TSLP regulates intestinal immunity and inflammation

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TSLP regulates intestinal immunity and inflammation

Abstract
This thesis characterizes and explores the role of thymic stromal lymphopoietin (TSLP) – TSLP receptor (TSLPR) interactions in the regulation of intestinal immunity and inflammation. TSLP is known to promote TH2 cytokine-mediated inflammation in the skin and the lung. Firstly, this thesis focuses on the characterization of TSLP and TSLPR expression within the gastrointestinal (GI) tract and in the immune cells of the associated lymphoid tissues. Our findings suggest that TSLP is primarily produced by intestinal epithelial cells (IECs) under homeostatic conditions. Expression of the heterodimeric TSLPR complex was exhibited on gut-associated lymphoid tissue (GALT) CD4+ T cells and CD11c+ dendritic cells (DCs) as well as on splenic mast cells and basophils in naïve mice. Second, this thesis explores the role of TSLP in the generation of a TH2 cytokine-mediated host-protective immune response to the gastrointestinal helminth pathogen Trichuris muris. We demonstrate that in an intact animal TSLP-TSLPR interactions are critical for resistance to Trichuris as disruption of the TSLP-TSLPR pathway either through genetic deletion of the TSLPR, or antibody-mediated depletion of TSLP, resulted in susceptibility to infection. Further, these mice exhibited increased infection-induced proinflammatory cytokine production and inflammation. However, TSLP-TSLPR interactions were not required for the generation of a host-protective TH2 cytokine-mediated immune response to Trichuris in the absence of IFN-γ, indicating TSLP-independent intestinal TH2 immune responses. Third, we examine the potential for TSLP-TSLPR interactions to regulate intestinal proinflammatory cytokine production. Examination of naïve TSLPR-/- mice revealed a trend towards increased CD4+ T cell production of IFN-γ in the GALT compared to TSLPR+/+ mice. Further, TSLPR-/- mice were more susceptible to DSS-induced colitis, exhibiting increased proinflammatory cytokine production and more severe intestinal inflammation compared to control mice. We demonstrate that TSLP can regulate DC-derived IL-12/23p40 production both in vitro and in vivo and that rTSLP is able to inhibit CD4+ T cell production of IFN-γ in splenocyte cultures in the absence of IL-4. Taken as a whole, this thesis identifies an important role for TSLP-TSLPR interactions in regulating intestinal immunity and inflammation and in the maintenance of intestinal immune homeostasis.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Immunology

First Advisor
David Artis

Keywords
mucosal immunology, intestinal epithelial cells, TSLP, Trichuris muris, inflammatory bowel disease

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/71
TSLP REGULATES INTESTINAL IMMUNITY AND INFLAMMATION

Betsy C. Taylor

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2009

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Dedication

I would like to dedicate this thesis firstly to my brother Zack Taylor, without whose unending, unwavering, and unconditional love and support this would not have been possible. Secondly, I dedicate this thesis to the wonderful women of WEBS, you brought light, laughter, and happiness into grad school; something I had never thought possible.
Acknowledgements

I would like to thank my advisor, David Artis. I would also like to thank every member of the Artis Lab; you guys made lab a great, supportive, fun place to work. I would especially like to acknowledge Amy Troy, I am eternally grateful for all of your help, kindness, and patience in training me. You will always be “my postdoc”. Thanks also to Colby Zaph, whose support and words of advice have been invaluable to me. Additionally, I would like to thank my thesis committee members — Jan Burkhardt, Yasmine Belkaid, Edward Pearce, John Wherry, and Warren Pear for their support and mentorship during this process. Special thanks also to Jan Burkhardt whose mentorship, guidance, and support in all things has meant the world to me. I am also grateful for my discussions with the members of the Pathobiology Department, which have helped make me a better scientist. And finally, I would like to thank my friends and family, without whom I would never have survived graduate school.
**Publications**


Saenz, S., Taylor, B.C., and Artis, D. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. *Immunological Reviews, 2008 Dec; 226(1): 172-190*

Zaph, C., Troy, A.E., Taylor, B.C., Berman-Booty, L.D., Guild, K.J., Du, Y., Yost, E.A.,
Gruber, A.D., May, M.J., Greten, F.R., Eckmann, L., Karin, M. and Artis, D. Epithelial
Mar. 29; 446 (7135): 552-556
Attributions

This thesis contains work from the manuscript entitled, “TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis”, published in JEM March 2009, with the following author list: Betsy C. Taylor, Colby Zaph, Amy E. Troy, Yurong Du, Katherine J. Guild, Michael R. Comeau, and David Artis.

The contributions of authors: Colby Zaph and Amy Troy assisted in mouse infections and tissue and cell preparations in multiple experiments, Yurong Du performed the Western blots depicted in Figure 10E, 11D, and 17D. Katherine Guild assisted with mouse infections and the IF staining depicted in Figure 10 D and Figure 11C. Michael Comeau provided reagents.

Data depicted in Figure 7D is modified from the manuscript entitled, “MHC class II-dependent basophil-CD4+ T cell interactions promote Th2 cytokine-dependent immunity”, published in NI July 2009, with the following author list: Jacqueline G. Perrigoue, Steven A. Saenz, Mark C. Siracusa, Eric J. Allenspach, Betsy C. Taylor, Paul G. Giacomin, Meera G. Nair, Yurong Du, Colby Zaph, Nico van Rooijen, Michael R. Comeau, Edward J. Pearce, Terri M. Laufer, and David Artis. Figure 7D of this thesis was modified from Figure 5 of the paper and Steven Saenz performed the individual experiment shown.
Abstract

TSLP regulates intestinal immunity and inflammation

Betsy C. Taylor
David Artis

This thesis characterizes and explores the role of thymic stromal lymphopoietin (TSLP) – TSLP receptor (TSLPR) interactions in the regulation of intestinal immunity and inflammation. TSLP is known to promote T_h2 cytokine-mediated inflammation in the skin and the lung. Firstly, this thesis focuses on the characterization of TSLP and TSLPR expression within the gastrointestinal (GI) tract and in the immune cells of the associated lymphoid tissues. Our findings suggest that TSLP is primarily produced by intestinal epithelial cells (IECs) under homeostatic conditions. Expression of the heterodimeric TSLPR complex was exhibited on gut-associated lymphoid tissue (GALT) CD4^+ T cells and CD11c^+ dendritic cells (DCs) as well as on splenic mast cells and basophils in naïve mice. Second, this thesis explores the role of TSLP in the generation of a T_h2 cytokine-mediated host-protective immune response to the gastrointestinal helminth pathogen *Trichuris muris*. We demonstrate that in an intact animal TSLP-TSLPR interactions are critical for resistance to *Trichuris* as disruption of the TSLP-TSLPR pathway either through genetic deletion of the TSLPR, or antibody-mediated depletion of TSLP, resulted in susceptibility to infection. Further, these mice exhibited increased infection-induced proinflammatory cytokine production and inflammation. However, TSLP-TSLPR interactions were not required for the generation of a host-protective T_h2 cytokine-mediated immune response to *Trichuris* in the absence of IFN-γ, indicating TSLP-
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Table of contents

Dedication .................................................................................................................. ii
Acknowledgements .................................................................................................... iii
Publications ................................................................................................................ iv
Attributions ................................................................................................................ vi
Abstract .................................................................................................................... vii
Table of contents ........................................................................................................ ix
List of figures ............................................................................................................. xi

Chapter 1: Literature Review ..................................................................................... 1

The intestinal immune system .................................................................................. 1
Balancing immunity and inflammation in the intestine .............................................. 1
Intestinal epithelial cells (IECs) form a physical barrier ........................................... 2
IECs actively modulate intestinal immune responses .............................................. 3

Thymic stromal lymphopoietin (TSLP) ................................................................... 5
Cellular sources and regulation of TSLP ................................................................. 5
Cellular targets of TSLP ............................................................................................ 12
Role of TSLP in immune system development ......................................................... 19
Functional biology of TSLP in the skin and lung ....................................................... 23
TSLP in the intestine .................................................................................................. 27

Model Systems .......................................................................................................... 30
Trichuris muris .......................................................................................................... 30
Life cycle and transmission ...................................................................................... 30
Public health impact .................................................................................................. 31
Trichuris muris as a model of protective Th2 cytokine-dependent immune responses... 32
Dextran Sulfate Sodium (DSS) colitis: a murine model of inflammatory bowel disease (IBD) ............................................................................................................................ 32
IBD: Disease and public health impact ...................................................................... 36
DSS colitis: A chemical-induced experimental colitis model .................................... 37
Conclusion .................................................................................................................. 38

CHAPTER 2: Characterization of TSLP-TSLPR expression in the gastrointestinal lymphoid tissues (GALT) .................................................................................. 40

Abstract ...................................................................................................................... 40
Introduction .................................................................................................................. 41
Materials and Methods .............................................................................................. 43

Results ......................................................................................................................... 47
Constitutive expression of TSLP in intestinal epithelial cells ................................... 47
TSLPR and IL-7Rα (CD127) are co-expressed on CD11c+ DCs .............................. 49
TSLPR complex expression on macrophages and granulocytes .............................. 54
CD3+ CD4+ T cells co-express TSLPR and CD127 ............................................... 57
Recombinant TSLP induces phospho-STAT5 in CD11c+ and CD4+ T cells directly ex vivo 59
CHAPTER 3: TSLP is required for immunity to Trichuris muris, however TSLP independent T_{h}2 cytokine dependent immune responses can develop in the absence of IFN-\gamma

Abstract

Introduction

Results

In vivo neutralization of TSLP in WT mice results in susceptibility to Trichuris
TSLP-TSLPR interactions are critical for immunity to Trichuris
Increased infection-induced inflammation and proinflammatory cytokine production in the absence of TSLP-TSLPR interactions
TSLP is not required to initiate T_{h}2 cytokine responses in the intestine

Discussion

CHAPTER 4: A role for TSLP-TSLPR interactions in the inhibition of proinflammatory cytokine production in the intestine

Abstract

Introduction

Materials and Methods

Results

Naïve TSLPR^{-/-} mice exhibit increased frequencies of CD4^{+} IFN-\gamma^{+} T cells in the GALT
TSLP regulates DC production of IL-12/23p40 both in vitro and in vivo
TSLPR^{-/-} mice exhibit an increased susceptibility to DSS-induced colitis

Discussion

CHAPTER 5: Discussion and Future Directions

Introduction

Summary, discussion, and future directions
TSLP and TSLPR complex expression in the intestine
The role for TSLP in intestinal T_{h}2 cytokine-mediated immune responses
TSLP and the regulation of proinflammatory cytokine production and inflammation in the intestine

Concluding remarks

References
List of figures

Figure 1 Regulation of TSLP production ................................................................. 11
Figure 2 TSLPR signaling pathways .................................................................. 17
Figure 3 TSLP promotes Th2 cytokine responses ............................................. 29
Figure 4 Trichuris muris life cycle and immune response ..................................... 35
Figure 5 TSLP is constitutively expressed in intestinal epithelial cells .............. 48
Figure 6 GALT DCs co-express TSLPR-α chain and CD127 ............................ 52
Figure 7 TSLPR complex expression on macrophage and granulocyte cell populations ........................................................................................................ 56
Figure 8 TSLPR and CD127 co-expression on CD3+ CD4+ T cells ..................... 58
Figure 9 rTSLP activates phospho-STAT5 in CD4+ T cells and CD11c+ DCs .... 61
Figure 10 In vivo neutralization of TSLP in WT mice results in susceptibility to Trichuris muris ........................................................................................................ 76
Figure 11 TSLP−/− mice interactions are critical for immunity to Trichuris muris .. 80
Figure 12 Anti-TSLP treatment in WT mice results in chronic infection .......... 81
Figure 13 TSLPR−/− mice have increased infection-induced inflammation and proinflammatory cytokine production ................................................................. 85
Figure 14 Anti-TSLP treatment in WT mice results in increased infection-induced inflammation ................................................................. 86
Figure 15 Anti-TSLP treatment in WT mice results in increased proinflammatory cytokine production ................................................................. 87
Figure 16 TSLPR−/− mice exhibit elevated frequencies of CD4+ IFN-γ+ T cells in the MLN at day 34 post-infection ............................................................................. 88
Figure 17 Neutralization of IFN-γ in susceptible TSLPR−/− mice restores Th2 cytokine production and immunity ................................................................. 91
Figure 18 Anti-IFN-γ treatment increases serum IgE levels ................................ 93
Figure 19 Naïve TSLPR−/− have increased frequencies of CD4+ IFN-γ+ T cells in the GALT ............................................................................................................... 108
Figure 20 Frequencies of GALT CD4+ IL-10+ and CD4+ FoxP3+ T cells in naïve TSLPR−/− mice ........................................................................................................ 109
Figure 21 Treatment of splenocyte cultures with rTSLP inhibits IFN-γ production .................................................................................................................. 112
Figure 22 TSLP regulates DC production of IL-12/23p40 in vitro and in vivo ...... 115
Figure 23 TSLPR−/− mice exhibit an increased susceptibility to DSS colitis ...... 119
Figure 24 TSLP is a central regulator of intestinal immune homeostasis .......... 143
Chapter 1: Literature Review

The intestinal immune system

Balancing immunity and inflammation in the intestine

The intestinal tract is the largest mucosal surface of the human body, with a surface area of approximately 100-200 m$^2$ (Mason 2008), and presents an immense immunologic challenge to the host. The large amount of exposed surface area is vulnerable to invasion by pathogenic bacteria, viruses, fungi, and parasites. However, despite the need to remain responsive against pathogenic microbes, the gastrointestinal (GI) tract must also remain hyporesponsive to innocuous stimuli. Due to its function in the absorption of nutrients and minerals, the intestine is continually exposed to large quantities of food and environmental antigens. Additionally, the adult human intestine is colonized by an estimated $10^{14}$ “commensal” bacteria (Ley, Peterson et al. 2006; Frank and Pace 2008), which exist in a mutualistic relationship with the host. In fact, the presence of commensal bacteria is required for normal development of the immune system, and they play important roles in the supply of essential nutrients, the breakdown of indigestible materials, and in the defense against opportunistic pathogens (Artis 2008; Round and Mazmanian 2009). Loss of hyporesponsiveness to environmental, food, and commensal-derived antigens leads to inflammation and disease states ranging from food allergy to inflammatory bowel disease (IBD) (Bouma and Strober 2003; Macdonald and Monteleone 2005; Karin, Lawrence et al. 2006).

In order to contend with these diverse challenges, the intestinal immune system has developed a complex combination of both anatomic and immunological adaptations that help maintain host immune homeostasis. One of the most important anatomic features
of the intestine is the separation of the external environment from the sterile tissues of
the body by a single layer of intestinal epithelial cells.

*Intestinal epithelial cells (IECs) form a physical barrier*

IECs have developed numerous adaptations that allow them to physically separate the
contents of the lumen of the gut from the cells of the lamina propria. The apical surface
of IECs is covered in microvilli, a dense network of actin filaments and membrane
invaginations that combine to form the brush border. The brush border not only
increases the surface area of the cell allowing maximal absorption of nutrients, but also
serves to separate luminal contents from the main IEC cell body (Sansonetti 2004).
Additionally, IECs are directly interconnected through a series transmembrane proteins
including claudins that form tight junctions. Tight junctions prevent the translocation of
bacteria between cells and are actively regulated to allow selective permeability of the
barrier (Sansonetti 2004). The cellular barrier is also reinforced chemically, as goblet
cell-derived mucins bind the apical surface of IECs to form a glycocalyx, and further
mucin production results in a thick layer of mucus that acts as a semi-permeable barrier
that traps bacteria and sequesters them away from direct contact with IECs (Sansonetti
2004). IECs also possess antimicrobial properties: they secrete defensins, cathelicidins,
and calprotectins whose amphipathic nature allows for insertion into and disruption of
bacterial cell walls (Ganz 2003; Salzman, Ghosh et al. 2003; Agerberth and
Gudmundsson 2006). IEC production of antimicrobial products helps to control and
regulate bacterial growth, while also helping to prevent live bacteria from directly
contacting IECs. Together, these IEC adaptations combine to make a substantial
physical barrier separating potentially pathogenic external stimuli from the lamina propria and are critical in the maintenance of intestinal immune homeostasis.

**IECs actively modulate intestinal immune responses**

In addition to providing a physical barrier, IECs also actively respond to and interact with their environment. IECs are known to express a wide variety of pattern-recognition receptors including members of the Toll-like receptor (TLR) family and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Artis 2008; Gribar, Richardson et al. 2008), which have been demonstrated to be important in the maintenance of intestinal immune homeostasis (Rakoff-Nahoum, Paglino et al. 2004). Importantly, IECs can discriminate between commensal and pathogenic organisms, helping to maintain the balance between immunity and inflammation. Possible mechanisms that allow for the selective activation of IECs by pathogenic bacteria compared to commensal bacteria include the selective and regulated expression of TLRs and NLRs, apical versus basolateral expression of TLRs, and the modulation of IEC signaling pathways by commensal bacteria (Neish, Gewirtz et al. 2000; Kelly, Campbell et al. 2004; Artis 2008).

When infected by enteropathogenic bacteria, IECs maintain the ability to respond and upregulate a diverse array of immune response genes. Activation results in the induction of cytokines including IL-8 and TNF-α as well as the expression of chemokines such as CCL2 (Eckmann, Kagnoff et al. 1993; Jung, Eckmann et al. 1995; Yang, Eckmann et al. 1997). These factors act to activate resident immune cells and to recruit monocytes to the site of infection, thus initiating the immune response. IEC recognition
of pathogenic bacteria is important in the initiation of protective immune responses. Deletion of the pattern recognition receptors TLR4 or NOD1 or the central TLR signaling molecule MyD88 in non-haematopoietic cells resulted in impaired immunity following bacterial infection (Schilling, Martin et al. 2003; Brandl, Plitas et al. 2007; Lebeis, Bommarius et al. 2007). The NF-κB signaling pathway appears to be a central regulator in IEC activation as multiple types of enteroinvasive bacteria activate the pathway and induce nuclear localization and DNA binding of NF-κB in IECs (Elewaut, DiDonato et al. 1999).

In addition to responding to bacterial invasion, IEC-intrinsic NF-κB signaling is critical to maintaining intestinal immune homeostasis. Mice with an IEC-specific deletion of IKK-γ (also known as NEMO), a critical regulator protein of the NF-κB pathway that is essential to both classical and non-classical NF-κB signaling, develop spontaneous colitis (Nenci, Becker et al. 2007). Loss of NEMO within IECs results in impaired IEC barrier function, with increased colonic IEC apoptosis, decreased antimicrobial peptide production, and translocation of bacteria into the lamina propria. Conditional ablation of either IKK-β or IKK-α specifically in IECs did not result in the development of spontaneous colitis in mice, indicating that suboptimal NF-κB signaling is sufficient to maintain barrier integrity (Nenci, Becker et al. 2007). However, when challenged with the intestinal helminth pathogen Trichuris muris, mice specifically lacking IKK-β in IECs exhibited increased susceptibility, increased proinflammatory cytokine production, and severe infection-induced inflammation compared to littermate control mice (Zaph, Troy et al. 2007). These results implicate the NF-κB pathway as a central regulator of intestinal immune...
homeostasis and in the development of host-protective immune responses to a broad spectrum of intestinal pathogens.

IECs modulate downstream responses through the regulated secretion of a number of immunomodulatory factors including IL-10, prostaglandin E$_2$ (PGE$_2$), transforming growth factor-$
\beta$ (TGF-$\beta$), and thymic stromal lymphopoietin (TSLP). IECs have been demonstrated to produce the anti-inflammatory cytokine IL-10 constitutively (Jarry, Bossard et al. 2008), which can inhibit proinflammatory cytokine production from macrophages and dendritic cells (DCs) (Mosser and Zhang 2008). PGE$_2$ inhibits DC production of IL-12 if present during priming, favoring the differentiation of naïve CD4$^+$ T cells into T$_H$2 cells. Further, PGE$_2$ can act directly on CD4$^+$ T cells to down modulate their production of IFN-$\gamma$ (Harris, Padilla et al. 2002). IECs constitutively secrete large amounts of TGF-$\beta$ (Barnard, Warwick et al. 1993), which potently inhibits proinflammatory cytokine production from macrophages and DCs and plays a role in the differentiation of both regulatory T cells and T$_H$17 cells (Rubtsov and Rudensky 2007). IECs also express TSLP, a cytokine implicated in the maintenance of intestinal immune homeostasis through its modulation of DC function (Rimoldi, Chieppa et al. 2005). This thesis will investigate the role of TSLP in the intestinal immune system.

Thymic stromal lymphopoietin (TSLP)

Cellular sources and regulation of TSLP

While TSLP was initially identified and characterized as a novel cytokine produced by thymic stromal cells (Friend, Hosier et al. 1994; Sims, Williams et al. 2000; Quentmeier, Drexler et al. 2001), it is predominantly expressed in epithelial cells of the skin
(keratinocytes), lung (small airway epithelial cells), and intestine (IECs) (Sims, Williams et al. 2000; Reche, Soumelis et al. 2001; Soumelis, Reche et al. 2002; Liu, Soumelis et al. 2007). Basal expression of TSLP mRNA in the steady state has been detected in epithelial cells of the tonsils, skin, lung, and intestine (Soumelis, Reche et al. 2002; Liu, Soumelis et al. 2007; Zaph, Troy et al. 2007); however, the basal production of TSLP protein has only been found in the lung, tonsils, and intestine (Watanabe, Hanabuchi et al. 2004; Rimoldi, Chieppa et al. 2005; Zhang, Shan et al. 2007). Structurally, TSLP resembles IL-7 and is a four-helix bundle cytokine. There is no cross-species reactivity between human and murine TSLP; they exhibit only 43% amino acid sequence identity (Liu, Soumelis et al. 2007). However, despite these substantial molecular differences, human and murine TSLP exert similar biological functions.

Increased TSLP expression in epithelial cells can be induced through exposure to viral, bacterial, or parasitic pathogens as well as ligation of Toll-like receptors (TLRs) (Allakhverdi, Comeau et al. 2007; Kato, Favoreto et al. 2007; Humphreys, Xu et al. 2008; Zeuthen, Fink et al. 2008; Kinoshita, Takai et al. 2009; Ramalingam, Pesce et al. 2009). Treatment with extract from the common environmental fungus, *Alternaria alternata*, resulted in TSLP induction in airway epithelial cells (Kouzaki, O’Grady et al. 2009), indicating allergen-driven TSLP expression. Exposure to proinflammatory cytokines such as IL-1β and TNF-α, or to Th2 cell-associated cytokines IL-4 and IL-13 alone or in combination has also been shown to induce the expression of TSLP (Bogiatzi, Fernandez et al. 2007; Kato, Favoreto et al. 2007; Lee and Ziegler 2007; Zhang, Shan et al. 2007; Miyata, Nakamura et al. 2009; Tanaka, Saga et al. 2009). Interestingly, administration of IL-13 was able to induce STAT6-dependent TSLP production in murine
epithelial cells from nasal, tracheal, lung, and skin explants, but not from the intestine (Miyata, Nakamura et al. 2009), indicating the possibility that tissue-specific regulatory mechanisms may exist. In addition to IL-13, treatment of a lung epithelial cell line, MLE12, with IL-25, another cytokine associated with the promotion of T_{H}2 cytokine-dependent immune responses, resulted in increased TSLP expression (Angkasekwinai, Park et al. 2007). Further, treatment with the T_{H}2-associated cytokine IL-33 increased TSLP mRNA expression in cecal tissues of mice infected with *Trichuris muris* (Humphreys, Xu et al. 2008).

Examination of the promoter regions for both human and murine TSLP revealed an NF-κB-binding site that is responsible for increased TSLP expression following exposure to IL-1β and TNF-α (Lee and Ziegler 2007). Recent studies focusing on keratinocyte biology have found that epidermal kallikrein (KLK5), a serine protease, upregulates TSLP expression in human keratinocytes through the activation of proteinase-activated receptor 2 (PAR2) in a NF-κB-dependent manner (Briot, Deraison et al. 2009). PAR2 was additionally shown to be important since siRNA-mediated knockdown of PAR2 expression inhibited TSLP production in response to both the allergen-derived cysteine protease papain and the prototypic protease trypsin in airway epithelial cells (Kouzaki, O'Grady et al. 2009). However, papain-induced TSLP expression was not entirely dependent upon PAR2, indicating the presence of additional mechanisms (Kouzaki, O'Grady et al. 2009). Consistent with a role for allergens in the induction of TSLP, both TSLP mRNA and protein were found to be dynamically upregulated in the dermis of atopic patients during allergen-induced late phase cutaneous responses (Corrigan, Jayaratnam et al. 2009). Clinical studies examining genetic variants of the *tslp* gene in
bronchial epithelial cells from 36 patients identified a single nucleotide polymorphism (SNP) in the promoter region of the long-form *tslp* that enhanced binding of the transcription factor activating protein (AP)-1 and increased promoter-reporter activity in response to stimulation (Harada, Hirota et al. 2009). These findings implicate for the first time a role for genetic polymorphisms in the regulation of TSLP expression.

