CXCL8, CCL2, and CCL5 in the supernatants of allergen-specific T cells (Fig 5a). When combined, IL-1 β , IL-6, and CXCL8 together significantly enhanced TSLP expression in PBEC (Fig 5b). Of note, we did not examine the combination of IL-6 and CXCL8 on the induction of TSLP, and therefore, it is still unclear what the combined role of IL-6 and CXCL8 is and remains to be elucidated. Furthermore, in the current study, we did not examine the expression of CXCL8 receptors, CXCR1 and CXCR2, in bronchial epithelial cells. However, CXCR1 and CXCR2 expression has been demonstrated on bronchial epithelial cells. Farkas et al. [38] previously reported that human bronchial epithelial cell lines (BEAS2B) and PBEC from patients with chronic obstructive pulmonary disease and healthy subjects express both CXCR1 and CXCR2. This finding was confirmed by Di Stefano et al. [39] and Schulz et al. [40]. Collectively, these findings along with our data suggest that CXCL8, together with IL-1 β and IL-6, can directly act on PBEC, resulting in the up-regulation of TSLP secretion. The lack of action through STAT5/6 and anti-T_H2 intervention leads to the conclusion that the major drivers of TSLP are not a result of the combination of IL-4, IL-5, and IL-13, supporting our finding that a cocktail of antibodies to the major T_H2 cytokines was unable to suppress TSLP production. However, further studies are needed to elucidate the possible combined effects of IL-13 and IL-1 β . Together, our data provide new evidence that allergen-specific T cells are a positive regulator of TSLP production by epithelial cells, via pro-inflammatory cytokines IL-1 β , IL-6, and CXCL8, suggesting T cell–airway epithelium interactions that may lead to maintenance and amplification of allergic inflammation.

The current study presents a TSLP-driven model of inflammation demonstrating the ability of PBEC-derived TSLP to potently activate moDC, inducing the release of CCL17 (Figs 6 and 7). Although several groups have shown that it is the induction of OX40L on TSLP-treated DC that drive T_H2 responses, we examined CCL17 induction, which recruits memory CCR4⁺ T_H2 cells [4, 6, 7], to investigate whether differential expression of TSLP between asthmatic and healthy PBEC translated to CCL17 expression. We found an increased release of TSLP from asthmatic PBEC compared to healthy controls in response to TLR3-ligand or allergen-specific T cells that resulted in a correspondingly increased release of CCL17, which was markedly inhibited by the addition of an anti-TSLP mAb demonstrating the dependency of TSLP signalling in the expression of CCL17 (Figs 6 and 7). To the best of our knowledge, this is the first study to demonstrate enhanced allergen-specific T cell-mediated CCL17 expression by DC *in vitro* in relation to clinical asthmatic status. Together, our data support the notion that TSLP partakes in allergen-specific T cell-mediated mucosal inflammatory response, which is increased in patients with asthma. Of note, CCL17 is expressed in the bronchial epithelium under physiological and pathological conditions, such as bronchial asthma [14, 41]. Cytokines such as IL-4, TNF α , and IFN γ have been previously shown to induce CCL17 from bronchial epithelial cells [42, 43] and may therefore contribute to CCL17 expression in our functional assay. Furthermore, Miazgowiec et al. [35] recently demonstrated the ability of TSLP to drive the production of CCL17 from TSLPR⁺ epithelial cells. Taken together, it is plausible that the moDC supernatant may be contaminated with epithelial-derived CCL17, which may explain why anti-TSLP mAb did not completely block CCL17 expression in the functional assay. Nevertheless, Fig. 6a shows a > 50% reduction, and Fig. 6b shows an approximate >60% reduction; therefore, although complete inhibition of CCL17 was not achieved, the degree of inhibition is substantial, highlighting the biological activity and dependence on TSLP in CCL17 induction. Increased expression of CCL17 in the bronchial epithelium of patients with asthma has previously been reported [14, 41] and may result from the action of activated T cells and other stimuli on airway epithelium, leading to TSLP production as suggested by our findings. Furthermore, given that the magnitude of LAR following peptide challenge correlates with CCL17 expression and that peptide-induced LAR is mast cell independent [18], our data suggest that mast cell-derived TSLP may not be essential for CCL17 expression [44]. Together, this supports the notion that airway narrowing and airway hyper-reactivity 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.

leads to secretion of TSLP by bronchial epithelial cells and activation of local DC, resulting in secretion of CCL17 and recruitment and activation of CD3⁺/CD4⁺ T cells, production of inflammatory cytokines, and, ultimately, trigger of the paracrine regulation of TSLP secretion by the epithelium, leading to the amplification of allergic inflammation (Fig. 8). Given that our observations support the idea that T cell activation is capable of triggering the inflammatory cascade via TSLP secretion, our findings may have high implications in patients with chronic severe asthma where the role of T cell is prominent. The effect of inhaled peptide challenge on the production of TSLP in airway epithelial cells from patients *in vivo* merits investigation. TSLP is currently a candidate for therapeutic intervention in asthma, but defining the factors that drive TSLP expression (T cell-derived factors) will allow for a better understanding of allergic disease development and for identifying new therapeutic targets for atopic disorders.

Acknowledgements

The authors acknowledge the valuable assistance of the following: Dr. Tillie Hackett, University of British

Columbia (PBEC ALI cultures); Dr. Guy Delespesse, Université de Montréal (anti-TSLP mAb); and Dr. Paul O'Byrne, Heather Campbell, and Karen Howie; McMaster University (recruitment of patients with asthma and provision of epithelial cells). The study was funded through operating grants (to ML) from the Canadian Institutes for Health Research (CIHR) and (to Dr. Paul O'Byrne) from GlaxoSmithKline Inc. ML was supported by the Canada Research Chairs program, the Canada Foundation for Innovation, and the McMaster University/GSK Chair in Lung Immunology at St. Joseph's Healthcare.

Conflict of interests

ML is a stockholder and consultant of Circassia Ltd. and Adiga Life Sciences Inc. and received research support from both of these companies. DMM is the past recipient of a European Respiratory Society Fellowship. He has been on advisory boards for Novartis and Nycomed and received speaker's fees from MSD and Astra Zeneca. He also received research support from Novartis, MSD, and Pfizer. The rest of the authors declare no conflict of interest.

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CHAPTER 3: The effects of thymic stromal lymphopoietin and IL-3 on human eosinophil-basophil lineage commitment: relevance to atopic sensitization

Published in *Immunity, Inflammation and Disease* 2014; 2(1):44-55

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Seminal studies have highlighted a critical immunomodulatory role for TSLP in allergic inflammation, as well as TSLP effects on the differentiation of progenitors in animal models [142]. Recently, TSLP has been shown to enhance Th2 cytokine production from human progenitors [15], but the role of TSLP in ‘*in situ* hemopoiesis’ and its relevance to atopic sensitization have not been studied. We therefore investigated the effects of TSLP on the induction of Eo/B differentiation from HHP as well as the molecular mechanisms through which this TSLP-dependent hemopoiesis occurs, and their relationships to atopy. We demonstrated that CD34+ progenitors have the capacity to participate in TSLP-driven allergic response *in vitro* through enhanced Eo/B colony formation. Furthermore, we showed p38MAPK-dependent autocrine signaling by TNF α in TSLP-mediated human Eo/B differentiation *ex vivo*. Lastly, we demonstrated enhanced stimulatory effects of TSLP on peripheral blood Eo/B progenitors-derived from atopic individuals.

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ORIGINAL RESEARCH

The effects of thymic stromal lymphopoietin and IL-3 on human eosinophil–basophil lineage commitment: Relevance to atopic sensitization

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Keywords

Atopy, eosinophil–basophil progenitors, hemopoiesis, IL-3, TSLP

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Funding information

This work was supported by AllerGen NCE Inc. and CIHR MOP 111029

Received: 28 January 2014; Revised: 2 April 2014; Accepted: 3 April 2014

Immunity, Inflammation and Disease (2014)

doi: 10.1002/iid3.20

Abstract

An important immunopathological hallmark of allergic disease is tissue eosinophilic and basophilic inflammation, a phenomenon which originates from hemopoietic progenitors (HP). The fate of HP is determined by local inflammatory cytokines that permit “in situ hemopoiesis,” which leads to the accumulation of eosinophils and basophils (Eo/B). Given that recent evidence supports a critical immunomodulatory role for thymic stromal lymphopoietin (TSLP) in allergic inflammation, as well as TSLP effects on CD34+ progenitor cytokine and chemokine secretion, we investigated the role of TSLP in mediating eosinophilo- and basophilopoiesis, the mechanisms involved, and the association of these processes with atopic sensitisation. In the studies presented herein, we demonstrate a direct role for TSLP in Eo/B differentiation from human peripheral blood CD34+ cells. In the presence of IL-3, TSLP significantly promoted the formation of Eo/B colony forming units (CFU) (including both eosinophils and basophils) from human HP (HHP), which was dependent on TSLP–TSLPR interactions. IL-3/TSLP-stimulated HHP actively secreted an array of cytokines/chemokines, key among which was TNF α , which, together with IL-3, enhanced surface expression of TSLPR. Moreover, pre-stimulation of HHP with IL-3/TNF α further promoted TSLP-dependent Eo/B CFU formation. HHP isolated from atopic individuals were functionally and phenotypically more responsive to TSLP than those from nonatopic individuals. This is the first study to demonstrate enhanced TSLP-mediated hemopoiesis ex vivo in relation to clinical atopic status. The capacity of HHP to participate in TSLP-driven allergic inflammation points to the potential importance of “in situ hemopoiesis” in allergic inflammation initiated at the epithelial surface.

Introduction

The airway epithelium is an important initiator of the allergic response; it secretes cytokines/chemokines, which regulate innate immune cells [1]. One such cytokine is thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine [2], which can be elicited from human airway epithelial cells by cytokines and pathogen-associated molecular patterns [3, 4]. Many effector cells involved in allergic diseases such as eosinophils [5], basophils [6, 7], and mast cells [3, 8] have all been shown to respond to TSLP with increased survival, differentiation, and cytokine secretion. TSLP signals through

a heterodimeric receptor complex consisting of the IL-7R α chain and a TSLP binding chain (TSLPR) [9]. TSLP is known to signal through the JAK/STAT and MAPK pathways [10, 11], both of which are involved in the differentiation of hemopoietic progenitor cells into eosinophils [12, 13]. However, the pathways involved in basophilopoiesis remain unclear [7]. Nonetheless, human eosinophils and basophils are closely linked during development and share a common progenitor [14–16], while in the mouse these differentiative pathways appear distinct [17].

Human eosinophils and basophils differentiate from a common *committed* CD34+ hemopoietic progenitor cell,

the eosinophil–basophil (Eo/B) progenitor, found in bone marrow, cord blood, and peripheral blood (PB) [14]. We have previously provided evidence that allergic inflammation is, at least in part, a result of CD34+ progenitors homing to sites of inflammation where they differentiate, under the control of local inflammatory cytokines, into eosinophils and basophils, a process referred to as “in situ hemopoiesis” [18–20]. This overarching concept is supported by findings of many investigators: Siracusa *et al.* [6], demonstrated that cytokines found at sites of inflammation (IL-3 or TSLP) can differentially impact the differentiation of murine progenitors into effector cells (basophils), resulting in functional and phenotypic heterogeneity; Sergejeva *et al.* [21], reported that ~10% of the eosinophilic cells found in murine bronchial alveolar lavage fluid post-allergen exposure was derived from eosinophil-lineage committed precursor cells, or local production of eosinophils within the airway; Robinson *et al.* [22], Kim *et al.* [23], and Dorman *et al.* [24] collectively showed that human CD34+ progenitors are detected in the bronchial and nasal mucosa, and sputum, respectively, of patients with atopic asthma and nasal polyposis, with increased numbers of CD34+/IL-5R α + cells found in the airways and sputum of asthmatics following allergen challenge, suggesting that CD34+Eo/B lineage committed cells are found in the tissue [22, 24]; furthermore, Allakhverdi *et al.* [8] demonstrated that human CD34+ progenitors can be induced by TSLP to produce Th2 cytokines, principally IL-5 and IL-13, and that these double-positive CD34+ cells are present in sputum after airway allergen challenge of atopic asthmatics, suggesting that progenitors may act as proinflammatory effector cells and directly contribute to allergic inflammation.

Recent evidence supports a critical immunomodulatory role for TSLP in allergic inflammation, as well as TSLP effects on CD34+ progenitor cytokine and chemokine secretion [8], but the biological effects of TSLP on human PB CD34+ progenitor Eo/B lineage commitment have not been previously described. In this study, we examine the influence of TSLP on IL-3-dependent CD34+ progenitor differentiation via phenotypic and functional human hemopoietic progenitor (HHP)-related Eo/B lineage commitment. Additionally, we elucidate the mechanisms through which TSLP enhances IL-3-mediated eosinophilo- and basophilopoiesis, and the association of these processes with atopic sensitisation.

Methods

Subjects

This study was approved by the Hamilton Health Sciences Research Ethics Board (approval number 08-015) and all

subjects provided written informed consent. Atopy-unattributable subjects were initially recruited for the study (Figs. 1–4), following which, subjects with ($n=10$) or without ($n=10$) atopy were recruited (Fig. 5). Atopy was defined as a positive skin prick test response (>2-mm wheal) to at least one of 14 common aeroallergens. Further subject characteristics are shown in Table 1.

Blood collection and processing

One hundred mL of blood were collected through direct venipuncture into heparinized vacutainer tubes (Becton–Dickinson, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells were isolated by density centrifugation and CD34+ progenitors were enriched using EasySep™ Human Progenitor Cell Enrichment Kit with Platelet Depletion (STEMCELL Technologies, Vancouver, BC, Canada) as per manufacturer’s instructions.

Methylcellulose colony assays

Enriched CD34+ progenitors (8000 cells/well) were cultured in duplicates in 0.9% methylcellulose (Sigma Aldrich, St. Louis, MO, USA) with Iscove’s 2+ (modified Dulbecco’s medium (Gibco, Burlington, Ontario, Canada) supplemented with FBS, penicillin–streptomycin, and 2-ME) and IL-3 (1 ng/mL), IL-5 (1 ng/mL), or GM-CSF (10 ng/mL; BD Biosciences, Mississauga, ON, Canada) in the presence or absence of TSLP (10 ng/mL; PeproTech, Rocky Hill, NJ, USA) in 12-well plates (Corning Costar, Corning, NY, USA). In some experiments, cells were treated with anti-TSLP (Amgen, Seattle, WA, USA), anti-TSLPR (R&D Systems, Minneapolis, MN, USA), anti-TNF α (R&D), or isotype control (each at 10 μ g/mL). Treatment with the indicated stimulatory/inhibitory conditions had no effects on cell viability as determined by trypan blue exclusion. Cultures were incubated for 14 days (37°C, 5% CO $_2$). Eo/B CFU were enumerated using inverted light microscopy (colonies were defined as tight, granular clusters ≥ 40 cells).

