The Regulation and Function of Thymic Stromal Lymphopoietin Receptor (TSLPR) in the Airway Epithelium

Michael Miles Miazgowicz

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Reading Committee:

Steven F. Ziegler, Chair

Joan M. Goverman

Daniel J. Campbell

Program Authorized to Offer Degree:

Department of Immunology

University of Washington

Abstract

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Michael Miles Miazgowicz

Chair of the Supervisory Committee:
Affiliate Professor Steven F. Ziegler
Department of Immunology

The epithelially-derived cytokine thymic stromal lymphopoietin (TSLP) plays a key role in the development and progression of allergic disease and has notably been shown to directly promote the inflammatory responses that characterize asthma. Current models suggest that TSLP is produced by epithelial cells in response to inflammatory stimuli and acts upon hematopoietic cells to effect a T_H2-type inflammatory response. Recent reports, however, have shown that epithelial cells themselves are capable of expressing the TSLP receptor (TSLPR), and may thus directly contribute to a TSLP-dependent response. In this thesis, we review what is known about both the immunological and epithelial responses that contribute to the pathogenesis of asthma, and further present novel data indicating a role for epithelially expressed TSLPR to play in those responses.

We present data that epithelial cells indeed express a glycosylated form of the TSLP receptor. Furthermore, we find that beyond simply expressing TSLPR, epithelial cells are capable of dynamically regulating TSLPR in response to the same inflammatory cues that drive the production of TSLP. This induced receptor is functional, as epithelial cells produce CCL17, a T_H2-associated chemokine, in response to stimulation with TSLP. These data suggest that a direct autocrine or paracrine response to TSLP by epithelial cells may initiate the first rounds of chemotaxis during an allergic inflammatory response. Intriguingly, we find that the regulation of TSLPR, unlike TSLP, is NF-κB independent, suggesting that the cell may be able to independently regulate TSLP and TSLPR levels in order to properly modulate its response to TSLP. Finally, we show evidence for this dynamic regulation occurring following the viral infection of primary epithelial cells from asthmatic patients. Taken together, the data suggest that induction of TSLPR and a direct response to TSLP by epithelial cells may play a novel role in the development of allergic inflammation.

Table of Contents

List of Figures

Page

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Chapter 1: Introduction

Overview

Asthma is a chronic inflammatory disease of the airways with a complex etiology. It is a significant public health concern; Asthma affects 8% of the adult US population, and 10% of children, and is estimated to account for $56 billion in medical and related expenses.[1] Patients typically develop the disease during childhood and suffer from its effects throughout the remainder of their lives. For most patients, asthma's symptoms can be controlled by the use of β_2-adrenergic receptor agonists and steroidal drugs, however, up to 10% of asthmatics are unresponsive to therapeutic agents and are at high risk for asthma-related hospitalization and death.[2] Importantly, despite asthma's history and prevalence, none of the currently available pharmaceutical therapies are effective in preventing or curing the disease. There exists, thus, a clear need for research into the pathogenesis of asthma, both in its initiation and elaboration, in order to better design effective treatment strategies.

While the precise causative mechanisms are unknown, both the symptoms and triggers of asthma have been well studied. In many cases, acute exacerbations ("asthma attacks")– involving wheezing, coughing, and difficulty breathing– are triggered by exposure to allergen.[3] Indeed, allergic asthma, along with atopic dermatitis and allergic rhinitis, is part of the so-called "allergic triad" defines the atopic syndrome.[4] In a minority of cases, asthma attacks are triggered non-atopically, by chemicals, intense exercise, or other poorly defined or unknown factors. Regardless of the triggering stimulus, however, the underlying symptoms of the disease are well defined. Asthmatic

disease is marked by extensive remodeling and thickening of airway walls, goblet cell metaplasia and subsequent mucus hypersecretion, and cellular infiltration of the airways with a characteristic "T_H2-skewed" hematopoietic profile featuring strong eosinophilia. As a result of the chronic inflammation and tissue remodeling, airways become hypersensitive to triggering stimuli, resulting in the acute bronchoconstriction experienced during asthma attacks. Unmanaged, the airway remodeling is progressive, leading to a permanent and significant impairment of lung function over time.[5]

Investigations into the causes of asthma stretch back over 150 years,[6] but it wasn't until relatively recently that asthma was appreciated as an inflammatory disorder associated with a dysregulated immune response. The discovery that inflammatory processes persisted in asthmatic lungs even during asymptomatic periods coincided with the discovery of the allergy-associated T_H2 subset of CD4 T cells, and it was subsequently found that these cells were present in asthmatic airways.[5;7] Accordingly, investigations into how immune responses, particularly allergic responses, become dysregulated have dominated contemporary research into asthma. More recent research has suggested that epithelial cells from the airways play a prominent, if not central role in asthma pathogenesis.[8-15] Indeed, much of the most promising current research into asthma focuses on the interplay between the immune system and airway epithelium, and how the responses of the epithelium to environmental insults result in the conditioning of responding immune cells towards a pro-asthmatic phenotype. By identifying and understanding the molecular and cellular mechanisms which the two systems use to coordinate the asthmatic response, it is hoped that pharmacological interventions can be targeted at those mechanisms to cure or even outright prevent the development of asthma.

One of the key mediators of asthma that has been identified in recent years has been the epithelially-derived cytokine thymic stromal lymphopoietin (TSLP). Significantly, work from our lab and others has demonstrated that TSLP lies at the crucial axis between the response of the airway epithelium to environmental insults and the activation of a T_H2-skewed immune response towards those insults. TSLP has been shown to be both necessary and sufficient to induce disease in mouse models of asthma. Intriguingly, recent studies from our lab have shown that respiratory viruses, infections with which have long been known to be a significant risk factor for the development of asthma, induce production of TSLP and may thus directly promote subsequent asthmatic responses. Investigating TSLP, therefore, offers an exciting avenue of research into the pathogenesis of asthma.

The aim of this thesis is to better understand the mechanisms that control the pathogenesis of asthma. We will examine the physiological systems involved in asthma pathogenesis, as well as review what is known about both the triggers and risk factors for the disease. We will discuss in particular how TSLP is responsible for mediating multiple aspects of asthma, and draw attention to the role that the airway epithelium plays in regulating the actions of TSLP. We will present novel findings concerning the ability of the epithelium to respond to TSLP, and how by regulating this responsiveness to TSLP following exposure to inflammatory stimuli the epithelium may play a direct role in the earliest stages of the asthmatic response.

The Airway Epithelium

At an estimated 100 m^2, the epithelium of the human lung is the largest surface in the body that is directly and continuously exposed to the outside environment. The

degree of this exposure is quite obviously required for the purposes of gas exchange, but does present a challenge in maintaining a strict separation of external and internal environments. At the most fundamental level, cells of the airway epithelium form a tight physical interactions with one another in order to form an impermeable barrier to the outside environment. This barrier is complemented by a coating of mucus that traps particulate matter, and is cleared from the lung by the mechanical action of ciliated epithelial cells, together forming the so-called "mucociliary escalator." Together, these systems form an effective first line physical defense against harmful physical, chemical or biological agents entering the airways.[8]

Airway epithelial cells are broadly classified into three groups: ciliated, secretory and basal cells. Ciliated epithelial cells account for over half of the airway epithelial cells, and form the luminal surface of the airways. These cells have a pseudostratified columnar morphology, with apical cilia responsible for the mechanical clearance of mucus. Interspersed among the ciliated epithelial cells lie the secretory goblet cells. Goblet cells, named for their characteristic "goblet-like" morphology, are responsible for secreting mucus into the airway lumen as well as controlling the viscosity of the secreted mucus. Both ciliated epithelial cells and goblet cells are supported by basal cells. As their name implies, basal cells attach to the basement membrane and provide a stable anchor for the ciliated epithelium and goblet cells. In addition, basal cells are capable of differentiating into both ciliated epithelial cells and goblet cells, and serve as the progenitor population for these cell types. In the smaller airways, where basal cells are fewer in number or altogether absent, a specialized cell type known as Clara cells is thought to function as the resident stem cell population. In addition, Clara cells also have a secretory character, producing surfactants and other more specialized compounds that aid in bronchiolar function.[16;17]

The ability of airway epithelial cells to form an impermeable barrier is mediated by several kinds of molecular complexes. Tight junctions, the most apical of these complexes, are formed by a network of homotypic and heterotypic protein interactions that bring the plasma membranes of adjacent epithelial cells into close contact. Proteins including claudins, occuldin, junctional adhesion molecule (JAM), zona occludens proteins ZO-1, -2 and -3, as well as various other adhesion and adapter proteins form a tight, nearly completely occluded junction between adjacent cells.[18] More basolaterally, adherens junctions, made predominantly of cadherin/catenin complexes,[19] and desmosomes/hemidesmosomes, made predominantly of cadherins and integrins,[20] provide additional adhesional foci. Along the basolateral edge of the epithelium lies a dense network of collagen, laminin and fibronectin, referred to as the basement membrane, that serves as both an anchor and orienting surface for the epithelial cells.[16] Importantly, while these various molecular interactions are impermeable to most external environmental agents, they can be modified both structurally and dynamically to form pores and transport networks that allow for selective passage of solutes and even infiltrating immune cells across the epithelial barrier.[18]

In addition to purely physical barrier against the general external environment, airway epithelial cells have a variety of mechanisms to respond to specific environmental insults. In particular, infectious agents present a challenge to epithelial cells that simple physical exclusion is insufficient to address, and that requires coordination with the immune system to effectively overcome. To respond to these agents, airway epithelial cells express a variety of pattern recognition receptors (PRRs) that are capable of sensing specific evolutionarily conserved pathogen associated molecular patterns (PAMPs) shared by many pathogens. Expression of Toll-like receptors (TLRs), RIG-I like receptors (RLRs) and NOD-like receptors (NLRs) equip airway epithelial cells with the capacity to

initiate inflammatory responses following exposure to both intracellular and extracellular pathogens.[21-24] In addition, epithelial cells express protease activated receptors (PARs) that allow the cells to respond to exogenous protease activities common among certain classes of pathogens as well as certain common allergens[25] In response to stimulation of PRRs and PARs, as well as other more specialized receptors, epithelial cells upregulate a wide array of pro-inflammatory chemokines and cytokines, including TNF-α, IL-1, CCL17, CCL22, MCP-1, IL-25, IL-33 and TSLP.[22-31] These factors act to recruit and activate cells from both the innate and adaptive arms of the immune system, as well as triggering structural changes in the epithelium itself that facilitate clearance of the pathogen. The details of this interaction between the epithelial and immune systems will be discussed in the following sections.

The Immune System

The mechanisms used by the body to protect against and eliminate various pathogenic insults are collectively referred to as the immune system, and include such functions as the formation of epithelial barriers as discussed above. However, a particular lineage of cells, derived from hematopoietic stem cells, specialize in the recognition and elimination of pathogens, and are typically what is being referred to when speaking of the immune system. These hematopoietic cells can be subdivided into two general categories: cells of the innate immune system and cells of the adaptive immune system. Each subsystem is comprised of several distinct cell types, each with highly specialized roles in the immune response.

Cells of the innate immune system are the first to respond to pathogenic insults, and are involved in both the general effector stages of an immune response as well as in

the activation and tailoring of the more potent downstream adaptive responses. Innate immune cells are largely derived from myeloid hematopoietic precursors, and include neutrophils, eosinophils, basophils, macrophages, monocytes, dendritic cells, and mast cells. A few innate (or innate-like) immune cells are derived from lymphoid hematopoietic precursors, and include NK cells, NKT cells, and γδ-T cells. Innate immune cells detect pathogens largely by means of the PRRs previously mentioned, as well as other specialized receptors that recognize evolutionarily conserved pathogenic components or byproducts of pathogenic activity. Furthermore, innate immune cells can become activated in response to non-specific pro-inflammatory signals, or can interact with byproducts of the adaptive immune response to respond in a targeted fashion to specific pathogens. In response to activation, innate immune cells produce a wide array of pro-inflammatory mediators, including TNF-α, Type I interferons, and other chemokines and cytokines, as well as various microbicidal or cytotoxic factors. Some cells, namely neutrophils, macrophages, monocytes and dendritic cells, possess phagocytic activity, and macrophages and dendritic cells in particular play important roles as antigen presenting cells (APCs) that serve a crucial function in further activating the adaptive immune response.[32]

In contrast to the innate immune system, the adaptive immune system responds to pathogens in a highly coordinated and exquisitely specific fashion. Adaptive immunity is largely mediated by T and B cells, of the lymphoid hematopoietic lineage, acting through tailored, pathogen-specific surface receptors (the T cell receptor– TCR– and B cell receptor– BCR– respectively). T and B cells (collectively referred to as lymphocytes) possess the ability to stochastically modify the genetic structure of the TCR and BCR loci, respectively, in order to generate receptors with a nearly infinite variety of specificities. The adaptive immune response is initiated when APCs (typically dendritic cells and some

macrophage populations, although previously matured B cells and even basophils may also serve as APCs) present antigenic fragments of pathogens to immature lymphocytes in the context of inflammation-induced co-stimulatory signals. Lymphocytes with receptors capable of binding to the presented antigens become activated and mature into effector cells. After the clearance of a specific pathogen, a certain percentage of mature lymphocytes will persist as long-lived memory cells, allowing the body to maintain long-term protection against previously encountered pathogens. This process forms the basis of immunological memory.

Adaptive immune cells can play a diverse array of effector roles. B cells are responsible for secreting antibodies (which are simply soluble forms of the BCR) as well as playing the aforementioned role of an APC, and comprise what is termed the humoral immune response. Following initial activation, B cells undergo processes known as affinity maturation and class switch recombination, the effects of which are to potently increase the specificity of their antibodies and modify the structure of those antibodies to better fit specific effector responses. A terminally differentiated version of a B cell known as a plasma cell is specialized towards the production and secretion of these matured antibodies. T cells, by contrast, mediate more cellular effector responses. T cells expressing the CD8 co-receptor mature to become cytotoxic T cells, responsible for the controlled elimination of virally infected cells. T cells expressing the CD4 co-receptor mature to become helper T cells, responsible for producing specific cytokines that assist or modify the responses of other effector cells. These helper T cells can be further subdivided into T-helper (T_H) subsets depending on which cytokines they produce. Classically, helper T cells were believed to terminally adopt either the T_H1, T_H2 or T_H17 phenotype, producing IFN-γ, IL-4/IL-5/IL-13 or IL-17, respectively, although work in

recent years has described many more T_H subtypes and has indicated that there is a high degree of plasticity between the different types.[33-35]

Regulation of the Immune Response

One of the central challenges that the immune system faces is the distinction between self and non-self. The effector responses the immune system is capable of generating are often times dramatic in both their intensity and effectiveness, but if not carefully targeted at foreign pathogens they can result in significant harm to the body. The ability to accurately target the immune response is especially important given the function of the adaptive arm of the immune response; if responses are targeted against endogenous antigens or against harmless, non-pathogenic exogenous antigens, immunological memory can be generated against those antigens, leading to cycles of chronic, non-productive inflammation and tissue damage. Indeed, it is precisely these processes that underlie autoimmunity and allergy, respectively. To prevent the mis-targeting of immune responses, the body employs a variety of regulatory mechanisms, broadly classifiable as central and peripheral tolerance.