There is speculation that TSLP can influence wound healing as expression can be upregulated by damage or trauma to epithelial cells (Allakhverdi, Comeau et al. 2007) (Figure 1). Also, TSLP treatment of human nasal epithelial cells *in vitro* resulted in enhanced barrier function and increased expression of the tight-junction proteins claudin-1, -4, -7, and occludin (Kamekura, Kojima et al. 2009). Further, studies examining the link between cigarette smoking and asthma have found that intranasal administration of cigarette smoke extract to mice upregulates TSLP mRNA and protein in the lung in an oxidative-stress dependent manner (Nakamura, Miyata et al. 2008). Also, clinical studies utilizing both bronchial biopsies and bronchoalveolar lavage (BAL) fluid samples found significantly elevated levels of TSLP mRNA positive cells and protein in patients with asthma or chronic obstructive pulmonary disease (COPD) compared to healthy, never-smoking controls (Ying, O’Connor et al. 2008). Although the molecular mediators and mechanisms of action remain unknown, recent work suggests that mast cells may also induce TSLP expression in epithelial cells, as mast cell-deficient mice fail to upregulate TSLP in a model of allergic rhinitis (Miyata, Hatsushika et al. 2008). These findings indicate a role for communication between immune cells and epithelial cells in the induction of TSLP expression.
In addition to microbial-derived signals, host-derived cytokines, and cell damage, nuclear receptors may also play a role in the regulation of TSLP. Ablation of retinoid X receptor in keratinocytes induced increased TSLP expression in these cells (Li, Messaddeq et al. 2005). Supporting this, treatment of normal keratinocytes with the nuclear receptor agonist vitamin D3 or low-calcemic analogs elevated TSLP production (Li, Hener et al. 2006). A summary of factors known to induce TSLP expression is depicted in Figure 1 and included in Table 1.

While the majority of studies to date have focused on the identification of factors known to induce TSLP production in epithelial cells, several factors have been identified that may be able to negatively regulate TSLP expression. Treatment of airway epithelial cells or primary human keratinocytes with glucocorticoids was sufficient to inhibit dsRNA-induced TSLP expression (Le, Takai et al. 2009; Miyata, Nakamura et al. 2009). Interestingly, topical administration of glucocorticoids is commonly used to treat patients with atopic dermatitis (Norris 2005), suggesting that one potential mechanism of action of the drug is the inhibition of TSLP production. Also, one study has recently proposed that 9-cis-retinoic acid may act to negatively regulate TSLP expression in airway epithelial cells (Lee, Headley et al. 2008). Given the findings that TSLP can be induced by both exogenous stimuli (damage, infection, TLR ligation) and host-derived signals, TSLP production by epithelial cells and its regulation may represent a critical pathway through which epithelial cells and the immune system communicate (Figure 1).

Although the current literature indicates that epithelial cells are a dominant source of TSLP, reports have identified hematopoietic cell-derived TSLP as mast cells and
basophils have been shown to express TSLP following stimulation. Mast cells were first identified as a possible source of TSLP when TSLP mRNA expression was examined in cDNA libraries from different primary cells. In contrast to constitutive production of TSLP by IECs, TSLP expression in mast cells requires stimulation, as cross-linking of the IgE receptor on mast cells results in elevated levels of TSLP mRNA (Soumelis, Reche et al. 2002). More recent data suggest that mast cell production of TSLP may be specific to IgE cross-linking, as treatment of mast cells with LPS, poly:IC, and IL-4 failed to induce TSLP mRNA expression. However, preincubation with IL-4 prior to IgE cross-linking significantly increased mast cell TSLP expression (Okayama, Okumura et al. 2009). Examination of suppressor of cytokine signaling 7 (SOCS7) in mast cells revealed a novel regulatory role for SOCS7 in the inhibition of TSLP production, as bone marrow-derived mast cells from Socs7 deficient mice exhibit increased production of TSLP compared to wild type (WT) controls following FcεRI-mediated activation (Knisz, Banks et al. 2009). Similar to mast cells, basophils do not appear to express TSLP in a resting state. However, basophils stimulated with papain upregulate both TSLP mRNA and protein (Sokol, Barton et al. 2008). While mast cells and basophils make TSLP in vitro, the contribution of these cellular sources in vivo remains to be fully ascertained.
Figure 1 Regulation of TSLP production

Figure 1  Epithelial cells are a source of TSLP.  TSLP expression is induced following exposure to both external and host-derived stimuli.  Modified from Saenz, Taylor et al. 2008
**Cellular targets of TSLP**

**TSLP-TSLPR signaling**

High affinity ligand binding of TSLP and subsequent signaling requires a heterodimeric receptor consisting of the IL-7Rα chain (CD127) and a unique TSLPR-α chain that resembles the common cytokine receptor γ chain (γc) (Park, Martin et al. 2000; Reche, Soumelis et al. 2001). Despite poor sequence homology between human and mouse TSLPR, both activate similar signaling pathways. Ligation of TSLPR leads to the downstream phosphorylation and activation of signal transducer and activator of transcription (STAT) 5 and STAT3 in mice, and STAT5 in humans (Isaksen, Baumann et al. 1999; Levin, Koelling et al. 1999; Sebastian, Borowski et al. 2008). TSLP-mediated STAT5 activation appears to be independent of any of the four Janus kinases (JAKs), however, partial abrogation of TSLP-mediated STAT5 phosphorylation was observed in cells expressing a dominant negative form of Tec kinase, indicating possible Tec family kinase involvement in TSLP signaling (Isaksen, Baumann et al. 1999; Levin, Koelling et al. 1999; Reche, Soumelis et al. 2001; Liu, Soumelis et al. 2007) (Figure 2). Also, studies indicate that TSLP signaling does not result in the activation of either ERK1/2 or p70S6K (Quentmeier, Drexler et al. 2001). These findings suggest that neither the mitogen-activated protein kinase (MAPK) pathway nor the p70S6K pathway are involved in TSLP-elicited signal transduction. The specific involvement of tyrosine kinases, their association with TSLPR, and the subsequent gene targets of TSLP-TSLPR interactions remain poorly defined (Figure 2).

Initially, based on mRNA and TSLP responsiveness, TSLPR expression was thought to be limited to immature B cells and myeloid cells such as monocytes and DCs (Park,
Martin et al. 2000; Reche, Soumelis et al. 2001; Liu, Soumelis et al. 2007). However, more recent work has highlighted a wider pattern of TSLPR expression as mast cells and human and murine CD4\(^+\) T cells are responsive to treatment with TSLP alone, or in conjunction with other cytokines (Allakhverdi, Smith et al. 2007; Omori and Ziegler 2007; Rochman, Watanabe et al. 2007; Sokol, Barton et al. 2008; Lu, Wang et al. 2009) (Table 1). DCs and mast cells appear to express TSLPR constitutively (Reche, Soumelis et al. 2001; Soumelis, Reche et al. 2002; Allakhverdi, Comeau et al. 2007), however CD4\(^+\) T cells appear to require TCR stimulation to induce receptor expression and become sensitive to TSLP treatment (Al-Shami, Spolski et al. 2004; Omori and Ziegler 2007; Rochman, Watanabe et al. 2007; Lu, Wang et al. 2009). As high affinity ligand binding is dependent upon the dimerization of the TSLPR-\(\alpha\) chain with the IL-7R-\(\alpha\) chain, co-expression of CD127 must also be examined. While lymphoid cell expression of CD127 has been well documented and studied (Mazzucchelli and Durum 2007), DC expression of CD127 remains more controversial with findings varying between tissue, DC subset, and activation status (Guimond, Veenstra et al. 2009; Vogt, Link et al. 2009). The recent development of a fluorochrome-conjugated TSLPR-\(\alpha\) chain-specific mAb for flow cytometry has allowed for the investigation of TSLPR complex expression on a variety of cell types directly \textit{ex vivo}. Analysis of TSLPR complex expression on cells in the GALT will be presented in Chapter 2.

Initial reports characterizing the function of TSLP on myeloid cell populations demonstrated that human monocytes and CD11c\(^+\) DCs were sensitive to TSLP treatment \textit{in vitro} and were potently stimulated to produce CCL17, a known ligand for CCR4, which is expressed on polarized T\(_{h2}\) cells (Reche, Soumelis et al. 2001).
Further, TSLP enhanced the spontaneous maturation of blood-derived human CD11c+ cells, increasing their expression of co-stimulatory molecules and their ability to promote naïve CD4+ T cell proliferation (Reche, Soumelis et al. 2001). The putative link between TSLP and Th2 cytokine responses was solidified when TSLP-treated DCs were demonstrated to drive IL-4, IL-5, and IL-13 production from naïve CD4+ T cells upon co-culture (Soumelis, Reche et al. 2002).

Mechanistic analysis demonstrated that TSLP-treatment of DCs induced multiple changes in DC function to create a permissive environment for Th2 cell differentiation. TSLP-treatment of human monocyte-derived DCs (mDCs) was shown to alter DC maturation such that the ability to upregulate major histocompatibility complex (MHC) class II and the co-stimulatory molecules CD80 and CD86 was preserved while DC cytokine production was selectively altered, thus resulting in the ability to stimulate and influence T cell differentiation (Soumelis, Reche et al. 2002; Rimoldi, Chieppa et al. 2005). TSLP-treated mDCs do not produce IL-12 and TSLP treatment of either mDCs or murine bone marrow-derived DCs (BMDCs) results in the inhibition of IL-12 production following stimulation with TLR-ligands or whole bacteria (Rimoldi, Chieppa et al. 2005; Zaph, Troy et al. 2007). TSLP-treatment also induces the expression of OX40L, which promotes Th2 cell differentiation in the absence of IL-12 (Ito, Wang et al. 2005). The presence of OX40L on TSLP-treated DCs also induces production of TNF-α in Th2 cells (instead of IL-10), suggesting these cells may have altered functions compared to conventional Th2 polarized cells (Ito, Wang et al. 2005; Liu 2006; Liu 2007). Interestingly, treatment of human DCs activated by TSLP with the imidazoquinoline compound R848 was sufficient to revert the DCs to induce Th1 cell differentiation. The
T\textsubscript{H}1 cell-inducing capacity of the R848 treated TSLP-DCs was dependent upon the induction of IL-12 expression and the repression of OX40L (Torii, Ito et al. 2008), thus highlighting the opposing roles of IL-12 and OX40L in TSLP-induced DC function. Additionally, the reversibility of the effects of TSLP on DCs upon R848 treatment suggests plasticity and that TSLP may not represent an overriding signal on DCs for T cell differentiation. The capacity of TSLP to modulate DC and T cell-derived proinflammatory cytokines will be examined in Chapter 4.

The ability of TSLP to promote T\textsubscript{H}2 cytokine responses is not confined to its abilities to influence DC function, as it is also able act on mast cells and T cells. Human mast cells have been recently demonstrated to express the functional TSLPR complex, and following exposure to TSLP they produce the T\textsubscript{H}2 cytokines IL-5 and IL-13 as well as the proinflammatory cytokines IL-6 and GM-CSF (Allakhverdi, Comeau et al. 2007). However, it is important to note that mast cells were unable to respond to TSLP alone and required IL-1 or TNF-\textalpha to induce cytokine expression (Allakhverdi, Smith et al. 2007) suggesting that a proinflammatory environment must be present for mast cells to be responsive to TSLP. Similarly, exposure to IL-33 is also able to sensitize mast cells to TSLP stimulation, resulting in mast cell production of IL-5, IL-13, GM-CSF, and enhanced production of several chemokines (Allakhverdi, Smith et al. 2007). However, IL-1, TNF-\textalpha, or IL-33 in conjunction with TSLP did not result in mast cell degranulation or mediator release (Allakhverdi, Comeau et al. 2007; Allakhverdi, Smith et al. 2007). TSLP, in conjunction with either IL-1/TNF-\textalpha or IL-33, is also able to act upon CD34\textsuperscript{+} haematopoietic progenitor cells to induce IL-5 and IL-13 production (Allakhverdi, Comeau et al. 2009). The ability of TSLP to influence CD34\textsuperscript{+} progenitor cells, which are
released from the bone marrow and following circulation into peripheral tissues differentiate into mast cells, eosinophils, or basophils indicates that TSLP may act on innate cells at multiple time points during Th2 cytokine-mediated inflammation.

*In vitro* studies demonstrated that following TCR stimulation human CD4+ T cells upregulate TSLPR expression and become sensitive to TSLP (Rochman, Watanabe et al. 2007; Lu, Wang et al. 2009). TSLP treatment of stimulated murine CD4+ T cells *in vitro* also results in the expression of IL-4 (Omori and Ziegler 2007). Collectively, these reports identify several cellular targets for TSLP *in vivo* and provide multiple mechanisms for TSLP to influence both innate and adaptive immune responses. A summary of known cellular targets in presented in Table 1.
Figure 2 TSLPR signaling pathways

Figure 2  Downstream signaling pathways activated following TSLP receptor ligation. Modified from Saenz, Taylor et al. 2008
Table 1 Summary of cellular sources, stimuli that induce expression, and cellular targets of TSLP

<table>
<thead>
<tr>
<th>Cellular sources</th>
<th>Inducing stimuli</th>
<th>Target cell populations</th>
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<tr>
<td>Thymic stromal cells</td>
<td>TLR ligands</td>
<td>Monocytes</td>
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<td>Lung ECs</td>
<td>Viruses</td>
<td>Dendritic cells</td>
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<td>Tonsilular crypt EC</td>
<td>Bacteria</td>
<td>CD4⁺ T cells</td>
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<tr>
<td>Intestinal ECs</td>
<td>Helminth parasites</td>
<td>CD8⁺ T cells</td>
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<td>Keratinocytes</td>
<td>Environmental airborne fungi</td>
<td>Mast cells</td>
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<td>Mast cells</td>
<td>IL-1, TNF-α</td>
<td>B cells</td>
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<tr>
<td>Basophils</td>
<td>IL-4, IL-13</td>
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<td></td>
<td>FcE cross linking</td>
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<td></td>
<td>Papain</td>
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<td>Trypsin</td>
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<td></td>
<td>Epidermal kallikrein (KLK5)</td>
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<td></td>
<td>Cigarette smoke extract</td>
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Role of TSLP in immune system development

Initially described as a factor able to support and promote the development of the NAG8/7 B cell line in vitro, TSLP was characterized as a B lymphocyte growth factor (Friend, Hosier et al. 1994; Sims, Williams et al. 2000). Subsequent in vitro studies demonstrated that B cell precursors must differentiate to the large pre-B stage and express the pre-B cell receptor in order to respond to TSLP treatment (Vosshenrich, Cumano et al. 2003; Vosshenrich, Cumano et al. 2004). However, in vivo studies utilizing TSLPR\(^{-/-}\) mice demonstrated no role for the TSLP pathway in B lymphopoiesis. TSLPR\(^{-/-}\) mice were indistinguishable from littermate control animals and had normal populations of B cells in adult animals (Carpino, Thierfelder et al. 2004). Despite unaltered B cell development in the absence of TSLP responsiveness, systemic overexpression of TSLP in vivo results in altered composition and frequency of B cell populations (Taneda, Segerer et al. 2001; Osborn, Ryan et al. 2004; Astrakhan, Omori et al. 2007). For example, targeted overexpression of TSLP in the skin increased circulating levels of TSLP that resulted in enhanced numbers of immature and naïve B cells in the periphery, loss of splenic marginal zone B cells, and increased numbers of peritoneal B-1b B cells (Astrakhan, Omori et al. 2007). B cell development was also impacted in a TSLP-dependent manner in a study examining the importance of Notch signaling in maintaining epidermal integrity in mice. Demehri et al. demonstrated that successive loss of Notch signaling specifically in the epidermis results in increased serum levels of TSLP, and that the increase in TSLP production was responsible for the lethal B-lymphoproliferative disorder observed in these mice (Demehri, Liu et al. 2008). While there was no evidence suggesting direct regulation of TSLP by Notch in epithelial cells, the authors link the heightened TSLP production to the inability to form fully
differentiated keratinocytes capable of barrier formation (Demehri, Liu et al. 2008). Similar to the skin transgenic model, the mice with heightened TSLP exhibited greatly expanded pre- and immature B cell compartments in the periphery (Demehri, Liu et al. 2008). Taken together, these data suggest that while TSLP is dispensable for B lymphopoiesis under normal conditions, excess production of TSLP can influence B cell development in vivo.

Despite the similarity of TSLP to IL-7 and the TSLPR chain to the $\gamma_c$, the TSLP-TSLPR pathway does not seem to be strictly required for T cell development. Examination of TSLPR$^{-/-}$ mice revealed normal T cell lymphopoiesis and normal T cell populations in adult mice (Carpino, Thierfelder et al. 2004). However, increased lymphoid defects seen in $\gamma_c$-TSLPR double deficient over $\gamma_c$ single deficient mice and the ability of transgenically overexpressed TSLP to restore lymphoid development in IL-7 deficient mice indicate that TSLP maintains the ability to support or promote T cell lymphopoiesis (Al-Shami, Spolski et al. 2004; Chappaz, Flueck et al. 2007). In vitro, TSLP was demonstrated to increase thymopoiesis in murine fetal thymic organ culture (Jiang, Coffield et al. 2007). Further, TSLP appears to influence the proliferative capacity of CD4$^+$ T cells, as addition of TSLP to TCR stimulated CD4$^+$ T cells in vitro resulted in increased cell division (Al-Shami, Spolski et al. 2004). TSLP acting on DCs may also play a role in CD4$^+$ T cell homeostasis as TSLP-treated DCs stimulated increased expansion of autologous CD4$^+$ T cells compared to control DCs (Watanabe, Hanabuchi et al. 2004). Additionally, CD4$^+$ T cells from TSLPR$^{-/-}$ mice expand less efficiently than WT CD4$^+$ T cells in irradiated hosts (Al-Shami, Spolski et al. 2004). Thus, similar to B cell development, TSLP is not
critical for T lymphopoiesis under normal conditions but alterations in its availability may impact T cell development or optimize T cell expansion under certain circumstances.

Thymic expression of TSLP has been demonstrated to function in DC activation and regulatory T cell (Treg) development. Expression of TSLP within the epithelial cells of the Hassal’s corpuscles region of the human thymic medulla correlates with DC activation and the subsequent positive selection of high-affinity self-reactive Tregs (Watanabe, Wang et al. 2005). Although mice lack the Hassal’s corpuscle structure within the thymus, there is evidence to support a potential role for murine TSLP in the direct promotion of Treg development. Supplementation of TSLP within fetal thymic organ cultures increased expression of FoxP3 and culture of thymocytes with TSLP promoted the differentiation of CD4+CD25+FoxP3+ cells (Jiang, Su et al. 2006; Lee, Lim et al. 2008). Depletion of IL-7 in the presence or absence of TSLPR was shown to produce a more severe defect in Treg development in the thymus when mice were lacking both IL-7 and TSLPR (Mazzucchelli, Hixon et al. 2008). These results suggest that TSLP and IL-7 may have some redundant and/or overlapping function in thymic Treg development.

In addition to influencing thymic Treg development, TSLP has also been proposed to influence peripheral conversion of naïve CD4+ T cells to FoxP3+ Treg cells. Studies found that TSLP-treated DCs co-cultured in vitro with total splenic CD4+ T cells increased the frequency of CD4+CD25+FoxP3+ T cells compared to either unstimulated or LPS-stimulated DCs (Besin, Gaudreau et al. 2008). Further, in vitro work using
human epithelial cells indicate that IEC-derived TSLP may be important for optimal peripheral conversion of CD4⁺ T cells into FoxP3⁺ Tregs (Iliev, Spadoni et al. 2009).

Despite these findings, the relative importance of TSLP influencing either natural or adaptive Treg populations in vivo remains controversial. Examination of TSLPR−/− mice found normal numbers of circulating CD4⁺CD25⁺FoxP3⁺ Tregs (Sun, Hall et al. 2007), indicating that TSLP is not essential for the development of natural Tregs. Additionally, analysis of surface marker expression on developing CD4⁺FoxP3⁺ Tregs in the thymus failed to show TSLPR-α chain expression, and these cells did not phosphorylate STAT-5 in response to TSLP stimulation (Vang, Yang et al. 2008). Further, lamina propria DCs isolated from TSLPR−/− mice did not exhibit any defect in mediating FoxP3 Treg conversion in vitro (Sun, Hall et al. 2007), suggesting that TSLP is also not required for adaptive Treg development in the periphery. Studies focusing on mature Treg cell survival in the periphery also failed to define a role for peripheral Treg homeostasis (Mazzucchelli, Hixon et al. 2008). Taken as a whole, these finding suggest that TSLP is not essential for B or T cell lymphopoiesis or the development/selection/maintenance of CD4⁺FoxP3⁺ Tregs in vivo. However, TSLP may be able to modulate these processes in cases of increased or altered availability.
**Functional biology of TSLP in the skin and lung**

TSLP was first associated with dysregulated T\(_H\)2 cytokine production in the human disease of atopic dermatitis. Skin biopsies from atopic dermatitis lesions showed greatly enhanced levels of TSLP compared to non-lesion biopsies (Soumelis, Reche et al. 2002). Heightened TSLP expression was also detected in the airways of asthmatic patients and correlated with disease severity (Ying, O'Connor et al. 2005). A more causal relationship between TSLP and T\(_H\)2 cytokine-mediated disease was established with transgenic over-expression models. Targeted overexpression of TSLP in epithelial cells of the skin or lung results in the development of severe T\(_H\)2-cytokine mediated inflammation resembling atopic dermatitis and asthma, respectively (Li, Messaddeq et al. 2005; Yoo, Omori et al. 2005; Zhou, Comeau et al. 2005). In both tissues, the increased expression of TSLP resulted in the development of CD4\(^+\) T\(_H\)2 cells, inflammatory cell infiltration, eosinophil infiltration, increased IgE production, and tissue damage. The CD4\(^+\) T\(_H\)2 cells that developed were highly polarized, producing high levels of IL-4 with little to no IFN-\(\gamma\) production being detected (Li, Messaddeq et al. 2005; Yoo, Omori et al. 2005; Zhou, Comeau et al. 2005).

The link between increased TSLP expression and disease has also been strengthened by the phenotype of transgenic mice whose gene alterations lead to the dysregulated overproduction of TSLP. Mice lacking nuclear hormone receptor retinoid X receptor (RXR) isotypes RXR-\(\alpha\) and RXR-\(\beta\) expression specifically in keratinocytes have significantly increased production of TSLP and develop a T\(_H\)2 cytokine-mediated chronic dermatitis that mirrors human atopic dermatitis (Li, Messaddeq et al. 2005). Further, application of topical vitamin D3 and other agonists for the RXR-\(\alpha\) and RXR-\(\beta\) signaling
pathways lead to increased TSLP expression and in turn severe chronic dermatitis (Li, Hener et al. 2006). Consistent with the connection between dysregulated TSLP production and atopic disease, mice lacking responsiveness to TSLP (TSLPR\textsuperscript{-/-}) mice are less susceptible to an OVA-induced model of allergic asthma. In this model, TSLPR\textsuperscript{-/-} animals failed to produce T\textsubscript{H}2 cell-associated cytokines and did not exhibit lung inflammation (Al-Shami, Spolski et al. 2005; Zhou, Comeau et al. 2005).

The expression of TSLP is largely restricted to barrier surfaces and it has been proposed that TSLP acts primarily in the microenvironment surrounding its site of production. While tissue-specific overexpression of TSLP in the lung resulted in increased levels of circulating total IgE, for example, the inflammatory response was predominately restricted to the lung. Activated, cytokine producing CD4\textsuperscript{+} T cells isolated from this site expressed the skin/lung homing marker CCR4. Furthermore, while CD4\textsuperscript{+} IL-4\textsuperscript{+} cells were detectable in the lung and draining lymph nodes, few cytokine positive cells were detected in peripheral lymph nodes (Zhou, Comeau et al. 2005).

While TSLP appears to primarily act within its localized microenvironment under physiological conditions, heightened systemic levels of TSLP have been demonstrated to alter systemic immune responses. For instance, examination of the K5-TSLP or K14-TSLP transgenic mice, which despite being under keratinocyte-specific promoters have overproduction of TSLP with high levels of circulating TSLP detected in the serum (Astrakhan, Omori et al. 2007; Chappaz, Flueck et al. 2007), revealed altered systemic immune responses. These mice display increased serum levels of IL-5 and elevated numbers of circulating eosinophils (Li, Messaddeq et al. 2005), as well the presence of
IL-4-producing CD4$^+$ T cells in non-skin draining lymph nodes (Zhou, Comeau et al. 2005). Further, repeated intradermal injection of recombinant TSLP into WT mice induces increased levels of serum IgE and IgG1 (Jessup, Brewer et al. 2008). Additionally, studies investigating the link between atopic dermatitis and the predisposition to the development of asthma (also known as the “atopic march”) have found that keratinocyte-derived systemic levels of TSLP increased the severity and onset of experimental asthma (Demehri, Morimoto et al. 2009; Zhang, Hener et al. 2009). Taken together, these findings suggest that increased production of TSLP extends its influence beyond barrier surfaces and may lead to predisposition of the host to multiple T$_{h2}$ cytokine-mediated pathologies.