Antibodies

Antibodies used included CD123-PE, TSLPR-PE, IL-7R α -APC, CD34-PerCP, and CD45-eFluor 450. IL-3R and TSLPR/IL-7R α and their respective isotype controls were purchased from BD Biosciences, Mississauga, ON, Canada and eBioscience, San Diego, CA, USA, respectively. CD34 and CD45 antibodies were purchased from BioLegend and eBioscience, San Diego, CA, USA, respectively.

Cell staining

Isolated CD34+ progenitors (10⁶ cells/mL) were stimulated overnight as indicated. Following which, CD34+

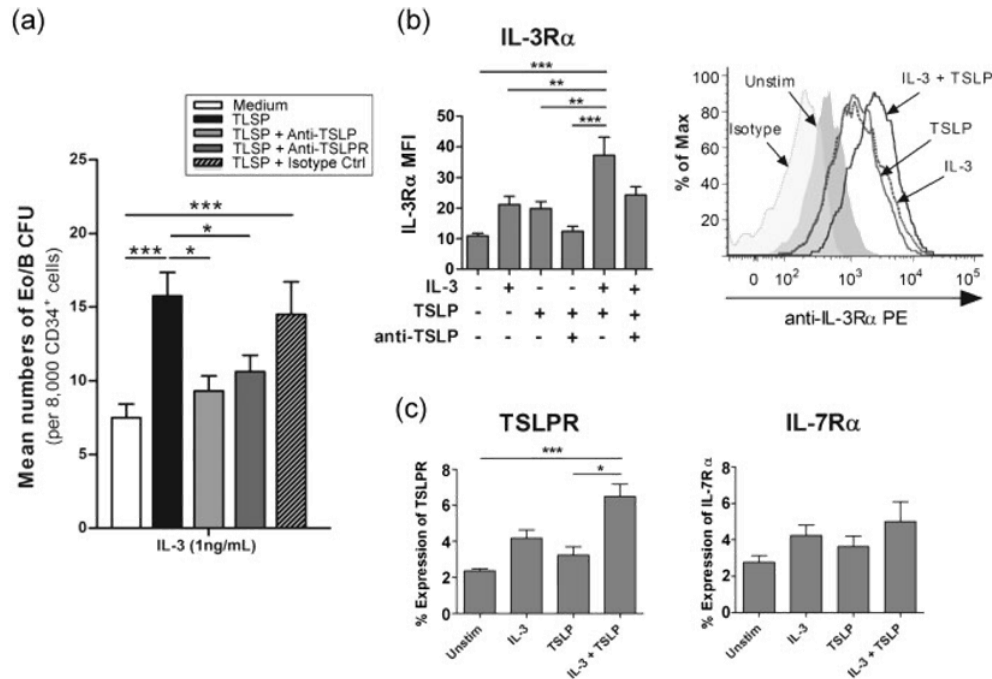


Figure 1. IL-3 and TSLP mediate Eo/B differentiation from PB HHP. PB CD34⁺ cells were stimulated as indicated and assessed for (a) Eo/B CFU by methylcellulose cultures ($n = 8$ in duplicates) and surface expression of (b) IL-3R α ($n = 6$), and (c) TSLPR ($n = 8$) and IL-7R α ($n = 7$) by flow cytometry. (b) A representative overlay histogram for expression of IL-3R α on unstimulated (shaded gray), IL-3 (dotted line), TSLP (gray line), and IL-3/TSLP (black line) stimulated CD34⁺ cells. Normal mouse IgG was used as an isotype control (shaded-light gray). Percent expression is the percent of CD34⁺ cells expressing a given antigen at the 98% confidence limit (i.e., relative to a quadrant marker set to include 2% of cells stained with isotype control antibody). Results shown are mean \pm SEM. One independent experiment performed per subject. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

progenitors were stained as previously described with modification [18]. Briefly, cells were washed with fluorescence-activated cell sorting buffer (PBS containing 0.1% sodium azide) and resuspended in murine block (1×10^5 cells/tube) and incubated in the dark (15 min, 4°C). Next, optimal amounts of isotype controls or test antibodies were added and incubated in the dark (30 min, 4°C). Cells were then washed, fixed in cytofix (BD), and stored in the dark at 4°C until ready for acquisition.

Acquisition and analysis

Stained cells were acquired with a LSR II flow cytometer (BD Biosciences) using the FACSDiva software (BD Biosciences). Offline analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA). CD34⁺ cells were enumerated using a previously established multi-parameter sequential gating strategy [18]. CD34⁺ progenitor cells were identified as having high CD34 expression, low-intermediate CD45 expression, and low forward and side scatter (Supplementary Fig. S1). Receptor expression data were collected as the

percentage of positive cells at the 98% confidence limit (i.e., relative to a quadrant marker set to include 2% of cells stained with isotype control antibody). Median fluorescence intensity (MFI) is defined as the MFI of the receptor of interest divided by the MFI of the isotype control. Due to the use of an enrichment protocol, absolute numbers of CD34⁺ cells were not determined.

Cytokine and chemokine secretion

CD34⁺ cells (10^6 cells/mL) were stimulated overnight as indicated and cell-free supernatant was harvested and IL-1 β , IL-4, IL-6, IL-9, IL-10, IL-13, GM-CSF, IFN γ , TNF α , CXCL8, eotaxin, CCL2 (MCP-1), CCL5 (RANTES), and CCL17 (TARC) were assessed using Bio-Plex assays (Bio-Rad, Hercules, CA, USA) according to manufacturer's recommendation. The detection limits for these cytokines were 3.2 (IL-1 β), 2.2 (IL-4), 2.3 (IL-6), 2.1 (IL-9), 2.2 (IL-10), 3.7 (IL-13), 2.2 (GM-CSF), 92.6 (IFN γ), 5.8 (TNF α), 1.0 (CXCL8), 40.9 (eotaxin), 2.1 (CCL2), 2.2 (CCL5), and 1.7 pg/mL (CCL17).

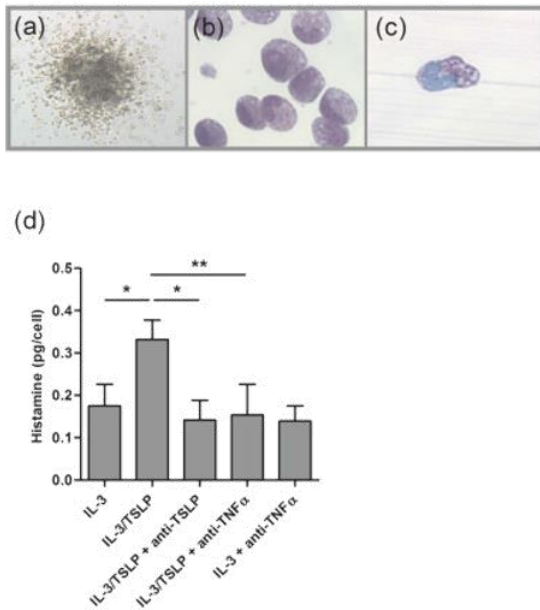


Figure 2. TSLP enhances IL-3-induced basophilopoiesis from PB HHP. (a) Representative pictograph of a colony on day 14 of a methylcellulose colony culture, which was then plucked and cytopsin preparations made and stained for (b) eosinophils and (c) basophils with Diff-Quik solution and toluidine blue respectively. (d) Histamine assay was performed on individual colonies on day 14 of methylcellulose colony cultures ($n = 15$). Results shown are mean \pm SEM. One independent experiment performed per subject. * $P < 0.05$; ** $P < 0.01$.

Histochemical stains

Individual Eo/B CFU cells were picked from methylcellulose and placed into PBS. Cytopsin preparations were made on glass slides using Shandon Cyto centrifuge 3 (Shandon Southern Instruments, Cambridge, UK). Eosinophils were identified using Diff-Quik (Siemens, Erlangen, Germany) and basophils identified using toluidine blue stain (Sigma).

Histamine assay

The total number of cells in each individual colony was enumerated using inverted light microscopy before being picked from methylcellulose and placed into PBS, boiled (99°C, 5 min), centrifuged, and cell-free supernatant harvested and measured for histamine content using Histamine Enzyme Immunoassay Kit (Bertin Pharma, Montigny-le Bretonneux, France) according to manufacturer’s recommendation. The detection limit of this assay is 55 pg/mL.

Statistical analysis

All data are expressed as the mean \pm SEM. Significance was assumed at $P < 0.05$. All analyses were performed with Prism version 5 (GraphPad Software, La Jolla, CA, USA) using non-parametric tests. Differences within groups were assessed by Friedman test with Dunnett post hoc test. Between-group comparisons (nonatopic vs. atopic) were made using the Mann–Whitney U -test.

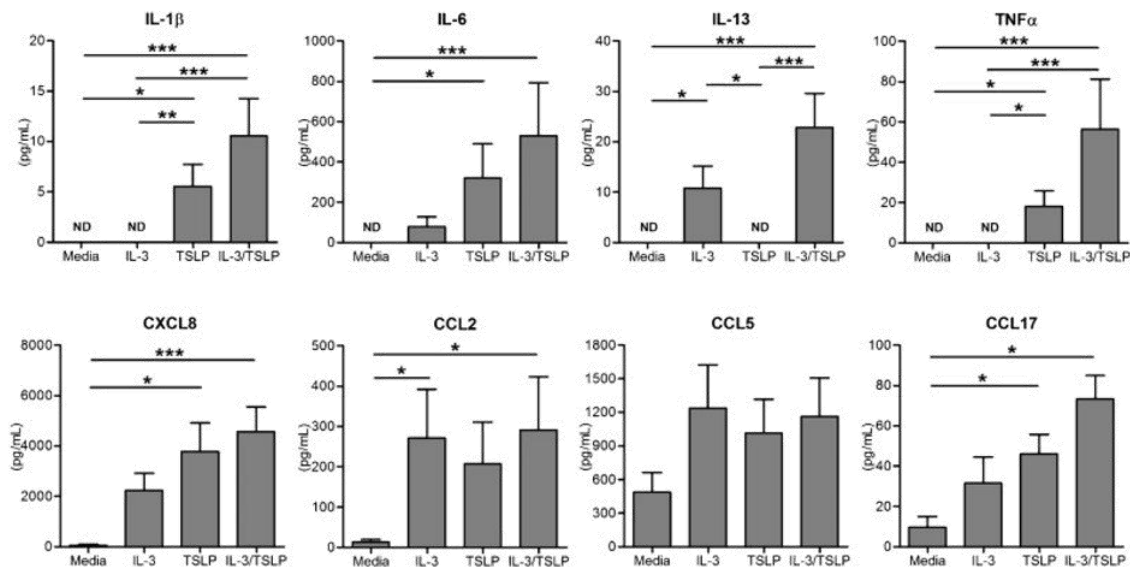


Figure 3. IL-3 and TSLP increase cytokine and chemokine secretion by PB HHP. PB CD34+ cells were stimulated overnight as indicated and cell-free supernatants were collected and measured for cytokine/chemokine secretion (pg/mL) using Luminex. Results shown are mean \pm SEM in duplicates ($n = 8$). One independent experiment performed per subject. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ND; not detected.

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TSLP enhances eosinophil and basophil differentiation

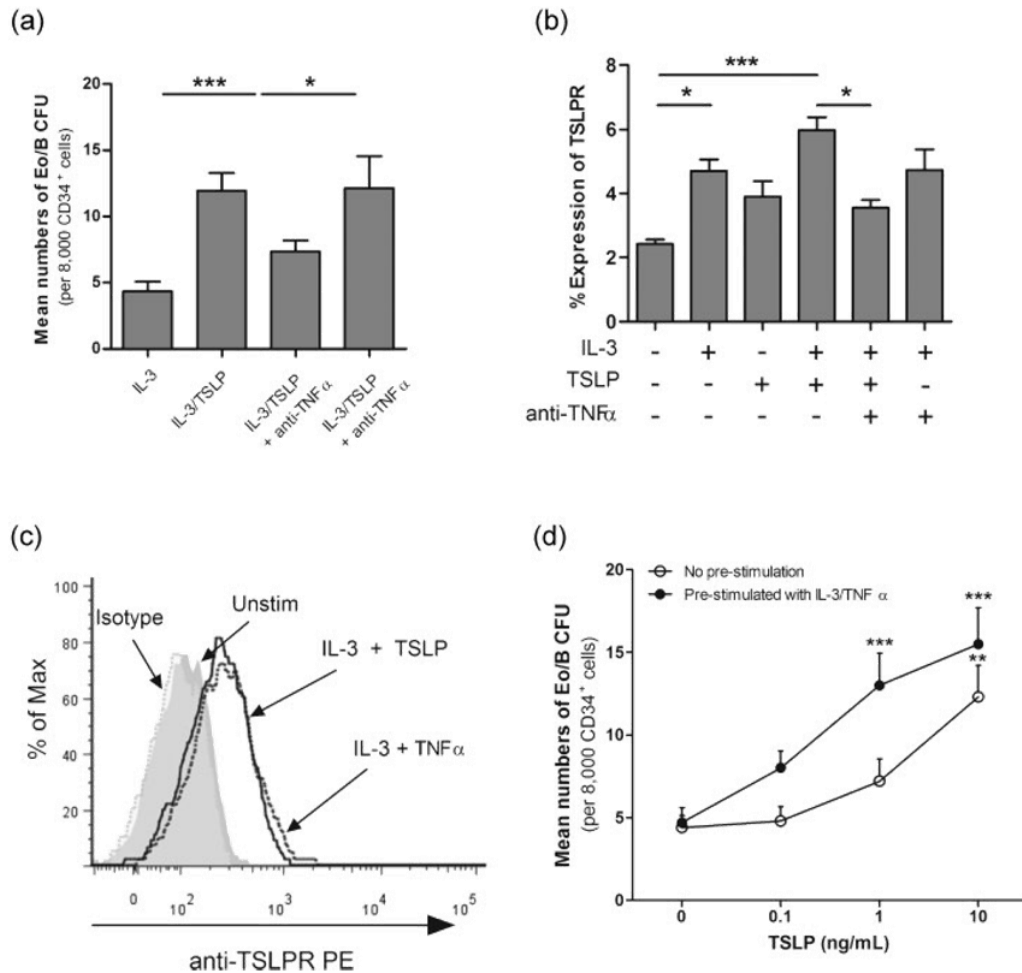


Figure 4. IL-3 and TNF α increase TSLPR expression and sensitivity of PB HHP to TSLP. PB CD34⁺ cells were treated with IL-3 (1 ng/mL), TSLP (10 ng/mL), and anti-TNF α (10 μ g/mL). (a) Eo/B CFU (defined as tight, granular clusters ≥ 40 cells) were enumerated at the end of 14 days methylcellulose cultures ($n=6$ in duplicates). (b) surface expression of TSLPR on PB CD34⁺ cells were analyzed by flow cytometry following 24 h stimulation ($n=7$). (c) A representative overlay histogram for expression of TSLPR on unstimulated (shaded gray), IL-3 and TNF α (gray line) and IL-3 and TSLP (black line) stimulated CD34⁺ cells. Normal mouse IgG was used as an isotype control (shaded-light gray). Percent expression is the percent of CD34⁺ cells expressing a given antigen at the 98% confidence limit (i.e., relative to a quadrant marker set to include 2% of cells stained with isotype control antibody). This experiment was performed four independent times with similar results. (d) PB CD34⁺ cells were pre-stimulated with IL-3 (1 ng/mL) and TNF α (50 pg/mL) for 24 h and cultured in methylcellulose colony assays in the presence of IL-3 (1 ng/mL) and increasing doses of TSLP (0.1, 1, 10 ng/mL). On day 14, Eo/B CFU were enumerated ($n=5$ in duplicates, ** $P < 0.01$; *** $P < 0.001$ compared with TSLP (0 ng/mL) within group). Results shown are mean \pm SEM. One independent experiment performed per subject. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Results

TSLP preferentially enhances IL-3-dependent Eo/B differentiation from PB HHP

The addition of TSLP significantly increased the formation of IL-3-responsive Eo/B CFU from 7.56 ± 0.9 to 15.8 ± 1.6 ($P < 0.001$; Fig. 1a), which was inhibited by the addition of neutralizing anti-TSLP (9.3 ± 1.0 per 8000 CD34⁺ cells;

$P < 0.05$) and anti-TSLPR (10.6 ± 1.1 per 8000 CD34⁺ cells; $P < 0.05$). TSLP did not have any effects on either IL-5- or GM-CSF-responsive Eo/B CFU (Supplementary Fig. S2). Moreover, effects of TSLP on HHP were not enhanced by IL-33 (data not shown).