Central tolerance includes the mechanisms that make the "first pass" against mis-targeted adaptive immune cells. Due to the stochastic nature by which the TCR and BCR genetic loci are rearranged to form full receptors, receptors can be generated that are non-functional, functional and specific for non-self antigens, or functional and specific for self antigens. To select for only cells that express functional receptors that target non-self antigens, developing lymphocytes undergo both positive and negative selection. These processes occur in specialized– or central– anatomical locations (namely, the thymus for T cells, and the bone marrow and spleen, to a lesser extent, for B cells),

providing controlled environment for selection to occur. In positive selection, lymphocytes are allowed to bind non-specifically to the cognate receptors utilized by APCs, and only cells with receptors functional enough to recognize those cognate receptors are permitted to survive. In negative selection, the newly positively selected lymphocytes are exposed to self antigens. Cells that become activated during negative selection are killed, leaving behind (theoretically) only those cells with a functional receptor that does not recognize self-antigens. Once thus selected, lymphocytes can travel from the thymus/bone marrow/spleen out into the peripheral tissues, ready to participate in an immunological response.[36;37]

Despite being highly effective, central tolerance does not eliminate every mis-targeted lymphocyte. Thus, the body relies upon a different set of tolerance mechanisms– peripheral tolerance– to eliminate or control these cells once they begin circulation through the body. At the simplest level, the aforementioned requirement that lymphocytes be exposed to inflammation-induced co-stimulation to become fully activated serves as a passive tolerance mechanism. Indeed, cells that encounter their antigen in the periphery in the absence of proper co-stimulation can become anergic, and lose the ability to respond to future stimulation.[38] Nevertheless, it is possible for mis-targeted lymphocytes to encounter their antigen within a "proper" context and become activated. Thus active mechanisms of peripheral tolerance are required to handle any inappropriate responses that may arise. A specialized class of CD4+ T cells known as regulatory T cells (T_{reg}) function to actively inhibit the effector responses of activated T cells. T_{reg} cells can be derived directly from T cells undergoing thymic selection, or can be induced in the peripheral under the correct circumstances, and they are believed to act both by producing inhibitory factors that act on activated T cells as well as interfering with the ability of activated T cells to receive the proper survival

signals.[39;40] Between the mechanisms of central tolerance, passive peripheral tolerance and active peripheral tolerance, inappropriate immune responses can be completely avoided under normal circumstances.

It is worthwhile to note that due to the static nature of the PRRs and other related receptors, the innate immune cells do not suffer to the same degree the propensity to respond inappropriately to non-pathological stimuli. Interactions between self-antigens and PRRs would be selected against evolutionarily, for obvious reasons, as would interactions with innocuous antigens. This evolutionary protection is not perfect however; certain allergens are thought to mimic the structure of PAMPs sufficiently to induce an (inappropriate) response.[41] Likewise certain self antigens, particularly nucleic acids, must be kept sequestered by various means from innate cell PRRs due to their similarity with pathogenic antigens (ie. viral nucleic acids). Inappropriate responses can be generated if that sequestration ever breaks down, as is believed to happen during the pathogenesis of systemic lupus erythematosus (SLE).[42] Furthermore, if adaptive immunity is generated against an inappropriate antigen, the effector responses of the adaptive immune cells can feed back to innate cells and drive them to respond to the same antigen, regardless of its nature. Inappropriate or excessive innate immune responses are typically controlled by an extensive variety of anti-inflammatory factors.

If the mechanisms of tolerance are broken, then significant pathologies can result. The ways in which tolerance can be broken and inappropriate immune responses generated are myriad, but depending on the nature of the antigen or antigens the responses are targeted against, different types of pathologies can be generated. If tolerance is broken against a self antigen, autoimmunity results. Examples of autoimmune disorders include type I diabetes (caused by a cytotoxic immune response directed at pancreatic β-islet cells), IPEX (caused by a mutation in the transcription

factor required for T_{reg} development), or the aforementioned SLE.[42-44] If the immune responses are instead generated against an innocuous foreign antigen, allergy results. Allergies include such diseases as atopic dermatitis, allergic rhinitis, and, of course, asthma. While both autoimmunity and allergy can involve any aspect of the immune system, autoimmunity– typically directed against specific tissues or cellular antigens– tends to involve a strongly cytotoxic, T_H1-, or T_H17-skewed effector response, and can be inhibited by T_H2 effector responses. Conversely, allergy has a strong T_H2 character that can be inhibited by T_H1-skewed responses.[45;46] As asthma is strongly associated with allergic responses, we will focus upon the mechanisms by which T_H2-type immune responses are generated within the context of the disease.

The Asthmatic Response

As discussed previously, asthma can be caused by a wide variety of factors, including non-atopic triggers such as synthetic chemicals and intense exercise. Nevertheless, the allergic form of asthma is widespread, accounting for up to 80% of all cases of asthma.[5] Interestingly, even in cases of non-atopic asthma, the underlying physiological response is largely the same as in atopic asthma. In both cases the physiological reaction includes airway inflammation and remodeling along with infiltration of the airway walls and bronchoalveolar spaces with immune effector cells of a distinct, pro-allergic "inflammatory T_H2" character. By studying the processes involved in allergen-dependent asthma, we can gain crucial insight into the general mechanisms of the disease.

Studies on asthma in human patients have yielded important and clearly relevant advancements in our understanding of the disease; indeed, the formulation of the link

between asthma and a T_H2-associated immune response was borne out of studies finding an enriched T_H2 lymphocyte population in the lungs of patients with atopic asthma.[7] However, murine models of asthma have allowed researchers to more carefully and precisely explore the identities and roles of the cells involved in the asthmatic response. An asthma-like disease, featuring all of the hallmarks of asthma in humans, can be induced in mice by sensitizing mice against an experimental antigen– frequently ovalbumin (OVA)– then rechallenging the mice intranasally with the same antigen. By examining the mice during all stages of the response, researchers have been able to map the roles that the different cell types play in the pathology of asthma. These roles are reviewed below.

Resident Antigen Presenting Cells

Antigen presenting cells are essential for the initiation of a full adaptive immune response, providing the bridge between the initial innate immune response and the activation of adaptive immunity. In mouse models of asthma, it has been shown that a response by CD4+ T cells to an antigen is required to cause disease,[47;48] implicating APCs in this requirement. In the lung, macrophages and dendritic cells are the primary cells responsible for APC activity, while more recent evidence suggests that basophils may additionally contribute to these duties. B cells may play a minor role, but to date the extent of their involvement in the pathogenesis of asthma has been unclear.[49]

A subset of macrophages known as alveolar macrophages (AMs) are a prominent constituent of the total cell population found in the airways from healthy lungs. Normally these cells are quiescent, existing in close contact with the airway epithelium, exhibiting poor phagocytic activity and producing anti-inflammatory cytokines. Indeed,

experiments depleting AMs worsened asthmatic responses.[50] However, under certain circumstances, their quiescence can be reversed, and AMs can contribute to inflammatory responses. Activation of PRRs, though, for example, viral infection, can induce AMs to begin secreting TNF-α.[51] Significantly, AMs can both recruit activated neutrophils during a response as well as phagocytose the apoptotic neutrophils following the response, playing a role both in the initiation and resolution of inflammation.[52-54]

While AMs can take up and present antigen to allergen-specific T cells,[54] it is believed that their primary role during an inflammatory response is purely phagocytic. In contrast, lung resident dendritic cells (DCs) are believed to be the primary antigen presenting cell.[55;56] Like AMs, resident DCs are thought to be largely tolerogenic in non-inflamed lungs, being able to induce the generation of T_{reg} populations through production of TGF-β as well as through other mechanisms[57;58]. In the context of an inflammatory response, however, DCs respond both directly to pathogenic insults as well as to epithelially-derived signals of inflammation, becoming activated and capable of presenting antigen to naïve T cells. In particular, DCs responding to TSLP produced by the lung epithelium upregulate the co-stimulatory molecules OX40L and CD86, which allows them to specifically promote the development T_H2-skewed T cells.[59-63] In addition to directly influencing T_H2 T cell generation, DCs also participate in recruiting T_H2 cells to the lung through the expression of the T_H2-tropic CCL17 and CCL22 chemokines.

In addition to macrophages and dendritic cells, recent evidence has indicated that basophils may participate in the allergic priming of T cells. Basophils are capable of expressing MHCII and the appropriate co-stimulatory molecules required for antigen presentation, and have been demonstrated to prime T_H2 T cells in models of papain-induced or OVA-induced allergic response. In addition, the normally robust T_H2 responses seen following challenge with papain or after infection with the intestinal

parasite *Trichuris muris* were both significantly attenuated following depletion of basophils.[64-67] While to date, no studies have examined whether basophils function as APCs in an asthmatic context, their presence in asthmatic lungs implies that they do, in fact, play a role in initiating an allergic response to antigen in the lungs.

T Lymphocytes

While APCs, and DCs in particular, play the primary role in initiating the asthmatic response, T lymphocytes play the primary role in coordinating the various aspects of the response. Mice lacking a functional T cell compartment, either through genetic modification (as is the case in *Rag2*-deficient animals) or through active T cell depletion, are unable to mount a full asthmatic response to antigenic challenge. It has been demonstrated furthermore that CD4+ T cells in particular are required to mediate a full asthmatic response, as reconstitution of T cell deficient animals with adoptively transferred CD4+ T cells was able to reconstitute the sensitivity of the animals to antigenic challenge.[47] These effects are furthermore reproducible in human patients; depletion of CD4+ T cells was successful in reducing asthma symptoms in a clinical trial.[68]

As was previously discussed, the predominant subtype of T cells present in asthmatic lungs is the T_H2 lineage. These cells are traditionally defined by their production of IL-4, IL-13 and IL-5, and have been shown to play a prominent role in a wide range of allergic diseases. Significantly, T_H2 cells have been demonstrated to drive asthmatic inflammation themselves; adoptive transfer of T_H2-skewed transgenic T cells into naïve, healthy mice is sufficient to allow the mice to develop all the hallmarks of

asthmatic disease when the animals were later challenged with the cognate antigen.[69;70] The cytokines produced by T_H2 cells provide the mechanism for their activity.

The cytokines IL-4 and IL-13 are thought to be the dominant effector cytokines in the asthmatic response. Signaling by IL-4 through the IL-4 receptor (composed of IL-4Rα and common gamma receptor chains) and by IL-13 through the IL-13 receptor (composed of IL-4Rα and IL-13Rα1 receptor chains) both activate the transcription factor STAT6. STAT6, in turn, activates a plethora of downstream targets and responses, many of which are directly involved in the pathology of asthma. In addition to mediating downstream responses, IL-4 and IL-13 also play key roles in the skewing of T cells towards the T_H2 lineage, and thus form a positive feedback circuit that reinforces the T_H2 response.[71] Ablation of STAT6 can prevent both T_H2 skewing and development of asthmatic responses,[72] although, interestingly, ablation of either IL-4 or IL-13 alone blocks only portions of those responses (Headley, Xu and Ziegler, unpublished data). IL-4, in particular, has been shown to be essential in inducing B cells to undergo class switching to IgE, which in turn is a primary component of the humoral allergic response.[73]

In addition to IL-4 and IL-13, T_H2 lymphocytes also produce cytokines that target more specific effector responses. While not a feature of all T_H2 cells, the T_H2 cells in the asthmatic lung can produce TNF-α, which activates inflammatory processes in the epithelium and can enhance both the recruitment of and phagocytosis by various innate immune cells.[74;75] Eotaxin, a potent eosinophil chemoattractant, is induced downstream of IL-4,[76] while the canonical T_H2 cytokine IL-5 additionally induces the activation and accumulation of eosinophils. Mice lacking IL-5 do not develop eosinophila following

antigenic challenge and, significantly are protected from the development of airway hyperresponsiveness.[77]

In contrast to the T_H2 lineage, other T cell lineages can play minor or alternative roles in asthma development. T_H1 cells, long considered the functional opposite of T_H2 cells, have been shown to inhibit the development of the asthmatic response under normal circumstances.[78] However, in settings of severe or chronic asthmatic inflammation, both T_H1 cells and CD8+ T cells can be recruited to the lungs and contribute to tissue damage.[79;80] In contrast, T_H17 cells and γδ-T cells both can produce large amounts of IL-17, which has been variously shown to both inhibit and exacerbate asthmatic symptoms.[81;82] Recent evidence has implicated T_H17 cells in particular in the development of a particularly severe form of asthma marked by a neutrophilic as opposed to eosinophilic cell infilitration.[82]

Eosinophils, Neutrophils, and Mast Cells

During an allergic asthmatic response, eosinophils make up the bulk of the infiltrating cells. Eosinophils are recruited from the bone marrow to the lungs and activated by the actions of IL-5 and eotaxin (among other factors), and can survive in the lung tissue for weeks in the presence of IL-3, IL-5 and GM-CSF. At the site of inflammation, activated eosinophils release granules containing a potent mix of cytotoxic and microbicidal agents, including major basic protein, eosinophil cationic protein and eosinophil peroxidase. Release of these agents can cause significant damage to airway epithelial cells, and are believed to significantly contribute to asthma pathology. In addition, eosinophils can themselves secrete various cytokines and chemokines, including TNF-α, IL-4, IL-13, IL-5, GM-CSF, IL-3, RANTES, platelet-activating factor,

and eotaxin, which form a positive feedback loop to recruit more T_H2 lymphocytes and eosinophils.[83;84] Several of these factors, notably IL-13, can act on the airway epithelium to induce wound repair, compensating for the cytotoxic effects of eosinophil activation, but also contributing to the airway remodeling seen in asthma.[85;86] There are some indications that the microenvironmental localization of eosinophils within the airways can lead to subtle differences in their regulation and behavior, illustrating the complexity of the eosinophil mediated response.[5]

Neutrophils, like eosinophils, can be rapidly recruited to sites of inflammation, and possess the ability to secrete cytotoxic and pro-inflammatory mediators. Unlike eosinophils however, neutrophils do not play a primary role in most cases of asthma. There is evidence that neutrophils are transiently recruited to the lungs after the initial inflammatory insult, but die or leave within 24-48 hours.[87] In mouse models of asthma, neutrophils are rarely found in the bronchoalveolar lavage fluid of asthmatic animals, but can be induced if IL-4 signaling is blocked (Headley, Xu and Ziegler, unpublished data). Neutrophil recruitment is associated with strong IL-17 signals and, as mentioned previously, is characteristic in certain severe cases of asthma.[82]