Given the ability of TSLP to influence DC responses in vitro, TSLP may target DCs to modulate localized immune responses. Either skin or lung resident DCs could become conditioned or activated by locally produced TSLP, migrate to the draining lymph node, initiate T$_{h2}$ cell differentiation, and imprint on the T cells the proper receptors to return to sites of inflammation. The expression of TSLPR on activated CD4$^+$ T cells also raises the possibility that TSLP could act directly on infiltrating CD4$^+$ T cells to promote T$_{h2}$ cell development. These possibilities are not mutually exclusive, however, and the ability of DCs to produce the T$_{h2}$ cell attracting chemokines, CCL17 and CCL22, may also act to help initiate and/or promote local T$_{h2}$ cytokine responses. However, as DC responses in vivo have not been examined directly in any of the existing literature and cell-specific TSLPR$^{-/-}$ mice have not been reported, it remains uncertain as to which cells TSLP is acting on in vivo to initiate T$_{h2}$-cytokine mediated inflammation.
Although it remains unclear which cells TSLP targets in vivo during allergic responses, evidence exists to suggest that an innate cell component is also involved. In the K5-TSLP skin transgenic model, the contribution of T cells to the inflammatory phenotype was determined by generating K5-TSLP transgenic mice that were also T cell deficient. In the absence of T cells, immune pathology still developed and inflammatory cell infiltration was observed (Yoo, Omori et al. 2005). Also, RAG\(^{-/-}\) mice treated on the ear with the vitamin D3 low-calcemic analog MC903, which is known to induce TSLP production and atopic dermatitis, still developed epidermal hyperplasia and dermal cell infiltrate including increased numbers of eosinophils, mast cells, and dermal dendritic cells (Li, Hener et al. 2006). Thus, treatment with MC903 is able to induce TSLP expression in the absence of adaptive immune cells, and mature B and T cells are also not required for the induction of MC903-mediated inflammation in the ear (Li, Hener et al. 2006). Given the ability of TSLP, in the context of either TNF-\(\alpha\) or IL-1, to induce mast cell cytokine production (Allakhverdi, Smith et al. 2007) it is possible that mast cells play a dynamic role in either initiating or perpetuating the inflammation caused by TSLP overexpression. However, studies utilizing intradermal injection of recombinant TSLP into RAG\(^{-/-}\), athymic nude mice, mast cell deficient mice (Kit\(^{W-sh}\)), and eosinophil deficient mice (\(\Delta dblGATA\)), highlighted the importance of T cells and eosinophils in the development of TSLP-mediated skin inflammation while suggesting that mast cells played a non crucial role (Jessup, Brewer et al. 2008). It is clear that more work is required to determine whether innate cells play a role in either the initiation or the maintenance of TSLP-mediated inflammation in the skin, and whether these roles are conserved in other tissue sites of TSLP-mediated inflammation such as the lung.
While reports of TSLP-induced Th2 cytokine-mediated diseases have been largely restricted to the skin and the lung, there are new reports emerging that TSLP may play a role in the pathogenesis of other allergic diseases. Increased TSLP expression has been reported in the nasal epithelium in a mouse model of allergic rhinitis and neutralization of TSLP using a monoclonal anti-TSLP antibody inhibited the development of disease (Miyata, Hatsushika et al. 2008). Also, increased TSLP expression has been documented in the synovial fluid of arthritic joints of human patients with rheumatoid arthritis (Koyama, Ozawa et al. 2007).

**TSLP in the intestine**

Although TSLP expression in the skin and the lung has been linked to pathologic Th2 cytokine-mediated responses, TSLP expression in the intestine appears to play an important role in protective host immunity. Recent work has highlighted the importance of IECs in influencing immune cell homeostasis and immunity to helminth parasites (Rimoldi, Chieppa et al. 2005; Nenci, Becker et al. 2007; Zaph, Troy et al. 2007). IEC-specific deletion of IKKβ resulted in susceptibility to the intestinal dwelling nematode *Trichuris* and was correlated to decreased expression of TSLP (Zaph, Troy et al. 2007). Despite being on a genetically resistant background, TSLPR−/− mice challenged with *Trichuris* failed to clear worms at day 21 post-infection and produced the non-protective Th1 cytokine IFN-γ (Zaph, Troy et al. 2007). The strong induction of TSLP mRNA upon infection (Humphreys, Xu et al. 2008), and its requirement for efficient early worm clearance (Zaph, Troy et al. 2007), indicate that TSLP is a critical component of protective Th2 cytokine-mediated immune responses in the intestine.
Despite these advances in the understanding of the potential role of TSLP-TSLPR interactions in the intestine, many basic questions remain undefined and will form the focus of this thesis research. Chapter 2 will be dedicated to defining and characterizing the cellular sources and targets of TSLP in the GALT and large intestine under naïve, homeostatic conditions. Chapter 3 will explore the requirement for TSLP in the generation of a host-protective $T_{H2}$ cytokine-mediated immune response to the intestinal helminth pathogen *Trichuris muris*. Finally, Chapter 4 will investigate the potential of TSLP to regulate proinflammatory cytokine production in the intestine.
Figure 3 TSLP promotes Th2 cytokine responses. Epithelial cell-derived TSLP (1) can act to promote Th2 cytokine responses through interactions with DCs (2), naïve CD4+ T cells (3), and granulocyte populations (4).
Model Systems

Trichuris muris

Life cycle and transmission

Trichuris muris is a gastrointestinal nematode pathogen of mice that is commonly utilized to model the human whipworm pathogen, Trichuris trichiura (Cliffe and Grencis 2004). T. muris transmission occurs through the ingestion of infective eggs present in fecal contamination. It is thought that the embryonated eggs hatch in the cecum (Panesar and Croll 1980), and L1 larvae can be detected within the cecum within hours of infection (Wakelin 1967). Larvae enter into the mucosal tissue of the large intestine living within the epithelial cell layer, never penetrating through the basement membrane into the lamina propria (Lee and Wright 1978; Tilney, Connelly et al. 2005). Larvae undergo a series of four molts, reaching sexual maturity and adulthood after the fourth molt occurring on day thirty-two (Cliffe and Grencis 2004). The anterior end of the worm remains embedded within the epithelial cell layer; scanning and transmission electron micrograph studies have revealed that the worm creates syncitial tunnels within intestinal epithelial cells (Lee and Wright 1978). Though the anterior head of the worm remains burrowed within the epithelial cell layer of the large intestine throughout the life cycle, the body of the worm extends freely into the lumen of the intestine and in adulthood can be visualized by the naked human eye. The extension of the body of the worm outside of the epithelial cell layer permits copulation (Panesar and Croll 1980). The life cycle is completed when following mating the female worm releases eggs into the intestine, which are then shed within the feces of the host. The eggs must then embryonate over a period of several months to become infective (Cliffe and Grencis 2004) (Figure 4A).
Public health impact

An estimated two billion individuals worldwide are infected with soil-transmitted helminths (Colley, LoVerde et al. 2001; WHO 2009). Recent studies have estimated the global prevalence of *Trichuris trichiura* at 604 million with an additional 3.2 billion individuals at risk of infection (de Silva, Brooker et al. 2003; Bethony, Brooker et al. 2006). Children remain especially susceptible to infection and other risk factors include living within a tropical climate, and poor sanitation (Hotez, Molyneux et al. 2007). Currently, developing regions of Asia, Africa and Latin America have the highest rates of *Trichuris trichiura* infection, with the highest incident rates being focused in areas of rural poverty (Hotez, Brindley et al. 2008)

While trichuriasis itself is rarely fatal, clinical manifestations of the disease range from impaired nutritional status, growth retardation, and lower educational achievement (Callender, Grantham-McGregor et al. 1992; Nokes, Grantham-McGregor et al. 1992; Nokes and Bundy 1994) to the more severe protein-losing enteropathy, chronic dysentery, anemia, and rectal prolapse (Symons 1969; Bundy and Cooper 1989; Grencis and Cooper 1996; Albonico, Crompton et al. 1999). Additionally, 1.6 - 6.4 million disability-adjusted life years (DALY) are attributed to trichuriasis (Hotez, Fenwick et al. 2009) indicating a substantial disease burden. Treatment with the anti-helminth drugs albendazole or mebendazole is effective to induce the expulsion of the infective worms (Hotez, Brindley et al. 2008), however the procurement and distribution of these drugs remains an issue in the global fight to eradicate trichuriasis (Hotez, Fenwick et al.
The World Health Organization (WHO) estimates that less than half of the drugs needed to treat trichuriasis globally are currently available (WHO 2008).

Trichuris muris as a model of protective T\(_{\text{H}2}\) cytokine-dependent immune responses

Investigation into Trichuris muris infection began as early as 1940 (Shikhobalova 1940) but it was not until the 1960s that the importance of the immune response to infection was appreciated (Campbell 1963; Wakelin 1967). Studies comparing Trichuris muris infection across multiple mouse strains also revealed a host genetic component to resistance, as worm expulsion varies between strains, with some strains demonstrating resistance to infection and others failing to expel the worms and remaining chronically infected for the lifetime of the host (Wakelin 1975). Although variation exists between strains, the peak of the effector immune response in genetically-resistant mice is at day 21, and mice expel their worms between days 18-24 with complete clearance by day 32 post-infection.

CD4\(^+\) T cells are required for immunity to Trichuris muris. Adoptive transfer studies demonstrated that transfer of CD4\(^+\) T cells into severe combined immunodeficient mice (SCID) was sufficient to mediate resistance to infection (Else and Grencis 1996). Antibody-mediated depletion studies have demonstrated that immunity to infection can occur in the absence of CD8\(^+\) T cells (Koyama, Tamauchi et al. 1995). B cells also do not appear to be required for host resistance in an intact animal. Trichuris-infected SCID mice lacking all B and T lymphocytes were able to clear infection with the transfer of CD4\(^+\) T cells alone (Else and Grencis 1996). Additionally, \(\mu\)MT mice, which are deficient in B cells, displayed intact T\(_{\text{H}2}\) cytokine-mediated host-protective immunity
Thus while CD8⁺ T cells and B cells may contribute to resistance within an intact animal, worm expulsion and immunity can occur in their absence.

Subsequent studies analyzing the role of CD4⁺ T cells in infection have revealed that immunity to *Trichuris muris* is critically dependent on the generation of CD4⁺ T₉₂ cells (reviewed in (Abbas, Murphy et al. 1996; O'Garra 1998; Murphy and Reiner 2002)) and the production of IL-4, IL-9, and IL-13 (Else, Entwistle et al. 1993; Else, Finkelman et al. 1994; Koyama, Tamauchi et al. 1995; Else and Grencis 1996) (Figure 4B). T₉₂ cytokine production in the intestine is associated with a number of physiologic changes that correlate with worm expulsion, including mastocytosis, eosinophilia, smooth muscle contraction and changes in epithelial cell turnover, all of which have been implicated in expulsion of multiple helminth parasites (reviewed in (Finkelman, Shea-Donohue et al. 1997; Grencis 1997; Anthony, Rutitzky et al. 2007). However, mastocytosis and eosinophilia are not required for immunity to *Trichuris muris* infection, as either genetic or antibody-mediated depletion of these cell subsets failed to alter worm expulsion (Betts and Else 1999; Koyama and Ito 2000). Instead, parasite expulsion is correlated to T₉₂ cytokine-induced goblet cell hyperplasia and the production of goblet cell-specific factors such as RELMβ and Gob5 (Zhou, Dong et al. 2001; Artis, Wang et al. 2004; Zaph, Troy et al. 2007), smooth muscle hypercontractility (Khan, Richard et al. 2003), and increased epithelial cell turnover (Cliffe, Humphreys et al. 2005).

In contrast to T₉₂ cytokine-dependent resistance, susceptibility to infection is characterized by the development of CD4⁺ T₉₁ cells (reviewed in (Abbas, Murphy et al. 1996; O'Garra 1998; Murphy and Reiner 2002)).
1996; O'Garra 1998; Murphy and Reiner 2002) and is critically associated with the production of IL-12 and IFN-γ (Else, Hultner et al. 1992; Else, Entwistle et al. 1993; Else, Finkelman et al. 1994) (Figure 4B). In fact, in vivo antibody-mediated depletion of IFN-γ in susceptible strains of mice is sufficient to induce resistance and worm expulsion (Else, Finkelman et al. 1994). Conversely, administration of recombinant IL-12 to genetically resistant strains of mice results in susceptibility and chronic infection (Bancroft, Else et al. 1997).
Figure 4 *Trichuris muris* life cycle and immune response

(A) Eggs are ingested orally and hatch in the cecum (1). Larval parasites then invade IECs that line the crypts of the cecum and proximal colon (2), where they undergo four molts to become sexually mature adults and release eggs into the environment (3, 4). (B) Different *Trichuris*-specific immune responses determine resistance and susceptibility to infection in inbred mice. *T*H1 associated cytokines (IL-12, IFN-γ) promote chronic infection in AKR mice, while *T*H2 associated cytokines (IL-2, IL-9, IL-13) promote larval expulsion and immunity in resistant strains such as C57/BL6, and Balb/c.
Dextran Sulfate Sodium (DSS) colitis: a murine model of inflammatory bowel disease (IBD)

IBD: Disease and public health impact

IBD in humans comprises multiple pathologies and disorders, the two main forms being Crohn's disease and ulcerative colitis (Cho 2008). Both diseases present with systemic symptoms such as weight loss, fever, and fatigue as well as gastrointestinal symptoms ranging from abdominal pain and diarrhea to rectal bleeding (Strober, Fuss et al. 2007). Clinical onset generally occurs between age 20-30 and both diseases are characterized by their chronic and relapsing nature (Cobrin and Abreu 2005; Xavier and Podolsky 2007). Disease incidence is generally low in developing nations, with substantially higher disease rates in first world countries (Gismera and Aladren 2008). It is estimated that between 7,000 - 46,000 cases of ulcerative colitis and 10,000 - 47,000 cases of Crohn's diseases are newly diagnosed each year in North America alone (Loftus 2004).

The increasing disease rates in developed nations indicate a role for environmental factors in driving IBD (Xavier and Podolsky 2007; Gismera and Aladren 2008). Genome-wide screens have also identified a genetic component to IBD susceptibility with multiple IBD-susceptibility loci being identified. These loci include immune response genes, with mutations in the genes encoding the bacterial sensing protein NOD2 and the IL-23 receptor complex being inked to increased incidence of Crohn's disease (Xavier and Podolsky 2007).

While Crohn's disease and ulcerative colitis present with similar symptoms, they are separate diseases. Crohn's disease may target any section of the gastrointestinal tract and is characterized histopathogically by transmural inflammation, macrophage
aggregation, and the presence of non-caseating granulomas (Xavier and Podolsky 2007). In contrast, ulcerative colitis is restricted to the large intestine and results in extensive superficial ulceration with depletion of goblet cell-derived mucin, neutrophilia in the lamina propria, and micro-abscesses in the crypt (Xavier and Podolsky 2007). Immunologically, the pathology associated with Crohn's disease is characterized by increased production of IL-12, IL-23, IFN-γ, and IL-17, while ulcerative colitis is associated with excess IL-13 production (Strober, Fuss et al. 2007).

Although the exact etiology of IBD remains poorly defined, there is strong evidence to suggest that inappropriate immune responses to innocuous commensal bacteria help to drive disease (Strober, Fuss et al. 2007; Xavier and Podolsky 2007; Cho 2008; Round and Mazmanian 2009). Increased barrier permeability is associated with disease, and subsets of patients respond to antibiotic therapy (Xavier and Podolsky 2007). Additionally, multiple murine models of IBD are ameliorated if animals are either raised in germ-free conditions or treated with antibiotics (Round and Mazmanian 2009). Further, while it is difficult to determine cause versus effect, patients with IBD have altered commensal flora compared to healthy control subjects (Frank, St Amand et al. 2007).

**DSS colitis: A chemical-induced experimental colitis model**

Dextran sulfate sodium (DSS) is a 30 - 50 kDa polyanionic derivative of dextran that when administered orally at concentrations between 3 – 10% in the drinking water induces colitis in mice (Okayasu, Hatakeyama et al. 1990). DSS is thought to be toxic to IECs (Kitajima, Takuma et al. 1999), and induces IEC damage and increased barrier
permeability (Kitajima, Takuma et al. 1999). DSS-colitis presents with weight loss, alterations in fecal consistency, rectal bleeding, and increases in mouse morbidity (Okayasu, Hatakeyama et al. 1990). Histopathologically, DSS colitis is characterized by colonic shortening, immune cell recruitment, neutrophilic infiltration, loss of crypt architecture, goblet cell hypoplasia, and the formation of focal ulcerations (Okayasu, Hatakeyama et al. 1990; Melgar, Karlsson et al. 2005).

Lymphocytes do not appear to be required for the onset of colitis, as DSS-treated SCID mice still develop disease (Dieleman, Ridwan et al. 1994). However, lymphocytes are recruited to the site of inflammation and produce cytokines that contribute to the maintenance and severity of disease. For example, RAG-1 deficient mice exhibited reduced disease symptoms and severity compared to WT mice following treatment with 1.5% DSS (Kim, Seo et al. 2006). Acute colitis is characterized by CD4⁺ T cell production of IFN-γ; however, if the colitis is allowed to progress and become chronic both Th1 and Th2 CD4⁺ T cells are present (Dieleman, Palmen et al. 1998). The proinflammatory cytokines TNF-α, IL-6, and IFN-γ are though to contribute to tissue damage (Strober, Fuss et al. 2002). However, more recent work has also indicated an important role for IL-23 and IL-17 (Ogawa, Andoh et al. 2004).

Conclusion
Together Trichuris muris infection and DSS-induced colitis provide relevant model systems in which the importance of TSLP-TSLPR interactions in the intestine can be assessed in vivo. Chapter 3 of this thesis will employ the Trichuris muris infection model to explore the requirement for TSLP-TSLPR interactions in the generation of a host-
protective T\(_{\text{H}2}\) cytokine response in the intestine. The DSS colitis model will be used in Chapter 4 to examine the ability of TSLP to influence intestinal proinflammatory cytokine production and inflammation.
CHAPTER 2: Characterization of TSLP-TSLPR expression in the gastrointestinal lymphoid tissues (GALT)

Abstract
While TSLP-TSLPR interactions have been shown to influence intestinal immune homeostasis, to date the cellular sources and targets of TSLP in the GALT and the large intestine have not been comprehensively analyzed. We found that under resting steady state conditions, IECs from WT naïve mice constitutively express TSLP at both the mRNA and protein level in the cecum and large intestine. Analysis of surface co-expression of TSLP-α chain and CD127, which form the functional TSLPR complex, found TSLPR complex to be expressed on splenic and GALT CD11c+ DCs, irrespective of DC subset or activation status. In vitro studies utilizing BMDCs found the TSLPR-α chain to be dynamically upregulated by externally-derived factors and a variety of host-derived cytokines. Additionally, TSLPR complex expression was exhibited by mast cells and basophils in the spleen and by CD3+ CD4+ T cells in the spleen and GALT. Functionality of receptor expression was assessed by direct ex vivo stimulation of splenocyte or MLN cultures with rTSLP. We found that both splenic and MLN CD11c+ DCs and CD4+ T cells responded to TSLP and upregulated phospho-STAT5. Collectively these results identify potential cellular targets of TSLP-TSLPR interactions in the intestine and highlight the ability of TSLP to influence components of both the innate and adaptive immune system.
Introduction

TSLP was originally isolated and identified as a product of the thymic stromal cell clone Z210R.1, which induced the proliferation of the nonadherent B lymphoid cell line NAG8/7 (Friend, Hosier et al. 1994). Subsequent work utilizing cDNA libraries from different cells, cell lines, or purified primary cells demonstrated TSLP to be most highly expressed in the skin and lung (Soumelis, Reche et al. 2002). Although TSLP mRNA expression was noted in the small intestine during initial characterization studies (Sims, Williams et al. 2000), Rimoldi et al. were the first to demonstrate expression of TSLP protein in the large intestine. Their work identified a role TSLP in the maintenance of intestinal immune homeostasis through the preferential promotion of T\textsubscript{H}2 cell differentiation (Rimoldi, Chieppa et al. 2005). However, the expression pattern of TSLP throughout the different microenvironments of the intestinal tract remains poorly defined.

While both chains of the heterodimeric TSLPR have been previously defined (Park, Martin et al. 2000), detection of cells expressing the unique TSLPR-\(\alpha\) chain has been limited by lack of a reliable monoclonal antibody (mAb) to detect cellular expression by flow cytometry. Initial experiments utilizing binding studies identified macrophages and monocytes as having high binding affinities (Park, Martin et al. 2000). These results were confirmed and expanded to include CD11c\(^+\) DC as having high levels of expression of TSLPR, using TSLPR mRNA levels as a surrogate for surface expression (Reche, Soumelis et al. 2001). Expression of TSLPR on DCs was shown to be functional as TSLP potently activates human monocyte-derived DCs, upregulating surface expression of co-stimulatory molecules such as CD80 and CD86 and inducing the production of the chemokines CCL17 and CCL22 (Reche, Soumelis et al. 2001; Soumelis, Reche et al.
2002). Functional readouts such as activation of cytokine production have also identified mast cell and CD4\(^+\) T cell expression of TSLPR (Allakhverdi, Comeau et al. 2007; Omori and Ziegler 2007; Rochman, Watanabe et al. 2007). Despite these advances in identifying possible cellular targets of TSLP, the majority of these studies were conducted \textit{in vitro} on cultured cells. For example, although purified naïve CD4\(^+\) T cells maintained in short term culture have been shown to be responsive to TSLP (Omori and Ziegler 2007), the expression of TSLPR on CD4\(^+\) T cells taken directly \textit{ex vivo} has not been assessed. Thus, the cellular targets of TSLP \textit{in vivo} remain largely unknown; further it is not clear whether the cellular expression pattern of the TSLPR complex is altered or influenced by tissue specific environments. In this chapter, we characterize for the first time the expression of both TSLP and the TSLPR complex within the GALT and large intestine. We demonstrate constitutive production of TSLP mRNA and protein in large intestine IECs. Further, our findings identify TSLPR complex expression on DCs, mast cells, basophils, and CD4\(^+\) T cells isolated from naïve WT mice.
Materials and Methods

Animals

Wild type C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). TSLPR\(^{+/+}\) and TSLPR\(^{-/-}\) mice were obtained from Dr. James Ihle at St. Jude’s Children’s Hospital, Nashville TN. Mice were bred and maintained in a specific-pathogen free environment at the University of Pennsylvania. Animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and all experiments were carried out following the guidelines of the University of Pennsylvania IACUC.

Parasites and antigens

*Trichuris muris* was maintained in genetically susceptible animals (Wakelin 1967; Wakelin 1969); isolation of *Trichuris* excretory-secretory (ES) antigen (Ag) and eggs was carried out as previously described (Artis, Villarino et al. 2004).

Isolation and preparation of cells

At necropsy, spleen, MLN, cecal patch, peyer’s patch, were harvested and single-cell suspensions were prepared in complete T cell medium (CTCM) (DMEM supplemented with 10% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 25mM HEPES, and 5 x 10\(^{-5}\) M 2-ME). If dendritic cells were to be examined, tissues were first minced and incubated in 5 mls of digestive enzyme solution (0.5 mg/ml collagenase and 0.05% DNAse in CTCM) for 25 minutes at 37 °C while shaking prior to the creation of single-cell suspensions.
Isolation of intraepithelial and lamina propria lymphocytes

At necropsy, small intestine and large intestine were removed and put into separate Petri-dishes with pre-warmed CTCM. Cecal patch and Peyer’s patches were removed and placed in 2 mls CTCM on ice. Intestines were cut longitudinally and feces were removed by shaking in a large Petri dish of PBS. Intestines were further cleaned and mucus removed by shaking in a clean Petri dish until PBS remained clear. Intestines were then cut into small sections and placed in 20 mls prewarmed epithelial strip buffer (1mM EDTA, 1 mM DTT, 5% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin) and incubated for 20 minutes at 37 °C while shaking. Tissue was allowed to settle and supernatant was filtered through a cell strainer for isolation of intraepithelial lymphocytes (IELs). Purification of IELs was conducted through utilization of a Percoll gradient. Briefly, cells were resuspended in 5 mls of 40% Percoll, underlayed with 70% Percoll, and the spun for 20 minutes at 600 g with the brake removed. Cells were then pipetted from the interface and washed twice with CTCM, before resuspending in final volume. For isolation of lamina propria lymphocytes (LPLs), intestines were washed with serum-free media 3 times, and then minced using a razor blade in a small Petri dish. Intestines were then resuspended in 15 mls of digestive enzyme solution (0.5 mg/ml collagenase and 0.05% DNAse in CTCM) and incubated for 30 minutes 37 °C while shaking. The entire contents of each tube were then filtered through a cell strainer, and intestines were mashed in the cell strainer using the back end of a 3 ml syringe. The strainers were then washed with 15 mls CTCM. Cells were then pelleted and washed with CTCM before the Percoll purification was performed as described above.
Flow cytometry and intracellular cytokine staining

For ex vivo surface staining, cells were washed in PBS and stained with the fixable aqua blue viability dye (Invitrogen) to assess viability. Cells were then washed and stained with fluorochrome-conjugated antibodies against CD3, CD19, CD4, CD8α, CD11c, CD11b, CD103, Gr-1, FcεRI, ckit, CD49b (DX5), CD127, and TSLPR. Cells were analyzed by flow cytometry on a FACS Canto II using CellQuest Pro software (BD Pharmingen); further analysis was then carried out using FlowJo software (Treestar, Inc.). For phospho-STAT5 staining, cells were rested overnight in 2% fetal bovine CTCM at 37 °C. Cells were then stimulated with rTSLP (final concentration 100 ng/ml) for various amounts of time at 37 °C. Cells were fixed for 10 minutes at 37 °C by adding in 16% PFA for a final concentration of 2%. Cells were then washed with PBS and permeabilized for 30 minutes on ice with the addition of 1 ml of 90% ice-cold methanol. Following permeabilization, cells were washed twice with FACs buffer and stained for 1 hour at room temperature with fluorochrome-conjugated antibodies against CD4, CD11c, and Phospho-STAT5 (BD Pharmingen). Cells were then analyzed by flow cytometry as described above.

Bone marrow-derived dendritic cells

Bone marrow derived-dendritic cells (BMDCs) were derived as follows. Bone marrow from WT C57BL/6 mice was plated in 6 well cell culture plates in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 5 x 10^{-5} M 2-ME, 10% FBS, 2mM L-glutamine, and 20 ng/ml GM-CSF (Peprotech). Media was changed at days 3, 6 and 8; cells were harvested at day 9. BMDCs were plated out at 4 x 10^6 cells/ml and mixed 1:1 with either media alone, LPS (final concentration 100 ng/ml), CpG
(final concentration 10 µg/ml), *Trichuris* ES antigen, *S. mansoni* egg antigen, papain (final concentration 50ng/ml), IL-4 (final concentration 40 ng/ml), IFN-γ (final concentration 100 U/ml), IL-10 (final concentration 10 ng/ml), TGF-β (final concentration 1 ng/ml), IL-6 (final concentration 10 ng/ml), or rTSLP (final concentration 10 or 100 ng/ml from R & D), for 8 h.

