The Functional Biology of IL-25

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The Functional Biology of IL-25

Abstract
CD4+ T helper (Th) 2 cells secrete interleukin (IL)-4, IL-5 and IL-13 and promote immunity to gastrointestinal helminth infections and chronic inflammation associated with asthma and allergic disorders. However, the innate immune pathways that promote Th2 cell responses remain poorly characterized. The non-hematopoietic cell-derived cytokines thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 (IL-17E) have been implicated in promoting Th2 cell-dependent inflammation at mucosal sites [1-4], but how these cytokines influence innate immune responses are less well defined. Studies in Chapter 2 and 3 examined the cellular mechanisms through which IL-25 promotes Th2 cell responses and demonstrated that IL-25 promotes the accumulation of a previously unrecognized non-B/non-T cell (NBNT) c-kit+ cell population in the gut-associated lymphoid tissue (GALT). Adoptive transfer of IL-25-elicited c-kit+ cells promoted Th2 cytokine responses and conferred protective immunity to helminth infection in normally susceptible Il17e−/− mice. In Chapter 3, characterization of the IL-25-elicited c-kit+ cells revealed these cells to be a lineage negative (Linneg) multi-potent progenitor (MPP) cell population. This cell population, termed MPPtype2 cells, exhibited multi-potent capacity, giving rise to cells of monocyte/macrophage and granulocyte lineages both in vitro and in vivo. These data indicate the IL-25-elicited MPPtype2 cells may contribute to extramedullary hematopoiesis in vivo. The relationships between MPPtype2 cells and other recently identified innate cell populations, including natural helper cells (NHCs), nuocytes, and innate type 2 helper (Ih2) cells were examined in Chapter 4. MPPtype2 cells were found to be phenotypically distinct from nuocytes, NHCs and Ih2 cells. Further, stimulation of IL-25-elicited MPPtype2 cells with TSLP and IL-33 resulted in the differentiation of these cells into a T1/ST2+ IL7Ra+ NHC/nuocyte-like cell population, indicating that MPPtype2 cells could be progenitors of NHC/nuocytes. Combined, the results presented in this thesis demonstrate that IL-25 induces the emergence of a previously unrecognized multi-potent progenitor cell population and suggests that extramedullary hematopoiesis is an evolutionary conserved pathway that promotes Th2 cytokine responses at mucosal sites.

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THE FUNCTIONAL BIOLOGY OF IL-25

Steven A. Saenz

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In
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Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2011

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ABSTRACT

THE FUNCTIONAL BIOLOGY OF IL-25

Steven A. Saenz

David Artis, PhD

CD4+ T helper (Th) 2 cells secrete interleukin (IL)-4, IL-5 and IL-13 and promote immunity to gastrointestinal helminth infections and chronic inflammation associated with asthma and allergic disorders. However, the innate immune pathways that promote Th2 cell responses remain poorly characterized. The non-hematopoietic cell-derived cytokines thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 (IL-17E) have been implicated in promoting Th2 cell-dependent inflammation at mucosal sites [1-4], but how these cytokines influence innate immune responses are less well defined. Studies in Chapter 2 and 3 examined the cellular mechanisms through which IL-25 promotes Th2 cell responses and demonstrated that IL-25 promotes the accumulation of a previously unrecognized non-B/non-T cell (NBNT) c-kit+ cell population in the gut-associated lymphoid tissue (GALT). Adoptive transfer of IL-25-elicited c-kit+ cells promoted Th2 cytokine responses and conferred protective immunity to helminth infection in normally susceptible Il17e−/− mice. In Chapter 3, characterization of the IL-25-elicited c-kit+ cells revealed these cells to be a lineage negative (Linneg) multi-potent progenitor (MPP) cell population. This cell population, termed MPPtype2 cells, exhibited multi-potent capacity, giving rise to cells of monocyte/macrophage and granulocyte lineages both in vitro and in vivo. These data indicate the IL-25-elicited MPPtype2 cells may contribute to extramedullary hematopoiesis in vivo. The relationships between MPPtype2 cells and
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Chapter 1

Introduction

Innate and adaptive immune cells located at the mucosal surfaces of the airways and the gastrointestinal tract play pivotal roles in the detection and elimination of invading pathogens. However, in the steady state these immune cell populations remain hyporesponsive to a vast array of innocuous environmental stimuli to which they are simultaneously exposed including allergens, pollutants and beneficial microbial communities [5]. The consequences of dysregulated immunologic hyporesponsiveness can result in inflammatory responses associated with food allergy, inflammatory bowel disease and cancer [6-10].