In contrast to both neutrophils and eosinophils, mast cells are largely resident within the lung tissue during both inflammatory and non-inflammatory conditions. Mast cell precursors are recruited from the bone marrow, and only mature once established within the lung tissue. The factors that mediate these events are unknown, but are believed to include eotaxin, among other chemokines and cytokines.[88] Once matured, mast cells are incredibly long lived. Like most other effector cells, mast cells express a range of PRRs that they use to respond to pathogenic insults. Importantly, however, mast cells also express FCεRI, the receptor for IgE antibody isotype, which enables mast cells to respond to specific pathogen in a highly sensitive manner. Once activated, mast

cells release granules containing a wide variety of cytokines, chemokines and other mediators. Included in these granules are the previously discussed cytokines TNF-α, IL-13, IL-5, and GM-CSF. More importantly, however, mast cell granules contain a number of acute mediators such as leukotrienes, prostaglandins and, famously, histamine, which can induce vasodialation and bronchoconstriction, hallmarks of acute asthma attacks.[89] Despite these functions, mast cells do not appear to be absolutely required for asthmatic responses, as mice lacking either mast cells or IgE can still mount an eosinophilic response to an allergic challenge.[90]

Airway Epithelial Cells

The last cell type we will consider within the context of the asthmatic response is by no means of the least importance; airway epithelial cells provide both the initial response to environmental insults as well as the stratum upon which the entire allergic immune response is built. As mentioned previously, airway epithelial cells express a wide variety of PRRs, PARs and other receptors. They are typically the first cells to physically interact with the triggering environmental insult, and are a source of many of the cytokines, chemokines, and other factors previously discussed. Frequently, the disruption of epithelial cells' barrier function precedes and facilitates the response to a pathogenic insult. It has been found that epithelial cells taken from individuals with asthmatic disease display a deficiency in the ability to reconstitute tight junctions *in vitro* compared to healthy epithelial cells.[91] This defect correlates with an increased propensity to produce inflammatory mediators. The epithelial layer is frequently damaged in asthmatic lungs– whether the damage is caused by or causative of asthmatic disease remains an open question– and the degree of damage is positively correlated

with disease severity. It is believed that the weakening of tight junctions along with the epithelial damage makes the epithelium "leaky," allowing normally excluded antigens access to underlying immune cells. In combination with the inflammation caused by the epithelial damage, immune responses can be generated against these antigens, even if the antigens themselves are innocuous.[8]

Epithelial cells possess a potent ability to undergo self-repair; epithelial cells can initiate the processes of dedifferentiation, migration and proliferation required to close a wound in as little as 15 minutes following the creation of that wound.[92] This process is controlled in part by IL-13. Indeed, it may be that the increase in production of IL-13 by most of the cells involved in the asthmatic response, including the airway epithelial cells themselves, is in part an attempt to repair the chronic injuries to the epithelium. Certainly, IL-13 controls the rate of goblet cell metaplasia and mucus hypersecretion, and is responsible for the related pathology.[8] Over time, the persistent activation of the wound repair responses and production of IL-13 contribute to the airway remodeling, thickening and hypersensitization characteristic to the asthmatic response. Indeed, the response of the epithelium to IL-13, as well as other cytokines, produces the majority of the physiological changes in the lung that are experienced by patients as the primary symptoms of asthma.

The Pathogenesis of Asthma

Asthma is a progressive disease. Over time, the coordinated action between the airway epithelial cells, APCs, lymphocytes and other effector cells creates a state of chronic inflammation punctuated by acute response episodes. In the early stages of disease, the inflammation and tissue remodeling are largely confined to the conducting

airways of the lung, but as the asthmatic condition persists, that inflammation and remodeling spread to both the large airways, including even the trachea and larynx, as well as the small airways.[93] In some cases, the symptoms of the disease wane with time, while in most other cases it is only the careful management of symptoms and avoidance of triggering stimuli can prevent serious or fatal complications from developing. Typically, by the time asthmatic symptoms begin to present, the disease is well established and largely non-reversible.[5]

It is for these reasons that there is intense interest in uncovering the how the conditions that drive asthma first arise. It has been long appreciated that asthma, at least in some cases, has a clear genetic component, even if the degree of that genetic contribution is highly variable. To date, genome-wide association studies have identified a handful of single-nucleotide polymorphisms (SNPs) that may predispose individuals to the development of asthma. These polymorphisms are unsurprisingly associated with genes known to play a role in the pathology of asthma, including IL-4, IL-13, IL-4Rα, TSLP, and STAT6, as well as the T_H1-associated IFN-γ and IL-12. PRRs, such as TLR4, TLR9, TLR10 and NOD1 have been implicated, as have proteinases such as ADAM33 and anti-inflammatory factors such as CTLA4, TGF-β and IL-10.[94;95]

More significant, however, are the roles thought to be played by environmental risk factors. It has been recognized for as long as asthma has been studied clinically that there is a strong association between prior lung disease and development of asthma; as was stated in a report on the etiology of asthma from 1859:

> *There is one circumstance in the history of asthma that is strongly suggestive of the idea that some organic injury of the lung is at the root of the asthmatic tendency: it is that, in the narrative of cases of asthma, it will be so frequently found that the asthma dates from some disease*

> *that implicate the lungs, and in such a way as to imply injury of an organic nature, though apparently temporary, as, for example, whooping-cough, bronchitis, and measles.*[6]

The idea that prior airway disease can predispose an individual towards the development of asthma is a powerful one, and consistent with what is understood about the link between epithelial cell responses and engagement of allergic immune responses. Indeed, exposure to fungi and helminth infections as well as chronic exposure to cigarette smoke, asbestos, or other chemical agents have all been linked to the asthma pathogenesis.[96;97] In particular, infection with respiratory viruses, especially early in life, is considered to be one of the most important risk factors for the development of asthma later in life.[98;99]

Respiratory virus infection is the leading cause of asthma exacerbation, accounting for up to 80% of exacerbations in some studies.[3] Of particular interest is respiratory syncytial virus (RSV). In longitudinal studies it was found that children who experienced severe RSV infections were at significant risk for developing asthma later in life.[98] RSV itself is a respiratory virus of the *Paramyxoviridae* family, which includes the parainfluenza viruses and the model murine respiratory virus Sendai virus. The virus has an RNA genome encoding several viral proteins, including the N, P, L, M2-1, M, G, F, NS1, NS2 and SH proteins. Of particular note are the G and F proteins, which mediate attachment and fusion of the virus, respectively, and which have been shown to respectively promote either T_H1- or T_H2-skewed CD4+ T cell responses.[100] What drives the distinction between the two possible responses is poorly understood. Responses against other viral proteins are largely mediated by CD8+ T cells, although the action of viral proteins NS1 and NS2 can inhibit general immune responses by interfering with

type I interferon function.[101;102] RSV primarily infects airway epithelial cell, but can also infect alveolar macrophages and dendritic cells.[51;103]

RSV is detected by a variety of PRRs, most significant of which is the cytoplasmic RNA sensor RIG-I. RSV-induced RIG-I signaling triggers a strong type I interferon response, as well as activating IRF3, IRF7, NF-κB, and various MAPK-mediated signaling pathways. Importantly, the culmination of these pathways upregulate many cytokines and chemokines previously discusses as being associated with the asthmatic response, including TNF-α, IL-1, TSLP, RANTES, and CCL17.[104-107] In murine systems, RSV infection has been shown to potently upregulate T_H2 under certain circumstances.[108] Exposing RSV-infected mice to a model of cockroach allergen-induced asthma produced an exacerbated disease that was dependent on IL-13.[109] These studies support the notion that RSV infection can create the conditions necessary for asthma development or exacerbation, although the precise mechanisms for how it does this are still incompletely understood.

Thymic Stromal Lymphopoietin

One of the more significant recent advances in understanding asthma's pathology and etiology has come from the discovery of the epithelially-derived cytokine TSLP. Originally discovered as a weak growth factor for B and T cells derived from a murine thymic stromal cell line,[110] TSLP was subsequently found to play a critical role in mediating allergic responses in the skin, gut, and both upper and lower airways.[62;63;111-114] TSLP is most closely related to IL-7, and signals through a heterodimeric receptor composed of the cytokine-specific TSLPR chain and the shared IL-7Rα chain. TSLPR is

an atypical type I cytokine receptor, having a heavily modified WSXWS motif and lacking the cytoplasmic Box 2 motif frequently present in other type I cytokine receptors.

Both TSLP and TSLPR share only ~40% amino acid identity between mouse and human, but nevertheless display strong functional homology. In both mouse and human, TSLP signals through a STAT5-dependent pathway, and in humans other STAT pathways have also been implicated in its function.[115-117] Initial studies indicated that TSLP-dependent STAT5 activation occurred in a JAK-independent fashion, although more recent studies have shown that both human and murine DCs activate JAK1 and JAK2 in response to TSLP.[117] TSLP stimulation was furthermore found to lead to the activation various NF-κB subunits, but only after a delay of approximately 48h, indicating a potential secondary signaling event.[117] Downstream gene targets of TSLP include OX40L, CD80, CD86, IL-13, SLPI and CCL17.[60-63;118-120]

Expression and Regulation

Since its discovery, a significant amount of work has been done to understand the cellular provenance and molecular regulation of TSLP. TSLP is predominantly expressed by cells of epithelial origin, and TSLP expression has been detected in the skin, gut, lung, heart and thymus.[121] In addition to the epithelium, TSLP has been shown to be expressed by basophils, following a response to papain,[64] and by bone marrow-derived dendritic cells (Lee, Headley and Ziegler, unpublished data). Regulation of TSLP expression is controlled by the p65 subunit of NF-κB.[27] Both human and mouse TSLP promoters contain an NF-κB element responsive to both TNF-α and IL-1β, and epithelial cells have been shown to upregulate TSLP following stimulation with agonists for TLR2, TLR3,

TLR8, and TLR9, as well as TNF-α and IL-1β.[22;27] This expression can be blocked by the action of the retinoid X receptor.[122]

In contrast to TSLP, the regulation of TSLPR has not been extensively studied. TSLPR was originally cloned from myleomonocytic cells, and based on functional studies of TSLP was implied to be expressed on B cells.[123-125] The initial reports of its native expression found that TSLPR transcripts were present predominantly in DC and monocytes, with lower expression in T cells, NK cells and mast cells.[118] Since those reports, TSLPR protein has been detected on a wide array of hematopoietic cells, including DCs, monocytes, CD4+ and CD8+ T cells, mast cells, B cells, macrophages and basophils.[119;126-132] More recently, several groups have detected TSLPR protein on cells of the gut and airways epithelium.[119;120] What factors regulate the expression of TSLPR are completely unknown.

Immunological Function

TSLPR's expression on cells of nearly every single hematopoietic lineage points clearly to TSLP playing a prominent role in immune responses. Initially, TSLP was shown to promote the growth and maturation of T and B cells, driving, in particular, the pro- to pre-B cell maturation.[110;125] Nevertheless, mice lacking TSLPR have essentially normal B and T cell compartments, indicating that its role as a general growth factor was modest at best. TSLP may play a direct role in some aspects of T cell function; some studies suggest that it can support the survival of T_{reg} cells,[133] while others have shown that it can drive naïve CD4+ T cells towards a T_H2 fate.[134] It is, however, clear that TSLP has potent effects on DCs. Stimulation of DCs with TSLP promotes their maturation, upregulating CD80, CD86 and MHCII. In particular, TSLP-stimulated DCs upregulate

OX40L, which allows them to efficiently promote T_H2 development.[60-62] In addition, TSLP drives production of CCL17 and CCL22 by DCs, and suppresses the expression of IL-12.[62;118] Basophils and eosinophils have both been shown to respond in various ways to TSLP,[128;135] and hematopoietic progenitor cells are capable of responding to TSLP in combination with IL-33 by producing a range of T_H2 cytokines.[136] Finally some studies have shown that TSLP treated NKT cells are capable of producing IL-13.[137]

That TSLP induces T_H2-associated factors in so many different cell types, it is perhaps unsurprising that there is a strong association between TSLP and allergic responses. In humans, high levels of TSLP have been detected in the diseased tissue of patients with atopic dermatitis, allergic rhinitis, and asthma.[62;113;114] In mouse models of atopic dermatitis and asthma, TSLP has been shown to be both necessary and sufficient to drive disease,[47;63;112] while in the gut, TSLP has been shown to play important roles in helminth infection, immune homeostasis, and food allergy.[45;111;138] The precise role that TSLP plays in each of these diseases varies in important ways, and it is beyond the scope of this review to detail the intricacies of each. TSLP's role in asthma, however, does merit further discussion.

Work in our lab as well as by other groups have shown that TSLP plays a central role in the pathology of asthma using various mouse models of the disease. Mice expressing TSLP under the control of Surfactant Protein C (SPC) restrict the overexpression of TSLP specifically to the airway epithelium. These mice were found to develop spontaneous airway inflammation consistent with an asthmatic response to an environmental antigen. Mice lacking TSLPR, conversely, are resistant to development of asthma using the OVA-driven model of disease induction.[63] Interestingly, mice given serial intranasal treatments of exogenous TSLP in combination with foreign antigen developed acute asthmatic disease, indicating that TSLP stimulation is sufficient to drive

disease, and may act at the earliest stages of an asthmatic response.[47] These effects are mediated by T_H2 CD4+ T cells, as ablation of either CD4+ T cells, IL-4 signaling or STAT6 signaling abolishes or significantly diminishes TSLP's ability to drive disease.[72]

Role in Asthma Pathogenesis

It is clear that by playing an integral role in the generation of a T_H2-skewed immune response TSLP plays an integral role in the pathology of asthma. Beyond that, however, there is mounting evidence that TSLP may play an important role in the mechanisms that underpin susceptibility to asthma, and may thus contribute directly to the pathogenesis of asthma itself. Genetic polymorphisms in TSLP or its receptor complex have been associated with the development of allergic airway disease; TSLP variant alleles have been linked to decreased sensitivity to cockroach allergen in a Costa Rican cohort,[139] and a SNP significantly linked to TSLP correlates with asthmatic disease in an Icelandic population.[140] In addition, allelic variations in IL-7Rα have been associated with sensitivity to inhalation allergy.[141]

In another study, a SNP in the TSLP promoter that forms a novel functional AP-1 site has been shown to lead to an upregulation in promoter activity following stimulation with a viral mimetic.[142] This report underscores a significant feature of the regulation of TSLP: infection of epithelial cells by respiratory viruses is a potent stimulator of TSLP expression. That these infections are also known risk factors for the development of asthma strongly implies a causal association between the upregulation of TSLP and the enhanced propensity to develop asthmatic disease mediated by respiratory viruses. In a recent study from our lab, it was assessed whether infection of primary airway epithelial cells with RSV lead to the enhancement of a TSLP response. Intriguingly, it was found

that not only were epithelial cells capable of upregulating TSLP in response to RSV infection, but that primary cells collected from asthmatic patients were uniquely susceptible to RSV-induced TSLP upregulation. We speculate that viral infection may exacerbate underlying defects within the epithelium, leading to enhanced TSLP production. These increased levels of TSLP, in turn, may then in turn be sufficient to trigger an inflammatory T_H2 response and initiate a positive feedback loop that ultimately leads to the development of asthmatic disease.[143]

The work in this thesis continues to explore the link between TSLP, the airway epithelium and the development of asthma. Our attention is focused upon the receptor for TSLP, and we show that much like TSLP, TSLPR expression in epithelial cells is induced by RSV infection, and that epithelium from asthmatic airways is uniquely susceptible to these effects. Significantly, our work shows that in addition to mediating its effects through responding hematopoietic cells, TSLP can act directly on the epithelium itself. We present evidence that the epithelium can independently regulate the expression of both TSLP and TSLPR, and we speculate that by doing so may be able to initiate the early asthmatic response in an autonomous fashion.