**RNA isolation and real-time PCR**

RNA was isolated from intestinal tissues of mice using a Trizol extraction and from BMDCs using RNEasy Spin Columns (Qiagen). Tissues were first disrupted in a tissue homogenizer (TissueLyzer, Qiagen). RNA was extracted from purified cells using RNeasy spin columns (Qiagen). cDNA was synthesized from the isolated RNA using Superscript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out on cDNA samples using commercial primer sets (Qiagen) and SYBR Green chemistry. All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Samples are normalized to naïve controls unless otherwise stated.

**Tissue staining**

At necropsy, cecal and proximal large intestine sections were removed and snap-frozen in OCT medium (Tissue Tek, Sakura Inc.). 5 µm sections were cut and stained for TSLP using a biotinylated monoclonal anti-TSLP antibody (eBioscience, San Diego, CA).
Results

Constitutive expression of TSLP in intestinal epithelial cells

The GI tract is composed of region-specific microenvironments defined by the presence of distinct accessory cell and lymphocyte populations and varied populations of commensal bacteria (Mowat 2003; Macdonald and Monteleone 2005; Artis 2008). The large intestine differs from the small intestine in immuno-regulatory pressures as it carries a higher level of commensal bacteria and is a common site for intestinal inflammatory diseases like ulcerative colitis (Tlaskalova-Hogenova, Ste Hankova et al. 2004). We sought to examine whether basal expression of TSLP differed in these distinct microenvironments. There was low expression of TSLP in the small intestine of naïve mice (Figure 5A). TSLP mRNA expression was also detected throughout the large intestine, with the highest expression levels in the proximal large intestine (Figure 5A). TSLP protein expression was examined by immunofluorescent (IF) staining of cryosections of cecum and large intestine. In the cecum, TSLP was found predominately in mature enterocytes located at the crypt table whereas in the proximal large intestine, TSLP was expressed throughout the length of the crypt (Figure 5B). The variable expression of TSLP throughout the length of GI tract suggests differential regulation depending on microenvironment. Although the mechanisms regulating TSLP expression remain undefined, our results demonstrate constitutive IEC TSLP production in the large intestine.
Figure 5. TSLP is constitutively expressed in intestinal epithelial cells.

(A) RNA was isolated from sections of the intestine and expression of TSLP was analyzed by RT-PCR. Values represent % of actin expression in each sample. DSI, distal small intestine, Ce, cecum, prox, proximal, dis, distal. Error bars indicate s.e.m. (B) IF staining of TSLP (red) in intestinal cryosections, co-stained with DAPI (blue). Bar, 50 µm. Data represents two individual experiments with 2-3 mice per group.
**TSLPR and IL-7Rα (CD127) are co-expressed on CD11c⁺ DCs**

While both human and murine CD11c⁺ DCs have been shown to be responsive to TSLP in vitro (Soumelis, Reche et al. 2002; Rimoldi, Chieppa et al. 2005; Zaph, Troy et al. 2007), expression of TSLPR-α chain and CD127 on DCs directly ex vivo has not been extensively characterized. Further, it is not known if signals present in the tissue microenvironment can influence expression of either chain of the TSLPR complex. In order to fully characterize DC expression of TSLPR and CD127 within the GALT, cells were isolated from the spleen, mesenteric lymph node (MLN), cecal patch (CP), and the lamina propria of the large intestine (large intestine lamina propria lymphocytes LI LPLs) of naïve, WT C57/BL6 mice, stained directly ex vivo and examined by flow cytometry; DCs were gated as CD3⁻CD19⁻ CD11c⁺. The specificity of the APC-labeled anti-TSLP mAb was assessed and no cross reactivity was observed in splenic DCs isolated from TSLPR⁻/⁻ mice (Figure 6A). We found that TSLPR expression was detected on DCs isolated from the spleen and each compartment of the GALT examined, (Figure 6B upper panel). DCs isolated from the large intestine lamina propria (LI LPL) had the lowest expression levels of TSLPR. As high affinity ligand binding and signaling of TSLP requires pairing of the TSLPR-α chain with CD127, DC co-expression of CD127 was examined. CD11c⁺ TSLPR⁺ DCs co-expressed CD127 in each tissue examined (Figure 6B lower panel), indicating heterodimeric TSLPR expression. Intestinal DCs can be further separated into subsets based on expression of CD8α, CD11b, and CD103 with specific functions being ascribed to each phenotype (Kelsall 2008). Analysis of TSLPR complex expression in CD8α⁺ CD11b⁻, CD8α⁻ CD11b⁺, CD8α⁺ CD11b⁻, and CD103⁺ DCs did not reveal substantial differences in between subsets in any of the GALT tissues analyzed (data not shown). Additionally, TSLPR expression does not appear to be
linked to the activation state of DCs, as high expression of MHC class II did not correlate to increased expression of TSLPR in GALT DCs (data not shown). Taken together, these data suggest that DCs from peripheral sites such as the spleen and from the GALT tissue express the functional heterodimeric TSLPR irrespective of activation status as measured by MHC class II expression levels.

While the dynamic expression of TSLP from EC cells is known to be regulated by a number of external and host-derived factors (Summarized in Figure 1 and Table 1) (Saenz, Taylor et al. 2008), the regulation of DC TSLPR expression remains largely undefined. In order to elucidate factors that could impact DC TSLPR expression, we utilized bone marrow-derived dendritic cells (BMDCs) as in vitro model system. BMDCs were generated and found to express the TSLPR-α chain under resting conditions (Figure 6C). First we assessed whether stimulation with externally derived products such as TLR ligands, secreted helminth products, or allergens influenced TSLPR expression. Stimulation with the TLR agonists LPS and CpG resulted in little to no increase in TSLPR expression above steady state levels (Figure 6C). However, exposure of BDMCs to helminth-derived products such as Trichuris ES antigen or Schistosoma mansoni egg antigen (SEA) resulted in a small but consistent elevation of TSLPR expression (Figure 6C). These finding are consistent with increased levels of TSLPR mRNA found in whole intestinal tissue samples following Trichuris infection (Humphreys, Xu et al. 2008) or in lung tissue after exposure to Schistosoma mansoni eggs (Ramalingam, Pesce et al. 2009). Treatment with the cysteine protease allergen papain resulted in strong upregulation of TSLPR (Figure 6C). Next we tested whether cytokine signaling on the BMDCs could regulate TSLPR expression. While stimulation
with IL-4, TGF-β, and IL-6 resulted in moderate upregulation of TSLPR (Figure 6D), treatment with IFN-γ and IL-10 resulted in strong upregulation of TSLPR in BMDCs (Figure 6D). BMDCs were also treated with the T<sub>H</sub>2-associated cytokine IL-25 which failed to increase TSLPR expression (data not shown). Stimulation with rTSLP resulted in a down-regulation of surface TSLPR expression (Figure 6E). Further studies need to be done to determine if this decrease is representative of receptor endocytosis. BMDC expression of CD127 was also examined following stimulation and failed to show dynamic regulation in surface expression (data not shown). As BMDC expression of CD127 is lower than on CD11c<sup>+</sup> DCs isolated directly <em>ex vivo</em>, these results were also confirmed using RTPCR, which showed no significant changes in CD127 mRNA levels following stimulation (data not shown).

Additionally, in order to investigate the relationship between DC activation and TSLPR expression, surface expression of the co-stimulatory molecule CD86 was examined. As expected, stimulation of BMDCs with LPS and IFN-γ strongly increased CD86 expression (Figure 6C,D lower panel). However, the increase in activation status (as measured by the upregulation of co-stimulatory molecules) did not directly correlate to increased TSLPR expression, as strong upregulation of TSLPR was seen following IFN-γ but not LPS stimulation (Figure 6C,D). Further supporting this, TSLPR expression was increased in response to IL-10 and TGF-β (Figure 6C, D), two cytokines that do not activate BMDCs. These results indicate that TSLPR expression is not directly linked to the activation status of the DC.
Figure 6 GALT DCs co-express TSLPR-α chain and CD127
Figure 6 GALT DCs co-express TSLPR-α chain and CD127

Figure 6 CD11c+ DCs from the GALT co-express TSLPR and CD127. (A) Splenic CD11c+ DCs from WT and TSLPR−/− mice expression of TSLPR assayed by flow cytometry. (B) Expression of TSLPR complex on DCs isolated from the spleen, mesenteric lymph node (MLN), cecal patch (CP), large intestine lamina propria (LI LP). Top panel, TSLPR expression, plots are gated on viable CD3− CD19− CD11c+ cells. Numbers are frequencies of TSLPR+ cells. Bottom panel, CD127 co-expression, plots are gated on CD3− CD19− CD11c+ TSLPR+ cells. WT, black line, unstained controlled, solid gray histogram. (C-E) BMDC expression of TSLPR following 8 hr stimulation. Top panel TSLPR expression, plots are gated on CD11c+ cells. FMO control, solid gray histogram, media alone, black line, stimulation, dashed gray line. Numbers equal mean fluorescent intensity. Bottom panel CD86 expression, media, black line, stimulation, gray dashed line. (C) Externally-derived stimuli. (D) Host-derived stimuli. (E) rTSLP. Data represents three individual experiments with 3-4 mice per group (A, B) or two independent experiments with conditions run in duplicate (D-E).
TSLPR complex expression on macrophages and granulocytes

Although the majority of studies to date have focused on the ability of TSLP to influence DC cell function, recent reports have highlighted that TSLP may also act upon other innate cell populations such as mast cells (Allakhverdi, Comeau et al. 2007). However, whether other cells of the innate immune system express the TSLPR complex remains unknown. To investigate this, TSLPR and CD127 surface expression was analyzed on macrophages (SSCA$^\text{hi}$, F4/80$^+$), neutrophils (Gr1$^+$), eosinophils (SiglecF$^+$), basophils (CD3$^-$ CD19$^-$ FcεR1$^+$ CD49b$^+$), and mast cells (CD3$^-$ CD19$^-$ FcεR1$^+$ ckit$^+$). Cells were isolated from the blood, spleen, MLN, CP, and LI LP, and examined directly ex vivo for TSLPR complex surface expression. Surface expression did not appear to vary between tissue types examined regardless of cell population (data not shown) however, due to the rarity of certain cell subsets in GALT tissues under steady state conditions; the most robust data was generated from the spleen (Figure 7). While macrophages, neutrophils, and eosinophils expressed low levels of TSLPR, they failed to co-express CD127 (Figure 7A), indicating these cells are not responsive to TSLP in the steady state. Consistent with previous reports of mast cell sensitivity to TSLP (Allakhverdi, Comeau et al. 2007; Allakhverdi, Smith et al. 2007), mast cells exhibited co-expression of TSLPR and CD127 (Figure 7B). While basophils have been recently identified as a cellular source of TSLP (Sokol, Barton et al. 2008), they have not previously been reported to be sensitive to TSLP stimulation. However, examination of both splenic (Figure 7B) and circulating basophils in the blood (data not shown) revealed co-expression of TSLPR and CD127. In order to determine if TSLP could impact the homeostasis of either mast cell or basophil populations, rTSLP was administered i.p. for four consecutive days. Administration of rTSLP did not influence mast cell populations but resulted in a
selective increase in basophils (Figure 7C) (Perrigoue, Saenz et al. 2009). At present it remains undetermined if TSLP is inducing either increased basophil production/release from the bone marrow or if TSLP is acting to increase basophil survival in the periphery. Taken together, these findings suggest that TSLP has the ability to influence innate immunity by impacting mast cells and basophils.
Figure 7 TSLPR complex expression on macrophage and granulocyte cell populations

(A) Splenic macrophage, neutrophil, and eosinophil expression of TSLPR (top panel) and CD127 (bottom panel). WT, black histogram, FMO, gray histogram, numbers indicate mean fluorescent intensity. (B) Splenic mast cell and basophil expression of TSLPR (top panel) and CD127 (bottom panel). WT, black histogram, FMO, gray histogram, numbers indicate mean fluorescent intensity. (C) Administration of rTSLP results in an increased frequency of basophils in the blood. Flow cytometry of basophil frequencies in blood of mice treated daily for 4 d with rTSLP. Top panel, expression of IL-4-eGFP by non-B, non-T cells from the peripheral blood. Bottom panel basophil frequencies in IL-4-eGFP+, non-B, non-T cell populations. Numbers adjacent to outlined areas indicate percent among total cells of gated population. Data represents three independent experiments with 2-4 mice per group.
**CD3⁺ CD4⁺ T cells co-express TSLPR and CD127**

While TSLP maintains the ability to modulate CD4⁺ T cell function by influencing DC-derived signals (Ziegler and Liu 2006; Liu 2007; Liu, Soumelis et al. 2007), TSLP can also directly impact CD4⁺ T cell function (Omori and Ziegler 2007; Rochman, Watanabe et al. 2007; He, Oyoshi et al. 2008; Lu, Wang et al. 2009). However, the majority of these studies have been conducted *in vitro*, and it remains unclear whether CD4⁺ T cells express the TSLPR complex *in vivo* under steady state conditions. Also, it is unknown whether different tissue microenvironments can impact CD4⁺ T cell TSLPR expression.

To investigate this, lymphocytes were isolated from the spleen, MLN, CP, and LI LP and examined for TSLPR and CD127 surface co-expression by flow cytometry. Viable, CD3⁺ CD4⁺ T cells isolated from the spleen, MLN, and CP showed low but detectable levels of TSLPR expression (Figure 8). CD4⁺ T cell expression of TSLPR in the LI LP was extremely low, with only marginal staining detected above CD4⁺ T cells isolated from the LP of TSLPR⁻/⁻ mice (Figure 8). Similar to LI LP DCs, it is unknown whether this lack of expression is a result of tissue specific regulation or receptor endocytosis upon ligand binding. Co-expression of the second chain of the TSLPR complex, CD127, was also examined on CD4⁺ TSLPR⁺ cells. All CD4⁺ TSLPR⁺ T cells co-expressed CD127 irrespective of the tissue they were isolated from (Figure 8 bottom panel). As CD127 binds with the common γ chain (CD132) to form the functional IL-7 receptor, and IL-7 is a critical survival signal for CD4⁺ T cells (Mazzucchelli and Durum 2007; Rochman, Spolski et al. 2009), this result is largely expected. However, detection of the heterodimeric TSLPR complex on CD4⁺ T cells from WT naïve mice under steady state conditions indicates that TSLP may be able to influence CD4⁺ T cell function without other accessory signals or prior stimulation.
Figure 8 TSLPR and CD127 co-expression on CD3⁺ CD4⁺ T cells

Figure 8 TSLPR and CD127 co-expression on CD3⁺ CD4⁺ T cells. Top panel, TSLPR expression on CD4⁺ T cells isolated from the spleen, mesenteric lymph node (MLN), cecal patch (CP), large intestine lamina propria (LI LP). Plots are gated on viable CD3⁺ CD4⁺ cells. Numbers indicate frequencies of TSLPR⁺ cells. Solid gray histogram TSLPR⁺, black line, WT. Bottom panel, CD127 co-expression, plots are gated on CD3⁺CD4⁺TSLPR⁺ cells. Solid gray histogram, unstained controlled, WT, black line. Data represent three independent experiments with 3-4 mice per group.
**Recombinant TSLP induces phospho-STAT5 in CD11c⁺ and CD4⁺ T cells directly ex vivo**

Initial characterization of TSLP-TSLPR interactions found that binding of TSLP ligand to the TSLPR complex results in activation of STAT-5a and STAT5b in the murine B lymphoid cell line NAG8/7 and in human HepG2 cells transfected to express both chains of the receptor (Isaksen, Baumann et al. 1999; Levin, Koelling et al. 1999). More recent work has shown that following *in vitro* stimulation, both human and murine CD4⁺ T cells respond to TSLP with increased levels of phospho-STAT5 being observed (Omori and Ziegler 2007; Rochman, Watanabe et al. 2007). In order to investigate whether the co-expression of TSLPR and CD127 on both CD4⁺ T cells and DCs isolated from the GALT resulted in the formation of a functional TSLPR complex, we sought to determine if these cells could respond directly *ex vivo* to TSLP and phosphorylate STAT5.

To examine the functionality of DC TSLPR complex expression, spleens and MLNs were harvested from WT C57/B6 mice and TSLPR⁻/⁻ mice and whole unfractionated cell cultures were rested overnight in low serum media prior to stimulation with rTSLP for 15 or 30 minutes. Following stimulation, cells were immediately fixed and stained for phospho-STAT5. Stimulation with GMCSF was utilized as a positive control for DC phospho-STAT5 staining (Figure 9A, B bottom panel), as it is known to signal through STAT5 (Martinez-Moczygemba and Huston 2003). As expected, stimulation with TSLP did not result in any upregulation of phospho-STAT5 in splenic or MLN DCs isolated from TSLPR⁻/⁻ mice at any time point (Figure 9A, B top panel and data not shown). However, TSLP stimulation did result in a small but consistent upregulation of phospho-STAT5 in WT DCs in both tissues examined at 15 minutes post stimulation (Figure 9A, B
middle panel). Further, the same level of phosphorylation persisted at 30 minutes in both the spleen and MLN (data not shown), indicating sustained signaling. These results indicate that TSLPR expression on both splenic and MLN DCs is functional and that these DCs are capable of responding to TSLP without prior stimulation.

We next examined whether CD4<sup>+</sup> T cells could respond to TSLP directly ex vivo without any activation or additional stimuli. Cells were isolated and rested as previously stated prior to TSLP stimulation. Consistent with the DC results (Figure 9A, B) addition of rTSLP to spleen and MLN cell cultures from TSLPR<sup>−/−</sup> mice failed to result in the upregulation of phospho-STAT5 at any time point (Figure 9C, D and data not shown). CD4<sup>+</sup> T cells from both the spleen and the MLN responded to rTSLP with between 15-20% of CD4<sup>+</sup> T cells staining positive for phospho-STAT5 (Figure 9C, D). This level of phosphorylation was not sustained as a reduction in phospho-STAT5 cells was observed at 30 minutes following stimulation (data not shown). However, CD4<sup>+</sup> phospho-STAT5<sup>+</sup> cells were detected at time points as late as ninety minutes post-stimulation in some experiments, potentially indicating sustained signaling in some cells (data not shown). Taken together, these data demonstrate that CD4<sup>+</sup> T cells from naïve WT mice express the functional TSLPR complex and can respond to TSLP without in vitro activation or stimulation.
Figure 9 rTSLP activates phospho-STAT5 in CD4⁺ T cells and CD11c⁺ DCs

Figure 9 rTSLP activates phospho-STAT5 in CD11c⁺ and CD4⁺ T cells isolated from the GALT. Whole splenocyte or MLN cultures were rested overnight prior to stimulation with rTSLP or GMCSF. Cells were fixed and intracellularly stained and were analyzed by flow cytometry. (A) Whole splenocyte cultures, gated on CD11c⁺ cells. (B) Whole MLN, gated on CD11c⁺ cells. (C) Whole splenocyte cultures, gated on CD4⁺. (D) Whole MLN cultures, gated on CD4⁺. Numbers represent frequency of phospho-STAT5⁺ cells. Data represent one experiment with 3 mice per group.
Discussion

Collectively, these results identify the cellular sources and targets of TSLP in the GALT and large intestine. We show for the first time that TSLP is constitutively expressed throughout the large intestine at both the mRNA and protein level under homeostatic conditions. Immunofluorescent staining indicates IECs as the dominant source of TSLP. Further, we demonstrate that DCs isolated from GALT tissues co-express both chains of the TSLPR complex and can respond to rTSLP stimulation directly ex vivo. We confirm mast cell expression of the TSLPR complex and identify receptor complex expression on basophils. Additionally, administration of rTSLP selectively increases the frequency of circulating basophils. Examination of CD4+ T cells from the GALT revealed TSLPR complex expression and the ability to respond to TSLP and phosphorylate STAT5 without the presence of additional stimuli. These results indicate the complex nature of TSLP-TSLPR interactions in GALT tissues, and indicate that TSLP may directly impact both innate and adaptive immune cell populations under steady state conditions.

While the ability of TSLP to influence immune-mediated inflammation in the skin and the lung has been well characterized (Liu, Soumelis et al. 2007), the functional biology of TSLP in the intestine has only recently begun to be investigated. TSLP expression in the small intestine was noted early (Sims, Williams et al. 2000), but it was not until the work of Rescigno and colleagues that TSLP was appreciated to participate in intestinal immune homeostasis (Rimoldi, Chieppa et al. 2005). Human colonic epithelial cells were demonstrated to produce bioactive TSLP that could influence DC function (Rimoldi, Chieppa et al. 2005). Our findings in the murine system also identify IECs as a dominant source of TSLP in the intestine, but additionally highlight possible differences
in expression between different intestinal microenvironments (Figure 5). As TSLP expression can be induced through exposure to TLR ligands (Rimoldi, Chieppa et al. 2005; Allakhverdi, Comeau et al. 2007; Kato, Favoreto et al. 2007; Zeuthen, Fink et al. 2008) and commensal bacteria have been demonstrated to influence cytokine production in the intestine (Ivanov, Frutos Rde et al. 2008; Zaph, Du et al. 2008), it is possible that alterations in the abundance or composition of communal bacterial populations in the distinct regions in the GI tract may influence TSLP mRNA expression either directly or indirectly through the cytokines they induce. As epithelial cell TSLP expression can be regulated by both external and host-derived stimuli, it will be important to determine the overriding factors that regulate intestinal TSLP expression both in the steady state and in the case of disease. Not withstanding this, the high levels of basal expression and the extensive distribution pattern of TSLP in the crypts of the proximal large intestine suggests a possible regulatory function for IEC-derived TSLP in intestinal immune homeostasis.

Previously, due to the unavailability of reliable reagents to detect TSLPR surface expression, the ability to identify cellular targets of TSLP was greatly restricted. *In vitro* studies relied upon the detection of phenotypic or functional changes in TSLP-treated purified cell populations. *In vivo* studies noted alterations in mixed cell populations in the presence or absence of TSLP, making it difficult to ascertain direct versus secondary effects. The development of the fluorochrome-conjugated specific anti-TSLPR mAb has allowed for the detection of TSLPR⁺ cells directly *ex vivo* without cell manipulation or stimulation.
Consistent with previous literature we found high levels of TSLPR complex expression in CD11c+ DCs (Park, Martin et al. 2000; Reche, Soumelis et al. 2001; Liu, Soumelis et al. 2007) isolated from the spleen. Importantly, we were also able to detect TSLPR complex expression on DCs isolated from GALT tissues (Figure 6), and verify its functionality (Figure 9), supporting a role for IEC-derived TSLP modulating intestinal DC function (Rimoldi, Chieppa et al. 2005). However, DCs isolated from the LP, the most proximal GALT tissue to the IEC-derived TSLP, demonstrated reduced surface expression of TSLPR (Figure 6A). Consistent with this, stimulation of BMDCs with rTSLP also resulted in downregulation of TSLPR (Figure 6E). More studies need to be conducted to understand if the lower surface expression is a result of receptor endocytosis following ligand binding or the result of a regulatory mechanism following stimulation. Conversely, the microenvironment and cytokine milieu of the LP is unique and distinct from the other tissues sampled and it is possible that other signals are acting to regulate LP DC TSLPR expression.

Our in vitro BMDC studies examining the regulation of TSLPR-α chain expression indicate that receptor surface expression can be modulated by a variety of stimuli. Stimulation with TLR agonists LPS and CpG failed to upregulate TSLPR expression (Figure 6C) indicating that the TLR signaling pathway may not influence receptor expression. However, as TSLP expression is more strongly upregulated by dsRNA stimulation and TLR3 signaling (Kato, Favoreto et al. 2007), a wider variety of TLR agonists should be sampled as possible stimuli to regulate TSLPR expression. Consistent with in vivo infection studies (Humphreys, Xu et al. 2008; Ramalingam, Pesce et al. 2009), Ag products derived from Trichuris and S. mansoni stimulated a
small but detectable up regulation of TSLPR expression in BMDCs (Figure 6C). These results indicate that in situations of infection, pathogens may directly modulate TSLPR expression. However, as these products remain unfractionated, the actual proteins or molecules responsible for the upregulation are unknown. Stimulation with the cysteine protease allergen papain resulted in strong upregulation of TSLPR expression in BMDCs (Figure 6C). As previous reports have shown papain to also target basophils (Sokol, Barton et al. 2008; Sokol, Chu et al. 2009), it is possible that papain may also influence basophil TSLPR expression.

Our BMDC studies also demonstrate that both pro and anti-inflammatory cytokines can impact surface levels of TSLPR expression. While treatment with IL-4, IL-6, and TGF-β all resulted in moderate upregulation of TSLPR expression, stimulation with IFN-γ and IL-10 resulted in the strongest increase in receptor expression (Figure 6D). These results indicate that at least in BMDCs, regulation of TSLPR expression is not exclusive to T_{H1}, T_{H2}, or anti-inflammatory cytokines. As the tested cytokines signal through a variety of pathways, and multiple JAKs and STATs are involved it remains difficult to determine the mechanism of regulation. Nevertheless, these findings indicate another layer of regulation on DC TSLP-TSLPR interactions. Taken together with our in vivo studies, these results highlight CD11c^{+} DCs as important cellular targets of TSLP within the GALT.