Overnight stimulation of HHP with IL-3/TSLP together enhanced the MFI of IL-3R α compared to unstimulated (3.4-fold; $P < 0.001$) and TSLP-stimulated (1.9-fold;

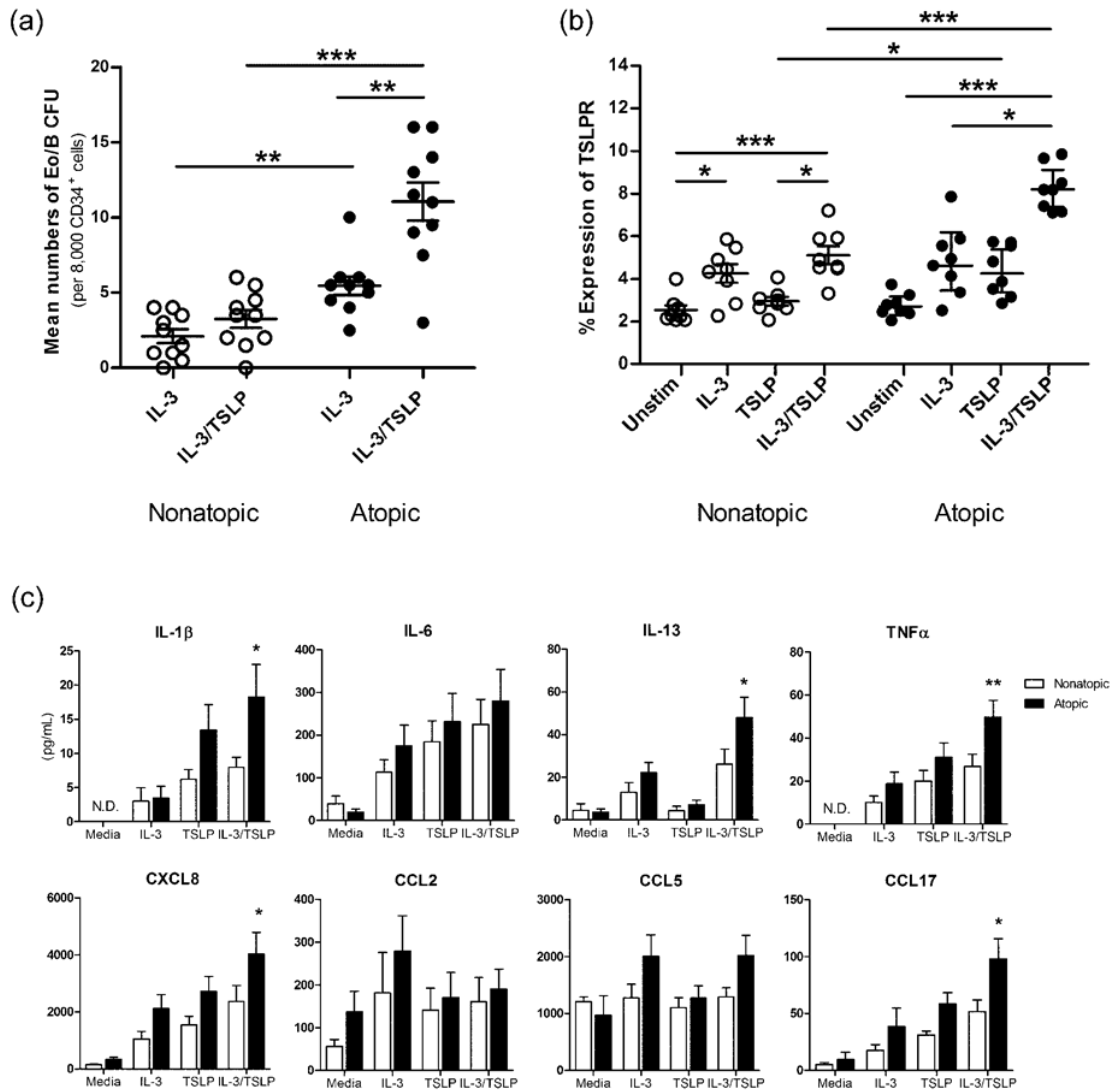


Figure 5. Differential Eo/B CFU, TSLPR expression, and cytokine/chemokine secretion from PB HHP from nonatopic and atopic individuals. PB CD34⁺ cells from nonatopic (open circles) and atopic (closed circles) subjects were stimulated as indicated and assessed for (a) Eo/B CFU by methylcellulose cultures ($n = 10$ in duplicates), (b) surface expression of TSLPR by flow cytometry ($n = 8$), and (c) cytokine/chemokine secretion by Luminex ($n = 8$ in duplicates). Percent expression is the percent of CD34⁺ cells expressing a given antigen at the 98% confidence limit (i.e., relative to a quadrant marker set to include 2% of cells stained with isotype control antibody). Results shown are mean \pm SEM. One independent experiment performed per subject. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ND; not detected.

$P < 0.01$; Fig. 1b). Overnight stimulation with IL-3/TSLP significantly increased percent expression of TSLPR compared to unstimulated ($P < 0.001$) and TSLP-stimulated HHP ($P < 0.05$). A similar trend was seen for IL-7 α expression, although not significant (Fig. 1c). No significant difference in TSLPR expression was observed following IL-5/GM-CSF and/or TSLP stimulation (Supplementary Fig. S3).

TSLP enhances IL-3-induced basophilopoiesis from PB HHP

Histochemical staining of individual colonies with DiffQuik and toluidine blue revealed the presence of cells with either eosinophilic granular cytoplasm or metachromatic granules, morphologically consistent with eosinophils and basophils,

Table 1. Subject demographics.

Characteristics	Nonatopics (n = 10)	Atopics (n = 10)
Sex		
Male	5 (50)	7 (70)
Female	5 (50)	3 (30)
Age		
18 to <30	6 (60)	4 (40)
30 to <60	3 (30)	6 (60)
≥60	1 (10)	0
Mean*	29.9 (13.6)	28.5 (7.4)

Values in parentheses indicate percentages.

*Mean (SD).

respectively (Fig. 2b,c). To further confirm the presence of basophils, colony histamine assay was performed. Taking colony size (cell number) into account, the mean (\pm SEM) calculated amount of histamine (pg/cell) was significantly higher in IL-3/TSLP-induced Eo/B CFU compared to IL-3-induced Eo/B CFU (48%; $P < 0.05$). In the presence of neutralizing anti-TSLP or neutralizing anti-TNF α , the histamine content was significantly lower compared to IL-3/TSLP stimulated colonies (57%; $P < 0.05$ and 54%; $P < 0.01$, respectively; Fig. 2d).

IL-3 and TSLP increase cytokine and chemokine secretion by PB HHP

TSLP alone induced significant levels of IL-1 β , IL-6, TNF α , CXCL8, and CCL17 from PB HHP, compared to unstimulated controls ($P < 0.05$; Fig. 3). IL-3/TSLP-stimulated HHP released significant levels of IL-1 β , IL-6, IL-13, TNF α , CXCL8, CCL2, and CCL17 compared to unstimulated controls ($P < 0.001$). PB HHP failed to secrete detectable levels of IL-4, IL-9, IL-10, GM-CSF, IFN γ , and eotaxin; while CCL5 was highly secreted by PB HHP cells under all conditions.

TNF α plays a key role in IL-3/TSLP-mediated Eo/B CFU formation and TSLPR expression

Next, we investigated whether TSLP-induced TNF α could support Eo/B CFU formation given that IL-3 and TNF α have previously been reported to upregulate TSLPR on human eosinophils [5]. The addition of neutralizing anti-TNF α significantly reduced TSLP-induced IL-3-responsive Eo/B CFU (38%; $P < 0.05$; Fig. 4a) and surface expression of TSLPR on HHP (40.1%; $P < 0.05$; Fig. 4b) compared to IL-3/TSLP-stimulated HHP. Overnight stimulation of PB HHP with IL-3 and TNF α (50 pg/mL) increased TSLPR expression to comparable levels post TSLP/IL-3-stimulation (Fig. 4c) and promoted Eo/B CFU formation at lower concentrations of TSLP, which was statistically significant compared to IL-3-stimulated HHP ($P < 0.001$; Fig. 4d).

PB HHP from atopic individuals exhibit enhanced Eo/B differentiation, TSLPR expression, and cytokine/chemokine secretion

We next examined responses of HHP in relation to atopic sensitization. PB HHP derived from atopic individuals produced significantly higher numbers of Eo/B CFU compared to those from nonatopic individuals following stimulation with IL-3 (5.4 ± 0.6 vs. 2.1 ± 0.5 per 8000 CD34+ cells; $P < 0.01$) and IL-3/TSLP (11.1 ± 1.3 vs. 3.3 ± 0.6 per 8000 CD34+ cells; $P < 0.001$; Fig. 5a). Within the atopic group, TSLP significantly increased the formation of IL-3-responsive Eo/B CFU (twofold; $P < 0.01$; Fig. 5a). No differences in TSLPR expression were observed between nonatopic and atopic individuals at baseline or after IL-3-stimulation (Fig. 5b). However, TSLPR expression was significantly higher in atopic subjects compared to nonatopic subjects following stimulation with TSLP ($4.40 \pm 0.42\%$ expression vs. $2.95 \pm 0.20\%$ expression; $P < 0.05$) and IL-3/TSLP ($8.26 \pm 0.38\%$ expression vs. $5.10 \pm 0.42\%$ expression; $P < 0.001$; Fig. 5b). Furthermore, PB HHP derived from atopic individuals produced significantly higher levels of IL-1 β ($P < 0.05$), IL-13 ($P < 0.05$), TNF α ($P < 0.01$), CXCL8 ($P < 0.05$), and CCL17 ($P < 0.05$), compared to PB HHP from nonatopic individuals, post IL-3/TSLP-stimulation (Fig. 5c).

DISCUSSION

We demonstrate for the first time that PB HHP respond directly to TSLP *in vitro* with enhanced Eo/B colony formation and TSLPR/IL-7R α expression, specifically upon co-stimulation with IL-3, and not IL-5 or GM-CSF. This differentiation process appears dependent on autocrine and/or paracrine signaling by TNF α -producing progenitors. IL-3 and TNF α , cytokines which are found at sites of allergic inflammation, may help explain the increase the sensitivity of HHP to TSLP-mediated Eo/B differentiation. Finally, we demonstrate enhanced stimulatory effects of IL-3 and TSLP on PB CD34+ progenitors derived from atopic individuals. To the best of our knowledge, this is the first study to demonstrate such findings in humans *ex vivo*. These findings provide a novel mechanism underlying eosinophil and basophil accumulation in tissues during allergic inflammation, linked as it is to TSLP and its known amplification of Th2 immune responses in atopic individuals.

TSLP–TSLPR interactions are crucial to the development of eosinophilia [25] and basophilia [6] in mice; however, its role in humans is unclear. We show for the first time the importance of TSLP–TSLPR in Eo/B lineage commitment of IL-3-responsive HHP. Our group and others have previously reported the presence of both eosinophils and basophils in IL-3-stimulated hemopoietic progenitor cultures [14–16];

our current findings therefore demonstrate that TSLP can serve the role of a key epithelial-derived factor in this process of human Eo/B differentiation. Differential counts of colony cells were not formally performed; therefore, relative proportions of eosinophils versus basophils in these colonies are unclear. However, methylcellulose colony assays have been used for many years by us and others, to both enumerate and assess progenitors and their progeny in response to hemopoietic cytokines (and other stimuli), as quantitated by Eo/B CFU [14, 19, 26]. We have elected to use the CFU assay, and add histamine assays, as previously extensively documented by us and others, to represent a specific, surrogate biomarker of basophil “content” within these Eo/B colonies, which are each derived from a single progenitor, given that histidine decarboxylase is only present within the basophil, not the eosinophil in these mixed basophil–eosinophil colonies [14, 20, 27–29]. Since cells within these Eo/B colonies are rather immature and often possess dual phenotypes – including cells with “hybrid” eosinophilic–basophilic granulation by standard morphological–histochemical assessments – differentiating the cells using histochemical stains is not reliable. When the latter are routinely performed, both toluidine blue-positive granules as well as eosinophilic staining granules are found in many cells simultaneously [14–16]. As such, our key (and to our judgment, more robust) arbiter of basophil content remains the colony histamine content. Indeed, our group has previously reported on the close correlation between the basophil numbers and the histamine content in Eo/B CFU [14, 20, 30]. Additionally, in the current study, the histamine assay allowed for comparative analyses, demonstrating marked differences in histamine content between colonies grown in the presence or absence of TSLP and/or neutralizing anti-TSLP or anti-TNF α . While further analyses are required to examine precisely how TSLP may alter colony histamine content, it appears unique among the epithelial-derived cytokines in its ability to promote basophilia in peripheral tissues [6]. Of note, Siracusa *et al.* [6] recently reported the ability of TSLP to induce IL-3-independent basophils from bone marrow-resident precursors in mice. However, we were unable to observe TSLP alone-mediated Eo/B CFU, suggesting that TSLP must work in concert with IL-3 to induce Eo/B differentiation. We speculate that this discrepancy may reflect inter-species differences in hemopoiesis due to the distinct surface phenotype of progenitors in mice and humans [31], or to the distinct differentiative pathways of eosinophil and basophil development in humans and mice [14, 17], or to a combination of these factors. As such, rather than examining the relative proportions of eosinophils versus basophils in these colonies of nascent eosinophils and basophils of mixed granulation (which one also sees in liquid cultures), we have focused on PB Eo/B CFU (and thus, HHP) production after TSLP

stimulation, providing novel evidence that TSLPR engagement on PB CD34+ cells has the capacity to enhance Eo/B lineage priming of myeloid progenitors, increasing the likelihood of development of allergic eosinophilic/basophilic inflammation, in addition to disease maintenance or progression.