Chapter 2: Materials and Methods

Reagents and constructs

Recombinant human tumor necrosis factor-alpha (TNF-α), PE-conjugated murine α-human TSLPR (Clone 1A6), FITC-conjugated α-human CD4 and PerCP-Cy5.5 conjugated α-human CD3 were purchased from eBioscience (San Diego, CA). Recombinant human TSLP and blocking α-human TSLP antibody was kindly provided by Dr Michael Cuomo (Amgen, Seattle, WA). BAY-11-7082 was purchased from EMD Millipore (Billerica, MD). Copper(II) sulfate ($CuSO_4$) and anisomycin were purchased from Sigma-Aldrich (St. Louis, MO). Endo-H, neuraminidase (NeuA), and PNGaseF were purchaced from New England Biolabs (Ipswitch, MA) and used according to manufacturer's protocol. Full length TSLPR and interleukin-7 receptor alpha (IL-7Rα), lacking a stop codon, were amplified from cDNA obtained from TNF-α A549 cells, and cloned into a mammalian expression vector using the pcDNA3.1/V5-His TOPO TA Expression Kit from Invitrogen (Carlsbad, CA). Expression vectors containing full length nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 or p50 subunits were created as previously described.[27] The -404 CIS promoter-luciferase (cis-luc) and pRSV-β-galactosidase reporter (pRSV-β-gal) were described previously.[27;144]

Submerged cell culture and viral infection

The human lung epithelial cell line A549 was obtained from ATCC, and maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FBS and 1% penicillin/streptomycin, non-essential amino acids, and sodium pyruvate.

Normal human bronchial epithelial cells (NHBECs) were obtained from Lonza (Walkersville, Md.) and maintained according to the manufacturer's instructions. RSV (Line 19, A strain) was obtained and propagated as previously described.[143] Virus was added to A549 or NHBECs at the titers indicated. Cells were washed and received fresh media after 2h, then harvested for analysis 24h following the initial infection.

Air-liquid interface culture and viral infection

Primary bronchial epithelial cells (BECs) were obtained from healthy and asthmatic children, cultured under air-liquid interface (ALI) conditions, and infected with RSV as previously described.[143] Briefly, BECs were collected by unsheathed cytologic brushings performed via an endotracheal tube during elective surgical procedures. Cells at passage 2 or 3 were subsequently seeded onto transwells and grown under ALI conditions. BECs were exposed at the apical surface to RSV Line 19 at MOI of 0.5 or an equivalent volume of fluid from the control Vero cell supernatant for two hours, and cells collected for analysis 96 hours post-infection. The protocol was approved by the Seattle Children's Hospital Institutional Review Board.

Real-time quantitative polymerase chain reaction (qPCR) analysis

Total RNA and cDNA preparation were performed as previously described.[27] Human primers used were purchased from Sigma-Aldrich (St. Louis, MO), and include TSLPR (forward, 5'-CAGAGCAGCGAGACGACATT-3'; reverse, 5'-GGTACTGAACCTCATAGAGG-3'), IL-7Rα (forward, 5'-GCTCAGGGGAGATGGATCCT-3'; reverse, 5'-GTCTTCTTATGATCGGGGAG-3'), CXCL5 (forward, 5'-

CTCCAAGGTGGAAGTGGTAG-3'; reverse, 5'-CTTGTCTTCCCTGGGTTCAG-3'), and HPRT (forward, 5'-TGGCGTCGTGATTAGTGATG-3'; reverse 5'-GCACACAGAGGGCTACAATG-3'). Primer sequences for TSLP and chemokine CCL17 were previously published.[122;145]

Western blots

Samples were lysed with 1x lysis buffer (50mM Tris-8.0, 150mM NaCL, 1.0% Triton-X100), and total protein concentration determined using the Bio Rad Protein Assay (Bio Rad, Hercules, CA), according to the manufacturer's protocol. Equal amounts of protein were electrophoresed on NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA), and transferred onto membranes. TSLPR was detected with murine α-human TSLPR clone 1A6 or polyclonal goat α-human TSLPR AF981 (R&D, Minneapolis, MN), and membranes stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), detected via rabbit α-human GAPDH (Trevigen, Gaithersburg, MD). TSLPR expression constructs were detected with mouse α-V5 antibody (Invitrogen).

Transfections

Prior to transfection, cell were seeded in wells and allowed to reach a confluency between 50%-70%. Plasmid constructs were mixed with Mirus TransIT-LT1 transfection reagent (Mirus, Madison, WI) according to manufacturer's protocol and applied to cell cultures. All transfections were allowed to rest at 12-16 hours prior to further experimental manipulation. For the STAT5 luciferase assays, expression vectors containing TSLPR and IL-7Rα, the cis-luc promoter-reporter construct, and pRSV-β-gal

control plasmid were transfected into cells at a ratio of 1:1:2:1, respectively. For transfections of TSLPR and IL-7Rα expression vectors alone, constructs were added in equal amounts.

Luciferase assays

A549 cells were transfected and rested as described. Cells were subsequently stimulated for eight hours with recombinant TSLP, washed with PBS, and immediately lysed in 200μL lysis buffer (Promega, Madison, WI). Lysates were analyzed for luciferase activity using Luciferase Assay Reagent (Promega), and read using a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany). β-galactosidase levels were detected using the β-galactosidase Enzyme Assay System (Promega), following manufacturer's protocol.

Human CD4+ T cell isolation and flow cytometry

Blood was collected from normal healthy donors by the Benaroya Research Institute Translational Core. Buffy coats were collected, and CD4+ T cells isolated using Naïve Human CD4+ T cell Isolation Kit II from Miltenyi (Auburn, CA) according to manufacturer's protocol. CD4+ T cells were cultured on tissue culture plates coated with α-CD3 (OKT3) and α-CD28 (BD Biosciences, San Jose, CA) for 3 days. Cells were stained with FITC-CD4, PerCP-Cy5.5-CD3 and PE-TSLPR and analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). PE-TSLPR stained cells were isolated using α-PE MACS beads from Miltenyi, according to manufacturer's protocol.

Statistical Analysis

All experiments were analyzed for statistical significance. Where samples were compared to an internal control and reported as a “Fold Change”, statistical significance was calculated with a one-sample t-test using the null hypothesis that the population mean is equal to one. In all other cases, two samples were compared using Student’s t-test. Statistical significance is indicated on figures, where $P = 0.05$ is used as the minimum threshold for significance.

Chapter 3: Respiratory syncytial virus induces functional thymic stromal lymphopoietin receptor in airway epithelial cells

Introduction

Asthma is a chronic inflammatory disease of the airways, classically associated with strong T_H2-type immune responses including airway hyperresponsiveness, eosinophilic cell infiltration, goblet cell metaplasia, and mucus hypersecretion.[5] While the etiology of asthma is complex, it is now appreciated that the epithelial-derived cytokine thymic stromal lymphopoietin (TSLP) plays a key role in mediating various aspects of the disease. In mouse models, overexpression of TSLP, or exogenous administration via the airways, is sufficient to drive the development of all the classical hallmarks of asthmatic disease. In addition, inhibiting TSLP signaling is also sufficient to prevent disease manifestation in mouse models of induced asthma.[72;146] TSLP appears to act both to initiate and to sustain an inflammatory response against foreign antigens when it acts at the appropriate stages of that response.[47]

In humans, high levels of TSLP are believed to drive asthmatic responses in a similar fashion. Elevated levels of TSLP are present in the lungs of asthmatic patients,[113] and the induced expression of TSLP has been linked to several known risk factors for the development of asthma. Of particular note, severe childhood infections with respiratory syncytial virus (RSV) have long been known to positively correlate with the subsequent development of childhood asthma.[98] Recent studies have shown that RSV is capable of inducing high levels of TSLP production in airway epithelial cells.[143] Intriguingly, this induction is enhanced in airway epithelial cells from asthmatic individuals, implying that

the inappropriate sensitivity of these cells to inflammatory stimuli may be linked to a TSLP-driven asthmatic response.[143]

While much attention has recently been naturally focused on TSLP and its role in allergic disease, less is known about the regulation and expression of its receptor. Recent reports have shown that the TSLP receptor (TSLPR) is expressed on cells of both the airway and colonic epithelium, where it has been shown to play a role in the wound-healing response.[119;120] We show here that the TSLP receptor is inducibly expressed on the cells of the airway epithelium in response to inflammatory stimuli including TNF-α treatment or infection with respiratory viruses. TSLPR expressed by epithelial cells is glycosylated at four different N-linked glycosylation sites on its extracellular domain. We present the novel observation that this induction is driven by same stimuli that drive TSLP production, albeit by a parallel mechanism that may include p38-mediated, JNK-mediated or other related intracellular signaling pathway. We furthermore show that this induced receptor is capable of functionally responding to TSLP in an autocrine/paracrine fashion. Finally, we show that much as they displayed an enhanced induction of TSLP, airway epithelial cells from asthmatic individuals also have an enhanced induction of the TSLP receptor in response to infection by RSV. We speculate that this enhanced induction in addition to allowing the epithelial cells to directly mediate a TSLP-dependent inflammatory response may also be symptomatic of an underlying sensitivity that had originally predisposed these individuals to asthma, and that by upregulating both the cytokine and it's receptor, epithelial cells can establish an autocrine/paracrine feedback loop that may serve to enhance and sustain the TSLP-dependent inflammatory response.

Results

TNF–α induces TSLPR and IL-7Rα expression in human airway epithelial cells.

The TSLP receptor is a heterodimer consisting of the cytokine-specific TSLPR chain and IL-7Rα chain that it shares with the IL-7 receptor. While it is well established that the receptor is expressed on cells of hematopoietic origin, recent reports have suggested that both TSLPR and IL-7Rα are expressed by epithelial cells in both the lungs and gut.[119;120] While we have previously shown that proinflammatory stimuli can induce the production of TSLP by lung epithelial cells,[27;143] it is not known whether TSLP receptor can be modulated under similar conditions. To assess whether the TSLP receptor can be induced under inflammatory conditions, we stimulated the human airway epithelial cell line A549 cells with the cytokine TNF-α. Upregulation of TSLPR mRNA was detectable by four hours, rising to a steady increase of 4-fold over unstimulated cells by eight hours (Figure 3.1A). Likewise, TNF-α induced IL-7Rα mRNA as early as one hour (data not shown), reaching a peak of 8-fold upregulation over unstimulated cells by four hours, and dropping off to a 2-fold upregulation at 24 hours post-stimulation (Figure 3.1A). These results demonstrate that TSLPR and IL-7Rα, like TSLP, are subject to dynamic regulation under inflammatory conditions.

Infection of epithelial cells with RSV potently induces both TSLPR and IL-7Rα.

RSV is a common respiratory virus and a known risk factor for the development of asthma[98;99]. We have previously shown that RSV infection potently upregulates TSLP in both primary human airways epithelial cells and epithelial cell lines.[143] To determine whether RSV infection could also induce TSLPR and IL-7Rα, we infected A549 cells with

various titers of RSV for two hours, and assessed mRNA levels 24 hours post-infection. Compared to mock-infected cells, at titers of $2x10^4$ and $2x10^5$ pfu, RSV induced a 2-fold and 7-fold upregulation, respectively, of TSLPR and a 2-fold and 25-fold upregulation, respectively, of IL-7Rα (Figure 3.1B). TSLPR protein was readily detectable at these levels (Figure 3.1C).

These effects were even more pronounced in primary epithelial cells; NHBECs were similarly infected with different titers of RSV and showed strong upregulation of both TSLPR and IL-7Rα. Induction of TSLPR was detectable at as low as $2x10^3$ pfu RSV, upregulating levels by 2-fold over mock infected cells, and increasing to up to 23-fold upregulation at $2x10^5$ pfu. Likewise, IL-7Rα mRNA was upregulated between 4- and 150-fold, respectively, at the same range of titers (Figure 3.1D). Taken together, these results demonstrate that TSLPR and IL-7Rα can be and are induced by the same inflammatory stimuli that drive TSLP production in airway epithelial cells.

Epithelially expressed TSLPR is glycosylated

In analyzing the protein expression patterns, we observed that TSLPR was running at a higher apparent molecular weight than expected from its amino acid sequence. Examination of its structure revealed four N-linked glycosylation motifs, marked by the sequence *N-x-S/T*, present within the extracellular domain of TSLPR. To determine whether these sites were glycosylated in epithelial cells, and account for the high apparent molecular weight, we created TSLPR overexpression constructs containing targeted mutations of the individual motifs. These mutations are diagrammed in Figure 3.2A. By mutating the asparagine residue in the motif to a glutamine, the ability to glycosylate the site is abolished while largely preserving the other structural

properties of the residue. We transfected A549 cells with the constructs, and analyzed the apparent molecular weight of each construct. The migration pattern of the constructs indicated that each of the four glycosylation motifs contribute to the molecular weight of the overall protein (Figure 3.2B). Significantly, mutation of all the glycosylation motifs resulted in a protein running close to the molecular weight predicted by TSLPR's amino acid sequence. To conclusively demonstrate that glycosylation accounts for the additional molecular weight, A549 cells were transfected with WT TSLPR and lysates were digested with various glycosidases. Digestion with either Endo-H and PNGaseF, which target N-linked glycosyl groups, reduced the apparent molecular weight of TSLPR, while digestion with neuraminidase, which cleaves sialic acid moieties, had no effect (Figure 3.2C). These results indicate that TSLPR is glycosylated on each of its four extracellular N-linked glycosylation motifs.

Because the presence of glycosylation was unexpected, we wanted to verify that the antibody we were using to detect TSLPR was indeed detecting a physiologically relevant isoform of the protein. Rare populations of circulating CD4+ T cells have been previously demonstrated to express TSLPR.[147] We isolated CD4+ T cells from human blood, and stained the cells with a PE-conjugated α-TSLPR, matching the clone that we used for our Western blots (clone 1A6). Approximately 0.2% of unstimulated CD4+ T cells were TSLPR+ (data not shown). To expand these numbers, we activated bulk CD4+ T cells with plate bound α-CD3/α-CD28 for 3 days, and repeated the staining. We detected TSLPR on approximately 2.0% of the activated T cells (Figure 3.3A). We enriched these cells using α -PE MACS beads, and analyzed the cells via Western. Using both the 1A6 clone as well as a separate polyclonal α-TSLPR antibody, TSLPR was detected strongly in lysates from TSLPR+ cells, and ran at the previously observed molecular weight (Figure 3.3B). To test whether the 1A6 antibody could detect other

potential isoforms of TSLPR, we transfected A549 cells with WT TSLPR or the mutant TSLPR lacking all glycosylation sites. We treated the lysates from these cells with PNGaseF, and analyzed the proteins via Western blot. While both undigested and PNGaseF treated TSLPR from both WT and mutant construct transfected cells was detectable using an antibody directed at the expression constructs' V5 epitope tag, the 1A6 α-TSLPR clone was only able to detect undigested WT TSLPR (Figure 3.3C), indicating it is highly specific for the glycosylated form. From this data, we can conclude that our reagents are detecting a physiologically relevant glycosylated isoform of TSLPR.