TSLP is also able to impact other innate immune cells as both mast cells and basophils express the TSLPR complex (Figure 7). Mast cells have previously been recognized to express both chains of the TSLPR complex at the mRNA level and to respond to
stimulation with TSLP when in the presence of accessory signals such as IL-1β and TNF-α (Allakhverdi, Comeau et al. 2007). We confirm mast cell expression of the TSLPR complex and also show that basophils express TSLPR (Figure 7). Further, administration of rTSLP resulted in a selective increase in frequencies of basophils, but not mast cells in the spleen and the blood (Figure 7) (Perrigoue, Saenz et al. 2009). The mechanism responsible for the increased accumulation of basophils remains unclear. TSLP may be acting to increase basophil survival in the periphery or to increase basophil release from the bone marrow. While it remains unknown if TSLP is directly signaling on basophils or is acting through an accessory cell population, expansion of the basophil population indicates a potential mechanism for TSLP to influence innate immune responses. As both mast cells and basophils are known to be important effectors in T helper 2 (Th2) cytokine-mediated inflammation and immune responses (Galli 2000; Galli, Kalesnikoff et al. 2005; Min 2008; Min and Paul 2008) and TSLP is known to promote Th2 cytokine-mediated inflammation (Liu 2006; Liu, Soumelis et al. 2007), TSLP may in part be acting through the activation or recruitment of these cell populations. Importantly, in situations of overexpression, TSLP has been shown to promote Th2 cytokine-mediated in the absence of T or T and B cells (Yoo, Omori et al. 2005; Li, Hener et al. 2006). Collectively, these findings implicate basophils and mast cells as relevant in vivo cellular targets of TSLP that must taken into account when analyzing the impact of TSLP-TSLPR interactions on innate and adaptive immunity.

TSLP has been demonstrated to act on CD4+ T cells in vitro following stimulation/activation (Omori and Ziegler 2007; Rochman, Watanabe et al. 2007; Lu, Wang et al. 2009). Our findings suggest that CD4+ T cells isolated from the spleen and
GALT express both chains of the TSLPR complex under steady state conditions \textit{in vivo} (Figure 8) and can also respond directly \textit{ex vivo} to stimulation with rTSLP (Figure 9). These results indicate that in addition to potentially influencing activated CD4^+ T effector cells during an immune response; TSLP may also directly act on CD4^+ T cells during homeostatic conditions. While the contribution of TSLP signaling on CD4^+ T cells \textit{in vivo} remains unknown, the ability of TSLP to directly influence T cell function must be recognized and accounted for in future experiments.

Overall, these data indicate that within the intestinal immune system, IEC-derived TSLP maintains the ability to target and impact cellular components of both the innate and adaptive immune response. Thus, the TSLP-TSLPR interactions in the GALT represent a complex network with many potential players.
CHAPTER 3: TSLP is required for immunity to Trichuris muris, however TSLP independent \(\text{T}_h 2\) cytokine dependent immune responses can develop in the absence of IFN-\(\gamma\)

Abstract

Intestinal epithelial cells (IECs) produce thymic stromal lymphopoietin (TSLP), however the \textit{in vivo} influence of TSLP-TSLP receptor (TSLPR) interactions on immunity and inflammation in the intestine remains unclear. Here we show that TSLP-TSLPR interactions are critical for immunity to the intestinal pathogen \textit{Trichuris}. Monoclonal antibody-mediated neutralization of TSLP or deletion of the TSLPR in normally resistant mice resulted in defective expression of \(\text{T}_h 2\) cytokines and persistent infection. Susceptibility was accompanied by elevated production of IFN-\(\gamma\) and IL-17A and the development of severe intestinal inflammation. Critically, neutralization of IFN-\(\gamma\) in \textit{Trichuris}-infected TSLPR\(^{-/-}\) mice restored \(\text{T}_h 2\) cytokine responses and resulted in worm expulsion, providing the first demonstration of TSLPR-independent pathways for \(\text{T}_h 2\) cytokine production.
**Introduction**

Originally characterized as a B cell growth factor, TSLP is now known for its ability to promote T\(_{H2}\) cytokine responses both *in vitro* and *in vivo*. Clinically, TSLP has been linked to the T\(_{H2}\) cytokine-mediated pathologies of atopic dermatitis and asthma. Skin biopsies from atopic dermatitis lesions showed greatly enhanced levels of TSLP compared with non-lesion biopsies (Soumelis, Reche et al. 2002), and heightened TSLP expression was detected in the airways of asthmatic patients and correlated with disease severity (Ying, O'Connor et al. 2005).

*In vitro* studies demonstrated that TSLP-conditioned human DCs can promote T\(_{H2}\) cell responses (Soumelis, Reche et al. 2002; Al-Shami, Spolski et al. 2005; Ito, Wang et al. 2005; Rimoldi, Chieppa et al. 2005). Mechanistically, TSLP treatment of DCs induces T\(_{H2}\) cell differentiation by inhibiting IL-12 production while simultaneously inducing OX40L expression (Ito, Wang et al. 2005; Rimoldi, Chieppa et al. 2005; Liu 2007). The *in vivo* functions of TSLP have been most extensively studied in the skin and the lung (Soumelis, Reche et al. 2002; Al-Shami, Spolski et al. 2005; Yoo, Omori et al. 2005; Zhou, Comeau et al. 2005). Transgenic over-expression of TSLP in cutaneous or pulmonary epithelial cells results in the onset of T\(_{H2}\) cytokine-mediated inflammation resembling atopic dermatitis or asthma respectively (Yoo, Omori et al. 2005; Zhou, Comeau et al. 2005). Based on these studies it has been proposed that TSLP is both necessary and sufficient for the initiation of T\(_{H2}\) cytokine-driven inflammation (Liu 2006; Ziegler and Liu 2006; Liu, Soumelis et al. 2007).
While TSLP is clearly linked to the promotion of pathogenic Th2 cytokine-mediated immune responses in the skin and the lung, the role for TSLP in the intestine remains less defined. Expression of TSLP under steady state conditions has been shown to be important in maintaining intestinal immune hyporesponsiveness (Rimoldi, Chieppa et al. 2005). Additionally, we recently showed that TSLP responsiveness is an important component of early immunity to the intestinal nematode pathogen *Trichuris* (Zaph, Troy et al. 2007). However, the mechanisms and absolute requirements for TSLP-TSLPR interactions in the regulation of intestinal immunity and inflammation *in vivo* remain unknown. In this chapter we characterize the role of TSLP-TSLPR interactions in immunity to *Trichuris* infection. We demonstrate that in the absence of TSLP-TSLPR interactions mice become susceptible to infection and develop severe infection-induced inflammation accompanied by increased production of proinflammatory cytokines. However, in the absence of IFN-γ, TSLP is not required for resistance to *Trichuris*, indicating TSLP-independent Th2 cytokine responses in the intestine.
Materials and Methods

Animals
Wild type C57BL/6J transgenic mice were obtained from Jackson Laboratories (Bar Harbor, ME). TSLPR\(^{+/+}\) and TSLPR\(^{-/-}\) mice were obtained from Dr. James Ihle at St. Jude’s Children’s Hospital, Nashville TN. Mice were bred and maintained in a specific-pathogen free environment at the University of Pennsylvania. Animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and all experiments were carried out following the guidelines of the University of Pennsylvania IACUC.

Parasites and antigens
Trichuris muris was maintained in genetically susceptible animals (Wakelin 1967; Wakelin 1969); isolation of Trichuris excretory-secretory (ES) antigen (Ag) and eggs was carried out as previously described (Artis, Villarino et al. 2004). Mice were infected on day 0 with 150-200 embryonated eggs and parasite burdens were assessed at various time points following infection. Serum was analyzed by ELISA for Trichuris-specific IgG2a as previously described (Artis, Shapira et al. 2002).

Cytokines and monoclonal antibodies
Neutralizing monoclonal antibody against IFN-\(\gamma\) (XMG-6) was purified from ascites by ammonium sulfate precipitation and dialyzed against PBS. Mice were injected i.p. with 1 mg antibody at the time of infection and every five days post-infection. Neutralizing monoclonal antibody against murine TSLP (M702) was obtained from Amgen. Mice
were injected i.p. with 1 mg antibody at the time of infection and every five days post-infection.

**Isolation of cells and flow cytometry staining**

At necropsy, MLN were harvested and single-cell suspensions were prepared in DMEM supplemented with 10% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25mM HEPES, and 5 x 10⁻⁶ M 2-ME. A portion of cells was analyzed *ex vivo* for cytokine production by stimulating with 10 µg/ml brefeldin A, 50 ng/ml PMA, and 750 ng/ml ionomycin for 4-5 h. For DC experiments, MLN cells were restimulated in the presence of *T. muris* ES antigen (50 µg/ml) and BFA (10 µg/ml). Cells were then harvested and stained with fluorochrome-conjugated antibodies against CD4, IFN-γ, IL-13, CD11c, CD8α, CD11b, TNF-α and IL-12/23p40. Cells were analyzed by flow cytometry on a FACScalibur using CellQuest Pro software (BD Pharmingen); further analysis was then carried out using FlowJo software (Treestar, Inc.). MLN cells were restimulated by plating cells in medium alone, in the presence of *T. muris* ES Ag (50 µg/ml), or with 1 µg/ml each of soluble α-CD3 and α-CD28 antibodies. Following 72 h in culture, cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 4-5 h. Cell-free supernatants were then harvested and analyzed for cytokine secretion by sandwich ELISA (eBioscience, San Diego, CA). The cells were then harvested and analyzed for cytokine production as described for the *ex vivo* analysis.

**Tissue staining and RELMβ responses**

At necropsy, cecal sections were removed and fixed in 4% paraformaldehyde or snap-frozen in OCT medium (Tissue Tek, Sakura Inc.). 5 µm paraffin-embedded sections
were cut and stained with H&E, PAS/Alcian Blue, or by IF for RELMβ and GOB5 for visualization of goblet cell responses. IF staining was carried out as previously described (Zaph, Troy et al. 2007). Fecal protein isolation was performed as previously described (Artis, Wang et al. 2004). Samples were equalized by protein content and analyzed by SDS-PAGE and then immunoblotted for RELMβ using a polyclonal rabbit α-murine RELMβ antibody (Peprotech, Rocky Hill, NJ).

RNA isolation and real-time PCR

RNA was isolated from intestinal tissues of mice using a Trizol extraction and from BMDCs using RNEasy Spin Columns (Qiagen). Tissues were first disrupted in a tissue homogenizer (TissueLyzer, Qiagen). cDNA was synthesized from the isolated RNA using Superscript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out on cDNA samples using commercial primer sets (Qiagen) and SYBR Green chemistry. All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Samples are normalized to naïve controls unless otherwise stated.
Results

In vivo neutralization of TSLP in WT mice results in susceptibility to Trichuris

Challenge of genetically resistant mice with the intestinal nematode pathogen *Trichuris* results in the dynamic upregulation of TSLP mRNA in the large intestine early during infection (Humphreys, Xu et al. 2008). Further, we recently demonstrated that in the absence of the TSLP receptor, genetically resistant mice show an early defect in the clearance of *Trichuris* (Zaph, Troy et al. 2007). However, TSLP has been shown to influence lymphocyte development both *in vivo* and *in vitro* (Levin, Koelling et al. 1999; Sims, Williams et al. 2000; Chappaz, Flueck et al. 2007) and previous work has established that alterations in the circulating levels of TSLP can influence the frequency and composition of B cell populations *in vivo* (Astrakhan, Omori et al. 2007). To investigate whether the impaired early immunity to infection in the TSLPR$^-$ mice was due to alterations in immune cell development or a definitive requirement for TSLP in protective immunity, we neutralized endogenous TSLP in genetically resistant animals during *Trichuris* infection using a neutralizing anti-TSLP monoclonal antibody (mAb). While mesenteric lymph node (MLN) cells isolated from control treated WT mice at day 21 post-infection produced IL-4 and IL-13 following antigen-specific restimulation, MLN cells isolated from anti-TSLP mAb-treated mice exhibited significantly reduced expression of these cytokines (Figure 10A). Consistent with a defect in T$_H^2$ cell differentiation *in vivo*, the frequency of IL-13$^+$ CD4$^+$ T cells was lower in MLN isolated from anti-TSLP mAb-treated than in control-treated mice (Figure 10B). Expression of T$_H^2$ cytokines in the intestine leads to physiological changes in the intestinal epithelium including increased cell turnover, goblet cell hyperplasia, and the elevated expression of goblet cell-associated genes that are correlated with worm expulsion (Theodoropoulos,
Hicks et al. 2001; Artis, Wang et al. 2004; Artis 2006; Nair, Guild et al. 2006; Wilson, Zaph et al. 2006; Zaph, Rook et al. 2006; Anthony, Rutitzky et al. 2007). Histological examination of ceca isolated from infected WT animals revealed goblet cell hyperplasia and increased mucin staining, consistent with the presence of $T_h2$ cytokines (Figure 10C). In contrast, mucin staining of cecal tissue sections from anti-TSLP mAb-treated mice failed to show detectable goblet cell responses (Figure 10C). Expression of the goblet cell-specific proteins RELMβ and GOB5 were also decreased in the anti-TSLP mAb treated mice (Figure 10D). Further, RELMβ secretion, as determined by protein analysis of fecal samples, was also defective in infected mice treated with anti-TSLP mAb (Figure 10E). Consistent with these defective $T_h2$ cytokine responses, anti-TSLP mAb treated mice failed to exhibit worm expulsion at day 21 post-infection (Figure 10F). These results show that optimal expression of TSLP is critical for the development of pathogen-specific $T_h2$ cytokine responses and early immunity to *Trichuris*. 
Figure 10 *In vivo* neutralization of TSLP in WT mice results in susceptibility to *Trichuris muris*
Figure 10  *In vivo* neutralization of TSLP in WT mice results in susceptibility to *Trichurus muris*

Figure 10. *In vivo* neutralization of TSLP in WT mice results in susceptibility to *Trichurus*. (A) Antigen-specific cytokine production from restimulated MLN cells was determined by ELISA. (B) Frequencies of CD4^+^ IL-13^+^ T cells in the MLN following 72h Ag restimulation. Plots are gated on CD4^+^ T cells. (C - E) Anti-TSLP treated mice fail to induce goblet cell responses following infection. (C) Paraffin sections of cecal tissue were stained with PAS/Alcian blue for goblet cell visualization. (D) Paraffin sections of cecal tissue were IF stained for RELMβ and GOB5 (green) and co-stained with DAPI (blue). (E) Protein isolated from fecal pellets on various days post infection was analyzed by SDS-PAGE and immunoblotted for RELMβ, N – naïve, uninfected control. (F) Worm burdens were assessed at day 21 post-infection. Bar, 50 µm. Error bars indicate s.e.m. Data represents three individual experiments with 3-6 mice per group.
TSLP-TSLPR interactions are critical for immunity to *Trichuris*

Deletion of TSLPR in normally resistant mice resulted in increased IFN-γ production and elevated worm burdens at day 21 post-infection (Zaph, Troy et al. 2007). In genetically resistant strains of mice, CD4+ T<sub>H2</sub> cell-mediated expulsion of *Trichuris* occurs between days 18-21 while genetically susceptible mice develop persistent infection and retain parasites for the lifetime of the host (Else, Finkelman et al. 1994). However, impaired early worm expulsion is not always indicative of a failed host-protective response. For example, *Trichuris*-infected mice deficient in ICOS fail to expel worms at day 18 post-infection but are able to successfully mount a protective T<sub>H2</sub> cytokine-mediated response and clear infection by day 34 post-infection (Wilson, Zaph et al. 2006). Thus TSLP-TSLPR interactions could be required for optimal early T<sub>H2</sub> cytokine responses but not for subsequent T<sub>H2</sub> cell-mediated clearance of infection.

To determine whether blockade of the TSLP-TSLPR pathway resulted in persistent chronic infection or simply resulted in delayed worm expulsion, TSLPR<sup>+/+</sup> and TSLPR<sup>−/−</sup> mice were infected with *Trichuris* and the kinetics of worm expulsion were monitored over 34 days. MLN cells isolated from infected TSLPR<sup>−/−</sup> mice failed to produce detectable levels of T<sub>H2</sub> cytokines (Figure 11A). Histological examination of cecal sections also revealed defective T<sub>H2</sub> cytokine-dependent goblet cell hyperplasia in infected TSLPR<sup>−/−</sup> mice in comparison to infected TSLPR<sup>+/+</sup> mice (Figure 11B). Further, production of goblet cell-derived RELMβ and GOB5 was decreased in infected TSLPR<sup>−/−</sup> mice compared to infected TSLPR<sup>+/+</sup> mice (Figure 11C). Luminal secretion of RELMβ was also decreased and unsustained in infected TSLPR<sup>−/−</sup> mice (Figure 11D). The defective T<sub>H2</sub> cytokine responses functionally altered the outcome of infection, as
TSLPR−/− mice failed to mediate worm expulsion even at a late time points and remained heavily infected at day 34 post-infection (Figure 11E). In addition, chronic infection was also observed in WT mice treated with anti-TSLP mAb as they exhibited high worm burdens at day 34 post-infection (Figure 12). Taken together, neutralization of TSLP and deletion of the TSLPR identify a critical role for TSLP-TSLPR interactions in the development of a host protective T\textsubscript{H}2 cytokine-dependent immune response to *Trichuris*. 
Figure 11 TSLP-TSLPR interactions are critical for immunity to *Trichuris muris*

Figure 11. TSLP responsiveness is required for immunity to *Trichuris muris*. (A) TSLPR<sup>−/−</sup> mice are unable to induce T<sub>H</sub>2 cytokine production. Antigen-specific cytokine production from restimulated MLN cells was determined by ELISA. (B-D) TSLPR<sup>−/−</sup> mice have decreased goblet cell responses. (B) Cecal sections from TSLPR<sup>+/+</sup> or TSLPR<sup>−/−</sup> mice were taken at day 20 post-infection and stained PAS/Alcian blue. (C) Paraffin sections of cecal tissue were IF stained for RELM<sub>β</sub> and GOB5 (green) and co-stained with DAPI (blue). (D) Protein isolated from fecal pellets on various days post infection was analyzed by SDS-PAGE and immunoblotted for RELM<sub>β</sub>. (E) Worm burdens in TSLPR<sup>+/+</sup> and TSLPR<sup>−/−</sup> mice were assessed at days 20 and 34 post-infection. Bar, 50 µm. Error bars indicate s.e.m. Data represents three individual experiments with 3-4 mice per group.
Figure 12. *In vivo* depletion of TSLP in WT mice results in chronic *Trichuris* infection. Worm burdens were assessed at day 34 post-infection. Error bars indicate s.e.m. Results are representative of two individual experiments with 3-4 mice per group.
Increased infection-induced inflammation and proinflammatory cytokine production in the absence of TSLP-TSLPR interactions

The defective production of $T_h2$ cytokines and susceptibility to *Trichurus* following the disruption of the TSLP-TSLPR pathway could be the result of impaired responsiveness to infection or dysregulation of T helper cell responses. Histological examination of cecal sections taken at day 34 post-infection revealed immune-mediated alterations in both WT and TSLPR$^{−/−}$ mice (Figure 13A). Cecal sections from WT mice exhibited minimal to mild submucosal edema, mixed inflammatory cell infiltrate and mild crypt hyperplasia indicative of a recent infection. In contrast, TSLPR$^{−/−}$ mice exhibited severe infection-induced inflammation characterized by severe submucosal edema and transmural inflammation with lymphocytic infiltrate in the muscularis and mixed lymphocytic and neutrophilic infiltrate in the submucosa and lamina propria (Figure 13A). Additionally, IECs in the TSLPR$^{−/−}$ mice appeared activated, and numerous mitotic figures were observed (Figure 13B, arrows). TSLPR$^{−/−}$ mice also exhibited foci of inflammation with disruption of crypt architecture (Figure 13C). Depletion of TSLP in WT mice also resulted in severe-infection induced inflammation. Histological examination of day 34 post-infection cecal sections showed severe submucosal edema and transmural inflammation in the anti-TSLP treated mice, but not the WT mice (Figure 14 A, B). Further, anti-TSLP mice exhibited foci of inflammation (Figure 14 C, left panel) and immune cell infiltration in between the crypts (Figure 14 C, right panel), both of which were absent in WT mice (Figure 14 B). The severe infection-induced inflammation exhibited in the absence of TSLP-TSLPR interactions contrasts with the mild to moderate inflammation seen in genetically susceptible AKR mice that also exhibit
The presence of severe intestinal inflammation in the TSLPR$^{-/-}$ mice suggested that susceptibility to *Trichuris* was not solely due to a lack of T$_{H2}$ responsiveness, but involved a more general dysregulation of infection-induced innate and adaptive immune responses. Consistent with this hypothesis, there was a significant increase in the frequency of CD4$^{+}$ IFN-γ$^{+}$ T cells isolated from the MLN of TSLPR$^{-/-}$ mice at day 21 post-infection compared to WT mice (Figure 13D). Restimulated MLN cells isolated from infected TSLPR$^{-/-}$ mice exhibited significantly elevated production of IFN-γ and IL-17A in comparison to infected WT mice following either antigen-specific or polyclonal stimulation (Figure 13E, F). As anti-TSLP treated WT mice also developed severe infection-induced inflammation, we investigated proinflammatory cytokine production in these mice. Similar to the findings with the TSLPR$^{-/-}$ mice, increased frequencies of CD4$^{+}$ IFN-γ$^{+}$ T cells were detected in MLNs isolated from anti-TSLP treated WT mice compared to WT control mice at day 21 post-infection (Figure 15A). MLN cells from anti-TSLP treated mice also produced significantly increased levels of IFN-γ compared to WT mice following Ag-specific restimulation (Figure 15B). Consistent with the upregulation of IFN-γ protein, anti-TSLP treated mice also exhibited increased levels of IFN-γ mRNA in the MLN compared to controls (Figure 15C). Also, isolation of RNA from large intestine tissue samples revealed a trend towards increased levels of IL-17A mRNA in the anti-TSLP treated mice compared to WT mice (Figure 15D). Dysregulated proinflammatory cytokine expression persisted in chronic infection, as infected TSLPR$^{-/-}$ mice showed increased frequencies of CD4$^{+}$ IFN-γ$^{+}$ T cells in MLN compared to WT mice at day 34.
post-infection (Figure 16). The heightened expression of IFN-γ and IL-17A and severe intestinal inflammation exhibited upon disruption of the TSLP-TSLPR pathway demonstrates that susceptibility to infection is not solely due the impairment of T\textsubscript{H}2 cytokine responses but also due to the development of an unregulated proinflammatory cytokine response.
Figure 13 TSLPR<sup>-/-</sup> mice have increased infection-induced inflammation and proinflammatory cytokine production.

Paraffin-embedded cecal sections from day 34 post-infection were stained with H&E. (B) Epithelial cells in TSLPR<sup>-/-</sup> mice exhibit numerous mitotic figures (arrowheads). (C) TSLPR<sup>-/-</sup> mice exhibit foci of inflammation with loss of crypt architecture. (D-G) TSLPR<sup>-/-</sup> mice have increased proinflammatory cytokine production at day 20 post-infection. (D) Frequencies of CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells in the MLN at day 20 post-infection. (E) Antigen-specific IFN-γ production from restimulated MLN was determined by ELISA. (F) Polyclonal IL-17A production from restimulated MLN was determined by ELISA. Error bars indicate s.e.m. Data represent two to three individual experiments with 3–4 mice per group.
Figure 14 Anti-TSLP treatment in WT mice results in increased infection-induced inflammation

Figure 14. Depletion of TSLP in *Trichuris* infected WT mice results in infection-induced inflammation. Paraffin-embedded cecal sections from day 34 post-infection were stained with H&E. (A). Anti-TSLP mAb-treated mice displayed severe submucosal edema with mixed inflammatory cell infiltrate. Second panel represents a greater magnification of boxed areas in first panel. (B) Increased magnification of a representative cecal section from WT mice showing minimal submucosal edema. (C) Increased magnification of representative cecal cross sections of anti-TSLP treated mice. Sections show foci of inflammation (left panel) and lymphocytic infiltration between crypts (right). Bar, 50 µm. Results are representative of two individual experiments with 3-4 mice per group.
Figure 15 Anti-TSLP treatment in WT mice results in increased proinflammatory cytokine production.

Figure 15. Depletion of TSLP in *Trichuris* infected WT mice results in increased expression of IFN-γ and IL-17. (A) Anti-TSLP mAb treated mice have increased frequencies of CD4+ IFN-γ+ T cells in the MLN at day 21 post-infection. Plots are gated on CD4+ cells. (B) Antigen-specific IFN-γ from restimulated MLN cells by ELISA. (C) Anti-TSLP mAb treated mice have increased mRNA transcripts for IFN-γ in the MLN at day 21-post infection. (D) Anti-TSLP mAb treated mice have increased mRNA transcript for IL-17A in the large intestine at day 34 post-infection. Error bars indicate s.e.m. Results are representative of three individual experiments with 3-6 mice per group.
Figure 16 TSLPR<sup>−/−</sup> mice exhibit elevated frequencies of CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells in the MLN at day 34 post-infection.