Allakhverdi *et al.* recently demonstrated the ability of TSLP, together with IL-33, to promote “Th2-like” properties in human CD34+ progenitors, based on induction of Th2 cytokines (IL-6, IL-13, GM-CSF) and chemokines (CXCL8, CCL1, CCL17, CCL12) [8]. Likewise, we detected levels of IL-1 β , IL-6, IL-13, TNF α , CXCL8, CCL2, CCL5, and CCL17 following overnight stimulation of PB HHP with TSLP; IL-4, IL-9, IL-10, GM-CSF, IFN γ , and eotaxin were undetected under all conditions. However, contrary to findings by Allakhverdi *et al.* [8], we were unable to detect levels of GM-CSF in the supernatant and of the cytokines detected, the concentrations were comparatively low. The higher levels of cytokines reported by Allakhverdi *et al.* may be due to the use of stem cell factor (100 ng/mL) in their culture medium [32]. Of the Th2 cytokines and chemokines detected, of importance is the enhanced secretion of TNF α , which, in conjunction with TSLP/IL-33, has been previously shown to enhance cytokine secretion by human cord blood and PB-derived CD34+ progenitors [8]. Furthermore, Caux *et al.* [33] showed enhancing effects of TNF α on IL-3- and GM-CSF-dependent proliferation of CD34+ HHP. It is plausible that IL-3/TSLP-induced TNF α is a key event in HHP autocrine secretion of Th2-like cytokines/chemokines and in Eo/B differentiation. Our observation that inhibiting TNF α reduces TSLPR expression on HHP, which is in agreement with a study that showed enhanced TSLPR expression on mature eosinophils post IL-3/TNF α -stimulation [5], may explain the reduction in Eo/B CFU formation and colony histamine levels in our cultures. The relevance of TNF α antagonists in decreasing eosinophil and/or basophil counts *in vivo* is unclear; however, anti-TNF α agents have been reported to decrease sputum histamine levels and improve asthma outcomes, airway hyper-responsiveness, and exacerbation rates [34, 35]. Furthermore, in OVA-sensitized allergic rhinitis and bleomycin-induced pulmonary fibrosis murine models, TNF α antagonists have been shown to inhibit eosinophilia in the nasal mucosa and lung, respectively [36, 37].

Although we have not herein reported on which signaling pathways are involved in TSLP-induced Eo/B differentiation, we do have findings implicating the preferential dependence of p38MAPK signaling pathways in IL-3/TSLP-mediated Eo/B differentiation (Supplementary Fig. S4). Indeed, Fanat *et al.* [38] showed that supernatants from TNF α -, IL-1 β -, and IFN γ -stimulated human airway smooth muscle (HASM) cells drive eosinophilic differentiation from HHP *in vitro*, in a p38MAPK-dependent way.

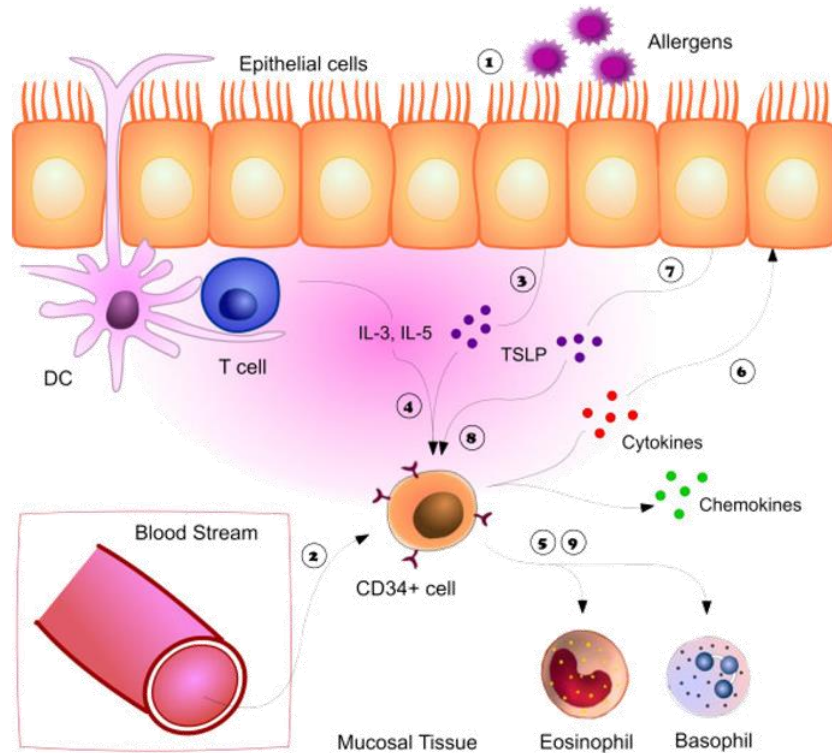


Figure 6. Autocrine/paracrine regulation of TSLP-mediated Eo/B differentiation. Following exposure to allergens (1), increase CD34⁺ progenitors are recruited and detected at sites of inflammation (2). Allergen induce epithelial-derived TSLP (3) along with local cytokines (IL-3) activate CD34⁺ cells with release of cytokines and chemokines (4) as well as induce in situ hemopoiesis – the differentiation of CD34⁺ progenitor cells into eosinophils and basophils (5). The cytokines secreted by CD34⁺ cells can directly activate the epithelium (6) resulting in the release of more TSLP (7), which can act cooperatively with local cytokines and/or CD34⁺ released cytokines to further enhance eosinophilo/basophilopoiesis in situ (8, 9).

TNF α /IL-1 β have been shown to induce TSLP production from HASM cells [39]; we therefore speculate the preferential dependence of TSLPR/p38MAPK signal transduction in TSLP-mediated Eo/B differentiation [38].

Atopic sensitization is a widely recognized risk factor for allergic diseases [40]. In the current study, we show that PB HHP from atopic individuals are more responsive to IL-3 and even more so to TSLP, resulting in elevated numbers of Eo/B CFU post IL-3/TSLP-stimulation, compared to HHP from nonatopic individuals. This novel finding related to TSLP effects is in keeping with previous observations that atopic sensitization and disease are associated with enhanced PB (and bone marrow) HHP Eo/B lineage commitment and tissue allergic inflammation related to more “classic” eosinophilopoietic cytokines such as IL-5 [18, 22, 23, 26]. Furthermore, our group has demonstrated increased levels of IL-5-responsive progenitors in atopic subjects, compared with nonatopic subjects [18]. Likewise, the observation that HHP from atopic individuals respond more robustly to TSLP/IL-3 may be related to increased

levels of IL-3-responsive progenitors. Alternatively, PB HHP from atopic subjects may have increased endogenous expression of IL-3, with consequent autocrine effects on Eo/B differentiation, as Kuo *et al.* [41] have shown with IL-5. The increased TSLP responsiveness of HHP from atopic individuals may be a reflection of increased production of TNF α compared to HHP from nonatopic individuals (Fig. 6c). We show in the current study that TNF α , together with IL-3, enhance TSLPR expression and HHP sensitivity to TSLP-mediated Eo/B differentiation. These findings are consistent with an atopic priming effect on HHP, such that they differentiate into Eo/B CFU more readily to IL-3/TSLP than HHP from nonatopic individuals, contributing to the development of tissue eosinophilia/basophilia. Of note, given the lack of any significant effects of TSLP on IL-5- and GM-CSF-Eo/B differentiation in non-attributable PB HHP (Fig. S2), we did not examine the combination of IL-5/TSLP nor GM-CSF/TSLP in the HHP of known atopic individuals, but rather concentrated on the TSLP/IL-3-mediated pathway.

There is also documented increased secretion of TSLP from bronchial epithelial cells of atopic, versus nonatopic individuals [4], suggesting that TSLP may play an important tissue role, such as enhancement of “in situ hemopoiesis” in atopy. Although the precise mechanisms are unclear, we postulate paracrine effects of epithelial cells on tissue-resident HHP, which have been detected at mucosal sites of allergic inflammation [22, 23]. We demonstrate in the current study that IL-3/TSLP induces TNF α and IL-1 β secretion, previously shown to mediate TSLP secretion from airway epithelial cells [3]. Moreover, our data illustrate that pre-stimulation of PB CD34+ cells with IL-3 and TNF α upregulates TSLPR expression, which significantly increases the sensitivity of CD34+ cells to TSLP-mediated Eo/B differentiation. It is possible that epithelial-derived TSLP, along with local hemopoietic factors (i.e., IL-3), both of which are elevated in atopic individuals [4, 42], may target HHP resident at mucosal sites, triggering the paracrine and/or autocrine differentiation of these recruited progenitor cells (Fig. 6). TNF α can be produced by various cell types other than CD34+ progenitors; however, in the current study, CD34+ progenitors were enriched using a protocol which virtually eliminated all T- and B-cells.

Although flow cytometry was not performed to check for residual T cells following separation, the ability of CD34+ cells themselves to produce Th2-like cytokines and TNF α following TSLP stimulation and toll-like receptor-ligation has previously been reported [3, 43].

Finally, the preferential synergy of TSLP with IL-3, and not IL-5 or GM-CSF, in driving Eo/B differentiation is consistent with previous work which demonstrates that the responsiveness of progenitors to these cytokines is highly dependent on the stage of differentiation [44, 45]. PB CD34+ cells consist of mainly immature progenitors, and therefore mostly respond to IL-3 and GM-CSF, early-acting cytokines [44, 46], which is consistent with our findings (Fig. 1a and Supplementary Fig. S2). Of note, IL-3 tends to drive CD34+ progenitor differentiation primarily along the basophil-lineage [47], while GM-CSF promotes the differentiation of a mixed population (Eo/B cells, macrophages, etc.) [46]. Therefore, the preferential differentiation of PB CD34+ progenitors to IL-3-responsive lineage suggests that the response of PB HHP to TSLP involves basophil-mediated inflammation [6].

In summary, our study uncovers a previously unrecognized role for TSLP in allergic inflammation – as a regulator of in situ hemopoiesis, by enhancing the process of HHP (CD34+ cell) differentiation within tissues. The enhanced TSLP-mediated hemopoiesis *ex vivo* in relation to clinical atopic status may help explain increased eosinophils and basophils at sites of inflammation in atopic individuals.

Acknowledgments

We thank Mike Comeau (Amgen) for the supply of anti-TSLP; Drs. Steven Smith and Tom Mu for technical assistance in histochemical staining and flow cytometry; Lesley Wiltshire for assistance in Luminex assays; and Lynne Larocque, for manuscript preparation. This work was supported by AllerGen NCE Inc. and CIHR MOP 111029 to J. A. Denburg. C. C. K. Hui is a recipient of the Canadian Allergy and Immune Diseases Advanced Training Initiative Award.

Conflict of Interest

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

Figure S1. Flow cytometric multi-gating strategy for receptor expression on PB CD34+ cells.

Figure S2. TSLP has no effect on IL-5- and GM-CSF-responsive Eo/B CFU.

Figure S3. IL-5 and GM-CSF has no effect on TSLPR expression.

Figure S4. p38MAPK signal transduction is preferentially involved in TSLP-mediated Eo/B differentiation.

SUPPLEMENTARY MATERIAL

The effects of thymic stromal lymphopoietin and IL-3 on human eosinophil-basophil lineage commitment: relevance to atopic sensitization

Claudia C.K. Hui, Sina Rusta-Sallehy, Ilan Asher, Delia Heroux, and Judah. A. Denburg

Supplementary Figure Legends

Fig. S1. Flow cytometric multi-gating strategy for receptor expression on PB CD34+ cells. Gating was based on high CD34 and low-intermediate CD45 positivity combined with low forward and side scatter, characteristics of CD34+ progenitor cells. (a) An initial gate (R1) was set to capture CD45+ cells (leukocytes). (b) A subsequent gate was made to capture CD34-bright cells (R2). (c) R3 was then gated to capture CD45+ cells with mononuclear morphology. (d) Cells with low forward and side scatter, consistent with mononuclear cell morphology, was gated (R4). Finally, cells in R4 (true CD34+ population) were further analyzed for staining with PE-linked TSLPR (f) or isotype control (e). Receptor expression data were collected as the percentage of positive cells at the 98% confidence limit (ie. relative to a quadrant marker set to include 2% of cells stained with isotype control antibody).

Fig. S2. TSLP has no effect on IL-5- and GM-CSF-responsive Eo/B CFU. PB CD34+ cells were stimulated with (a) IL-5 and (b) GM-CSF and assessed for Eo/B CFU by methylcellulose cultures. Results shown are mean \pm SEM in duplicates (n=5). One independent experiment performed per subject.

Fig. S3. IL-5 and GM-CSF has no effect on TSLPR expression. PB CD34+ cells were stimulated as indicated to examine the effects of (a) IL-5 (n=5) and (b) GM-CSF (n=4) on TSLPR expression. Results shown are mean \pm SEM. One independent experiment performed per subject.

Fig. S4. p38MAPK signal transduction is preferentially involved in TSLP-mediated Eo/B differentiation. PB CD34⁺ cells were incubated with STAT5 inhibitor (50 μ M), U0126 (ERK1/2 inhibitor; 10 μ M), SB203580 (p38MAPK inhibitor; 10 μ M), or SP600215 (JNK inhibitor; 50nM) for 1 h before stimulating with IL-3 (1ng/mL) and/or TSLP (10ng/mL). Eo/B CFU (defined as tight, granular clusters \geq 40 cells) were enumerated at the end of 14 d methylcellulose cultures. Results shown are mean \pm SEM in duplicates (n=6). One independent experiment performed per subject. * P<0.05; ** P<0.01; *** P<0.001.