Regulation of TSLPR and IL-7Rα is not mediated by NF-κB.

TNF-α and RSV, while distinctly different stimuli, utilize similar signaling pathway intermediates; both are capable of signaling through canonical NF-κB as well as p38-kinase, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) pathways.[148;149] Given that previous studies from our laboratory have shown that TSLP expression is dependent on the activity of NF-κB,[27] it was reasonable to infer that NF-κB also regulates TSLPR and IL-7Rα expression. To test if this is the case, we transfected A549 cells with expression vectors expressing either the p50 or p65 subunits of NF-κB, and assayed for gene expression 24 hours following transfection. Surprisingly, while we observed the previously reported p65-dependent TSLP induction,[27] neither TSLPR nor IL-7Rα were upregulated by either p50 or p65 (Figure 3.4A). To confirm these results, we pretreated A549 cells with the NF-κB inhibitor BAY-11-7082, followed by treatment with TNF-α and assayed 4h later for suppression of TSLP, TSLPR and IL-7Rα induction. Consistent with our previous results, the induction of TSLP was blocked

in the presence of the inhibitor, while TSLPR and IL-7Rα levels were indistinguishable from cells treated with TNF-α and vehicle alone (Figure 3.4B).

Both TNF-α and RSV can mediate their effects through p38- and JNK-dependent signaling pathways in addition to NF-κB, To see if stimulation of these pathways could drive expression of TSLPR and IL-7Rα, we treated A549 cells with the JNK/p38 agonist anisomycin. Intriguingly, anisomycin was not able to drive appreciable levels of either TSLPR and IL-7Rα expression over a wide range of concentrations (Figure 3.5A). In addition to anisomycin, we also treated cells with 0.5mM $CuSO_4$, another known agonist of p38 and JNK pathways. In contrast of anisomycin, $CuSO_4$ was able to produce a transient induction of TSLPR and a more sustained induction of IL-7Rα (Figure 3.5B). Significantly, the transient upregulation of TSLPR mRNA produced a upregulation of TSLPR protein over longer periods of time (Figure 3.5C). While we find it surprising that $CuSO_4$ is capable of inducing TSLPR and IL-7Rα where anisomycin is not, given that both are agonists of p38 and JNK pathways, we note that $CuSO_4$ is more promiscuous in its effects,[150] and speculate that there are more complex actions governing the regulation of TSLPR and IL-7Rα expression. Taken together, we can conclude from these data that in contrast to TSLP, TSLPR and IL-7Rα are not dependent on canonical NF-κB, and speculate that other intracellular signaling pathways may control their induction.

Epithelial cells expressing TSLPR can functionally respond to TSLP.

Much of the previous research on TSLP has focused on its effects on hematopoietic cells, where it has been shown to drive the maturation of both T_H2 CD4+ T cells and dendritic cells, and stimulate the production of chemokines such as CCL17.[63;113] Less well studied are the effects of TSLP on epithelial cells. While previous

studies have suggested a role for TSLP in assisting with an IL-13-mediated wound healing response in airway epithelial cells,[119] we wanted to assess whether epithelial cells could be directly involved with a TSLP-mediated T_H2-type response.

Our data suggests that by upregulating both the cytokine and its receptor, epithelial cells may be able to respond to TSLP in an autocrine or paracrine fashion. Stimulation of untreated A549 cells with TSLP alone is insufficient to produce a detectable response (data not shown), but treatment of cells with TNF-α to induce receptor expression also induces TSLP, complicating the analysis of the data. To address these effects, we treated A549 cells with an α-TSLP blocking antibody, and assessed the antibody's ability to block target gene upregulation. Previous data had indicated that A549 cells upregulate CCL17 following treatment with TNF-α and IL-4, and upregulate the neutrophil chemoattractant CXCL5 with TNF-α treatment alone. By treating A549 cells with TNF-α/IL-4 or TNF-α alone we observed that α-TSLP was able to inhibit approximately 50% of the observed upregulation of CCL17 (Figure 3.6A) or CXCL5 (Figure 3.6B). The inhibition was observed over a wide range of concentrations, and thus we speculate that the inability of α-TSLP to fully inhibit expression of CCL17 or CXCL5 may be due to compensation by TSLP-independent, TNF-α (or IL-4) dependent pathways. Consistent with this idea, transfection of A549 cells with NF-κB p65 resulted in an upregulation of CCL17 (Figure 3.6C). Taken together, we interpret this data to indicate that autocrine/paracrine signaling by TSLP may play a partial role in TNF-α dependent gene regulation.

In order to more directly test whether TSLPR is capable of signaling in epithelial cells, we made use of the WT TSLPR expression construct in combination with an IL-7Rα expression construct to bypass the requirement for TNF-α stimulation. Previous studies have shown that TSLP is an activator STAT5.[115;125;151] To determine whether TSLP

activates STAT5 in epithelial cells, we made use of a reporter construct containing luciferase under the control of a STAT5-responsive element from the human cytokine-inducible SH2-containing protein (CISH) promoter.[144] We transfected A549 cells with the reporter in combination with TSLPR and IL-7Rα expression constructs, and treated the cells for eight hours with varying concentrations of recombinant TSLP. Compared with untreated cells, TSLP treatment induced a 1.5-fold upregulation of STAT5-dependent luciferase activity at all concentrations of TSLP tested (Figure 3.7A). While modest, this effect was remarkably reproducible, and is consistent with similarly modest TSLP-dependent responses reported elsewhere.[119]

As previously mentioned, CCL17 is a known TSLP-target gene and plays a central role in recruiting many of the cell types associated with the canonical TSLP-dependent inflammatory response.[152] While our previous data showed that epithelial cells could express CCL17 in response to TNF-α and IL-4, we wanted to test whether TSLP alone could drive CCL17 expression in TSLPR+ epithelial cells. We transfected A549 cells with TSLPR and IL-7Rα expression constructs, enriched for TSLPR+ cells using an α-TSLPR antibody, and treated these TSLPR+ A549 cells with 50ng/mL of recombinant TSLP for eight hours. We found that TSLP treatment upregulated CCL17 levels by more than 2-fold compared to similarly enriched but untreated cells (Figure 3.7B). Taken together, these results indicate that epithelial cells can indeed respond to TSLP, and that they can do so by participating in known T_H2-associated response patterns.

Asthmatic BECs upregulate TSLPR and IL-7Rα following RSV infection.

While the above data strongly indicate that airway epithelial cells are capable of upregulating a functional TSLP receptor complex in response to common inflammatory

stimuli, we wanted to assess whether epithelial cells behaved the same way in a more physiological setting. In order to more closely mimic the microenvironmental conditions airway epithelium is naturally exposed to, we collected primary BECs from bronchial brushings of both healthy and asthmatic individuals, and cultured the cells under ALI conditions.[143]

We first assessed what the levels of TSLPR and IL-7Rα expression were under basal conditions in both healthy and asthmatic individuals. Expressed as a percentage of the levels of the housekeeping gene HPRT, we found no significant difference between the mean expression of TSLPR in healthy vs. asthmatic samples (Figure 3.8A). The overall level of TSLPR expression was comparable to the levels observed in submerged NHBEC cultures (data not shown). In contrast, we found that asthmatic samples did express significantly lower levels of IL-7Rα compared to healthy samples (Figure 3.8A), and overall levels from both healthy and asthmatic groups were between 3-5 times lower than IL-7Rα levels observed in submerged NHBEC cultures.

We next assessed whether BECs could upregulate TSLPR and IL-7Rα in response to RSV infection. Matching samples from each patient analyzed above were apically exposed to RSV, and harvested 4 days later. Expressed now as a fold change relative to each sample's respective basal expression, we found that asthmatic samples, but not healthy samples, significantly upregulated TSLPR levels over baseline. TSLPR levels were upregulated by an average of 2.6-fold with a 95% confidence interval of ±1.1 in the asthmatic group, in contrast to a 1.5-fold ±0.6 in the healthy group (Figure 3.8B). This data mirrors previous data showing TSLP levels are only increased following RSV infection in samples from asthmatic individuals.[143]

With regards to IL-7Rα, samples displayed a more heterogeneous response to RSV, with some samples upregulating IL-7Rα as much as 50-fold and others displaying no change in levels at all. Overall, both healthy and asthmatic samples displayed a trend towards upregulating IL-7Rα, but failed to meet the criteria for statistical significance with p-values of 0.086 and 0.059, respectively (Figure 3.8B). It is worthwhile to note, however, that the raw expression values of IL-7Rα in infected samples (as a percentage of HPRT) were not significantly different between healthy and asthmatic groups, in contrast to the differences seen in under basal conditions. We interpret this to indicate that IL-7Rα levels are truly upregulated, at least in the asthmatic group, and that the statistical significance is simply being masked by high variance between samples.

Discussion

Investigations into the interplay between the regulation of TSLP by epithelial cells and the development of T_H2-type inflammatory responses has provided keen insights into the etiology of atopic disease. More recently, however, it is becoming apparent that the regulation of the TSLP receptor in epithelial cells may also play an interesting role in how atopic responses are generated. We have shown here that both TSLPR and IL-7Rα can be upregulated in epithelial cells by both the general proinflammatory cytokine TNF-α as well as by infection with RSV. Both stimuli have previously been shown to upregulate TSLP and, more significantly, RSV infection in particular is known to correlate with the development and exacerbation of asthmatic disease.[98;99] Our data suggests that epithelial cells are capable of initiating an autocrine/paracrine TSLP signaling circuit, thereby directly initiating or propagating the

TSLP-driven T_H2-type inflammatory responses associated with RSV infection and other forms of airway inflammation. Indeed, we find that stimulation of TSLPR+ epithelial cells drives the production of the T_H2 chemokine CCL17, allowing epithelial cells to directly participate in a T_H2-type response.

Prior investigations of TSLPR have not examined the physical characteristics of the receptor in any detail. We report here that, at least on epithelial cells and CD4+ T cells, TSLPR exists in a highly glycosylated form. Many extracellular receptors, particularly those of the type I cytokine family, are glycosylated; glycosylation has been linked to a diverse array of effects including both receptor stabilization and enhancement of ligand binding in other receptor systems.[153] We have attempted to assess the functional consequences of TSLPR glycosylation, and while we have had some indications that glycosylation affects the sensitivity of the receptor to TSLP, our results have thus far been inconclusive (data not shown). We note that we have had difficulty detecting the receptor on human cells, other than T cells and epithelial cells. While this may simply be due to the relative rarity of those cells in our samples– much in the same way that TSLPR+ CD4+ T cells are rare– we also speculate that TSLPR may have different glycoforms that aren't detectable by the specific reagents we have at our disposal. Further research along these avenues may prove to be worthwhile.

Despite being downstream of the same kinds of stimuli, we have presented data indicating that TSLP and TSLPR expression are in fact regulated by different intracellular signaling pathways. While the precise pathway controlling TSLPR and IL-7Rα is still unknown, we have ruled out the NF-κB pathway as a contributor to their expression. That NF-κB is the central regulator of TSLP expression[27] creates an interesting situation where epithelial cells have the ability to selectively modulate their own responsiveness to TSLP without otherwise interrupting TSLP-dependent responses

in other cells. Indeed, while we have only looked at two representative stimuli in this study– TNF-α and RSV– there may be other more selective stimuli that drive the expression of only TSLPR and IL-7Rα without affecting TSLP or vice versa. By upregulating only the receptor, the epithelium may be able to prime itself for a rapid TSLP-driven response only after further environmental requirements have been met. Conversely, by only upregulating TSLP, the epithelium can delay such a response until after the responding cells have integrated the proper environmental cues.

We can see evidence for this type of regulation occurring when we examine primary BECs from healthy and asthmatic patients under basal conditions. Previous research has found that asthmatic epithelium produces significantly greater levels of TSLP,[143] while our data indicates that levels of TSLPR are equivalent between healthy and asthmatic cells. Furthermore, we find that IL-7Rα levels are depressed in asthmatic cells. Given that IL-7Rα is known in other cell types to undergo downregulation upon stimulation,[154;155] we believe that, taken together, our data indicates that epithelial cells from asthmatic individuals have previously responded to an inflammatory stimulus that was sufficient to elevate their production of TSLP, but insufficient to sustain expression of TSLPR. Since we know that epithelial cells are capable of directly participating in a T_H2-type response upon TSLP stimulation, downregulating the receptor may allow epithelium to limit the perpetuation of this response in the face of chronic inflammation.

The work presented in this thesis is consistent with the model of asthma pathogenesis in which dysregulations of the airway epithelium are the key initiating event of the disease.[156-158] Work from our lab using the ALI culture system has now shown that both TSLP and TSLPR expression is intrinsically dysregulated in epithelial cells from asthmatic individuals following exposure to respiratory viruses and pro-inflammatory cytokines. It remains an open question whether this regulation is the

result of an inherent difference between the asthmatic and healthy epithelium– a difference that exists independently of the airway's history of inflammation– or if the differences are acquired as a result of chronic inflammatory stimulation. It is known that asthmatic epithelium has both structural[157] and epigenetic[159] differences from healthy epithelium, even outside of the context of acute inflammation. By utilizing the ALI culture system, we remove the epithelial cells from *in situ* environmental cues, but still maintain any cell intrinsic modifications, and thus we can conclude that regardless of the cause, asthmatic epithelial cells respond fundamentally differently to environmental insult than healthy epithelial cells. Consistent with this hypothesis, prior work from our lab has demonstrated that asthmatic cells have altered levels of the viral RNA sensor RIG-I following infection with RSV,[143] and we suggested that RIG-I control of TSLP expression provided a link between RSV infection and asthma. Our work here indicates that TSLPR expression by the epithelium must also be taken into account when examining the mechanisms by which TSLP links viral infection with asthma pathogenesis. Taken as a whole, our data suggests that in addition to supporting the TSLP-dependent response by hematopoietic cells, epithelial cells themselves contribute directly to that response, and thus are central mediators of asthmatic disease.