Figure 16. Infected TSLPR<sup>−/−</sup> mice have increased frequencies of CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells in the MLN at day 34 post-infection. Plots are gated on CD4<sup>+</sup> cells. Results are representative of three individual experiments with 3-4 mice per group.
TSLP is not required to initiate Th2 cytokine responses in the intestine

Previous studies in both skin and lung models of Th2 cytokine-mediated allergic inflammation indicated that TSLP is both necessary and sufficient to drive the development of Th2 cytokine-dependent inflammation, suggesting that TSLP may be a ‘master switch’ for the development of Th2 cytokine responses (Yoo, Omori et al. 2005; Zhou, Comeau et al. 2005; Liu 2006). The impaired immunity to *Trichuris* exhibited following disruption of the TSLP-TSLPR pathway supports this model. However, anti-TSLP mAb treatment or deletion of TSLPR resulted in heightened CD4+ T cell-derived IFN-γ production. To test whether TSLP-TSLPR interactions were absolutely required or dispensable for *Trichuris*-induced Th2 cytokine responses, *Trichuris*-infected TSLPR−/− mice were treated with neutralizing monoclonal antibodies against IFN-γ throughout the course of infection. If TSLP-TSLPR interactions were necessary for Th2 cytokine responses, anti-IFN-γ treated TSLPR−/− mice would remain susceptible to *Trichuris*.

Following neutralization of IFN-γ in infected TSLPR−/− mice there was a marked reduction in the frequency of IFN-γ+ CD4+ T cells in the MLN at day 21 post-infection compared to control treated TSLPR+/− mice, consistent with the role of IFN-γ in promoting Th1 responses (Figure 17A). Coincident with decreased levels of IFN-γ there was an increase in levels of IL-4, IL-5, and IL-13 secreted from antigen-restimulated MLN cells from anti-IFN-γ treated TSLPR−/− mice compared to the control treated TSLPR+/− mice (Figure 17B). Critically, the levels of IL-4, IL-5, and IL-13 in the anti-IFN-γ treated TSLPR−/− mice were comparable to those seen in resistant TSLPR+/+ mice (Figure 17B). Blockade of IFN-γ did not impact *Trichuris*-specific IgG1 serum antibody levels (data not shown), but did inhibit the development of a *Trichuris*-specific IgG2a serum antibody
response, as serum levels of IgG2a in anti-IFN-γ treated TSLPR+/− mice were comparable to levels in TSLPR+/+ mice (Figure 17C). Goblet cell responses were restored as the expression of both GOB5 and RELMβ were increased in anti-IFN-γ treated TSLPR+/− mice (Figure 17D,E). Further, histological examination revealed that the severe crypt elongation, edema and inflammatory infiltrate present in the Trichuris-infected TSLPR+/− mice were absent following anti-IFN-γ treatment (Figure 17F). Critically, anti-IFN-γ treatment also recovered immunity in the TSLPR+/− mice. While control treated TSLPR+/− mice exhibited persistent infection, anti-IFN-γ treated mice exhibited reduced worm burdens at day 21 post-infection with subsequent worm expulsion and clearance by day 34 post-infection (Figure 17G). The delay in worm clearance from day 21 to day 34 post-infection in the anti-IFN-γ treated TSLPR+/− mice indicates that while TSLP-TSLPR interactions are not required for immunity, they may play an important role in the development of optimal protective immune responses following Trichuris infection. Anti-IFN-γ treatment also augmented levels of total serum IgE (Figure 18). These results demonstrate that in the absence of IFN-γ in an in vivo system, TSLP-independent mechanisms allow for the generation of a protective Th2 cytokine response in the intestine.
Figure 17 Neutralization of IFN-γ in susceptible TSLPR−/− mice restores T_{H}2 cytokine production and immunity
Figure 17 Neutralization of IFN-γ in susceptible TSLPR<sup>-/-</sup> mice restores T<sub>h</sub>2 cytokine production and immunity

Figure 17. TSLP is not required for the initiation of an intestinal T<sub>h</sub>2 cytokine response to *Trichuris*. (A) Frequencies of CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells isolated from the MLN and stimulated directly *ex vivo*. (B) Antigen-specific cytokine production from restimulated MLN cells was determined by ELISA. (C) Antigen-specific serum IgG2a levels. (D, E) Antibody treatment restored goblet cell function in TSLPR<sup>-/-</sup> mice. (D) Protein isolated from fecal pellets on various days post infection was analyzed by SDS-PAGE and immunoblotted for RELMβ. (E) Paraffin sections of cecal tissue were IF stained for RELMβ and GOB5 (green) and co-stained with DAPI (blue). (F) Day 34 cecal sections were stained with H&E. (G) Worm burdens were assessed at days 21 and 34 post-infection. Bars, 50 µm. Error bars indicate s.e.m. Data are representative of two individual experiments with 3-4 mice per group.
Figure 18. Anti-IFN-γ treatment of *Trichuris*-infected TSLPR<sup>+/−</sup> mice increases total serum IgE levels at day 21 post-infection. Error bars indicate s.e.m. Data is representative of two individual experiments with 3-4 mice per group.
Discussion

These findings indicate a key role for TSLP-TSLPR interactions in regulating intestinal immunity and inflammation. We show for the first time that the TSLP-TSLPR pathway within the intestine is critical for host-protective immunity, as disruption of TSLP-TSLPR interactions in vivo led to susceptibility to Trichuris infection. Following infection, anti-TSLP mAb-treated WT mice and TSLPR\(^{-/-}\) mice remained chronically infected, had elevated expression of IFN-\(\gamma\) and IL-17A, and exhibited severe infection-induced inflammation. However, unlike models of inflammation in the skin or the lung, we found that TSLP was not critically required for pathogen-specific T\(_{H2}\) cytokine responses in the intestine. Infected TSLPR\(^{-/-}\) mice treated with neutralizing anti-IFN-\(\gamma\) antibodies recovered pathogen-specific T\(_{H2}\) cytokine production and immunity, thus identifying TSLP-independent pathways for the initiation of intestinal T\(_{H2}\) cytokine responses.

The putative link between TSLP and the promotion of T\(_{H2}\) cytokine responses has been well developed in both in vitro and in vivo studies (Liu 2006; Ziegler and Liu 2006; Liu, Soumelis et al. 2007). Initial in vitro studies found that human monocyte-derived DCs treated with TSLP induced CD4\(^{+}\) T cells to produce IL-4 upon co-culture (Soumelis, Reche et al. 2002; Al-Shami, Spolski et al. 2005; Rimoldi, Chieppa et al. 2005), and that TSLP-induced upregulation of OX40L on DCs promoted CD4\(^{+}\) T\(_{H2}\) cell differentiation (Ito, Wang et al. 2005). Recent work also demonstrated the ability of TSLP to directly influence both human and murine CD4\(^{+}\) T cells (Omori and Ziegler 2007; Rochman, Spolski et al. 2009). In the murine system, in vitro treatment of purified CD4\(^{+}\) T cells with TSLP induced T cell IL-4 production and T\(_{H2}\) cell (Omori and Ziegler 2007). Further, in the presence of TNF-\(\alpha\) or IL-1, TSLP has been shown to induce production of the T\(_{H2}\)
cytokines IL-5 and IL-13 from mast cells (Allakhverdi, Comeau et al. 2007). Supporting these *in vitro* studies, the transgenic over-expression of TSLP by epithelial cells in the skin or the lung induces T\(\text{H}_2\) cytokine-mediated inflammation *in vivo* (Yoo, Omori et al. 2005; Zhou, Comeau et al. 2005). Thus, through its ability to influence both innate cell populations as well as CD4\(^+\) T cells, TSLP could contribute both directly and indirectly to T\(\text{H}_2\) cell differentiation *in vivo*.

TSLP may act to directly influence T\(\text{H}_2\) cell differentiation by directly influencing CD4\(^+\) T cell cytokine production. For example, TSLP has been shown to promote *il-4* gene transcription in CD4\(^+\) T cells (Omori and Ziegler 2007). While the exact mechanisms remain unknown, it is possible that TSLP-induced STAT5 activation plays a role in promoting *il-4* gene transcription independently of IL-4, STAT6, or GATA-3 (Zhu, Cote-Sierra et al. 2003; Takatori, Nakajima et al. 2005; Zhu, Yamane et al. 2006). Once transcription is initiated, IL-4 may act in an autocrine fashion on CD4\(^+\) T cell to activate STAT6 and upregulate GATA-3 expression, promoting and stabilizing T\(\text{H}_2\) cell differentiation (Murphy and Reiner 2002; Mowen and Glimcher 2004).

Indirectly, TSLP could promote T\(\text{H}_2\) cell differentiation by influencing innate cell responses. TSLP may induce mast cell production of IL-13, which could signal through STAT6 to promote proximal events including GATA-3 upregulation in T\(\text{H}_2\) cell differentiation (Murphy and Reiner 2002; Mowen and Glimcher 2004). Additionally, administration of rTSLP elicits increased frequencies of basophils (Chapter 2 and (Perrigoue, Saenz et al. 2009)), and basophils have recently been demonstrated to be important antigen presenting cells in the initiation of T\(\text{H}_2\) cytokine immune responses.
(Perrigoue, Saenz et al. 2009; Sokol, Chu et al. 2009; Yoshimoto, Yasuda et al. 2009). Neither of these possibilities is mutually exclusive however, and it is likely that TSLP acts through multiple pathways to promote T\(_H\)2 cell differentiation and T\(_H\)2 cytokine production.

Beyond an influence on T\(_H\)2 cytokine production, TSLP may also regulate recruitment and/or retention of immune cells at the site of infection or inflammation. For example, TSLP treatment of human mDCs induces expression of the chemokines CCL17 and CCL22, known ligands for CCR4, a chemokine receptor found on effector T\(_H\)2 cells (Soumelis, Reche et al. 2002; Liu 2006). Thus following exposure to *Trichuris*, IEC-derived TSLP could be acting on intestinal DCs to simultaneously create conditions permissive to T\(_H\)2 cell differentiation and to promote T\(_H\)2 effector cell recruitment or retention in the intestine.

While these data support a role for TSLP in directly influencing and promoting T\(_H\)2 cytokine responses, our finding that TSLP was not critically required for the initiation of a pathogen-specific T\(_H\)2 cytokine response following *Trichuris* infection demonstrates that there are TSLP-independent pathways for the generation of intestinal T\(_H\)2 cytokine responses. In the absence of TSLP it is possible that a combination of other T\(_H\)2-promoting factors present in the intestine is able to compensate and either act alone or in concert to create a microenvironment permissive for T\(_H\)2 cell differentiation. Candidates include cytokines such as IL-25 and IL-33 as they have each been demonstrated to promote T\(_H\)2 cytokine responses (Fort, Cheung et al. 2001; Schmitz, Owyang et al. 2005). Further, both IL-25 and IL-33 have been shown to play important
roles in immunity to *Trichuris* infection (Owyang, Zaph et al. 2006; Humphreys, Xu et al. 2008; Saenz 2008). The Notch-Notch ligand pathway may also play a role in the generation of TSLP-independent T\(_h\)2 cytokine responses in the intestine. Previous work has demonstrated that Notch-Notch ligand interactions are important in mediating immunity to *Trichuris* (Tu, Fang et al. 2005), and Notch may act either directly in the promotion of T\(_h\)2 cell differentiation (Ansel, Djuretic et al. 2006; Amsen, Antov et al. 2007; Fang, Yashiro-Ohtani et al. 2007; Kubo 2007; Perrigoue, Marshall et al. 2008) or through its influence on epithelial cell function (Demehri, Liu et al. 2008). Thus, TSLP may be another factor that, while not itself essential for the development of T\(_h\)2 cytokine responses in the intestine, is critical in combination with a number of other factors to create a T\(_h\)2 permissive microenvironment. Additionally, as anti-IFN-\(\gamma\) treated TSLPR\(^{-}\) mice displayed delayed worm clearance during *Trichuris* infection, TSLP-TSLPR interactions may also be important in determining the magnitude and/or kinetics of T\(_h\)2 cytokine responses in the intestine. As TSLP does not appear to be strictly required for intestinal T\(_h\)2 cytokine responses, and loss of TSLP-TSLPR interactions resulted in increased infection-induced proinflammatory cytokine production and inflammation, it is possible that TSLP may also have another regulatory role in the intestine. The hypothesis that TSLP is important in the regulation of intestinal proinflammatory cytokine production will be tested in Chapter 4.
CHAPTER 4: A role for TSLP-TSLPR interactions in the inhibition of proinflammatory cytokine production in the intestine

Abstract

The importance of TSLP-TSLPR interactions in the promotion of Th2 cytokine-mediated inflammation has been well documented and studied. However, we previously demonstrated that TSLP is not critically required for intestinal Th2 cytokine responses, and that in the absence of TSLP-TSLPR interactions mice infected with Trichuris exhibit elevated proinflammatory cytokine production and increased infection-induced inflammation in the large intestine. Despite these findings, little is known about the potential of TSLP-TSLPR interactions to regulate intestinal proinflammatory cytokine production. Here, we demonstrate that naïve TSLPR\textsuperscript{−/−} mice have a trend towards increased frequencies of CD4\textsuperscript{+} IFN-γ\textsuperscript{+} T cells in the GALT and intestine compared to naïve littermate control TSLPR\textsuperscript{+/+} mice. Introduction of rTSLP into whole splenocyte cultures was also sufficient to inhibit IFN-γ production from CD4\textsuperscript{+} T cells in the absence of IL-4, indicating an IL-4-independent pathway of TSLP-dependent inhibition of IFN-γ production by CD4\textsuperscript{+} T cells. TSLP was found to inhibit DC-derived IL-12/23p40 production both in vitro and in vivo. Additionally, TSLPR\textsuperscript{−/−} mice were found to be more susceptible to DSS-induced colitis, exhibiting increased proinflammatory cytokine production and more severe intestinal inflammation compared to DSS-treated WT mice. Collectively, these findings suggest that TSLP-TSLPR interactions in the GALT play a previously unrecognized role in the regulation of proinflammatory cytokine production and inflammation in the intestine.
**Introduction**

TSLP-TSLPR interactions maintain the ability to promote T\(_H\)2 cytokine-mediated immune responses through a variety of mechanisms. TSLP can influence DC (Soumelis, Reche et al. 2002; Ito, Wang et al. 2005; Rimoldi, Chieppa et al. 2005; Zaph, Troy et al. 2007), CD4\(^+\) T cell (Omori and Ziegler 2007; Rochman, Watanabe et al. 2007), and mast cell function (Allakhverdi, Comeau et al. 2007) to promote T\(_H\)2 cytokine production. As a result of these diverse abilities, TSLP has been recognized as a critical factor in the promotion of T\(_H\)2 cytokine-mediated inflammation in the skin and the lung (Soumelis, Reche et al. 2002; Al-Shami, Spolski et al. 2005; Yoo, Omori et al. 2005; Zhou, Comeau et al. 2005; Liu 2006; Liu, Soumelis et al. 2007). Further, TSLP-TSLPR interactions are important in the development of a host-protective T\(_H\)2 cytokine response to infection with intestinal pathogen *Trichuris muris* (Chapter 3, (Zaph, Troy et al. 2007).

In addition to these functions, there is also evidence to suggest that TSLP may play an immunoregulatory role in the inhibition of proinflammatory cytokine production within the intestine. The ability of TSLP to modulate DC production of the T\(_H\)1 cytokine-promoting factor IL-12 has been previously documented (Soumelis, Reche et al. 2002; Ito, Wang et al. 2005; Rimoldi, Chieppa et al. 2005; Zaph, Troy et al. 2007). Within the intestine, constitutive IEC production of TSLP was found to be important in the maintenance of intestinal immune hyporesponsiveness through the promotion of T\(_H\)2 cell differentiation and the inhibition of IFN-\(\gamma\) production (Rimoldi, Chieppa et al. 2005). Further, disruption of the TSLP-TSLPR pathway in mice results in increased infection-induced inflammation and elevated levels of IFN-\(\gamma\) and IL-17A following infection with *Trichuris* (Chapter 3 (Zaph, Troy et al. 2007)).
Additionally, while TSLP-TSLPR interactions were critical for the development of a host-protective T\textsubscript{H}2 cytokine-mediated immune response to infection with the intestinal parasite *Trichuris*, TSLP-independent T\textsubscript{H}2 cytokine immune responses developed in the absence of IFN-\textgamma (Chapter 3 (Taylor, Zaph et al. 2009)). TSLP was also not required for T\textsubscript{H}2 cytokine-mediated immunity to *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* (Massacand, Stettler et al. 2009). These data indicate that TSLP is not critically required for the development of intestinal host-protective T\textsubscript{H}2 cytokine responses. Taken together with the findings of dysregulated proinflammatory cytokine production in the absence of TSLP, these data suggest that TSLP may also have a regulatory function within the intestine.

In this chapter, we demonstrate dysregulated production of GALT DC-derived IL-12/23p40 and CD4\textsuperscript{+} T cell-derived IFN-\textgamma in naïve TSLPR\textsuperscript{-/-} mice. While this disruption of intestinal immune homeostasis was not sufficient to result in the development of spontaneous colitis, TSLPR\textsuperscript{-/-} mice do exhibit increased susceptibility to DSS-induced colitis. Further, we demonstrate that IEC-derived TSLP regulates DC production of IL-12/23p40 both *in vitro* and *in vivo*, and that addition of rTSLP into whole splenocyte cultures is able to inhibit CD4\textsuperscript{+} T cell production of IFN-\textgamma in the absence of IL-4. As a whole, these data indicate that in addition to its ability to promote T\textsubscript{H}2 cytokine responses, TSLP also acts to limit proinflammatory cytokine production and inflammation in the intestine.
Materials and Methods

Animals

Wild type C57BL/6J transgenic mice were obtained from Jackson Laboratories (Bar Harbor, ME). TSLPR\(^{+/+}\) and TSLPR\(^{-/-}\) mice were obtained from Dr. James Ihle at St. Jude’s Children’s Hospital, Nashville TN. Mice were bred and maintained in a specific-pathogen free environment at the University of Pennsylvania. Animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and all experiments were carried out following the guidelines of the University of Pennsylvania IACUC.

DSS-induced intestinal inflammation.

DSS (MP Biomedicals) was added to drinking water at 5% weight/volume for four days. Mice were monitored for weight loss, rectal bleeding and general appearance (piloerection, lethargy). Pathology was scored as follows: A) Weight loss, No change = 0; <5% = 1; 6–10% = 2; 11–20% = 3; >20% = 4; B) Feces, Normal = 0; Pasty, semi-formed = 2; Liquid, sticky or unable to defecate after five minutes = 4; C) Blood, No blood = 0; Visible blood in rectum = 1; Visible blood on fur = 2; D) General appearance, Normal = 0; Piloerection = 1; Lethargy and piloerection = 2; Motionless, sickly = 4.

Isolation and preparation of cells

At necropsy, spleen, MLN, cecal patch, peyer’s patch, were harvested and single-cell suspensions were prepared in complete T cell medium (CTCM) (DMEM supplemented with 10% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 25mM HEPES, and 5 \(\times\) 10\(^{-5}\) M 2-ME). If dendritic cells were to be
examined, tissues were first minced and incubated in 5 mls of digestive enzyme solution (0.5 mg/ml collagenase and 0.05% DNAse in CTCM) for 25 minutes at 37 °C while shaking prior to the creation of single-cell suspensions.

Isolation of intraepithelial and lamina propria lymphocytes

At necropsy, small intestine and large intestine were removed and put into separate Petri dishes with pre-warmed CTCM. Cecal patch and Peyer’s patches were removed and placed in 2 mls CTCM on ice. Intestines were cut longitudinally and feces were removed by shaking in a large Petri dish of PBS. Intestines were further cleaned and mucus removed by shaking in a clean Petri dish until PBS remained clear. Intestines were then cut into small sections and placed in 20 mls prewarmed epithelial strip buffer (1mM EDTA, 1 mM DTT, 5% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin) and incubated for 20 minutes at 37 °C while shaking. Tissue was allowed to settle and supernatant was filtered through a cell strainer for isolation of intraepithelial lymphocytes (IELs). Purification of IELs was conducted though utilization of a Percoll gradient. Briefly, cells were resuspended in 5 mls of 40% Percoll, underlayed with 70% Percoll, and the spun for 20 minutes at 600 g with the brake removed. Cells were then pipetted from the interface and washed twice with CTCM, before resuspending in final volume. For isolation of lamina propria lymphocytes (LPLs), intestines were washed with serum-free media 3 times, and then minced using a razor blade in a small Petri dish. Intestines were then resuspended in 15 mls of digestive enzyme solution (0.5 mg/ml collagenase and 0.05% DNAase in CTCM) and incubated for 30 minutes 37 °C while shaking. The entire contents of each tube were then filtered through a cell strainer, and intestines were mashed in the cell strainer using the back end of a 3 ml syringe. The strainers
were then washed with 15 mls CTCM. Cells were then pelleted and washed with CTCM before the Percoll purification was preformed as described above.

Flow cytometry and intracellular cytokine staining

A portion of cells was analyzed ex vivo for cytokine production by stimulating with 10 \( \mu \text{g/ml} \) brefeldin A, 50 ng/ml PMA, and 750 ng/ml ionomycin for 4-5 h. For DC experiments, MLN cells were restimulated in the presence of *T. muris* ES antigen (50 \( \mu \text{g/ml} \)) and BFA (10 \( \mu \text{g/ml} \)). Cells were then harvested, washed in PBS and stained with the fixable aqua blue viability dye (Invitrogen) to assess viability. Cells were then stained with fluorochrome-conjugated antibodies against CD3, CD4, IFN-\( \gamma \), IL-17A, IL-10, FoxP3, CD11c, CD8\( \alpha \), CD11b, TNF-\( \alpha \), and IL-12/23p40. Cells were analyzed by flow cytometry on a FACS Calibur or a FACS CANTOII using CellQuest Pro software (BD Pharmingen); further analysis was then carried out using FlowJo software (Treestar, Inc.). MLN cells were restimulated by plating cells in medium alone, in the presence of *T. muris* ES Ag (50 \( \mu \text{g/ml} \)), or with 1 \( \mu \text{g/ml} \) each of soluble \( \alpha \)-CD3 and \( \alpha \)-CD28 antibodies. Following 72 h in culture, cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 4-5 h. Cell-free supernatants were then harvested and analyzed for cytokine secretion by sandwich ELISA (eBioscience, San Diego, CA). The cells were then harvested and analyzed for cytokine production as described for the ex vivo analysis.

BMDCs and BMDC: T cell co-culture

Bone marrow derived-dendritic cells (BMDCs) were derived as follows. Bone marrow from WT C57BL/6 mice was plated in 6 well cell culture plates in RPMI 1640
supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 5 x 10^{-5} M 2-ME, 10% FBS, 2mM L-glutamine, and 20 ng/ml GM-CSF (Peprotech). Media was changed at days 3, 6 and 8; cells were harvested at day 9. BMDCs were plated out at 4 x 10^6 cells/ml and mixed 1:1 with either media alone, IEC supernatants (CMT-93 cell line), or rTSLP (final concentration 10 or 100 ng/ml from R & D), for 16-18 h. Cells were stimulated with LPS (10 ng/ml). At various time points cell-free supernatants were harvested for analysis by ELISA and cells were harvested either for analysis by flow cytometry or RT-PCR for mRNA expression. For BMDC: T cell co-culture, BMDCs were harvested and conditioned with CMT-93 supernatants or rTSLP as described above. They were then pulsed with 0.5 mg/ml ovalbumin (Worthington) and 1 µg/ml CpG (Coley Pharmaceutical Group) for 16-18 h. Purified OT-II CD4^+ T cells were cultured with the BMDCs for 4 days. Cells were stimulated with PMA, ionomycin, and brefeldin A for the final 4 h and analyzed by flow cytometry.

_Tissue staining_
At necropsy, sections of the proximal large intestine were removed, flushed with PBS, and fixed in 4% paraformaldehyde. 5 µm paraffin-embedded sections were cut and stained with H&E.

_RNA isolation and real-time PCR_
RNA was isolated from intestinal tissues of mice using a Trizol extraction and from BMDCs using RNEasy Spin Columns (Qiagen). Tissues were first disrupted in a tissue homogenizer (TissueLyzer, Qiagen). RNA was extracted from purified cells using RNeasy spin columns (Qiagen). cDNA was synthesized from the isolated RNA using
Superscript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out on cDNA samples using commercial primer sets (Qiagen) and SYBR Green chemistry. All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Samples are normalized to naïve controls unless otherwise stated.
Results

**Naïve TSLPR$$^-/-$$ mice exhibit increased frequencies of CD4$$^+$$ IFN-$$^\gamma$$+T cells in the GALT**

The increased levels of IFN-$$^\gamma$$ and IL-17A seen in TSLPR$$^-/-$$ mice following *Trichuris* infection is indicative of dysregulated T helper cell differentiation within the GALT. Further, IEC-derived TSLP has been previously demonstrated to be important in maintaining intestinal immune cell homeostasis (Rimoldi, Chieppa et al. 2005). To investigate whether TSLPR$$^-/-$$ mice have altered homeostasis of intestinal CD4$$^+$$ T cells, cells were isolated from the GALT and large intestine of naïve TSLPR$$^-/-$$ mice and TSLPR$$^{+/+}$$ controls, and examined ex vivo following PMA/Ionomycin stimulation for cytokine production. As the composition of the commensal bacteria within the intestine can greatly impact CD4$$^+$$ IL-17A production (Ivanov, Frutos Rde et al. 2008), experiments were performed with littermate control mice raised in the same SPF facility.