Supplementary Figures

Figure S1

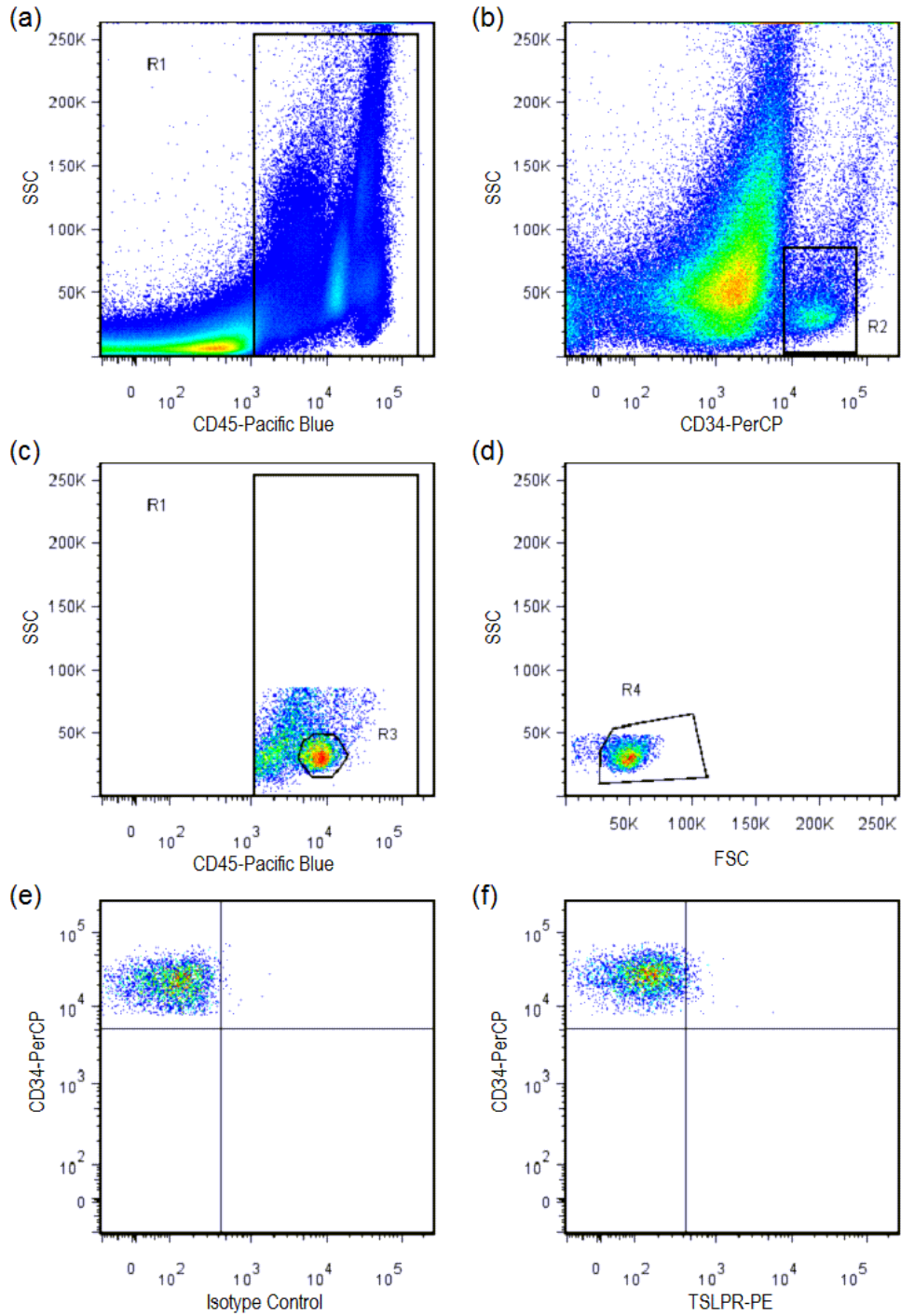


Figure S2

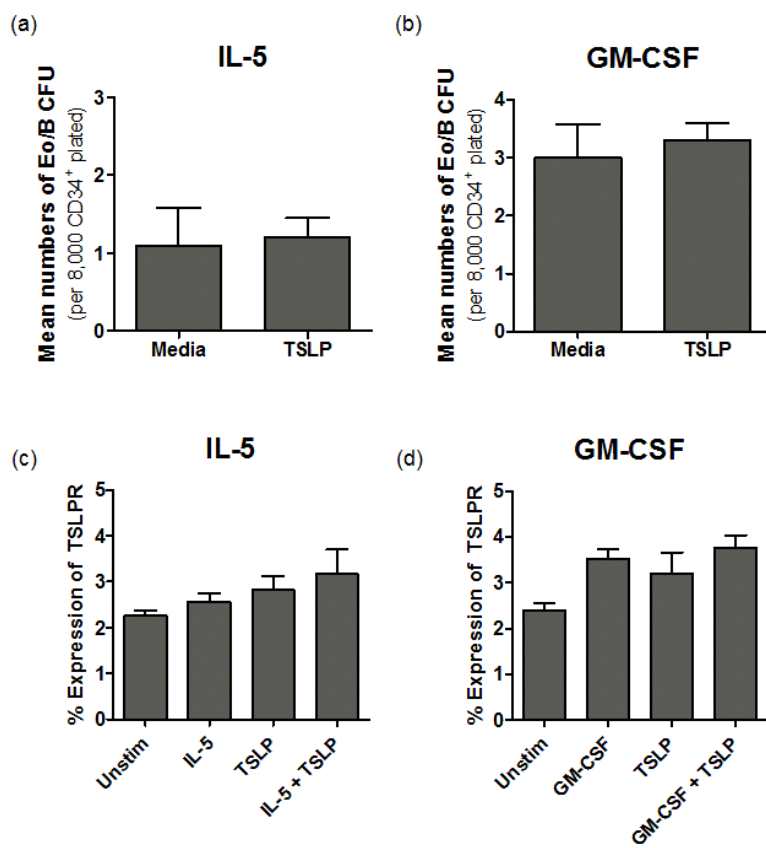


Figure S3

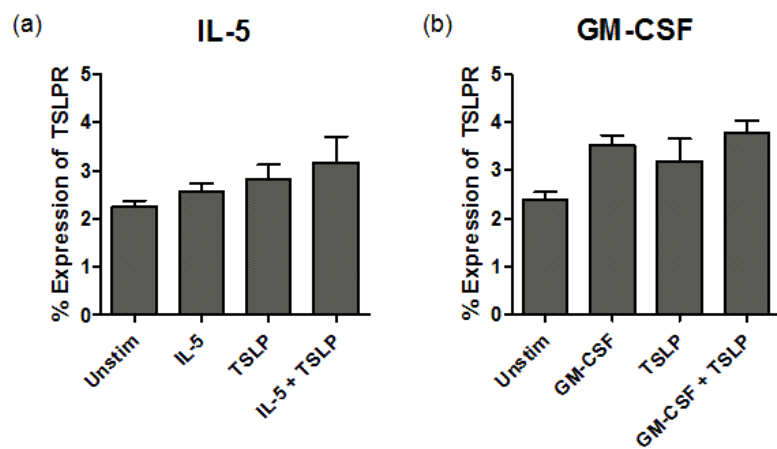
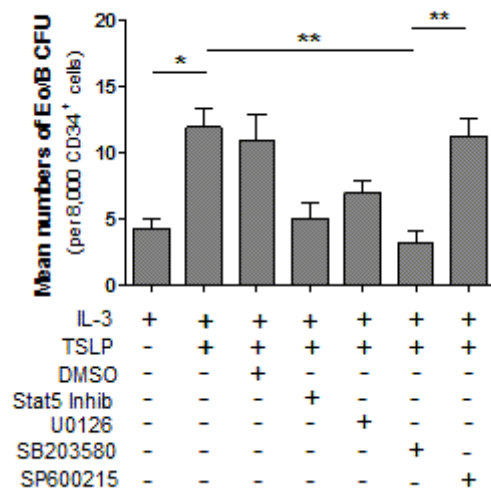


Figure S4



CHAPTER 4: Decreased nasal epithelial response to dsRNA in individuals with a thymic stromal lymphopoietin gene variant

Submitted to *Mucosal Immunology*. June 2014

TSLP, a cytokine highly implicated in allergic disease, can be differentially elicited from healthy vs. asthmatic-derived epithelial cells by stimuli such as allergens and viruses [235]. Emerging evidence supports a role for TSLP in promoting survival and differentiation of a range of effector cells involved in allergic diseases, such as eosinophils [203, 221]. Recently, SNPs in the TSLP pathway have been implicated in the pathogenesis of allergic disease [82, 190, 232]. The SNP rs1837253 of *TSLP* has been reported to be inversely associated with asthma and related-traits and suggested to have functional consequences on TSLP expression [9-11, 75, 236, 237]. To investigate whether rs1837253 is a genetic variant with functional effects, we evaluated the expression of TSLP and secretion of TSLP protein as a function of rs1837253 genotype using NEC cultured from atopic individuals. We demonstrated that while polyI:C-mediated TSLP secretion in NEC is not affected by atopic sensitization, individuals who are homozygous and heterozygous for minor allele secrete significantly less TSLP compared to individuals who are homozygous for the major allele.

Title: Thymic stromal lymphopoietin (TSLP) secretion from human nasal epithelium is a function of *TSLP* genotype

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Short title: TSLP SNP and TSLP secretion

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ABSTRACT

Recent candidate gene and genome-wide association studies have identified ‘protective’ associations between the single nucleotide polymorphism (SNP) rs1837253 in the *TSLP* gene and risk for allergy, asthma and airway hyper-responsiveness. The absence of linkage disequilibrium of rs1837253 with other SNPs in the region suggests it is likely a causal polymorphism for these associations, having functional consequences. We hypothesized that rs1837253 genotype would influence TSLP secretion from mucosal surfaces. We therefore evaluated the secretion of TSLP protein from primary nasal epithelial cells (NEC) of atopic and non-atopic individuals and its association with rs1837253 genotype. We found that while atopic sensitization does not affect the secretion of TSLP from NEC, there was decreased TSLP secretion in NEC obtained from heterozygous (CT; 1.8-fold) and homozygous minor allele (TT; 2.5-fold) individuals, compared to NEC from homozygous major allele individuals (CC; $p < 0.05$) post double-stranded (ds)RNA stimulation (50 $\mu\text{g/mL}$). Our novel results show 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.

INTRODUCTION

Allergic diseases are often triggered by environmental stimuli that induce Th2 immune responses. For many years, the airway epithelium was recognized purely for its function as a physical barrier; however, it is now seen as an important initiator of the allergic response by secreting cytokines/chemokines, which regulate innate immune cells.^{1,2} An epithelial cell-derived cytokine, thymic stromal lymphopoietin (TSLP), is IL-7-like,³ appears to be a key initiator of allergic inflammation, and also plays a role in the pathogenesis of allergic diseases – with effects on dendritic cells,⁴ T lymphocytes⁵ and other effector cells such as mature eosinophils^{6,7} and basophils⁸ as well as their progenitors.^{9,10} In asthmatics, TSLP is expressed at elevated levels in the lungs and is linked to disease severity.¹¹ Studies in murine models have demonstrated reduced airway disease in TSLP receptor (TSLPR)-deficient mice, while experimental lung-specific expression of a TSLP transgene induced experimental asthma (Th2 cytokine-associated inflammation of the airways).¹² In a recent clinical trial, treatment with a human anti-TSLP antibody (AMG 157) reduced allergen-induced bronchoconstriction and airway inflammation in mild atopic asthmatics.¹³

Genome-wide association studies have shown associations between genetic variants in *TSLP* and allergic disease and phenotypes, including asthma¹⁴⁻¹⁶ and eosinophilia.¹⁷ Furthermore, a single nucleotide polymorphism (SNP) rs1837253 in the *TSLP* gene has been shown to be associated with asthma, atopic asthma, and airway hyper-responsiveness.^{14-16, 18, 19} It has further been reported that the minor T allele of rs1837253 in *TSLP* is associated with reduced risk of allergic rhinitis and asthma in males.^{20, 21} The absence of linkage disequilibrium (LD) between rs1837253 and other SNPs, 2Mb up or

downstream in the chromosomal region, suggests that it is likely causal, having functional consequences.¹⁸

Recent evidence suggests that SNPs in the TSLP pathway may play a major role in the pathogenesis of allergic disease, but the functional role of the rs1837253 SNP in *TSLP* has not been previously described. Given that the T allele of the rs1837253 SNP in *TSLP* is associated with reduced risk of allergic disease, and since there are no other SNPs in LD with rs1837253, we hypothesized that there would be associations between rs1837253 genotype and *ex vivo* production of TSLP, which is a crucial cytokine for the induction of Th2 inflammatory responses. Specifically, we hypothesized that individuals with the minor allele would have a decreased propensity to induce a Th2 inflammatory response related to an altered mediator profile, leading to ‘protection’ from asthma and related traits. We therefore evaluated the double-stranded (ds)RNA-induced secretion of TSLP from primary nasal epithelial cells (NEC) from non-atopic and atopic individuals, examining associations among rs1837253 genotype, atopy, and TSLP secretion.

RESULTS

Subject Characteristics

From October 30, 2013 to February 10, 2014, 61 subjects were recruited. On Visit 1, all 61 subjects received skin prick tests and had mouthwash samples collected and genotyped for the SNP rs1837253 – 29 were homozygous for the major allele (CC), 9 were homozygous for the minor allele (TT), and 23 were heterozygous (CT). On Visit 2, only 43 of these subjects returned and received nasal scrapes. Of these latter 43 subjects, 36 long-term expansions of NEC *in vitro* were successful. Of these 36 subjects, the breakdown by genotype was as follows: 14 CC, 8 TT, and 14 CT. Of the 18 subjects that dropped out before Visit 2, 14 were due to studying/exam and holiday schedule conflicts and four were due to a common cold. Further subject characteristics are shown in Table 1.

PolyI:C induces TSLP expression in NEC

PolyI:C has been previously reported to induce TSLP production (messenger (m)RNA or protein) in human airway epithelial cells.²²⁻²⁴ To investigate whether NEC behave similarly, the epithelial nature of the cultured cells was confirmed using immunofluorescence staining for cytokeratin-5 (Fig. 1), and cultured cells were stimulated with polyI:C for 0, 3, 6 and 24 h. PolyI:C (25 µg/ml) induced a time-dependent expression of TSLP mRNA ($p < 0.001$ at 6h; Fig. 2A). Furthermore, a dose-dependent release of TSLP protein was observed following 24 h stimulation with polyI:C ($p < 0.01$; Fig. 2B).

Effects of atopy and *TSLP* rs1837253 genotype on TSLP expression

No statistically significant association was found between allergic sensitisation and production of TSLP by NEC at baseline. Furthermore, polyI:C did not induce differential TSLP secretion from NEC derived from non-atopic, compared with atopic, individuals (Fig. 3). However, polyI:C did induce differential TSLP secretion according to genotype (Fig. 4A). When subjects were stratified by genotype, decreased TSLP secretion was observed in NEC obtained from heterozygous (CT; 1.8-fold; $p < 0.05$) and homozygous minor allele (TT; 2.5-fold; $p < 0.05$) individuals, compared with NEC from homozygous major allele (CC) individuals, post stimulation with polyI:C (25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$; Fig. 4B, C).

DISCUSSION

There is increasing evidence implicating *TSLP* polymorphisms in the development of allergy and asthma.²⁵⁻²⁷ Previous work has documented inverse associations between the T allele of rs1837253 and asthma;^{18,20} however, the reasons for this association have remained unclear. Genetic polymorphisms are proposed to exert effects that can be measured through a variety of ‘intermediate’ steps and outcomes (‘phenotypes,’ which could be biological or clinical); however, information on these ‘intermediate phenotypes’ is currently lacking.