Figures

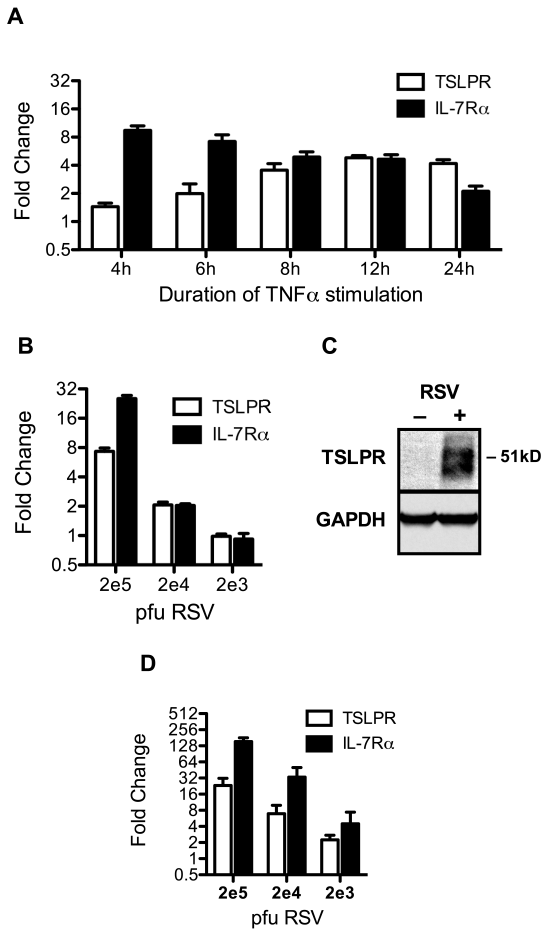

Figure 3.1 Induction of TSLPR and IL-7Rα in epithelial cells

(A) A549 cells were treated with 40ng/mL TNFα for the indicated times and subjected to qPCR analysis. **(B)** A549 cells were infected with the indicated titers of RSV for two hours and harvested 24 hours post-infection for qPCR analysis. **(C)** Representative Western blots of TSLPR and GAPDH from A549 cells mock infected or infected with $2x10^5$ pfu RSV. **(D)** NHBECs were infected with the indicated titers of RSV for two hours and harvested 24 hours post-infection for qPCR analysis. All qPCR data are reported as the mean ± SEM of at least three independent experiments. mRNA levels are normalized to HPRT and expressed relative to untreated or mock infected cells.

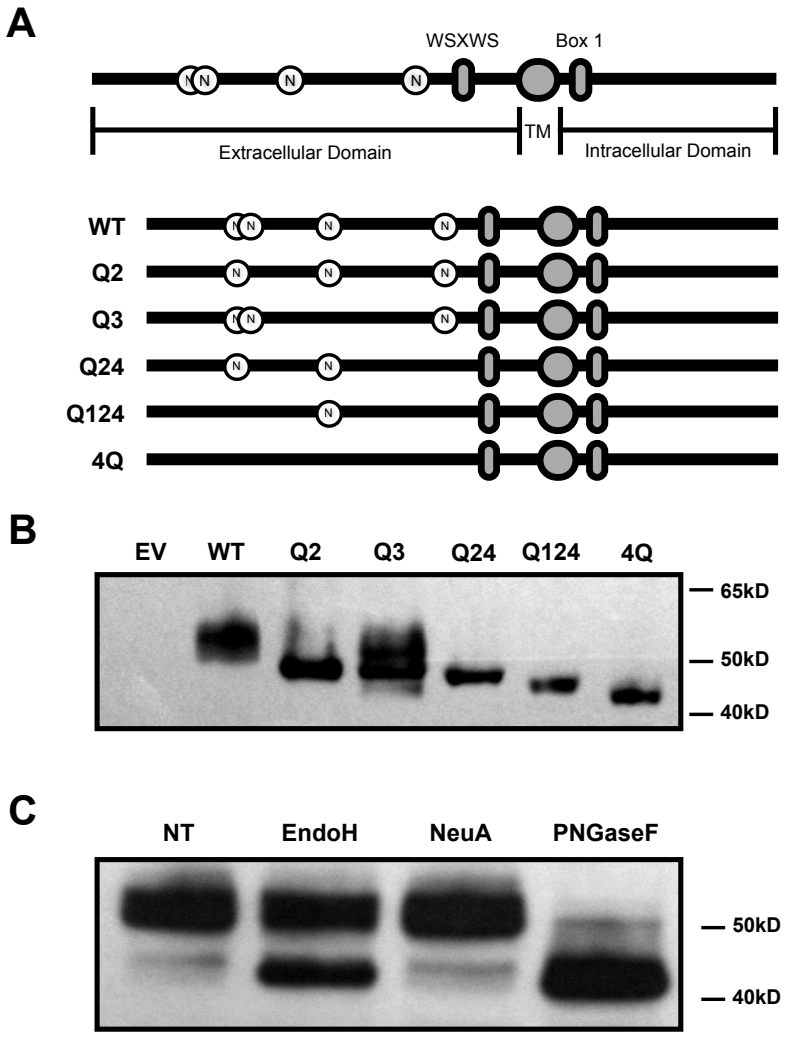

Figure 3.2 TSLPR is glycosylated on four extracellular glycosylation motifs

(A) A schematic of the protein structure of TSLPR. The N-x-S/T glycosylation motifs are marked as white circles, other structural motifs are marked as shaded shapes. Site-directed mutagenesis was used to create asparagine to glutamine mutations in each glycosylation motif, and schematics of the corresponding expression constructs are shown. **(B)** Western blot of A549 cells transfected with the aforementioned expression constructs. **(C)** A549 cells were transfected with WT TSLPR, and lysates treated with the indicated proteases. For all western blots construct expression was detected with an α-V5 tag antibody.

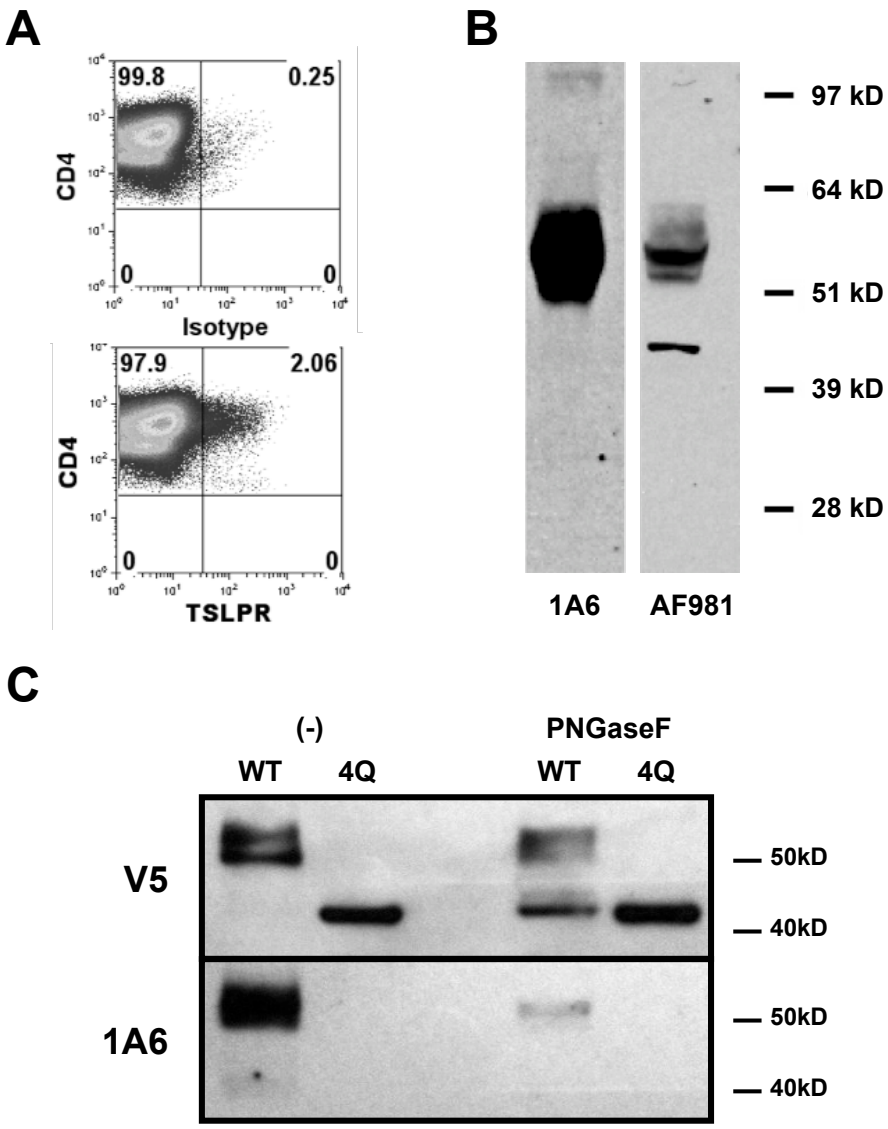

Figure 3.3 The α-TSLPR antibody 1A6 is specific for glycosylated TSLPR

(A) Human CD4+ T cells were isolated from blood and stained with an isotype control antibody (top) or 1A6 (bottom). **(B)** CD4+ cells were stained with 1A6 and sorted on a MACS column. TSLPR+ cells were analyzed by Western blot, using the indicated α-TSLPR antibodies. The lower band visible in the AF981 blot is non-specific. **(C)** A549 cells were transfected with WT or 4Q TSLPR constructs, lysates were treated with PNGaseF as indicated. Equal amounts of treated lysates were analyzed by Western blot, and protein detected with α -V5 (top) or 1A6 (bottom) .

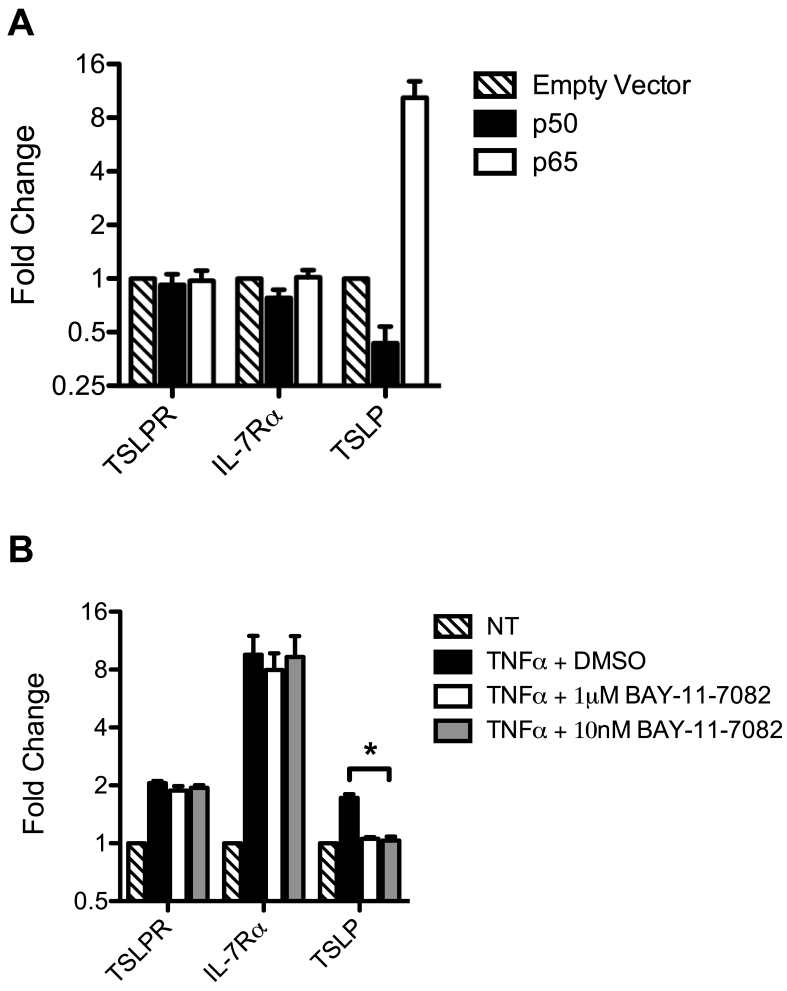

Figure 3.4 TSLPR and IL-7Rα are not regulated by NF-κB

(A) A549 were transfected with empty vector or expression vectors containing NF-κB-p50 or NF-κB-p65, and harvested 24 hours post- transfection for qPCR analysis. **(B)** A549 were pre-treated with DMSO, 1μM BAY-11-7082, or 10nm BAY-11-7082 for 30 minutes, then treated with 40ng/mL TNF-α for four hours and harvested for qPCR analysis. All data are reported as the mean ± SEM of at least three independent experiments. mRNA levels are normalized to HPRT and expressed relative to empty vector or untreated control samples, respectively.

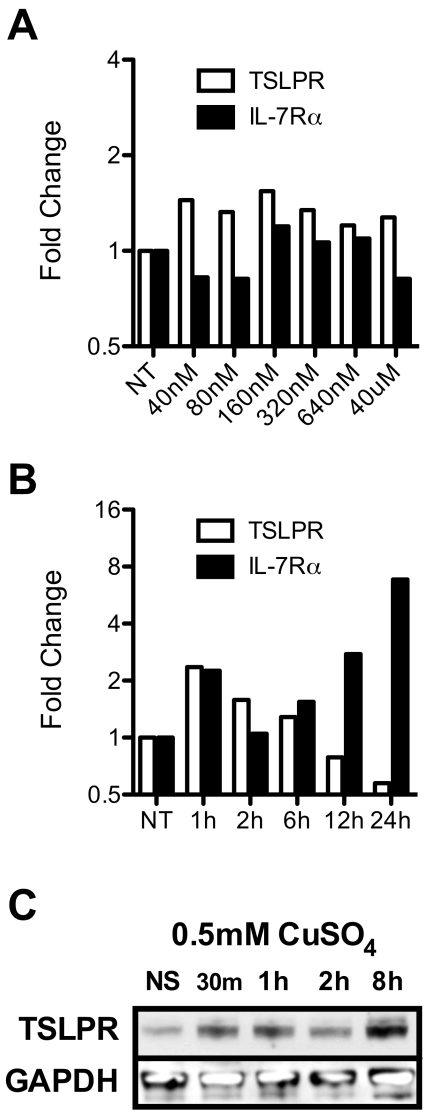

Figure 3.5 The mechanism of NF-κB independent regulation of TSLPR is unclear.

(A) A549 cells were treated with the indicated amounts of anisomycin and harvested after four hours for qPCR analysis. Values are reported as fold change of vehicle-treated cells. **(B)** A549 cells were treated with 0.5mM $CuSO_4$ for the indicated times. Values are reported as fold change of untreated cells. **(C)** A549 cells were treated with $CuSO_4$ as above for the indicated times, and harvested for protein analysis. Western blots were developed with α-TSLPR or α-GAPDH antibodies, as indicated.

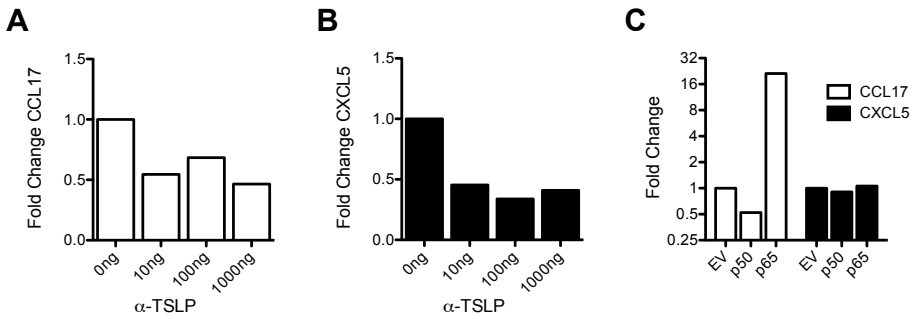

Figure 3.6 A blocking α-TSLP antibody partially inhibits TNF-α induced gene expression

(A) A549 cells were treated with 40ng/mL TNF-α + 10ng/mL IL-4 and the indicated amounts of a blocking α-TSLP antibody. Cells were harvested after four hours and analyzed via qPCR for expression of CCL17, reported relative to cells untreated with antibody. **(B)** A549 cells were treated with 40ng/mL TNF-α and the indicated amounts of a blocking α-TSLP antibody. Cells were harvested after four hours and analyzed via qPCR for expression of CXCL5, reported relative to cells untreated with antibody. **(C)** A549 were transfected with empty vector or expression vectors containing NF-κB-p50 or NF-κB-p65, and harvested 24 hours post-transfection for qPCR analysis.