A trend towards increased frequencies of CD4$$^+$$ IFN-$$^\gamma$$+ T cells was observed in the MLN and CP of TSLPR$$^-/-$$ mice compared to littermate controls (Figure 19A). This trend was most apparent in the MLN and was consistent among three individual experiments ($p = 0.04$, $p = 0.06$, $p = 0.1$). Although individual experiments did not reach statistical significance in the CP, combined data from 3 experiments representing a total of 10 mice show a trend towards increased frequencies of CD4$$^+$$ IFN-$$^\gamma$$+ T cells in the TSLPR$$^-/-$$ mice compared to TSLPR$$^{+/+}$$ mice ($p = 0.08$). While slight increases of CD4$$^+$$ IFN-$$^\gamma$$+ T cells were observed in the PP and in both the IEL and LPL compartments of the large intestine lamina propria of TSLPR$$^-/-$$ mice compared to TSLPR$$^{+/+}$$ littermate controls (Figure 19A), these numbers did not reach statistical significance ($p > 0.1$) due to
variability within groups and limited cell recovery from the lamina propria. Although preliminary in nature, these results indicate that in the steady state TSLP-TSLPR interactions may be playing a role in limiting frequencies of CD4+ IFN-γ+ T cells in the GALT. Frequencies of CD4+ IL-17A+ positive T cells in the GALT were also examined and revealed a slight trend towards increased frequencies of CD4+ IL-17A+ T cells in TSLPR−/− mice in the MLN, CP, and PP (Figure 19B). However, these differences did not reach statistical significance (p > 0.1) in any of the three experiments performed. Additionally, no differences were detected between TSLPR+/+ and TSLPR−/− mice in either the IEL or LPL compartments of the large intestine (Figure 19B).

As dysregulation of IFN-γ could be due to either alteration or impairment of other regulatory mechanisms known to be important in the intestine, we also examined the frequency of CD4+ IL-10+ and CD4+ FoxP3+ T cells in the GALT of naïve mice. We observed no significant differences in the frequency of CD4+ IL-10+ T cells in TSLPR−/− mice compared to TSLPR+/+ littermate controls in the MLN, CP, PP, or the IEL or LPL compartments of the large intestine (Figure 20A). Further, examination of the frequencies of CD4+ FoxP3+ T cells in GALT did not reveal any consistent decreases in the TSLPR−/− mice in comparison to littermate control TSLPR+/+ mice. (Figure 20B). Despite the preliminary nature of this work, these findings indicate that the dysregulation of proinflammatory cytokines was not due to an absence of CD4+ FoxP3+ or CD4+ IL-10+ cells. While TSLPR−/− mice do not develop spontaneous colitis, these data suggest that even in the steady state, TSLP-TSLPR interactions may play a role in the limitation of proinflammatory cytokine production in the intestine.
Naïve TSLPR<sup>−/−</sup> have increased frequencies of CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells in the GALT

Figure 19. Naïve TSLPR<sup>−/−</sup> mice have increased frequencies of CD4<sup>+</sup> IFN-γ<sup>+</sup> and CD4<sup>+</sup>IL-17A<sup>+</sup> cells in the GALT compared to naïve littermate control TSLPR<sup>+/+</sup> mice. Cells were isolated from the mesenteric lymph node (MLN), cecal patch (CP), peyer’s patch (PP), large intestine intraepithelial lymphoid cells (LI IEL), large intestine lamina propria (LI LPL), and are gated on viable, CD3<sup>+</sup>, CD4<sup>+</sup>. (A) IFN-γ. (B) IL-17A. Numbers are average of CD3<sup>+</sup>CD4<sup>+</sup> cytokine<sup>+</sup> cells from two mice per group and are representative of three independent experiments with 2-4 mice per group.
Figure 20. Frequencies of GALT CD4\(^+\) IL-10\(^+\) and CD4\(^+\) FoxP3\(^+\) T cells in naïve TSLPR\(^{+/−}\) mice.

(A) IL-10. (B) FoxP3. Numbers are average of CD3\(^+\) CD4\(^+\) cytokine\(^+\) cells from two mice per group and are representative of two independent experiments with 2-4 mice per group.
rTSLP treatment of whole splenocyte cultures results in decreased frequencies of CD4+ IFN-γ+ T cells

The increased frequencies of CD4+ IFN-γ+ T cells in the GALT of naïve TSLPR−/− mice suggests that TSLP maybe regulating CD4+ T cell production of IFN-γ. However, as the mice utilized in those experiments have a germ-line deletion of TSLPR, the findings may be confounded by developmental defects. In order to rule out the possibility of any confounding developmental defects, an in vitro culture system utilizing WT mice was employed. Splenocytes from WT C57/BL6 mice were cultured for three days in the presence or absence of rTSLP. Cells were cultured with soluble α-CD3/α-CD28 and either α-IL-4/α-IFN-γ to stimulate under neutral conditions or α-IL-4/rIL-12 for T_H1 permissive conditions. Under neutral conditions, the addition of rTSLP decreased the frequency of CD4+ IFN-γ+ T cells by a third compared to the untreated control (Figure 21A). Examination of the MFI of the IFN-γ+ cells also revealed a decrease with rTSLP supplementation, indicating that the cells treated with rTSLP were producing less cytokine on a per cell basis compared to cells in the control wells (Figure 21A). rTSLP treatment in the T_H1 permissive conditions also resulted in a decrease in both the frequency of CD4+ IFN-γ+ cells and the MFI of cytokine positive cells (Figure 21A). However, the inhibition of IFN-γ was not as striking as that seen in the neutral conditions. In order to ascertain if rTSLP had an impact on IFN-γ protein production under T_H1 polarizing conditions, cell free supernatants were harvested and analyzed by ELISA. Treatment with rTSLP significantly decreased the amount of IFN-γ protein secreted into the supernatants (Figure 21B), indicating that rTSLP was able to substantially inhibit IFN-γ production under T_H1 permissive conditions. It remains possible that the inhibition of IFN-γ was a secondary result of TSLP’s known ability to drive IL-4 production, which is
well known to counter-regulate CD4$^+$ T cell production of IFN-γ. However, the inhibition of IFN-γ occurred in the presence of IL-4 neutralizing mAb in both neutral and T$_{H}$$^1$ permissive conditions, indicating another mechanism of action. Taken together with our in vivo findings, these results indicate that rTSLP is able to modulate IFN-γ production by CD4$^+$ T cells.
Figure 21. Administration of rTSLP inhibits CD4⁺ IFN-γ production. Whole splenocytes were cultured with soluble αCD3/αCD28, rTSLP was supplemented daily at a concentration of 50 ng/ml. (A) Intracellular cytokine staining of cells harvested at day 3. Horizontal numbers indicate frequency of IFN-γ⁺CD4⁺ T cells, vertical numbers indicated MFI of IFN-γ⁺ cells. (B) Cell free supernatants of the Th1 polarizing conditions were assayed by ELISA for IFN-γ. Error bars indicate s.e.m. Data is representative of two independent experiments.
**TSLP regulates DC production of IL-12/23p40 both in vitro and in vivo**

The dysregulation of CD4$^+$ T cell production of IFN-γ observed in the GALT of TSLPR$^{-/-}$ mice both in the steady state and following *Trichuris* infection suggests a possible alteration in signals that influence T helper cell differentiation. As previous *in vitro* studies demonstrated that TSLP could inhibit IL-12 secretion from human DCs (Al-Shami, Spolski et al. 2005; Ito, Wang et al. 2005; Rimoldi, Chieppa et al. 2005), we next wanted to investigate whether dysregulated IL-12/23p40 production could be playing a role early following infection. Analysis of CD11c$^+$ CD11b$^+$ DCs isolated from MLNs of infected TSLPR$^{-/-}$ mice showed increased production of the proinflammatory cytokines TNF-α and IL-12/23p40 at day 10 post-infection (Figure 22 A, B). Additionally, levels of IL-12/23p40 mRNA were increased in the large intestine of TSLPR$^{-/-}$ mice at day 12 post-infection (Figure 22C). These data demonstrate an *in vivo* role for TSLP in the regulation of infection-induced IL-12/23p40 production from intestinal DCs.

To examine the ability of IEC-derived TSLP to modulate DC function, an *in vitro* conditioning system was employed. Bone marrow-derived DCs (BMDCs) were cultured overnight in the presence of supernatants taken from the IEC line CMT-93 (IEC-Sup), which is known to express TSLP (Zaph, Troy et al. 2007). Control and conditioned BMDCs were subsequently stimulated with LPS for 8 hours. Exposure to IEC-Sup did not impair the ability of DCs to respond to LPS as IEC-Sup-conditioned BMDCs exhibited normal upregulation of surface expression of MHC Class II and the costimulatory molecules CD80 and CD86 as compared to unconditioned BMDCs (Figure 22D). Further, conditioning with IEC-Sup did not alter or inhibit LPS-induced production of TNF-α (Figure 22 E, G). However, IEC-Sup-conditioned DCs exhibited reduced
production of IL-12/23p40 (Figure 22 F, H). As the IEC-Sup contains multiple IEC-derived factors, we sought to determine the contribution of TSLP to the regulation of DC responses by utilizing recombinant TSLP (rTSLP). Similar to the results with IEC-Sup, exposure of BMDCs to rTSLP did not alter their surface marker expression or production of TNF-α following LPS stimulation but did result in decreased IL-12/23p40 production in comparison to control treated BMDCs (Figure 22 I-K). These findings demonstrate the ability of TSLP to selectively regulate DC production of IL-12/23p40. Consistent with their inhibition of IL-12/23p40, both IEC-Sup- and rTSLP-conditioned BMDCs induced fewer CD4+ T cells to produce IFN-γ in antigen specific co-culture assays (Figure 22L). Taken as a whole, these results indicate that TSLP is important in the regulation of DC-derived IL-12/23p40 both in vitro and in vivo.
Figure 22 TSLP regulates DC production of IL-12/23p40 in vitro and in vivo
Figure 22 TSLP regulates DC production of IL-12/23p40 in vitro and in vivo

Figure 22. TSLP inhibits DC production of IL-12/23p40 in vivo and in vitro. (A-B) TSLPR<sup>−/−</sup> mice have increased DC-derived proinflammatory cytokine production at day 10 post-infection. Cells were isolated from MLN and restimulated directly ex vivo with Trichuris ES antigen. Cells are gated on CD11c<sup>+</sup> CD11b<sup>+</sup> populations. (A) TNF-α, (B) IL-12/23p40. (C) IL-12/23p40 mRNA expression in the colon at day 12 post-infection. (D-F) Supernatants from the IEC CMT-93 cell line specifically inhibit LPS-induced IL-12/23p40 production. (D) LPS-induced upregulation of MHC-II, CD80, and CD86. (E-F) BMDC cytokine production was measured using intracellular cytokine staining (ICS). (E) TNF-α, (F) IL-12/23p40. (G-H) Cytokine secretion was assayed by ELISA of the supernatants. (G) TNF-α, (H) IL-12/23p40. (I-J) rTSLP specifically inhibits LPS-induced IL-12/23p40 production. (I) LPS-induced upregulation of MHC-II, CD80, and CD86. (J-K) BMDC cytokine production as measured using ICS, (J) TNF-α, (K) IL-12/23p40. (L) IEC and rTSLP conditioning of BMDCs decreased CD4<sup>+</sup> T cell production of IFN-γ upon co-culture. Error bars indicate s.e.m. A-C represent two individual experiments with 3 mice per group. D-K represent three to five individual experiments, L two individual experiments.
**TSLPR<sup>−/−</sup> mice exhibit an increased susceptibility to DSS-induced colitis**

The dysregulation of DC-derived IL-12/23p40 and CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells in the intestine observed in the TSLPR<sup>−/−</sup> under both homeostatic and infection-induced settings suggests that TSLPR<sup>−/−</sup> mice may be predisposed to the development of severe intestinal inflammation following intestinal damage. To assess whether the ability of TSLP to influence intestinal proinflammatory cytokine extended beyond helminth infection, we utilized the dextran sodium sulfate (DSS) model of chemical-induced colitis. Oral administration of DSS is toxic to IECs and induces IEC damage, barrier leakage and exposure of sub-epithelial immune cells to commensal bacteria. Barrier breakdown results in immune cell recruitment, increased expression of proinflammatory cytokines, and the development of focal ulcerations in the large intestine (Byrne and Viney 2006).

WT and TSLPR<sup>−/−</sup> mice were exposed to 5% DSS in their drinking water for four days and disease was assessed daily. DSS-treated TSLPR<sup>−/−</sup> mice exhibited more rapid weight loss in comparison to treated WT mice, displaying weight loss as early as day 1 post-treatment (Figure 23A). Increased weight loss in the TSLPR<sup>−/−</sup> mice correlated with early disease onset and increased disease severity as measured by fecal consistency, rectal bleeding, and general appearance as well as weight loss (Figure 23B). At day four post-DSS treatment, sections from the mid colon of DSS-treated WT mice revealed mild thickening of the muscularis and minimal presence inflammatory cells. In contrast, tissue sections from DSS-treated TSLPR<sup>−/−</sup> mice exhibited severe thickening of the muscularis, a marked mucosal inflammatory cell infiltrate, and large numbers of focal lesions characterized by epithelial cell sloughing, increased inflammatory cell infiltrate and loss of crypt architecture (Figure 23C). Consistent with the increased severity of
disease score and inflammation, direct ex vivo organ culture of colonic segments displayed increased production of IL-12/23p40 in the DSS-treated TSLPR<sup>−/−</sup> mice (WT 0.18 ± 0.05 ng/ml/mg tissue; TSLPR<sup>−/−</sup> 0.33 ± 0.08 ng/ml/mg tissue). Ex vivo analysis of MLN cells from day four post-DSS treatment demonstrated an increased number of IFN-γ<sup>+</sup> CD4<sup>+</sup> T cells in DSS-treated TSLPR<sup>−/−</sup> mice compared to WT DSS-treated mice (Figure 23D). In addition, polyclonal restimulations of MLN cells harvested at day four post-DSS treated TSLPR<sup>−/−</sup> mice revealed increased levels of IFN-γ in comparison to DSS-treated WT mice (Figure 23E). DSS-treated TSLPR<sup>−/−</sup> mice also displayed exacerbated colonic shortening, thickening, and enteric bleeding at day 6 post treatment compared to DSS-treated WT mice (Figure 23 F, G). Collectively, these results demonstrate a previously unrecognized role for TSLP-TSLPR interactions in the regulation of intestinal inflammation in a murine model of IBD.
Figure 23. TSLPR<sup>−/−</sup> mice exhibit an increased susceptibility to DSS colitis. (A) Weight loss. (B) Total pathology score for WT and TSLPR<sup>−/−</sup> mice on 5% DSS. (C) Paraffin-embedded cecal sections from day 4 post-DSS administration were stained with H&E. Bar, 50 µm. (D) Total number of CD4<sup>+</sup> IFN-γ<sup>+</sup> T cells isolated from the MLN at day 4 post-DSS. (E) Cytokine production from anti-CD3 anti-CD28 restimulated MLN cells was determined by ELISA. (F) Colon of day 6 DSS-treated WT and TSLPR<sup>−/−</sup> mice, Bar, 1 cm. (G) Colonic shortening, data are presented as percent length of naïve. Error bars indicate s.e.m. * p ≤ 0.05 ** p ≤ 0.08. Data are representative of three individual experiments with 4-5 mice per group.
Discussion

Results presented in this chapter highlight a previously unrecognized role for TSLP-TSLPR interactions in the regulation of proinflammatory cytokine production and inflammation within the intestine. Though TSLPR−/− mice do not develop spontaneous colitis, analysis of GALT tissues from naïve TSLPR−/− mice revealed dysregulated DC-derived IL-12/23p40 production as well as increased frequencies of CD4+ IFN-γ+ T cells, indicating dysregulated intestinal immune homeostasis. The role of TSLP in this dysregulation was confirmed as in vitro addition of rTSLP into splenocyte cultures resulted in the inhibition of CD4+ T cell IFN-γ production. Further, TSLP was shown to modulate DC-derived IL-12/23p40 both in vitro and in vivo. The disruption in intestinal immune homeostasis in the absence of TSLP-TSLPR interactions has a functional consequence, as TSLPR−/− mice exhibited an increased susceptibility to DSS-induced colitis. TSLPR−/− mice displayed increased disease pathology, inflammatory cytokine production, and more severe intestinal inflammation compared to DSS-treated WT control animals. Taken together, these findings suggest that in addition to promoting T H 2 cytokine responses, a function of TSLP in the intestinal microenvironment may be to directly and/or indirectly inhibit proinflammatory cytokine production.

Although these data extend the function of TSLP beyond its well-established role in directly promoting T H 2 cytokine responses, the ability of TSLP to modulate proinflammatory cytokine production has been previously indicated. In vitro studies showed TSLP-treated DCs exhibit reduced production of IL-12/23p40 upon TLR ligation, and the ability of DC-derived OX40L to drive T H 2 cell differentiation is critically dependent upon the absence of IL-12 (Soumelis, Reche et al. 2002; Ito, Wang et al.
In addition, transgenic overexpression of TSLP in the skin decreased the frequency of CD4+ IFN-γ+ T cells in the transgenic mice in comparison to normal littermate controls, and TSLPR−/− mice exhibited significantly increased IL-12 mRNA levels in the lungs compared to WT animals following OVA-induced allergic inflammation (Al-Shami, Spolski et al. 2005; Yoo, Omori et al. 2005). These findings are consistent with a role for TSLP in negatively regulating proinflammatory cytokine production in DC populations in peripheral sites. Thus the exaggerated T\( _{\text{H}2} \) T cell-mediated diseases in mice with transgenic over-expression of TSLP may be a result of simultaneous increases in T\( _{\text{H}2} \) cytokine responses and decreased expression of proinflammatory cytokines including IL-12 and IFN-γ that would counter-regulate developing T\( _{\text{H}2} \) cytokine responses.

While our results highlight the ability of TSLP to modulate proinflammatory cytokine production in the intestine, the exact mechanisms of action and molecular pathways involved remain unclear. As TSLPR is functionally expressed on a variety of cell types including DCs, mast cells, basophils, and CD4+ T cells (see Chapter 2), it is not known if TSLP is acting either directly, or indirectly to modulate CD4+ T cell IFN-γ production. TSLP may be acting indirectly through DCs, as TSLP-treatment of DCs inhibits IL-12/23p40 production in a manner that results in decreased production of IFN-γ from co-cultured CD4+ T cells (Figure 23L). Although the ability of TSLP to inhibit DC production of IL-12/23p40 has been well documented (Soumelis, Reche et al. 2002; Ito, Wang et al. 2005; Rimoldi, Chieppa et al. 2005; Zaph, Troy et al. 2007), the signaling pathways induced by TSLP in DCs to regulate gene expression are unknown at present. Additionally, TSLP treatment of DCs may influence intestinal cytokine production by
altering the composition of immune cell populations within the intestine. As TSLP-treatment of DCs is known to induce expression of the Th2 cell-attracting chemokine CCL20, and the T cell-attracting chemokine CCL17 (Reche, Soumelis et al. 2001; Soumelis, Reche et al. 2002), TSLP could be increasing the recruitment of non-IFN-γ producing T cells to the intestine.

TSLP may also act directly on CD4+ T cells to limit Th1 cell differentiation. Although rTSLP was shown to inhibit CD4+ T cell production of IFN-γ in the absence of IL-4 (Figure 21), it remains possible that TSLP is regulating IFN-γ production through its promotion of Th2 cytokine responses. TSLP is known to promote expression of GATA-3 and IL-4 in CD4+ T cells (Omori and Ziegler 2007); pathways that inhibit expression of IFN-γ (Murphy and Reiner 2002; Szabo, Sullivan et al. 2003). Direct inhibition of CD4+ T cell IFN-γ production may also be possible. For instance, TSLP activates STAT5 (Isaksen, Baumann et al. 1999; Levin, Koelling et al. 1999), and STAT5a activation in CD4+ T cells has been shown to inhibit IL-12-induced STAT4 activation through the induction of SOCS3 (Takatori, Nakajima et al. 2005). However, it remains unknown which of these pathways, if any, contribute to the ability of TSLP to decrease CD4+ T cell production of IFN-γ.

TSLP may also act indirectly through innate cell production of IL-4 to influence CD4+ T cell production of IFN-γ. In conjunction with other cytokines, TSLP has been shown to induce mast cell production of Th2 cytokines (Soumelis, Reche et al. 2002; Allakhverdi, Comeau et al. 2007; Allakhverdi, Smith et al. 2007; Okayama, Okumura et al. 2009), indicating mast cells could represent an important source of IL-4. However, as mast
cells are not normally present within the GALT at high frequencies, and TSLP-induced T\textsubscript{H}2 cytokine production requires the presence of additional cytokines, it is possible that mast cell-derived IL-4 plays a more important role in the inhibition of CD4\textsuperscript{+} T cell IFN-\textgamma production under situations of inflammation or infection. Further studies must be done to determine the relative importance of TSLP acting either indirectly through DCs and/or mast cells, or directly through CD4\textsuperscript{+} T cells, to inhibit CD4\textsuperscript{+} T cell production of IFN-\textgamma. It is important to note that these possibilities are not mutually exclusive and that it is feasible that TSLP is utilizing multiple mechanisms to limit proinflammatory cytokine production and inflammation in the intestine.
CHAPTER 5: Discussion and Future Directions

Introduction

It has been fifteen years since the biological activity of TSLP was first described (Friend, Hosier et al. 1994) and ten years since the genes for both TSLP and TSLPR were cloned and sequenced (Park, Martin et al. 2000; Sims, Williams et al. 2000; Quentmeier, Drexler et al. 2001). Despite numerous and significant advances in understanding the role of TSLP in lymphoid development and in the promotion of T\textsubscript{H}2 cytokine-mediated inflammation in the skin and the lung (see Chapter 1), the understanding of the role of TSLP in the intestinal immune system is still in its infancy. While expression of TSLP mRNA was noted in the ileum of the small intestine in 2000 (Sims, Williams et al. 2000), a function for TSLP in the intestine was not proposed until 2005 when Rescigno and colleagues demonstrated that TSLP was important in maintaining intestinal immune hyporesponsiveness through the promotion of T\textsubscript{H}2 cell differentiation (Rimoldi, Chieppa et al. 2005). Despite advances in identifying both TSLP-expressing and TSLP-responsive cell populations \textit{in vitro}, the relevant cellular sources and targets of TSLP \textit{in vivo} remain largely unknown. In addition to these more basic questions, the biological function of TSLP-TSLPR interactions within the intestine has also not been fully defined. At the onset of this thesis, it was unknown if TSLP was critically required for the development of host-protective intestinal T\textsubscript{H}2 cytokine-mediated immune responses, or if the function of TSLP in the intestinal microenvironment extended beyond its ability to promote T\textsubscript{H}2 cytokine responses. Within this context, it was the goal of this thesis to begin to define and characterize the role of TSLP-TSLPR interactions in regulating intestinal immunity and inflammation.
Summary, discussion, and future directions

**TSLP and TSLPR complex expression in the intestine**

In Chapter 2 of this thesis, in order to achieve a more comprehensive understanding of the cellular components of TSLP-mediated regulation in the intestine, I characterized the cellular sources and targets of TSLP in the large intestine and GALT. Further, I determined the functionality of TSLPR complex expression on GALT-derived CD4+ T cells and CD11c+ DCs. From these studies, we demonstrated that under naïve homeostatic conditions, the dominant source of TSLP within the large intestine is IECs (Figure 5). Further, we found that surface expression of TSLPR complex is extensive throughout tissues of the GALT, being detected on CD11c+ DCs, CD4+ T cells, as well as the innate cell populations of basophils and mast cells (Figures 6-8). Also, we demonstrated that both CD11c+ DCs and CD4+ T cells from the GALT can respond to TSLP and upregulate phosph-STAT5 directly *ex vivo* without additional activation or stimulation (Figure 9).

**Regulation of TSLP expression in the intestine**

The constitutive expression of TSLP from IECs in the large intestine is consistent with previous findings (Rimoldi, Chieppa et al. 2005), however the cellular signals that mediate or induce this expression remain unidentified. *In vitro* studies have demonstrated that TSLP production from epithelial cells can be elicited by external factors such as TLR ligands and viruses as well as by host-derived factors such as cytokines (Saenz, Taylor et al. 2008), making it difficult to distinguish the relevant signals driving TSLP expression within the intestinal tract. The elevated expression of TSLP in the large intestine compared to the small intestine or cecum correlates with the
increasing population size of commensal bacteria in the distal portions of the GI tract. This makes it tempting to speculate that TSLP expression, may, at least in part, be driven by commensal-derived signals. However, preliminary studies comparing TSLP mRNA expression in large intestine tissue between conventionally housed mice and germ free mice showed no significant alteration in expression between experimental groups (Colby Zaph, personal communication, and data not shown), indicating that commensal-derived signals may not be essential for intestinal TSLP expression. These findings do not entirely rule out external stimuli in driving IEC TSLP expression, however, since sterilized mouse chow has been found to contain detectable levels of bacterial DNA (David Hill, personal communication), which could act as a stimulatory signal for TSLP expression in germ-free mice. Finally, it is possible that food antigens or the mechanical act of digestion and smooth muscle contraction could stimulate TSLP production by IECs.

In addition to externally-derived signals, host-derived cytokines could also play a role in stimulating TSLP expression in the intestine. As the intestine is an active immunologic site, continually being stimulated by environmental, food, and commensal bacteria-derived antigens, the steady state production of cytokines within the intestine could be sufficient to induce TSLP expression. Although many possible cytokine candidates exist, TSLP was induced following *Trichuris* infection in SCID mice (Humphreys, Xu et al. 2008), indicating an innate cell source. Additionally, IECs themselves must be considered as a source of factors that could regulate TSLP expression. It is possible that IEC-derived products could be acting in an autocrine fashion to induce constitutive TSLP expression. It is important to note that the possible mechanisms to stimulate IEC
TSLP production are not mutually exclusive and that many factors may act to regulate expression of TSLP within the intestine.