In the current study, our aim was to examine a key intermediate phenotype – specifically, TSLP secretion from NEC in response to stimulation *ex vivo* - which could shed insight on the underlying involvement of rs1837253 in the pathogenesis of allergic disease. We selected the *TSLP* SNP rs1837253, based on the fact that it was the most significant signal in a candidate gene association study on asthma and related phenotypes,¹⁸ which has been confirmed in recent global GWAS analyses.¹⁴⁻¹⁶

Consistent with previous studies on small airway epithelial cells,²² NEC,²⁸ and bronchial epithelial cells (BEC) from healthy and asthmatic individuals,^{23, 29, 30} we demonstrated that exposure to dsRNA *in vitro* induced a time-dependent expression of TSLP mRNA and a dose-dependent release of TSLP protein in NEC. We²⁹ and others³⁰⁻³² have previously 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.³¹ In the current study,

dsRNA did not induce differential TSLP secretion in NEC obtained from non-atopic compared to atopic individuals, suggesting that there is no discrepancy in RIG-1 expression in NEC between these two groups. Further study examining differential RIG-1 expression in relation to clinical phenotypes will provide additional mechanistic insights on the function of SNP rs1837253.

Previous data demonstrate that the long form of TSLP is highly inducible by polyI:C in BEC.²⁶ A functional SNP, rs3806933, has been identified in the regulatory region of the *TSLP* gene, which enhances the binding of activator protein-1 (AP-1), a prominent transcription factor in airway diseases that regulates the expression of multiple inflammatory proteins.³³ Furthermore, the rs3806933 SNP influences AP-1-driven promoter activity of the long-form of TSLP in BEC.²⁶ AP-1 expression is enhanced in the asthmatic airway,³³ consistent with reports that SNP rs3806933 is associated with adult asthma and childhood atopic asthma.²⁷

In the current study, we related the differential secretion of TSLP protein to the SNP rs1837253 genotype, and our data suggest that this SNP in the upstream region of *TSLP* has functional effects on TSLP protein production. SNP rs1837253 is located 5.7 kb upstream of the TSLP transcription start site and is predicted to disrupt a number of potential transcription factor binding sites.³⁴ It is possible the rs1837253 T allele down-regulates microbe-induced production of TSLP in NEC through inhibiting the binding of transcription factors to regulatory elements. In order to better understand the mechanism of SNP rs1837253 associations with TSLP secretion, potential differential binding of possible regulatory proteins to this important SNP should be examined.

In a recent proof-of-concept study,¹³ in addition to reducing allergen-induced bronchoconstriction in both early and late asthmatic responses, AMG 157, a human anti-TSLP monoclonal antibody, was demonstrated to reduce markers of systemic and airway inflammation measured as fraction of exhaled nitric oxide and sputum eosinophil levels, as well as levels of circulating eosinophils. The capacity for AMG 157 to decrease baseline blood eosinophil counts and exhaled nitric oxide¹³ suggests a pivotal role for TSLP in the development or persistence of asthma in the absence of allergen exposure. In support of this, we previously demonstrated the ability of TSLP to mediate eosinophil-basophil differentiation from human peripheral blood hemopoietic progenitor cells, a process which was dependent on TNF α and TSLP–TSLPR interactions¹⁰. In the current study, we demonstrate for the first time diminished TSLP secretion in NEC derived from individuals who are carriers of the rs1837253 T allele, a finding that provides key insight into allergic disease pathogenesis and explains, at least in part, the inverse association between rs1837253 and asthma and related phenotypes, and the recently reported therapeutic effects of TSLP antagonism. Several clinical outcomes and phenotypes of allergic diseases such as allergic rhinitis, total IgE and airway hyper-responsiveness, have also been reported to be associated with SNPs in *TSLP*.^{18, 20, 21, 25-27, 35} Collectively, these data suggest a critical role for TSLP in regulating multiple downstream effector pathways involved in allergic disease.

Finally, our findings may have implications in explaining virus-induced asthmatic exacerbations, especially those due to rhinovirus infections, a major cause of asthma exacerbations in both children and adults³⁶ resulting from these single-stranded RNA viruses which synthesize dsRNA during replication at the respiratory epithelium.³⁷ Therefore, the effects of dsRNA and/or TLR-mediated production of TSLP in NEC from

patients with or without asthma in relation to rs1837253 genotype merits investigation. Activation of the innate immune system in the epithelium involving rs1837253 may ultimately influence the development of allergic diseases by modulating TSLP's downstream effector pathways. Our findings in the current study provide further evidence for *TSLP* being an 'asthma gene', and help uncover the possible function of the rs1837253 variant.

METHODS

Study Subjects. All studies were approved by Hamilton Integrated Research Ethics Board and subjects provided written informed consent to participate. Eligible subjects were healthy, non-smoking individuals, 18 to 63 years of age, with no history of use of any nasal, oral, or inhaled corticosteroids within the previous three months. All recruited study participants had mouthwash samples collected and were identified as atopic using a positive skin prick test response (>2-mm wheal) to at least one of 14 common aeroallergens. Additionally, all subjects were confirmed to have no prior history of upper respiratory tract infection, within 6 weeks of sample collection. Further subject characteristics are shown in Table 1.

DNA extraction 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 LD with any other SNP.^{18, 20, 21} DNA was 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 performed using a commercially available TaqMan® genotyping assay for rs1837253 (Assay # C__11910823_20, Life Technologies Inc., Burlington, ON, Canada). The genotyping data were verified for Hardy-Weinberg equilibrium (HWE) by a Chi-square test.

Nasal epithelial cell isolation and culture. Primary NEC were derived from the inferior nasal turbinate using Rhino-Probe nasal curettes (Arlington Scientific, Springville, UT, USA). NEC were expanded and cultured as previously described with modification.²⁹ Briefly, NEC were maintained in bronchial epithelial growth medium (BEGM) - bronchial

epithelial basal medium (Clonetics, Allendale, NJ, USA) supplemented with SingleQuots (Clonetics). Collected cells were centrifuged and re-suspended in BEGM with 10% 100x DNase 1 (Sigma Aldrich, St. Louis, MO, USA; at RT, 20 mins). Cells were washed twice and plated on 35 mm culture dishes (Corning Costar, Corning, NY, USA), coated with Purecol (Inamed, Fremont, CA, USA) and incubated at 37°C, 5% CO₂. Media were changed 24 h post-plating; thereafter, media were replaced every second day until 80-90% confluence. Subsequently, cells were passaged (P1) using trypsin (Gibco, Burlington, ON, Canada) and further expanded in 75cm² flasks (Corning) in BEGM. In this study, second and third passaged cells were used. Viability was assessed by exclusion of trypan blue dye (Gibco) and the epithelial nature of cells assessed by immunocytochemistry as previously described with modification.³⁸ Briefly, selected cultures were fixed in 4% paraformaldehyde and permeabilized using Triton-X 100 (Sigma). Subsequently, cells were incubated with rabbit anti-cytokeratin 5 antibody (Abcam, Cambridge, UK) overnight at 4°C. Cells were then exposed to AlexaFluor 488 goat anti-rabbit IgG antibody (Life Technologies) at RT for 1 h in the dark. Nuclei were stained with DRAQ5 (Abcam) at RT for 15 minutes and slides were subsequently mounted using Fluoroshield Mounting Medium (Abcam). Negative control slides were prepared by omitting the primary antibody (Fig. 1). Fluorescent images were viewed using a Leica SP5 confocal inverted microscope (Leica Microsystems Inc., Concord, ON, Canada) using a 63X oil immersion objective. Images were captured and viewed using LAS AF (Leica) acquisition software.

PolyI:C stimulation. NEC were seeded onto 24-well plates (Corning) and allowed to grow to 80-90% confluence. Thereafter, BEGM was replaced with bronchial epithelial basal

media (Clonetics) and 1% fetal bovine serum (FBS; R&D Systems, Minneapolis, MN, USA) prior to experimental stimulation. NEC were treated with polyI:C (EMD Chemicals, Gibbstown, NJ, USA) as indicated or vehicle control for 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, 6 h) 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 cDNA were aliquoted and stored at -80 °C.

Quantitative reverse transcription polymerase reaction. The expression of TSLP was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA) with a MX4000 Stratagene detection system according to the manufacturer's instructions. The PrimePCR Human Reference Gene Panel (BioRad) was used to determine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) to be the optimal reference genes. We used the following primer set for qRT-PCR: (NM_033035.4 and NM_138551.4), 5'-CTAAGGCTGCCTTAGCTATC-3' and 5'-AAGCGACGCCACAATCCTTG-3' as previously described.²⁶ PrimePCR SYBR© green assay for Human GAPDH and YWHAZ

were used for reference gene primers (BioRad). All TSLP primer sets (Integrated DNA Technologies, Inc., Coralville, IA) were designed and evaluated as per the minimum information for publication of qRT-PCR experiments (MIQE) guidelines.³⁹ Pooled cDNA was used to optimize annealing temperature (found to be 60 °C for all primer sets). 8-point standard curves were used to verify amplification efficiency for each TSLP isoform primer sets and the GAPDH, YWHAZ reference genes using Sso Advanced SYBR green Supermix (BioRad). 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.⁴⁰ The standard $2^{(-\Delta\Delta Ct)}$ formula was used to calculate arbitrary TSLP mRNA concentrations.⁴⁰ The level of TSLP mRNA was normalized to the level of GAPDH/YWHAZ mRNA.

TSLP measurement. The release of TSLP in cell-free supernatant after 24 h stimulation with polyI:C was measured using DuoSet ELISA Development kits (R&D) as per the manufacturer's instructions.

Statistical Analysis. All data are expressed as the means \pm SEM. Significance was assumed at $p < 0.05$. All analyses were performed with Prism version 5 (GraphPad Software, La Jolla, CA, USA) using non-parametric tests. Differences within groups were assessed by Friedman test with Dunn's Post hoc test. Differences between groups (CC, CT, vs. TT) were assessed by Mann Whitney U test.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. Mark Larché (McMaster University) for his valuable scientific input and critical appraisal of the manuscript; and Adrian Baatjes (McMaster University) for his assistance with immunofluorescence staining. This work was supported by AllerGen NCE Inc. and Canadian Institutes for Health Research MOP 111029 to JAD. CCKH is a recipient of the AllerGen NCE Inc. Canadian Allergy and Immune Diseases Advanced Training Initiative (CAIDATI) Award. LA is the recipient of a UBC Four Year Doctoral Fellowship and an AllerGen NCE Inc. CAIDATI Award.

DISCLOSURE

The authors declare no conflict of interest.

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FIGURE LEGENDS**Table 1.** Subject Characteristics

Figure 1. Immunofluorescence staining of cultured primary NEC. (A) Representative image of primary NEC stained for cytokeratin (green staining with AlexaFluor 488) to assess for the epithelial nature of the cells. (B) Representative image of negative control of primary NEC stained without primary antibody. Nuclei in all images stained red with DRAQ5 (X63).

Figure 2. PolyI:C induces TSLP expression and secretion in human primary NEC. (A) Kinetics of TSLP expression in polyI:C (25 µg/ml)-stimulated NEC (n=8). Results shown are expressed as fold change in the TSLP mRNA level in polyI:C-stimulated NEC at 3h and 6h relative to the level in unstimulated NEC (0h). (B) Primary NEC were incubated for 24h with increasing concentrations of poly I:C (0-50 µg/ml). Concentrations of TSLP protein in the culture supernatant were measured using ELISA. Results shown are mean ± SEM of duplicates (n=8). One independent experiment performed per subject. Significant difference from unstimulated cells (*p<0.05; **p<0.01; ***p<0.001).

Figure 3. Relationship between atopy and polyI:C-induced TSLP secretion in NEC. Concentrations of TSLP protein in NEC culture supernatant from nonatopic (n=22) and atopic (n=14) individuals were measured using ELISA following overnight stimulation with (A) increasing concentrations of polyI:C (0-50 µg/ml); (B) 25 µg/ml of polyI:C; and (C) 50

µg/ml of polyI:C. Results shown are mean ± SEM of duplicates. One independent experiment performed per subject.

Figure 4. Relationship between *TSLP* rs1837253 genotype and polyI:C-induced TSLP secretion in nasal epithelial cells. Concentrations of TSLP protein in nasal epithelial cell culture supernatant from rs1837253 homozygous major allele (CC; n=14), heterozygous (CT; n=14), and homozygous minor allele (TT; n=8) individuals were measured using ELISA following overnight stimulation with (A) increasing concentrations of polyI:C (0-50 µg/ml); (B) 25 µg/ml of polyI:C; and (C) 50 µg/ml of polyI:C. Results shown are mean ± SEM of duplicates. One independent experiment performed per subject. Significant difference from CC genotype (*p<0.05).

TALBES & FIGURES**Table 1**

Characteristics	Homozygous Major Allele (CC)	Heterozygous (CT)	Homozygous Minor Allele (TT)
Sample size, <i>n</i>	14	14	8
Sex, male:female	5:9	11:3	3:5
Age*, years	28.4 (15.0)	30.4 (15.4)	27.0 (11.0)
Atopy, <i>n</i>	6	4	4
Ethnicity			
Asian	3 (21.4)	4 (28.6)	6 (75.0)
Caucasian	10 (71.4)	8 (57.1)	2 (25.0)
Other	1(7.2)	2 (14.3)	0 (0.0)

Values in parentheses indicate percentages.