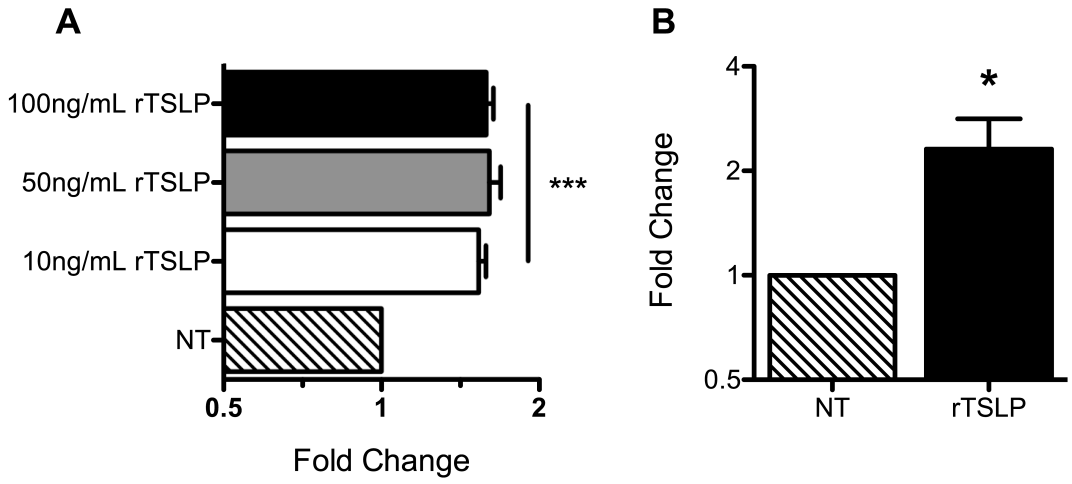

Figure 3.7 Epithelial cells functionally respond to TSLP

(A) A549 cells were co-transfected with TSLPR and IL-7Rα expression constructs along with the cis-luc reporter construct and the pRSV-β-gal control vector. Following transfection, cells were stimulated with indicated amounts of recombinant TSLP for eight hours, harvested and assayed for luciferase activity. Activity levels were normalized to β-gal levels, and expressed relative to untreated cells (***$P < 0.001$ versus untreated cells). **(B)** A549 cells were co-transfected with TSLPR and IL-7Rα expression constructs. Following transfection, TSLPR+ cells were enriched with an α -TSLPR antibody, and treated with 50ng/mL recombinant TSLP. Cells were harvested after eight hours, and analyzed via qPCR for expression of CCL17, normalized to HPRT and expressed relative to untreated cells (*$P = 0.031$ versus untreated cells). All data are reported as the mean ± SEM of at least six independent experiments.

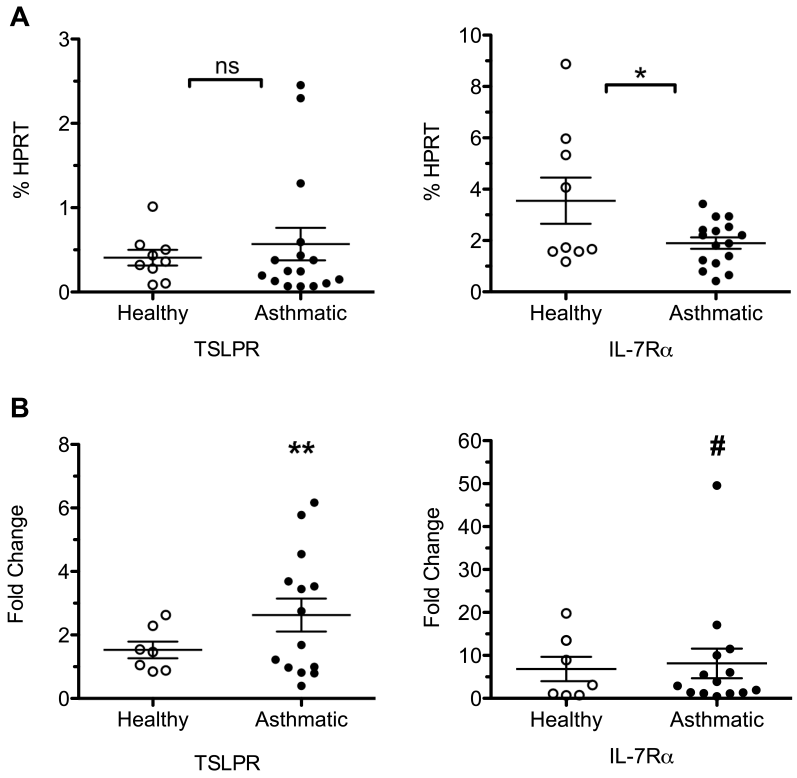

Figure 3.8 Primary BECs from asthmatic individuals upregulate TSLPR following infection with RSV

(A) Bronchial brushings from healthy and asthmatic patients were collected and grown in air-liquid interface (ALI) culture. Basal condition samples were apically exposed to control Vero cell supernatants, and collected for qPCR analysis. Levels of TSLPR and IL-7Rα are expressed as a percentage of HPRT levels from each sample. Each data point represents an individual patient, and mean values ± SEM are reported. Healthy and asthmatic patients were grouped and compared with Student's t-test (*P = 0.03; ns indicates no significant difference) **(B)** Matching ALI cultures from each patient were infected with RSV at an MOI of 0.5, and collected for qPCR analysis. Levels of Levels of TSLPR and IL-7Rα were normalized to HPRT, and reported as a fold change over the respective basal condition sample. Each data point represents an individual patient, and mean upregulation ± SEM are reported. Healthy and asthmatic groups were evaluated for significant upregulation at the 95% confidence interval (**P = 0.008; # indicates P = 0.059)

References

(1) AAAAI. American Academy of Allergy, Asthma, and Immunology: Asthma Statistics. http://www.aaaai.org/about-the-aaaai/newsroom/asthma-statistics.aspx. Accessed January 18, 2013.

(2) Levine SJ, Wenzel SE. Narrative review: the role of Th2 immune pathway modulation in the treatment of severe asthma and its phenotypes. *Ann Intern Med* 2010;152:232-237.

(3) Wark PA, Gibson PG. Asthma exacerbations . 3: Pathogenesis. *Thorax* 2006;61:909-915.

(4) Kapoor R, Menon C, Hoffstad O, Bilker W, Leclerc P, Margolis DJ. The prevalence of atopic triad in children with physician-confirmed atopic dermatitis. *J Am Acad Dermatol* 2008;58:68-73.

(5) Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* 2004;22:789-815.

(6) Salter H. On the Etiology of Asthma. *British Medical Journal* 1859;538-540.

(7) Robinson DS, Hamid Q, Ying S et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Eng J Med* 1992;326:298-304.

(8) Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 2011;242:205-219.

(9) Barbato A, Turato G, Baraldo S et al. Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* 2006;174:975-981.

(10) Bucchieri F, Puddicombe SM, Lordan JL et al. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am J Respir Cell Mol Biol* 2002;27:179-185.

(11) Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM. Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. *Am J Respir Crit Care Med* 2006;174:1110-1118.

(12) Kiss A, Montes M, Susarla S et al. A new mechanism regulating the initiation of allergic airway inflammation. *J Allergy Clin Immunol* 2007;120:334-342.

(13) Runswick S, Mitchell T, Davies P, Robinson C, Garrod DR. Pollen proteolytic enzymes degrade tight junctions. *Respirology* 2007;12:834-842.

(14) Wan H, Winton HL, Soeller C et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999;104:123-133.

(15) Ying S, Meng Q, Corrigan CJ, Lee TH. Lack of filaggrin expression in the human bronchial mucosa. *J Allergy Clin Immunol* 2006;118:1386-1388.

(16) Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. *Respirology* 2003;8:432-446.

(17) Crystal RG, Randell SH, Engelhardt JF, Voynow J, Sunday ME. Airway epithelial cells: current concepts and challenges. *Proc Am Thorac Soc* 2008;5:772-777.

(18) Steed E, Balda MS, Matter K. Dynamics and functions of tight junctions. *Trends Cell Biol* 2010;20:142-149.

(19) Harris TJ, Tepass U. Adherens junctions: from molecules to morphogenesis. *Nat Rev Mol Cell Biol* 2010;11:502-514.

(20) Green KJ, Jones JC. Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J* 1996;10:871-881.

(21) Greene CM, McElvaney NG. Toll-like receptor expression and function in airway epithelial cells. *Arch Immunol Ther Exp* 2005;53 :418-427.

(22) Kato A, Favoreto S, Jr., Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol* 2007;179:1080-1087.

(23) Sabbah A, Chang TH, Harnack R et al. Activation of innate immune antiviral responses by Nod2. *Nat Immunol* 2009;10:1073-1080.

(24) Le GR, Pothlichet J, Vitour D et al. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *J Immunol* 2007;178:3368-3372.

(25) Asokananthan N, Graham PT, Stewart DJ et al. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol* 2002;169:4572-4578.

(26) Allakhverdi Z, Comeau MR, Jessup HK et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med* 2007;204:253-258.

(27) Lee HC, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkappaB. *Proc Natl Acad Sci U S A* 2007;104:914-919.

(28) Nakamura Y, Miyata M, Ohba T et al. Cigarette smoke extract induces thymic stromal lymphopoietin expression, leading to T(H)2-type immune responses and airway inflammation. *J Allergy Clin Immunol* 2008;122:1208-1214.

(29) Reibman J, Hsu Y, Chen LC, Bleck B, Gordon T. Airway epithelial cells release MIP-3á/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol* 2003;28:648-654.

(30) Ebeling C, Lam T, Gordon JR, Hollenberg MD, Vliagoftis H. Proteinase-activated receptor-2 promotes allergic sensitization to an inhaled antigen through a TNF-mediated pathway. *J Immunol* 2007;179:2910-2917.

(31) Stumbles PA, Strickland DH, Pimm CL et al. Regulation of dendritic cell recruitment into resting and inflamed airway epithelium: use of alternative chemokine receptors as a function of inducing stimulus. *J Immunol* 2001;167:228-234.

(32) Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197-216.

(33) Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 2009;30:646-655.

(34) Mossmann TR, Coffman RL. Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;7:145-173.

(35) Harrington LE, Mangan PR, Weaver CT. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr Opin Immunol* 2006;18:349-356.

(36) Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 2005;5:772-782.

(37) Kyewski B, Klein L. A central role for central tolerance. *Annu Rev Immunol* 2006;24:571-606.

(38) Schwartz RH. T cell anergy. *Annu Rev Immunol* 2003;21:305-334.

(39) Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3(+) regulatory T cells. *Nat Rev Immunol* 2011;11:119-130.

(40) Thompson C, Powrie F. Regulatory T cells. *Curr Opin Pharmacol* 2004;4:408-414.

(41) Valenta R, Natter S, Seiberler S et al. Autoallergy: a pathogenetic factor in atopic dermatitis? *Curr Probl Dermatol* 1999;28:45-50.

(42) Barrat FJ, Meeker T, Gregorio J et al. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* 2005;202:1131-1139.

(43) Ochs HD, Gambineri E, Torgerson TR. IPEX, FOXP3 and regulatory T-cells: a model for autoimmunity. *Immunol Res* 2007;38:112-121.

(44) Kay TW, Thomas HE, Harrison LC, Allison J. The beta cell in autoimmune diabetes: many mechanisms and pathways of loss. *Trends Endocrinol Metab* 2000;11:11-15.

(45) Taylor BC, Zaph C, Troy AE et al. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* 2009;206:655-667.

(46) Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol Res* 1999;20:147-161.

(47) Headley MB, Zhou B, Shih WX, Aye T, Comeau MR, Ziegler SF. TSLP conditions the lung immune environment for the generation of pathogenic innate and antigen-specific adaptive immune responses. *J Immunol* 2009;182:1641-1647.

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

(49) Lindell DM, Berlin AA, Schaller MA, Lukacs NW. B cell antigen presentation promotes Th2 responses and immunopathology during chronic allergic lung disease. *PLoS ONE* 2008;3:e3129.

(50) Bang BR, Chun E, Shim EJ et al. Alveolar macrophages modulate allergic inflammation in a murine model of asthma. *Exp Mol Med* 2011;43:275-280.

(51) Becker S, Quay J, Soukup J. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. *J Immunol* 1991;147:4307-4312.

(52) Nomura H, Sato E, Koyama S et al. Histamine stimulates alveolar macrophages to release neutrophil and monocyte chemotactic activity. *J Lab Clin Med* 2001;138:226-235.

(53) Rubins JB. Alveolar macrophages: wielding the double-edged sword of inflammation. *Am J Respir Crit Care Med* 2003;167:103-104.

(54) Song C, Luo L, Lei Z et al. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol* 2008;181:6117-6124.

(55) Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;8:193-204.

(56) Lambrecht BN, Hammad H. Biology of lung dendritic cells at the origin of asthma. *Immunity* 2009;31:412-424.

(57) de Heer HJ, Hammad H, Soullie T et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 2004;200:89-98.

(58) Ostroukhova M, Seguin-Devaux C, Oriss TB et al. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest* 2004;114:28-38.

(59) Gilliet M, Soumelis V, Watanabe N et al. Human dendritic cells activated by TSLP and CD40L induce proallergic cytotoxic T cells. *J Exp Med* 2003;197:1059-1063.

(60) Ito T, Wang YH, Duramad O et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 2005;202:1213-1223.

(61) Seshasayee D, Lee WP, Zhou M et al. In vivo blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation. *J Clin Invest* 2007;117:3868-3878.

(62) Soumelis V, Reche PA, Kanzler H et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;3:673-680.

(63) Zhou B, Comeau MR, De Smedt T et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol* 2005;6:1047-1053.

(64) Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 2008;9:310-318.

(65) Sokol CL, Chu NQ, Yu S, Nish SA, Laufer TM, Medzhitov R. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat Immunol* 2009;10:713-720.

(66) Perrigoue JG, Saenz SA, Siracusa MC et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol* 2009;10:697-705.

(67) Yoshimoto T, Yasuda K, Tanaka H et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol* 2009;10:706-712.

(68) Kon OM, Sihra BS, Compton CH, Leonard TB, Kay AB, Barnes NC. Randomised, dose-ranging, placebo-controlled study of chimeric antibody to CD4 (keliximab) in chronic severe asthma. *Lancet* 1998;352:1109-1113.

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

(70) Hirahara K, Yamashita M, Iwamura C et al. Repressor of GATA regulates TH2-driven allergic airway inflammation and airway hyperresponsiveness. *J Allergy Clin Immunol* 2008;122:512-520.