Although IECs appear to be the main source of TSLP within the intestine, recent work has indicated that innate cells may also represent a source of TSLP. Studies have demonstrated that mast cells upregulate both TSLP mRNA and protein following FcεRI cross-linking (Soumelis, Reche et al. 2002; Okayama, Okumura et al. 2009). Additionally, basophils upregulate both TSLP mRNA and protein following stimulation with papain (Sokol, Barton et al. 2008). As both mast cells and basophils require stimulation to produce TSLP (Allakhverdi, Comeau et al. 2007; Sokol, Barton et al. 2008), it remains possible that they only function as sources of TSLP in situations of infection and/or inflammation. Also, as mast cells and basophils are normally present at very low frequencies within the lamina propria and GALT, infection and/or inflammation-derived signals may be required to recruit enough of these cells into the tissues to become a physiologically relevant source of TSLP. Given the recently discovered importance of antigen presenting basophils in the initiation of Th2 cytokine immune responses (Perrigoue, Saenz et al. 2009; Sokol, Chu et al. 2009; Yoshimoto, Yasuda et al. 2009), it is possible that basophil-derived TSLP may play an important role in the initiation of intestinal Th2 immune responses. However, more studies will need to be done to determine if basophil-derived or mast cell-derived TSLP is sufficient to influence intestinal immune responses, and if its expression can be controlled or regulated.

*Cellular targets of TSLP and regulation of TSLPR expression*
Identification of the cellular targets of TSLP has been hindered by a lack of reagents that are able to specifically and reliably detect surface protein expression of the TSLPR-α chain of the TSLPR complex. Previous studies have largely been limited to the examination of either TSLPR mRNA expression in whole tissues, cell lines, or in purified populations or by in vitro binding assays (Park, Martin et al. 2000; Reche, Soumelis et al. 2001; Allakhverdi, Comeau et al. 2007). Our findings of surface expression of TSLPR-α chain on GALT CD11c+ DCs and mast cells are consistent with the published literature (Reche, Soumelis et al. 2001; Soumelis, Reche et al. 2002; Rimoldi, Chieppa et al. 2005; Allakhverdi, Comeau et al. 2007); however, we extended these findings by demonstrating co-expression of the second, critically required chain of the heterodimeric TSLPR complex, CD127 (IL-7Rα) on these cell populations (Figure 6, 7). We also show that in addition to being cellular sources of TSLP (Sokol, Barton et al. 2008), basophils express the TSLPR complex (Figure 6). Our characterization of TSLPR complex expression in the GALT also revealed functional receptor surface expression on CD4+ T cells directly ex vivo. These findings are inconsistent with in vitro studies on human CD4+ T cells, which showed no surface expression on freshly isolated cells (Rochman, Watanabe et al. 2007; Lu, Wang et al. 2009). Although the source of the discrepancy is unknown, it is possible that there is species-specific regulation of TSLPR expression. Alternatively, the sensitivity of the reagents utilized in the studies could be different. Nonetheless, our data showing CD4+ T cell phospho-STAT5 activation following TSLP treatment indicate that within the murine model, the possibility of TSLP acting directly on naïve CD4+ T cells must be taken into account.
Although we have a basic understanding of the factors that can regulate TSLP expression (Chapter 1), very little is known about the factors that regulate TSLPR complex expression. On the cellular level, work focusing on human CD4+ T cells found upregulation of the TSLPR-α chain following polyclonal activation (Rochman, Watanabe et al. 2007; Lu, Wang et al. 2009). Studies examining TSLPR mRNA expression in whole tissues indicate that TSLPR expression is dynamically regulated during the course of helminth infection (Humphreys, Xu et al. 2008; Ramalingam, Pesce et al. 2009). However, due to the nature of these studies it is not known if the regulation of TSLPR expression was mediated directly by the pathogen, or was a result of the immune response following infection. Our in vitro findings utilizing BMDCs suggest that both methods of regulation may be possible as TSLPR expression was upregulated following treatment with helminth-derived products as well as with both pro- and anti-inflammatory cytokines (Figure 6). However, as treatment with LPS and CpG did not mediate TSLPR upregulation in BMDCs (Figure 6) and TSLPR expression has not been examined following infection with either bacterial or viral pathogens, it is possible that the ability of pathogens to modulate TSLPR expression is restricted to helminths. Treatment of BMDCs with papain induced TSLPR upregulation, indicating that the TSLPR complex may also be dynamically regulated in allergic responses. While these findings begin to hint at the signaling pathways that could potentially be involved, the exact mechanisms and mediators remain unknown, and it is unclear whether regulation of receptor expression differs between cell types.

**Future directions and challenges**
As TSLP is a relatively new cytokine, many basic and fundamental questions remain unanswered regarding both its production and cellular targets. Much of the work done characterizing the factors that regulate TSLP expression has been done in vitro, and the physiologic relevance of these findings remains to be determined. Further, it is unclear how the inherently different microenvironments of the skin, lung, and intestine influence either epithelial or innate cell production of TSLP. Recent advances in the development of reliable reagents to detect TSLPR-α chain surface expression have begun to allow analysis of target cell populations directly ex vivo. Despite this, it is unknown what role, if any, TSLPR regulation plays in modulating immune responses in vivo. Future studies focusing on TSLP and TSLPR expression must move in vivo to extrapolate the physiologically relevant signals that both positively and negatively regulate expression.

In addition to defining regulatory signals for both TSLP and TSLPR expression, a more in depth understanding of the TSLP signaling pathway is needed. The current understanding of TSLP signaling is limited (Sebastian, Borowski et al. 2008), with the kinases and target genes largely unresolved. Further, the majority of the studies examining TSLP signaling were conducted in cell lines transfected with components of the TSLPR, or in cell types that are now known to not be physiologically relevant targets of TSLP (Isaksen, Baumann et al. 1999; Levin, Koelling et al. 1999; Isaksen, Baumann et al. 2002). Thus it is difficult to ascertain the relevance of these findings and understand how it relates to TSLP signaling in primary cells. Also, it is unknown if TSLP signaling varies from one cell population to another, which, given the numerous downstream functions attributed to TSLP signaling, seems likely. For instance, while TSLP alone is sufficient to influence DC function (Reche, Soumelis et al. 2001;
Soumelis, Reche et al. 2002), treatment with TSLP alone does not impact mast cell function (Allakhverdi, Comeau et al. 2007). Despite constitutive TSLPR complex expression (Figure 7 and (Allakhverdi, Comeau et al. 2007)), mast cells require sensitization with either IL-1, TNF-α, or IL-33 for TSLP to induce Th2 cytokine release (Allakhverdi, Comeau et al. 2007; Allakhverdi, Smith et al. 2007). The mechanism behind this regulation is not clear. It is possible that the sensitization signal results in the de-repression of TSLP signaling, or that it alters the abundance of the required downstream signaling molecules. Further studies must be conducted focusing on TSLPR signaling in multiple cell types.

Although much progress has been made in understanding the functional biology of TSLP, it remains important to address fundamental questions regarding the regulation of both the ligand and the receptor. Additionally, the current limitations in the understanding of TSLP signaling make it difficult to identify and assess potential cross regulation from other cytokines. Answering these questions will be key to understanding the biology of TSLP in the intestine as well as in other tissues. Further, these findings may help identify components of the regulatory network that could potentially be exploited for therapeutic benefit.

**The role for TSLP in intestinal Th2 cytokine-mediated immune responses**

In Chapter 3, I directly tested the hypothesis that TSLP-TSLPR interactions would be required for the development of a host-protective Th2 cytokine-mediated immune response in the intestine utilizing infection with the model GI nematode pathogen *Trichuris*. From these studies, we demonstrated that while TSLP-TSLPR interactions
are required for immunity to *Trichuris*, TSLP-independent T_{H2} cytokine responses developed in the absence of IFN-γ (Figure 17). These findings demonstrate that TSLP is not critically required for the initiation of T_{H2} cytokine responses in the intestine.

*The requirement for TSLP in infection-induced T_{H2} cytokine responses*

Although TSLP is important in both the initiation and promotion of allergic T_{H2} cytokine-mediated inflammation in the skin and lung (Liu 2006; Liu, Soumelis et al. 2007), our findings indicate that TSLP is not critically required for the initiation of an infection-induced T_{H2} cytokine response in the intestine (Chapter 3). While it remains possible that tissue-specific differences exist in terms of TSLP function, more recent work suggests that the requirement for TSLP in the initiation and promotion of T_{H2} cytokine responses varies between situations of allergy and infection.

In addition to our findings, TSLP-independent intestinal T_{H2} cytokine responses have also been documented in situations of infection. TSLPR\textsuperscript{-/-} mice were demonstrated to mount T_{H2} cytokine responses following infection with the intestinal dwelling helminth *Heligmosomoides polygyrus* (*H. polygyrus*) (Massacand, Stettler et al. 2009). Further, TSLPR\textsuperscript{-/-} mice exhibited intact T_{H2} cytokine-mediated immunity to infection with *Nippostrongylus brasiliensis* (*N. brasiliensis*), a helminth pathogen with an intestinal phase of infection (Massacand, Stettler et al. 2009). Also, TSLP does not appear to be required for infection-induced T_{H2} cytokine responses in the liver or lung. Although TSLPR\textsuperscript{-/-} mice infected with *Schistosoma mansoni* (*S. mansoni*) cercariae exhibited reduced production of IL-13 and decreased liver fibrosis at 9 weeks post infection compared to WT controls, TSLPR\textsuperscript{-/-} mice produced equivalent levels of IL-4 and IL-5 to
WT mice. Also, TSLPR<sup>−/−</sup> mice still developed chronic infection and exhibited Th2-cytokine-dependent pathology in the liver that was indistinguishable from WT mice at 12 weeks post-infection (Ramalingam, Pesce et al. 2009). The role of TSLP in pathogen-induced airway inflammation was investigated utilizing infection with *N. brasiliensis*, whose migration through the lung induces Th2 cytokine-mediated inflammation and pathology. TSLPR<sup>−/−</sup> mice infected with *N. brasiliensis* not only cleared both primary and secondary infections, but also developed Th2 cytokine responses, and Th2 cytokine-dependent lung inflammation comparable to WT control mice (Massacand, Stettler et al. 2009).

Overall, these studies focusing on the role of TSLP in infection indicate that while TSLP may be important for optimal responses, it is not strictly required for the development of infection-induced Th2 cytokine responses. These data support the hypothesis that TSLP is required in allergen-driven Th2 cytokine responses, but not in situations of infection. However within the intestine, the corollary of TSLP involvement in allergen-driven responses remains unknown. As food allergy is associated with intestinal Th2 cytokine immune responses (Nagler-Anderson 2001; Devereux 2006), we hypothesize that TSLP will be important in food allergen-associated immune responses.

Allergy versus infection

It remains unclear why TSLP is differentially required in situations of allergy-driven Th2 cytokine responses and infection-induced Th2 cytokine responses. Although allergic responses and the subsequent Th2 cytokine-mediated inflammation are pathological, and infection-induced Th2 cytokine responses are usually host-protective, the line
between protective and pathologic becomes blurred when examining chronic helminth infections such as *S. mansoni*. TSLPR<sup>−/−</sup> mice still develop severe T<sub>H2</sub> cytokine-dependent pathology in the liver following *S. mansoni* infection (Ramalingam, Pesce et al. 2009). Ramalingam et al. propose that the different routes of Ag exposure may be sufficient to explain the difference between the requirement for TSLP in allergy and infection. As opposed to allergens, which primarily interact with epithelial cells at mucosal surfaces, helminth infection usually results in Ag exposure in and damage to multiple tissues as a result of parasite migration and maturation. It is possible therefore, that the increased damage and more systemic level of stimulation triggers other T<sub>H2</sub> cytokine-inducing factors, alleviating the need for TSLP (Ramalingam, Pesce et al. 2009).

This hypothesis of altered route of Ag exposure and increased tissue damage is also consistent with the observed differences in the requirement for TSLP in T<sub>H2</sub> cytokine responses between infection with *H. polygyrus* and *Trichuris*. TSLP is not required for the generation of a T<sub>H2</sub> cytokine response to infection with *H. polygyrus* (Massacand, Stettler et al. 2009), whose life cycle requires penetration of the intestinal submucosa and migration through intestinal muscle tissue (Finkelman, Shea-Donohue et al. 1997). In contrast, TSLP is required for T<sub>H2</sub> cytokine-mediated immunity to *Trichuris* (Chapter 3, (Massacand, Stettler et al. 2009), whose life cycle is entirely enteric, with worms only embedding their anterior ends within the epithelial cell layer of the intestine, never penetrating the submucosa or the muscle (Cliffe and Grencis 2004). Thus, it is possible that TSLP is required in response to *Trichuris* infection because the life cycle of the worm does cause sufficient tissue damage or Ag exposure outside of the intestinal
epithelium to trigger other T_{H2} inducing factors. Although more work needs to be done to determine the reason for the differential requirement of TSLP in allergen-induced and infection-induced T_{H2} cytokine responses, it is possible that signals derived from infection-induced tissue damage may play a role.

The functional biology of TSLP in Trichuris infection

The demonstration that TSLPR^{-/-} mice are capable of mounting T_{H2} cytokine responses to _N. brasiliensis_ and _H. polygyrus_ raises the question about the differential requirement for TSLP in _Trichuris_ infection. As TSLP is not required for the initiation of T_{H2} cytokine responses in other intestinal infections (Massacand, Stettler et al. 2009), it must serve another function during _Trichuris_ infection important in the creation of a T_{H2} permissive microenvironment. Work from Massacand et al. pinpoints the importance of TSLP in the inhibition of DC-derived IL-12/23p40 in _Trichuris_ infection. While ES products from _N. brasiliensis_ and _H. polygyrus_ inhibit BMDC production of IL-12/23p40, ES products from _Trichuris_ fail to block DC-derived IL-12/23p40 _in vitro_. Blockade of IL-12/23p40 _in vivo_ during _Trichuris_ infection is sufficient to restore immunity to TSLPR^{-/-} mice (Massacand, Stettler et al. 2009).

Taken together with our findings, these data suggest that primary function of TSLP in _Trichuris_ infection is to inhibit proinflammatory cytokine production, as TSLP-independent mechanisms are sufficient to drive T_{H2} cytokine responses in the absence of the T_{H1}-associated cytokine IL-12/23p40 and IFN-γ. Thus, in comparison to _N. brasiliensis_ and _H. polygyrus_, _Trichuris_ lacks the ability to modulate the intestinal immune response, and requires TSLP to take the place of a pathogen-derived factor to
limit proinflammatory cytokine production and create a Th2 permissive microenvironment.

Although TSLP is not required for the development of protective immunity to *Trichuris* in the absence of proinflammatory cytokines, the relevant cellular sources and targets of TSLP during infection remain undefined. Though IECs appear to be the dominant source of TSLP in the intestine, basophil and mast cell production of TSLP have been reported (Sokol, Barton et al. 2008; Okayama, Okumura et al. 2009). As discussed previously, it is possible that basophils and mast cells could be physiologically relevant sources of TSLP over the course on an infection. To address this issue, chimeric studies must be carried out to delineate the importance of haematopoietic versus non-haematopoietic cell expression of TSLP in vivo. Further, as previous work has been completed with germ line knockouts of TSLPR, we do not know the function or relative importance of TSLP acting on individual cell populations in vivo. While the studies from Massacand et al. indicate that DCs are the pertinent target cell population in *Trichuris* infection (Massacand, Stettler et al. 2009), our studies indicate that CD4+ T cells also express functional TSLPR complex (Figure 6) and could represent an additional cellular target, either for the inhibition of proinflammatory cytokine production (Figures 13, 16, 19), or for the initiation of Th2 cytokine production (Omori and Ziegler 2007). To address whether CD4+ T cell restricted or accessory cell restricted TSLPR expression is sufficient to promote a Th2 cytokine response to *Trichuris*, transfer studies isolating TSLPR expression on CD4+ T cells or accessory cells need to be carried out. Understanding of the relative contribution and importance of TSLP signaling on each cell type in vivo is important in understanding the complexity of the functional biology of TSLP.
Future directions and challenges

The finding of TSLP-independent infection-induced T\textsubscript{H}2 cytokine responses suggests that the concept that TSLP plays a central role in all T\textsubscript{H}2 cytokine responses (Liu, Soumelis et al. 2007) must be reevaluated. The demonstration of a differential requirement for TSLP in the initiation of allergen versus infection-induced T\textsubscript{H}2 cytokine response indicates that the functional biology of TSLP varies dependent on the immunological context. The fundamental differences between situations of allergy and infection that influence the requirement for TSLP in the initiation of T\textsubscript{H}2 cytokine responses remain to be elucidated. Studies examining increased routes of allergen exposure and coupling allergen exposure with tissue damage must be done to dissect the importance of increased Ag exposure or infection-induced tissue damage in the TSLP independence of infection-induced T\textsubscript{H}2 cytokine responses (Ramalingam, Pesce et al. 2009) these factors. Additionally, to examine the possibility that tissue-specific factors can influence the requirement for TSLP in allergic responses, the role of TSLP in food allergy must be investigated. Although we hypothesize that TSLP will be required for the generation of a food allergen-induced T\textsubscript{H}2 cytokine response, the distinct microenvironment of the intestine may be able to compensate for the loss of TSLP, resulting in a TSLP-independent T\textsubscript{H}2 cytokine response.
**TSLP and the regulation of proinflammatory cytokine production and inflammation in the intestine**

In Chapter 4 I investigated the hypothesis that TSLP was important in the regulation of proinflammatory cytokine production and inflammation within the intestine. Consistent with this, naïve TSLPR\(^{-/-}\) mice were found to have dysregulated production of DC-derived IL-12/23p40 and CD4\(^+\) T cell derived IFN-\(\gamma\) in the GALT (Figures 19, 21). Further, *in vitro* studies indicate that TSLP maintains the ability to regulate CD4\(^+\) T cell IFN-\(\gamma\) production in the absence of IL-4 (Figure 20). While it is not clear whether TSLP is acting solely through DCs to modulate CD4\(^+\) T cell IFN-\(\gamma\) production, TSLP regulates DC-derived IL-12/23p40 both *in vitro* and *in vivo* (Figure 22). Although the dysregulation of proinflammatory cytokines in the GALT does not lead to spontaneous colitis, TSLPR\(^{-/-}\) mice display an increased susceptibility to DSS-induced colitis, exhibiting increased proinflammatory cytokine production and more severe intestinal inflammation compared to WT control mice (Figure 23).

**TSLP and the regulation of epithelial cell barrier function**

Recent evidence suggests that TSLP could contribute to the regulation of intestinal inflammation by impacting barrier function. *In vitro* studies utilizing human nasal epithelial cells (HNECs), found that TSLP treatment upregulated the tight junction proteins claudin-1, -4, -7, and occludin within 6 -24 hours. Further, TSLP treatment impacted barrier function as TSLP-treated HNECs showed significantly increased transepithelial electrical resistance (TER), and decreased permeability of larger molecular weight molecules (Kamekura, Kojima et al. 2009). As TSLPR-\(\alpha\) chain was not found to be expressed on HNECs or human nasal mucosa *in vivo* (Kamekura, Kojima et
al. 2009), the changes in barrier permeability most likely did not result from TSLP-TSLPR signaling on the epithelial cells. However, these findings still indicate that TSLP can influence epithelial cell barrier function through an unknown mechanism.

It is possible that TSLP-mediated regulation of barrier function extends into the intestine, and that TSLP may play an important role in barrier maintenance. Crohn’s disease is characterized by compromised barrier function and the development of immune responses directed against commensal bacteria (Xavier and Podolsky 2007). Interestingly, loss of TSLP expression is observed in intestinal biopsies from Crohn’s disease patients (Rimoldi, Chieppa et al. 2005). It remains possible that the loss of TSLP expression observed in Crohn’s disease could contribute to the increased barrier permeability and bacterial translocation that results in immune cell activation and inflammation.

**TSLP in the maintenance of immune homeostasis**

Our findings of dysregulated intestinal proinflammatory cytokine production in the absence of TSLPR are consistent with work from Rescigno and colleagues which identified a role for TSLP in the maintenance of intestinal immune homeostasis (Rimoldi, Chieppa et al. 2005). As the intestinal microenvironment is constantly being immunologically stimulated by both food-derived and commensal bacteria-derived antigens (Chapter 1), TSLP may have developed as a regulatory mechanism to prevent the development of inflammation resultant from continual stimulation. However, the lack of spontaneous colitis in TSLPR−/− mice indicates TSLP is not as central to maintaining intestinal homeostasis as IL-10, whose deletion results in spontaneous disease (Strober,
Fuss et al. 2002; Elson, Konrad et al. 2004). Instead, TSLP in the intestine may be one of the many partially redundant immunoregulatory factors that act to maintain hyporesponsiveness and homeostasis.

Currently, it remains undetermined if the ability of TSLP to regulate proinflammatory cytokine production extends beyond the intestine to include other tissues. While there have been indications of TSLP-mediated regulation of proinflammatory cytokine production in both the skin and the lung (Al-Shami, Spolski et al. 2005; Yoo, Omori et al. 2005), the anti-inflammatory role of TSLP in these tissues has not yet been directly examined. As the level of antigenic stimulation and burden of commensal bacteria is substantially reduced in the skin and the lung, TSLP may not be required for, or important in, the maintenance of immune cell homeostasis in these tissues.

The increased susceptibility of TSLPR<sup>−/−</sup> mice to DSS-induced colitis suggests that TSLPR<sup>−/−</sup> mice may also have heightened T<sub>H1</sub> cytokine responses, possibly increasing their resistance to bacterial or viral pathogens. The immunobiology of TSLP in intestinal infections that require T<sub>H1</sub> cytokine responses for immunity such as Salmonella (Monack, Mueller et al. 2004; Coburn, Grassl et al. 2007) remains unknown. Our findings suggest that TSLPR<sup>−/−</sup> mice would be predisposed to intestinal CD<sub>4+</sub> T cell IFN-γ production upon infection, possibly increasing resistance and resulting in a faster kinetic of bacterial clearance. However, it is also possible that the predisposition and increased IFN-γ response may lead to unregulated immune-mediated pathologies and death of the TSLPR<sup>−/−</sup> mice. It will also be important to determine if TSLPR<sup>−/−</sup> mice have systemically dysregulated T<sub>H1</sub> cytokine responses. Studies with T<sub>H1</sub> cytokine-inducing parasitic
infection models such as *Leishmania major*, which can be localized to the footpad or ear, or intraperitoneally injected *Toxoplasma gondii* which results in systemic infection, have also not been done. Understanding the role of TSLP in T\textsubscript{H}1 cytokine responses is important as it might impact susceptibility or resistance to infection in human diseases where TSLP expression is dysregulated, such as Crohn’s disease (Rimoldi, Chieppa et al. 2005) or asthma (Ying, O’Connor et al. 2005).

**Future directions and challenges**

Taken together, our studies suggest an important role for TSLP in the regulation of intestinal inflammation. However, as the majority of studies completed on TSLP to date focus on the T\textsubscript{H}2 promoting functions of TSLP, many basic questions concerning TSLP-mediated regulation of proinflammatory cytokine production remain unanswered. The mechanisms and molecular mediators involved remain undefined, and it is unclear whether the mechanisms vary between homeostatic conditions and situations of infection or inflammation.

Understanding whether TSLP’s ability to inhibit proinflammatory cytokine production is a tissue-specific function restricted to the intestine is of critical importance if the TSLP-TSLPR pathway is to be manipulated for therapeutic benefit. While studies have indicated a therapeutic benefit to blocking either TSLP (Miyata, Nakamura et al. 2009) or OX40-OX40L interactions (Seshasayee, Lee et al. 2007; Wang and Liu 2007) in situations of atopy, our findings indicate that systemic targeting of TSLP may lead to a predisposition to develop inflammation within the intestine. The potential for negative consequences resulting from systemic targeting of the TSLP-TSLPR pathway highlight
the importance of fully characterizing the functionality of TSLP in each tissue. Although TSLP appears to be predominately associated with pathologic $T_\text{H}2$ cytokine responses in the skin and lung, the possible immunoregulatory function of TSLP in these tissues must not be discounted.

Concluding remarks

The results presented in this thesis provide new insights into the role that TSLP-TSLPR interactions play in regulating immunity and inflammation within the GI tract (summarized in Figure 24). Data presented in Chapter 2 reveal the complex nature of TSLP-TSLPR interactions within the intestine, as cells of both the innate and adaptive immune system express the TSLPR complex and respond to TSLP stimulation. In Chapter 3 we investigate the functional biology of TSLP in intestinal infection and identify TSLP-independent host-protective $T_\text{H}2$ cytokine responses, demonstrating that TSLP is not the critical factor required for the initiation of $T_\text{H}2$ immune responses. The potential regulatory function of TSLP is explored in Chapter 4. We demonstrate that TSLP is important in the maintenance of intestinal immune homeostasis and can regulate proinflammatory cytokine production \textit{in vitro} and \textit{in vivo}. Collectively these studies identify an important role for TSLP-TSLPR interactions in the regulation of the intestinal immune system and provide insights into how this pathway could be manipulated for therapeutic benefit when the balance of immunity and inflammation in the intestine is disrupted.
Figure 24 TSLP is a central regulator of intestinal immune homeostasis
Figure 24 TSLP as a central regulator of intestinal immune homeostasis

Figure 24 TSLP-TSLPR interactions are important in the maintenance of intestinal immune homeostasis. TSLP is produced by IECs and acts on DCs to promote $T_{h2}$ cell differentiation and inhibit $T_{h1}$ cell differentiation, partially through the inhibition of DC-derived IL-12/23p40. TSLP acts directly on naïve CD4$^+$ T cells to promote $T_{h2}$ cell differentiation and potentially inhibit IFN-$\gamma$ production. TSLP acts on mast cells in conjunction with other cytokines to induce $T_{h2}$ cytokine release. Treatment with TSLP elicits increased frequencies of basophils which act on naïve CD4$^+$ T cells to promote $T_{h2}$ cell differentiation. Mast cells and basophils produce TSLP following stimulation. TSLP may also act on IECs to promote barrier function.
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