*Mean (SD)

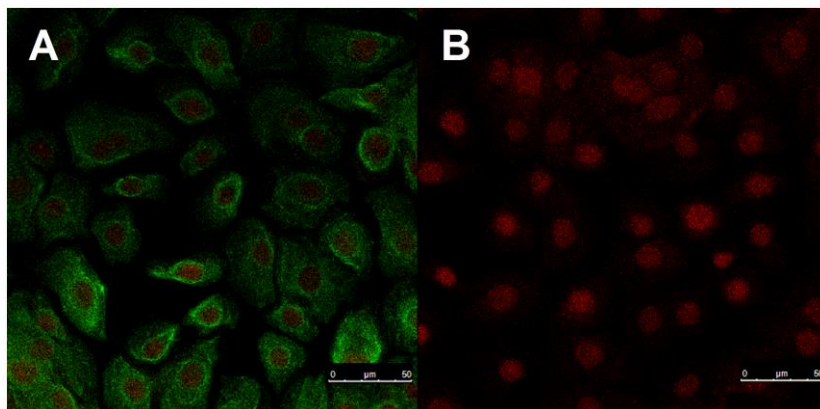
Figure 1

Figure 2

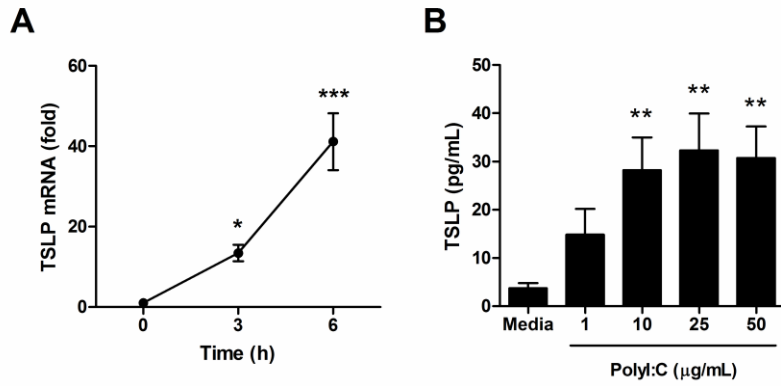


Figure 3

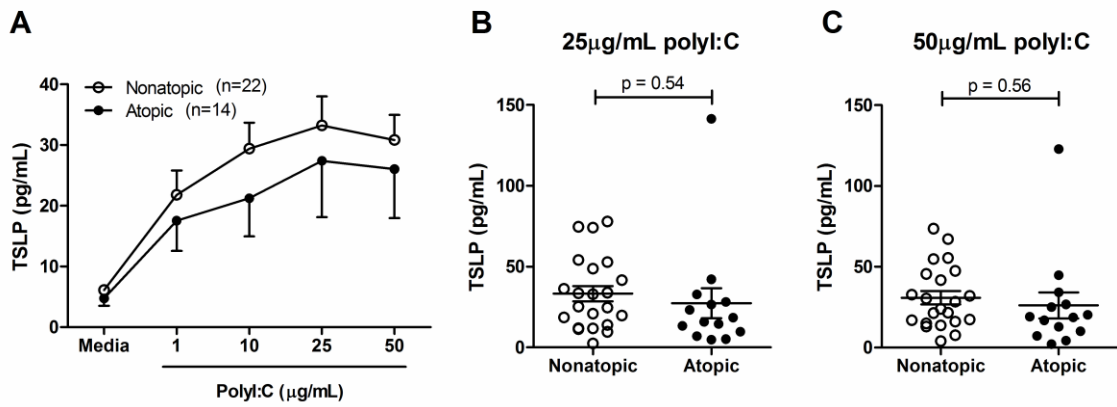
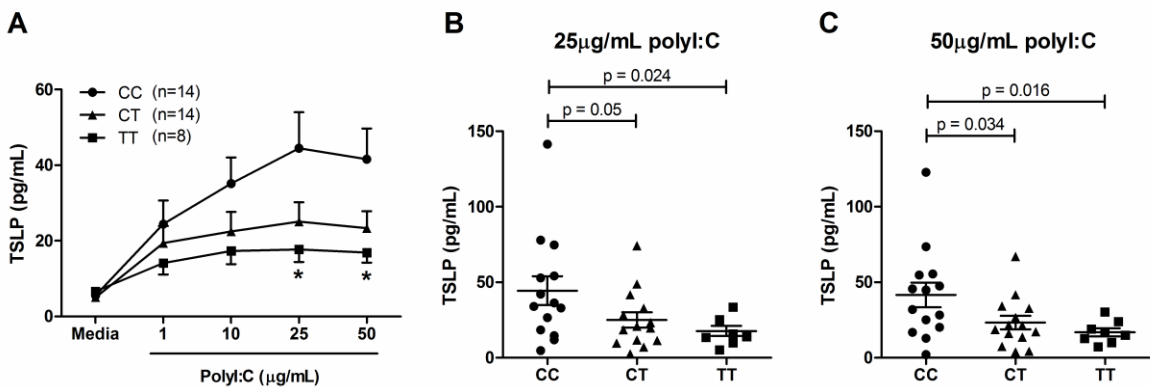


Figure 4



CHAPTER 5: DISCUSSION

An estimated 300 million individuals worldwide have been diagnosed with asthma, with an expected 100 million more by 2025 [28, 29]. In Canada, the cost of treating asthma alone is estimated to be \$4.2 billion by 2030 [238]. Given the rising socio-economic and health burdens of asthma, it is important to elucidate mechanisms underlying its development and thus discover novel treatments and preventive strategies. Although allergic sensitization is widely recognized as a risk factor for allergic diseases [17], not all sensitized individuals have symptoms. Studies have reported $\leq 50\%$ prevalence of atopic sensitization in asthma, suggesting that other factors contribute to the onset of disease [18]. With the completion of the human genome project in 2001, much effort in the last decade has centered on identifying novel genes as susceptible loci for asthma and related traits. Nonetheless, the precise roles of genetic factors in direct causation remain unclear, with all evidence pointing to complex gene-environment interactions.

With this in mind, the *TSLP* gene has emerged as one of the most important associations for the development of asthma and related traits in recent candidate gene [11] and GWAS [10, 236, 237] findings. The most established mechanism for TSLP-driven Th2 inflammation is via the TSLP-DC axis whereby CD4⁺ T cells are primed towards a Th2 biased immune response [14]. Aside from DC, the pleiotropic effects of TSLP on T lymphocytes [216], effector cells, such as mature eosinophils [203, 221] and basophils [142], as well as their progenitors [15, 143], and, most recently, ILC2 [239], have also been reported. The airway epithelium gives rise to TSLP, IL-33, and IL-25, a triad of cytokines that have emerged as key influencers of the allergic immune response. However, evidence

supporting a role for IL-33 and IL-25 in allergic inflammation is derived mainly from murine models [240, 241].

The overall objective of this thesis was to investigate the influence of innate (TLR3-mediated) or adaptive (allergen-specific T cell putative factors-mediated) immune stimuli on epithelial cell-derived TSLP production and the subsequent effector pathways leading to allergic inflammation and disease. Specifically, the studies described herein investigate two distinct TSLP-mediated pathways: a *TSLP-DC axis* and a *TSLP-Eo/B progenitor axis*. The hypothesis for the studies found herein is that variants in the *TSLP* gene are linked to alterations in TSLP secretion that then modulate downstream cellular events. These critical events include hemopoietic progenitor differentiation and DC functional effects on Th2 cell development, ultimately influencing the course of tissue allergic inflammation and thus, disease.

The data presented herein provide evidence for TSLP being involved in the regulation of multiple pathways of allergic immune response, and support *TSLP* being an “asthma gene.” In this regard, the data presented in Chapter 2 demonstrate enhanced TLR3- and allergen-specific T cell-mediated CCL17 expression *in vitro* in relation to clinical asthmatic status. Chapter 3 investigates an alternate downstream pathway of TSLP-mediated inflammatory response, and demonstrates enhanced TSLP-mediated hemopoiesis *ex vivo* in relation to clinical atopic status. Given the enhanced inflammatory response observed in our asthmatic and atopic group, we hypothesized that rs1837253 variant in the *TSLP* gene may influence TSLP secretion from mucosal surfaces, which might consequently influence the development of allergic responses. The data in Chapter 4 demonstrate that NEC derived from individuals with the minor protective allele have

diminished secretion of TSLP. Our findings not only provide key and novel insights into the possible function of rs1837253, but also explain, in part, the inverse association between rs1837253 and asthma and related phenotypes [9-11, 236, 237].

5.1 TSLP Mediates Crosstalk Between Epithelial Cells and Immune Cells

The airway epithelium acts as the first line of defense towards inhaled pathogens and other harmful substances [92]. However, persistent damage and/or exposure to numerous agents including viruses can cause the epithelium to lose its barrier function, through disrupted tight junctions and impaired innate immunity [93], which may contribute to increased sensitivity to infections and allergens. In the last few years, much work has been focused on the epithelium, especially due to the many cytokines and chemokines derived therefrom; in particular, there has been focus on the pleotropic effects of epithelial-derived TSLP, which can activate an array of immune cells [15, 203, 216, 221] such as DC [14] and hemopoietic progenitor cells [15], indicating that there is active TSLP-mediated crosstalk between epithelial cells and these immune cells.

5.1.1 Epithelial Cell-Dendritic Cell Interaction

In recent years, the roles of T cells and TSLP in allergic asthma have been extensively examined. However, very little is known about the interaction between the epithelial-derived TSLP and T cells. What role, if any, do allergen-specific T cells play in the activation of airway epithelium and the subsequent release of TSLP? In Chapter 2, the underlying concept is that in patients with asthma, there are increased allergen-specific T

cells which, upon their activation in the airway mucosa (after allergen or peptide epitope exposure), initiates the secretion of TSLP by BEC and activation of local DC. This results in secretion of CCL17 and recruitment and activation of CD3+/CD4+ T cells, production of inflammatory cytokines and, ultimately, triggering of the paracrine regulation of TSLP secretion by the epithelium, leading to the amplification of allergic inflammation. This series of events is also thought to be the basis for blood and airway eosinophilia in patients with allergic asthma [242]. The data in Chapter 2 show that allergen specific T cell-activated primary BEC culture supernatants induce TSLP-dependent CCL17 production from monocyte-derived DC, commensurate with clinical asthmatic status. CCL17 has been reported to be associated with CD4+ T cell accumulation during peptide-induced LAR as well as the magnitude of the peptide-induced LAR [85]. Our *in vitro* model provides further support for the concept that TSLP mediates crosstalk between the epithelium and DC during allergic inflammation. However, Kashyap *et al.* reported the ability of DC to produce TSLP post-TLR-ligation [194], which demonstrates a dual role for DC, to both respond to TSLP and to produce TSLP in response to pathogens/allergens. Their work raises the possibility that DCs themselves could bypass the need for epithelial cell-derived TSLP, by creating an autocrine loop, both producing and responding to TSLP, in addition to serving as a source of TSLP for other immune cells. Nevertheless, the airway epithelium acts as the first line of defense towards pathogens and other harmful substances, rendering the epithelium as the body's major source of TSLP [92, 243].

5.1.2 *Epithelial Cell-Eo/B Progenitor Interaction*

Recently, TSLP has been shown to directly activate CD34⁺ hemopoietic progenitors, with release of pro-inflammatory and Th2 cytokines, as well as chemokines in a dose-dependent manner [15]. Moreover, it has been suggested that TSLP may modulate the function of CD34⁺ cells via changes in hemopoietic cytokine receptors, which have been shown to be altered in cord blood CD34⁺ cells of atopic at-risk infants [244]. In line with this, the ability of TSLP to induce differentiation of progenitors in animal models has previously been demonstrated [142]; however, the effects of TSLP on human progenitor Eo/B lineage commitment remain poorly understood. The study reported in Chapter 3 was an attempt to better understand the link between TSLP and human progenitor Eo/B differentiation at the epithelial surface. Our data demonstrate that TSLP drives IL-3-mediated Eo/B differentiation through induction of autocrine TNF α secretion by HHP. Furthermore, we demonstrate enhanced TSLP-mediated hemopoiesis *ex vivo* in relation to clinical atopic status, which may help explain, in part, why atopic individuals may have increased eosinophil and basophil accumulation at sites of inflammation. Our novel findings demonstrate that besides the direct recruitment of inflammatory cells under the effects of inflammatory mediators [17, 22, 23], the airway epithelium can interact with hemopoietic progenitors to enhance Eo/B differentiation and induce an eosinophilic-basophilic inflammatory response.

5.1.1.1 *TSLP-Mediated in situ Hemopoiesis*

In Chapter 3, we defined a concept for a pathway which leads to the development of eosinophilic-basophilic inflammation in the absence of Th2 infiltration and accompanying

inflammatory mediators, under the term “*in situ* hemopoiesis.” This concept refers to the recruitment to, and differentiation at, mucosal sites of hemopoietic inflammatory cell progenitors [151]. There is indirect and direct evidence that undifferentiated CD34+ hemopoietic progenitors can home, and give rise to eosinophils and basophils, at sites of allergic inflammation, such as the bronchial mucosa of patients with asthma, the nasal mucosa in AR and nasal polyposis, and at other target tissue sites in related allergic inflammatory diseases [152, 153, 245]. In this regard, in 1988 Ohnishi *et al.* reported highly potent, but not totally defined, Eo/B colony-stimulating activities in conditioned medium derived from cultured human nasal polyp epithelial scrapings [246]. In line with this, we report augmented secretion of TSLP from asthmatic epithelial cells (Chapter 2) and demonstrate TSLP-mediated hemopoiesis *ex vivo*, which is especially enhanced in atopic individuals (Chapter 3). Based on these observations, we posit that human TSLP, which was only discovered in 2001, may be the key mediator responsible for the Eo/B colony-stimulating activities reported by Ohnishi *et al.* [246]. In further support of this hypothesis, TSLP is over-expressed in subjects with nasal polyposis [228] and AR [227], and is significantly correlated with disease severity. Moreover, intravenous administration of AMG 157, a human anti-TSLP mAb, in mild atopic asthmatics is accompanied by a decrease in baseline blood eosinophil counts [234], suggesting that eosinophil levels are controlled by epithelial-derived TSLP and/or TSLP-responsive factors. These findings further support our findings in Chapter 3, which demonstrated the ability of TSLP to mediate Eo/B differentiation from HHP, a process which was dependent on TSLP–TSLPR interactions and TNF α [143]. Further study examining airway epithelial cell-derived TSLP-

induced Eo/B differentiation in asthmatic subjects and controls, and its clinical relevance is warranted.

5.1.1.2 Hemopoietic Progenitor Cells or ILC2?

TSLP is thought to be important in the generation of eosinophilic inflammation in patients with allergic asthma, through activation of airway DC and promotion of increased numbers of Th2 cells, resulting in the production of specific inflammatory cytokines, including IL-5 and IL-13 [24, 207]. TSLP has also been demonstrated to influence the production of IL-5 and IL-13 from CD34⁺ progenitor cells and nascent mast cells [15]; in this context, TSLP induces IL-5⁺/IL-13⁺ CD34⁺ cells to appear in sputum after airway allergen challenge of atopic asthmatics [15]. Recently, nasal polyp epithelial-derived TSLP has been demonstrated to act on ILC2 [239], and in combination with IL-33, stimulates the production of Th2 cytokines, especially IL-5 and IL-13. The enhanced production of IL-5 may help to explain the induction of eosinophilic inflammation in the absence of Th2 cell activation in eosinophilic asthma [239, 247]. Given that TSLP induces surface expression of TSLPR, along with the capacity to produce high levels of IL-5 and IL-13 [59, 62, 205, 223], in both CD34⁺ cells and the recently identified ILC2, makes it difficult to functionally distinguish CD34⁺ hemopoietic progenitors from ILC2. The recent identification of ILC2 in the context of allergic inflammation has attracted a lot of attention, but whether human ILC2 are as important as CD34⁺ progenitors in triggering eosinophilic inflammation is still unclear. Nonetheless, the capacity of various epithelial cell-derived cytokines such as TSLP, IL-33 and IL25 to activate ILC2 suggests that, like CD34⁺ progenitors, the epithelium closely interacts with both these cell types.