(71) Shirakawa I, Deichmann KA, Izuhara I, Mao I, Adra CN, Hopkin JM. Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. *Immunol Today* 2000;21:60-64.

(72) Zhou B, Headley MB, Aye T, Tocker J, Comeau MR, Ziegler SF. Reversal of thymic stromal lymphopoietin-induced airway inflammation through inhibition of Th2 responses. *J Immunol* 2008;181:6557-6562.

(73) Altin J, Shen C, Liston A. Understanding the genetic regulation of IgE production. *Blood Rev* 2010;24:163-169.

(74) Drost EM, MacNee W. Potential role of IL-8, platelet-activating factor and TNF-alpha in the sequestration of neutrophils in the lung: effects on neutrophil deformability, adhesion receptor expression, and chemotaxis. *Eur J Immunol* 2002;32:393-403.

(75) Rahman I, Gilmour PS, Jimenez LA, MacNee W. Oxidative stress and TNF-alpha induce histone acetylation and NF-kappaB/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. *Mol Cell Biochem* 2002;234-235:239-248.

(76) Mochizuki M, Bartels J, Mallet AI, Christophers E, Schroder JM. IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J Immunol* 1998;160:60-68.

(77) Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195-201.

(78) Onari Y, Yokoyama A, Haruta Y et al. IL-12p40 is essential for the down-regulation of airway hyperresponsiveness in a mouse model of bronchial asthma with prolonged antigen exposure. *Clin Exp Allergy* 2009;39:290-298.

(79) Hamzaoui A, Chaouch N, Grairi H, Ammar J, Hamzaoui K. Inflammatory process of CD8+. *Mediators Inflamm* 2005;2005:160-166.

(80) O'Sullivan SM. Asthma death, CD8+ T cells, and viruses. *Proc Am Thorac Soc* 2005;2:162-165.

(81) Finkelman FD, Hogan SP, Hershey GK, Rothenberg ME, Wills-Karp M. Importance of cytokines in murine allergic airway disease and human asthma. *J Immunol* 2010;184:1663-1674.

(82) McKinley L, Alcorn JF, Peterson A et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol* 2008;181:4089-4097.

(83) Rothenberg ME. Eosinophilia. *N Engl J Med* 1998;338:1592-1600.

(84) Schmid-Grendelmeier P, Altznauer F, Fischer B et al. Eosinophils express functional IL-13 in eosinophilic inflammatory diseases. *J Immunol* 2002;169:1021-1027.

(85) Singhera GK, MacRedmond R, Dorscheid DR. Interleukin-9 and -13 inhibit spontaneous and corticosteroid induced apoptosis of normal airway epithelial cells. *Exp Lung Res* 2008;34:579-598.

(86) Allahverdian S, Harada N, Singhera GK, Knight DA, Dorscheid DR. Secretion of IL-13 by airway epithelial cells enhances epithelial repair via HB-EGF. *Am J Respir Cell Mol Biol* 2008;38:153-160.

(87) Lukacs NW, Hogaboam CM, Kunkel SL et al. Mast cells produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation. *J Leukoc Biol* 1998;63:746-751.

(88) Collington SJ, Williams TJ, Weller CL. Mechanisms underlying the localisation of mast cells in tissues. *Trends Immunol* 2011;32:478-485.

(89) Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* 2006;117:1277-1284.

(90) Takeda K, Hamelmann E, Joetham A et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997;186:449-454.

(91) Holgate ST, Roberts G, Arshad HS, Howarth PH, Davies DE. The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. *Proc Am Thorac Soc* 2009;6:655-659.

(92) Erjefalt JS, Erjefalt I, Sundler F, Persson CG. In vivo restitution of airway epithelium. *Cell Tissue Res* 1995;281:305-316.

(93) Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:1505-1510.

(94) Weidinger S, Gieger C, Rodriguez E et al. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. *PLoS Genet* 2008;4:e1000166.

(95) Weiss ST, Raby BA, Rogers A. Asthma genetics and genomics 2009. *Curr Opin Genet Dev* 2009;19:279-282.

(96) Fallon PG, Mangan NE. Suppression of TH2-type allergic reactions by helminth infection. *Nat Rev Immunol* 2007;7:220-230.

(97) Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008;454:445-454.

(98) Sigurs N, Aljassim F, Kjellman B et al. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. *Thorax* 2010;65:1045-1052.

(99) Johnston SL, Pattemore PK, Sanderson G et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995;310:1225-1229.

(100) Srikiatkhachorn A, Braciale TJ. Virus-specific memory and effector T lymphocytes exhibit different cytokine responses to antigens during experimental murine respiratory syncytial virus infection. *J Virol* 1997;71:678-685.

(101) Ling Z, Tran KC, Teng MN. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. *J Virol* 2009;83:3734-3742.

(102) Spann KM, Tran KC, Collins PL. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. *J Virol* 2005;79:5353-5362.

(103) Munir S, Le NC, Luongo C, Buchholz UJ, Collins PL, Bukreyev A. Nonstructural proteins 1 and 2 of respiratory syncytial virus suppress maturation of human dendritic cells. *J Virol* 2008;82:8780-8796.

(104) Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol* 2007;81:1401-1411.

(105) Loo Y-M, Fornek J, Crochet N et al. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol* 2008;82:335-345.

(106) Becker S, Soukup JM. Airway epithelial cell-induced activation of monocytes and eosinophils in respiratory syncytial viral infection. *Immunobiology* 1999;201:88-106.

(107) Klein KP, Tan L, Werkman W, van Bleek GM, Coenjaerts F. The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. *Crit Rev Immunol* 2009;29:531-550.

(108) Roman M, Calhoun WJ, Hinton KL et al. Respiratory syncytial virus infection in infants is associated with predominant Th2-like response. *Am J Respir Crit Care Med* 1997;156:190-195.

(109) Lukacs NW, Tekkanat KK, Berlin A et al. Respiratory syncytial virus predisposes mice to augmented allergic airway responses via IL-13-mediated mechanisms. *J Immunol* 2001;167:1060-1065.

(110) Friend SL, Hosier S, Nelson A, Foxworthe D, Williams DE, Farr A. A thymic stromal cell line supports in vitro development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells. *Exp Hematol* 1994;22:321-328.

(111) Blazquez AB, Mayer L, Berin MC. Thymic Stromal Lymphopoietin is Required for Gastrointestinal Allergy but not Oral Tolerance. *Gastroenterology* 2010;139:1301-1309.

(112) Larson RP, Zimmerli SC, Comeau MR et al. Dibutyl phthalate-induced thymic stromal lymphopoietin is required for th2 contact hypersensitivity responses. *J Immunol* 2010;184:2974-2984.

(113) Ying S, O'Connor B, Ratoff J et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005;174:8183-8190.

(114) Mou Z, Xia J, Tan Y et al. Overexpression of thymic stromal lymphopoietin in allergic rhinitis. *Acta Otolaryngol* 2009;129:297-301.

(115) Isaksen DE, Baumann H, Trobridge PA, Farr AG, Levin SD, Ziegler SF. Requirement for stat5 in thymic stromal lymphopoietin-mediated signal transduction. *J Immunol* 1999;163:5971-5977.

(116) Wohlmann A, Sebastian K, Borowski A, Krause S, Friedrich K. Signal transduction by the atopy-associated human thymic stromal lymphopoietin (TSLP) receptor depends on Janus kinase function. *Biol Chem* 2010;391:181-186.

(117) Arima K, Watanabe N, Hanabuchi S, Chang M, Sun SC, Liu YJ. Distinct signal codes generate dendritic cell functional plasticity. *Sci Signal* 2010;3:ra4.

(118) Reche PA, Soumelis V, Gorman DM et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J Immunol* 2001;167:336-343.

(119) Semlali A, Jacques E, Koussih L, Gounni AS, Chakir J. Thymic stromal lymphopoietin-induced human asthmatic airway epithelial cell proliferation through an IL-13-dependent pathway. *J Allergy Clin Immunol* 2010;125:844-850.

(120) Reardon C, Lechmann M, Brüstle A et al. Thymic stromal lymphopoetin-induced expression of the endogenous inhibitory enzyme SLPI mediates recovery from colonic inflammation. *Immunity* 2011;35:223-235.

(121) Sims JE, Williams DE, Morrissey PJ et al. Molecular cloning and biological characterization of a novel murine lymphoid growth factor. *J Exp Med* 2000;192:671-680.

(122) Lee HC, Headley MB, Iseki M, Ikuta K, Ziegler SF. Cutting edge: Inhibition of NF-kappaB-mediated TSLP expression by retinoid X receptor. *Cutting Edge J Immunol* 2008;181:5189-5193.

(123) Park LS, Martin U, Garka K et al. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med* 2000;192:659-670.

(124) Fujio K, Mosaka T, Kojima T et al. Molecular cloning of a novel type 1 cytokine receptor similar to the common gamma chain. *Blood* 2000;95:2210.

(125) Levin SD, Koelling RM, Friend SL et al. Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM+ B cells in vitro and signals via a novel mechanism. *J Immunol* 1999;162:677-683.

(126) Rochman I, Watanabe N, Arima K, Liu YJ, Leonard WJ. Cutting edge: direct action of thymic stromal lymphopoietin on activated human CD4+ T cells. *J Immunol* 2007;178:6720-6724.

(127) Rochman Y, Leonard WJ. The role of thymic stromal lymphpoietin in CD8+ T cell homeostasis. *J Immunol* 2008;181:7699-7705.

(128) Siracusa MC, Saenz SA, Hill DA et al. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature* 2011;477:229-233.

(129) Lu N, Wang YH, Wang YH, Arima K, Hanabuchi S, Liu YJ. TSLP and IL-7 use two different mechanisms to regulate human CD4+ T cell homeostasis. *J Exp Med* 2009;206:2111-2119.

(130) Han H, Headley MB, Xu W, Comeau MR, Zhou B, Ziegler SF. Thymic Stromal Lymphopoietin Amplifies the Differentiation of Alternatively Activated Macrophages. *J Immunol* 2012.

(131) Kaur D, Doe C, Woodman L et al. Mast cell-airway smooth muscle crosstalk: the role of thymic stromal lymphopoietin. *Chest* 2012;142:76-85.

(132) Scheeren FA, van Lent AU, Nagasawa M et al. Thymic stromal lymphopoietin induces early human B-cell proliferation and differentiation. *Eur J Immunol* 2010;40:955-965.

(133) Mazzucchelli R, Hixon JA, Spolski R et al. Development of regulatory T cells requires IL-7Ralpha stimulation by IL-7 or TSLP. *Blood* 2008;112:3283-3292.

(134) Omori M, Ziegler S. Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *J Immunol* 2007;178:1396-1404.

(135) Wong CK, Hu S, Cheung PF, Lam CW. TSLP Induces Chemotactic and Pro-survival Effects in Eosinophils: Implications in Allergic Inflammation. *Am J Respir Cell Mol Biol* 2009;Epub ahead of print.

(136) Allakhverdi Z, Comeau MR, Smith DE et al. CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation. *J Allergy Clin Immunol* 2009;123:472-478.

(137) Nagata Y, Kamijuku H, Taniguchi M, Ziegler S, Seino K. Differential role of thymic stromal lymphopoietin in the induction of airway hyperreactivity and Th2 immune response in antigen-induced asthma with respect to natural killer T cell function. *Int Arch Allergy Immunol* 2007;144:305-314.

(138) Zaph C, Troy AE, Taylor BC et al. Epithelial-cell-intrinsic IKK-á expression regulates intestinal immune homeostasis. *Nature* 2007;446:552-556.

(139) Hunninghake GM, Soto-Quiros ME, Avila L et al. TSLP polymorphisms are associated with asthma in a sex-specific fashion. *Allergy* 2010.

(140) Gudbjartsson DF, Bjornsdottir US, Halapi E et al. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. *Nat Genet* 2009;41:342-347.

(141) Shamim Z, Muller K, Svejgaard A, Poulsen LK, Bodtger U, Ryder LP. Association between genetic polymorphisms in the human interleukin-7 receptor alpha-chain and inhalation allergy. *Int J Immunogenet* 2007;34:149-151.

(142) Harada M, Hirota T, Jodo AI et al. Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2009;40:368-374.

(143) Lee HC, Headley MB, Loo YM et al. Thymic stromal lymphopoietin is induced by respiratory syncytial virus-infected airway epithelial cells and promotes a type 2 response to infection. *J Allergy Clin Immunol* 2012;130:1187-1196.

(144) Verdier F, Rabionet R, Gouilleux F et al. A sequence of the CIS gene promoter interacts preferentially with two associated STAT5A dimers: a distinct biochemical difference between STAT5A and STAT5B. *Mol Cell Biol* 1998;18:5852-5860.

(145) Kumagai N, Fukuda K, Nishida T. Synergistic effect of TNF-alpha and IL-4 on the expression of thymus- and activation-regulated chemokine in human corneal fibroblasts. *Biochem Biophys Res Commun* 2000;279:1-5.

(146) Al Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med* 2005;202:829-839.

(147) Reefer AJ, Hulse KE, Lannigan JA et al. Flow cytometry imaging identifies rare T(H)2 cells expressing thymic stromal lymphopoietin receptor in a "proallergic" milieu. *J Allergy Clin Immunol* 2010;126:1049-58, 1058.

(148) Baker SJ, Reddy EP. Modulation of life and death by the TNF receptor superfamily. *Oncogene* 1998;17:3261-3270.

(149) Mikkelsen SS, Jensen SB, Chiliveru S et al. RIG-I-mediated activation of p38 MAPK is essential for viral induction of interferon and activation of dendritic cells: dependence on TRAF2 and TAK1. *J Biol Chem* 2009;284:10774-10782.

(150) Mattie MD, McElwee MK, Freedman JH. Mechanism of copper-activated transcription: activation of AP-1, and the JNK/SAPK and p38 signal transduction pathways. *J Mol Biol* 2008;383:1008-1018.

(151) Isaksen DE, Baumann H, Zhou B et al. Uncoupling of proliferation and Stat5 activation in thymic stromal lymphopoietin-mediated signal transduction. *J Immunol* 2002;168:3288-3294.

(152) Homey B, Zlotnik A. Chemokines in allergy. *Curr Opin Immunol* 1999;11:626-634.

(153) Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell* 2006;126:855-867.

(154) Vranjkovic A, Crawley AM, Gee K, Kumar A, Angel JB. IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human CD8+ T cells. *Int Immunol* 2007;19:1329-1339.

(155) Hammerbeck CD, Mescher MF. Antigen controls IL-7R alpha expression levels on CD8 T cells during full activation or tolerance induction. *J Immunol* 2008;180:2107-2116.

(156) Holgate ST. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol* 2007;28:248-251.

(157) Holgate ST. Epithelial dysfunction in asthma. *J Allergy Clin Immunol* 2007;120:1233-1244.

(158) Kato A, Schleimer RP. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr Opin Immunol* 2007;19:711-720.

(159) Stefanowicz D, Hackett TL, Garmaroudi FS et al. DNA methylation profiles of airway epithelial cells and PBMCs from healthy, atopic and asthmatic children. *PLoS ONE* 2012;7:e44213.