5.2 Mechanisms Underlying Differential TSLP Secretion in Asthma and Allergic Diseases

Allergic diseases are complex inflammatory disorders highly influenced by both genetic predisposition and responses to environmental stimuli (e.g. infection, allergens, diet) [1]. In Chapter 2, we demonstrate enhanced TSLP secretion from asthmatic BEC following polyI:C stimulation. Others have shown that enhanced RIG-1 [105] and AP-1 [248] in asthmatic BEC compared to healthy controls is, in part, responsible for the enhanced TSLP secretion. However, gene expression is not solely determined by interactions between transcription factors and DNA sequences in promoters, but also by epigenetic alterations, such as DNA methylation [249]. DNA methylation is characterized by the addition of a methyl group to a cytosine base in a cytosine-guanine dinucleotide. Generally, increased DNA methylation of promoter sequences is associated with decreased gene expression [250]. *TSLP* has been demonstrated to be a methylation-sensitive gene [251, 252]. Along these lines, our findings demonstrate comparable basal levels of TSLP secretion in asthmatic and healthy control BEC, suggesting that BEC may be susceptible to epigenetic alterations induced by environmental factors such as microbial infection [235]. These epigenetic changes may, in turn, alter the threshold for epithelial activation in response to environmental stimuli such as viruses and allergens [235]. For example, TLR-ligands have been demonstrated to alter methylation processes [253]. Given that reduction of DNA methylation facilitates transcription by permitting transcription factors or co-activators to bind to regulatory elements (promoter or enhancer regions) [250, 254], we hypothesized that there would be diminished methylation of the TSLP promoter in asthmatic BEC following exposure to polyI:C [251, 252].

TSLP is an established susceptibility gene for asthma and allergic diseases across different ethnicities [9, 10, 232, 236] and its association with asthma and related phenotypes has been confirmed in both candidate gene [11] and GWAS [10, 236, 237] investigations - especially the rs1837253 polymorphism studied in this thesis [11]; though the reasons for this association have remained unclear. In Chapter 4, we demonstrate diminished *TSLP* expression in NEC obtained from individuals who were carriers for the protective minor allele of the rs1837253 polymorphism (T) compared to non-carriers (CC), post stimulation with polyI:C but not at baseline. Although the precise mechanisms underlying the regulation of *TSLP* secretion by rs1837253 remains elusive, an individual's genotype may modulate the effects of environmental exposure through epigenetic mechanisms [255]. Furthermore, it has been shown that rs3806933 (-847 C > T) in *TSLP* creates a binding site for AP-1, enhancing the transcriptional efficiency of the long-form *TSLP* induced by polyI:C in BEC [190]. Although the assessment of epigenetic modification was not the objective of these studies, we are now in a position to examine this hypothesis in prospective studies.

5.3 Clinical Implications: Viral-Induced Asthma Exacerbations

Asthma is a heterogeneous disease caused by multiple environmental factors in combination with genetic susceptibility [1], and has many distinguishable phenotypes. Eosinophilic inflammation is generally considered to be the main feature of allergic asthmatic airways and is presumed to be crucial in the pathogenesis of allergic asthma [27]. Approximately 50% of asthmatics have persistent eosinophilic asthma [256].

Asthma exacerbations are a major cause for mortality, and repeated exacerbations can cause permanent impairment of lung function [257]. Respiratory viruses are a significant cause of asthma exacerbations [258]. Rhinoviruses are single stranded RNA viruses that synthesize dsRNA during replication at the respiratory epithelium; therefore, mediating its effects via TLR3 signaling [259]. We [187] (Chapter 2 and 4) and others [13, 103-105] have shown that TLR3 ligation induces production of TSLP from epithelial cells, with enhanced secretion observed from epithelial cells obtained from asthmatic individuals compared to their healthy counterparts. With particular relevance to this thesis, the ligation of TLR3 by dsRNA has been shown to induce TNF α in airway epithelial cells [260]. Indeed, in Chapter 3 we demonstrate enhanced TSLPR expression on HHP following overnight stimulation with both IL-3 and TNF α , which significantly enhanced the sensitivity of CD34+ cells to TSLP-mediated Eo/B differentiation. These findings implicate TNF α , as a key mediator of epithelial-TSLP effects on Eo/B progenitors; and highlight a role for the innate immune response, initiated at the epithelial surface, in augmenting TSLP-mediated inflammation.

Virus-induced exacerbations are dependent in large part on the augmentation of pre-existing airway inflammation in asthma, leading to increased obstruction of the airway [261]. At baseline, *most* patients with allergic asthma have eosinophilia - increased eosinophils both in the periphery and at sites of inflammation [27]. Following viral infections, the airway epithelium secretes elevated levels of TSLP [13, 103-105, 187], which along with TLR3-induced TNF α , further promotes *in situ* hemopoiesis and augments the *eosinophilic* inflammatory response. Rhinovirus infection has been shown to induce airway eosinophilia in some individuals [262]. Our studies may thus help to explain some

mechanisms involved in virus-induced asthmatic exacerbations, highlighting, as they do, pathways through which the *eosinophilic* inflammatory response may be amplified and worsened following viral infections and/or viral-induced exacerbations of asthma.

5.4 Therapeutic Implications: TSLP Pathway as a Target in Asthma and Allergic Diseases

TSLP has been highly implicated in allergic diseases, with elevated levels correlating to disease phenotype [12, 218, 225, 226]. As we have demonstrated in Chapter 2, the TSLP–DC axis is critical in the development of a Th2 inflammatory response. TSLP-activated DCs permit Th2 development by upregulating OX40L, the Th2-polarizing signal, in the absence of IL-12 [208]. Data from murine models provide compelling evidence for a critical role for OX40/OX40L in the pathogenesis of asthma [263, 264]. However, support for the role of OX40/OX40L in humans is limited [208, 265]. Relatedly, a clinical trial using allergen inhalation challenge in mild atopic asthmatics reported that human monoclonal OX40L had no effects on allergen-induced early and late responses [266]. These findings suggest that the OX40/OX40L axis may not be critical in the pathogenesis of allergic inflammation in humans; rather, TSLP-induced secretion of CCL17 by DC may play a greater part by recruiting Th2 cells (Chapter 2).

Another clinical trial using AMG 157, a human TSLP mAb that inhibits its interaction with the TSLP receptor, was recently designed to prevent airway obstruction in mild atopic asthmatic by targeting the TSLP-TSLPR pathway [234]. AMG 157 was reported to reduce both the early and late responses following an allergen challenge, which, as the authors postulated, was likely due to the indirect effects of anti-TSLP treatment on

both mast cell activation and inflammatory cell recruitment, given that mediators from airway mast cells and basophils are major contributors to the early and LAR [267], and since the late response is also associated with the allergen-induced influx of inflammatory cells, such as basophils and eosinophils [267, 268]. The likelihood of this hypothesis is supported by the observed striking decreases in blood and sputum eosinophils and exhaled nitric oxide at baseline in the recruited subjects treated with the antibody [234]. However, as the authors mention, it is unclear whether these changes, reduced eosinophil numbers, were responsible for the inhibition of the allergen-induced responses or whether the reduction in eosinophils and subsequent changes in the FEV₁ were coexistent but not causal.

In Chapter 3 we demonstrated the ability of TSLP to mediate Eo/B differentiation from human peripheral blood hemopoietic progenitor cells *ex vivo*, which was dependent on TSLP–TSLPR interactions. Our findings provide a mechanism by which AMG 157 may operate to decrease eosinophils and basophils at sites of inflammation. However, it is as yet unclear whether the inhibition of the allergen-induced responses was due to the decreased numbers of eosinophils in the blood and sputum [234]; further mechanistic studies of AMG 157 are warranted.

To date, of all the available asthma treatments, inhaled glucocorticoids are the gold standard for asthma treatment, as they are the only agents known to attenuate baseline levels of blood eosinophil counts and exhaled nitric oxide, while all other available treatments can only attenuate components of allergen-induced airway responses [269]. Therefore, although the full clinical value of anti-TSLP therapy cannot yet be ascertained, the capacity of AMG 157 to attenuate baseline indices of inflammation appears promising. Collectively, these findings highlight a possible role for TSLP in the development or persistence of asthma by

initiating hemopoietic responses in the mucosa and/or promoting *in situ* hemopoiesis by providing signals necessary for lineage commitment into effector cells that contribute to inflammation at mucosal surfaces. Further clinical studies will be needed to carefully evaluate the potential clinical benefits and/or side effects of anti-TSLP therapy, given that the biological effects of TSLP are strongly dependent upon the local tissue microenvironment: while in the skin and airway TSLP induces Th2-type inflammation, in the intestinal mucosa it promotes tolerance [270].

5.5 Limitations

We acknowledge certain limitations in the studies presented herein. In Chapter 2, asthmatic BEC were obtained from asthmatic subjects recruited at McMaster University, while healthy BEC were commercially obtained from Clonetics, which may raise concerns of altered cell responses due to differential BEC collection and/or culture methods. Although we cannot be certain how healthy BEC samples were obtained, we took great care to culture the cells in exactly the same culture media and supplements so as to reduce variation between cultures of cells from asthmatic and healthy controls. Furthermore, while we acknowledge that smoking can be a confounding factor in the induction of TSLP [271], unfortunately we do not have data on the smoking status of our subjects. However, our observed differential expression of TSLP between asthmatic and healthy BEC is supported by previous studies, which have all shown increased TSLP expression from asthmatic BEC compared to healthy BEC [13, 105, 272]. Finally, it was only very recently discovered that IL-1 β , IL-6, and CXCL8 are the principle mediators responsible for allergen-specific T cell-

dependent induction of epithelial-derived TSLP. Unfortunately, we had exhausted all the asthmatic BEC on the earlier experiments and due to resource and subject recruitment constraints there was not enough asthmatic BEC to explore whether IL-1 β , IL-6, and CXCL8 induced more TSLP in BEC from asthmatics or not.

In Chapter 3, it is not clear how TSLP and/or any of the other manipulations (such as anti-TNF α) may alter the relative proportions of eosinophils vs. basophils in the Eo/B colonies. There are advantages and disadvantages to liquid cultures, in which enumeration of eosinophils and basophils can be performed, but progenitors cannot be accurately quantified as they can in Eo/B CFU assays. In light of this, we elected to use the CFU assay, and added colony histamine assays as a specific, surrogate biomarker of basophil ‘content’ within these Eo/B colonies, given that histidine decarboxylase is only present within the basophil, not the eosinophil in these mixed basophil-eosinophil colonies, each derived from a single progenitor, [132, 136, 144, 273, 274]. Rather than examine the relative proportions of eosinophils vs. basophils in these colonies of nascent eosinophils and basophils of mixed granulation (which one also sees in liquid cultures), we have focused on peripheral blood Eo/B CFU (and thus, HHP) production after TSLP stimulation, providing novel evidence that TSLPR engagement on PB CD34 $^+$ cells has the capacity to enhance *Eo/B lineage priming* of myeloid progenitors, increasing the likelihood of development of allergic eosinophilic/basophilic inflammation, in addition to disease maintenance or progression.

Finally, in Chapter 4, we describe the effects of dsRNA on NEC-derived TSLP (mRNA and protein) production. However, only very few cells are obtained from nasal scrapes, and consequently, there were not enough cells available to examine the expression of other inflammatory proteins, such as AP-1 and the retinoic acid-inducible gene 1 (RIG-1)

that are in part responsible for the differential secretion of TSLP between healthy and asthmatic airways, and among SNP rs1837253 genotypes [105, 248]. In addition, we had gathered limited subject characteristics, which restricted our examining the effects of rs1837253 on the differential production of TSLP amongst asthmatics.

5.6 Summary

The overall objective of this thesis was to investigate the influence of innate (TLR3-mediated) or adaptive (allergen-specific T cell putative factors) immune stimuli on epithelial cell-derived TSLP secretion and the subsequent effector pathways of allergic disease. Our initial objective was to determine whether activated allergen-specific T cells are able to induce the expression of TSLP in primary, differentiated BEC and the molecular mechanisms through which this T cell-dependent expression occurs. We demonstrated that, like polyI:C which has previously been shown to induce TSLP in airway epithelium [13, 104], supernatants from activated T cells (and individual cytokines) are able to induce TSLP and that production is higher in epithelial cells from asthmatic subjects. This study was the first to demonstrate enhanced allergen-specific T cell-mediated CCL17 expression *in vitro* in relation to clinical asthmatic status. These findings support allergen-specific T cells as positive regulators of TSLP secretion, suggesting T cell-airway epithelium interactions that may lead to maintenance and amplification of allergic inflammation.

We next examined whether TSLP is able to induce eosinophil and basophil differentiation from human hemopoietic progenitors and investigated the molecular mechanisms through which this TSLP-dependent hemopoiesis occurs. Unlike previous

studies of TSLP-induced differentiation, we used peripheral blood CD34+ progenitor cells isolated from atopic and nonatopic control subjects to address these questions *ex vivo*. We demonstrated that TSLP is able to drive IL-3-mediated Eo/B differentiation and that this process is through induction of autocrine TNF α secretion by CD34+ progenitor cells, both novel findings. Furthermore, this study was the first to demonstrate enhanced TSLP-mediated hemopoiesis *ex vivo* in relation to clinical atopic status. This study enhanced our understanding of the link between TSLP and human progenitor Eo/B differentiation at the epithelial surface and may help explain increased eosinophils and basophils at sites of inflammation in atopic individuals.

Finally, we attempted to elucidate the reasons for association between the rs1837253 and asthma by relating rs1837253 genotype differences to *ex vivo* TSLP production from NEC. We demonstrated *diminished* TSLP secretion in NEC from individuals who are carriers for the protective minor allele. This study sheds light on the functional role of rs1837253 and helps to explain, in part, the reported protective association rs1837253 has with asthma [9-11, 236, 237]. Collectively, the studies presented herein provide evidence that TSLP plays a pivotal role in regulating airway inflammatory responses, and support continued investigations into the functional mechanisms linking TSLP variants to allergic disease phenotype.

5.7 Future Direction: SNP rs1837253 Associated Epigenetic Regulation of Asthma and Allergic Disease

TSLP is currently a hot topic, which has refuelled interest in the role of the epithelium in asthma and allergic diseases. Novel findings have reported on the immunological effects of

TSLP on its downstream effector pathways [14, 142, 187, 203, 216, 221], with attention recently focused on the effects of *TSLP* polymorphisms on asthma and related phenotypes [9-11, 236, 237].

In our study, we demonstrated diminished TSLP secretion in NEC derived from individuals who are carriers of the protective rs1837253 T allele; however, the mechanism for reduced TSLP expression is unclear. *TSLP* has recently been shown to be a methylation-sensitive gene [251, 252]. 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 [252]. In a more recent study, Luo *et al* [251] reported DNA hypomethylation of the TSLP promoter region, which was accompanied by overexpression of TSLP in skin lesions from patients with AD compared with controls. 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 [11], continued research on the effects of rs1837253 genotype on TSLP secretion in different phenotypes (mild and severe) of asthma will help unravel the association. Although the obstruction of TSLP-TSLPR interaction seems promising as a therapy for allergic airway inflammation [234], 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.

CHAPTER 6: REFERENCES

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Editor-in-Chief Dr. Marc Veldhoen

Online ISSN: 2050-4527

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