Based on their location at the interface between host tissues and external environment, it is now recognized that epithelial cells (ECs) are a critical cell population that can influence the initiation, regulation and resolution of innate and adaptive immune cell responses at mucosal sites. There has been particular interest in EC-derived cytokines including thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 as recent data support roles for these cytokines in influencing Th2 cytokine responses at mucosal sites through the induction of distinct innate cell populations [11-17]. For instance, TSLP has been shown to selectively promote basophilia in the periphery, while IL-33 elicits eosinophils [11]. In contrast, the cellular mechanisms through which IL-25 promotes CD4+ T helper (Th) 2 cytokine responses remain poorly characterized. Increased understanding of early cellular and molecular mechanisms that promote Th2 cytokine responses, and the mechanisms by which TSLP, IL-33 and IL-25 influence this process, may offer insight
into novel therapeutic approaches to modulate Th2 cytokine-mediated inflammation associated with asthma, allergic disorders and helminth infections.

This chapter will provide an introduction to the global health impact of helminth infections and allergic disorders, the functions and development of CD4+ T helper cell subsets, and *Trichuris muris* as a model system of Th2 cytokine-dependent immunity. In addition, this chapter will discuss the cellular sources, targets, signaling pathways, functional biology and potential cross-regulatory pathways of the epithelial cell-derived cytokines TSLP, IL-33 and IL-25. Lastly, extramedullary hematopoiesis and recently identified innate lymphoid cell populations will be discussed as potential mechanisms for the initiation of rapid local immune responses at mucosal sites.

1.1 Helminth infections and allergic disorders

The mucosal lining of the lungs and gastrointestinal tract represent a vast interface between host tissues and the external environment. As such, the mucosal immune system plays a pivotal role in the detection and elimination of invading pathogens. However, not only must the immune system successfully recognize pathogens, it must also recognize innocuous antigens from food, commensal microbes and allergens while remaining hyporesponsive to these stimuli [5]. ECs located at barrier surfaces such as the intestines, lung and skin are a critical cell population that can initiate and regulate immune responses. Among the potential immune challenges encountered by ECs at barrier surfaces, helminth parasites and food allergies pose significant human health concerns. Understanding the pathways that regulate and initiate Th2 cytokine-mediated inflammation associated with these diseases will greatly benefit efforts aimed at
developing therapeutics to control and prevent helminth infections and allergic inflammatory diseases.

1.1.1 Helminth infections

Parasitic helminth infections such as roundworms, hookworms, whipworms, blood flukes and schistosomes are considered neglected tropical diseases (NTDs) that, collectively, represent the some of the most prevalent infectious diseases worldwide [18]. For instance, it is estimated that approximately two billion individuals worldwide are infected with soil-transmitted helminths [18-20]. While helminth infections typically do not have high mortality rates, they can elicit chronic morbidities including chronic anemia and inflammation, malnutrition, disfigurement, blindness, infertility, growth retardation, delays in cognitive development, enteropathy, chronic dysentery or rectal prolapse [21-27]. Moreover, soil-transmitted helminth infections and schistosomiasis impact agricultural productivity not only through human disease and morbidity, but also through infection of livestock, causing reduced food production and economic losses.

The human whipworm *Trichuris trichiura* infects approximately 600 million people worldwide and an additional three billion individuals are at risk of infection [28, 29]. Currently, developing regions of Asia, Africa and Latin America have the highest rates of *Trichuris trichiura* infection and the highest incident rates found in rural areas [19]. Treatment with anti-helminth drugs is effective to induce the expulsion of parasitic worms [19], however, to date there are no successful vaccines to prevent infection with soil-transmitted helminths and efforts aimed at controlling these diseases have fallen behind those aimed at eradicating HIV/AIDS and malaria. As a result, increased efforts to control helminth infections can have a dramatic impact on global health, especially
considering that helminth infections cause as significant a public health burden as HIV/AIDS and malaria in terms of the number of life years lost due to disability or death. For example, while 64 million disability adjusted life years (DALYs) have been attributed to HIV/AIDS, and soil-transmitted helminth account for 20 million DALYS and *T. trichiura* alone results in an estimated 6.4 million DALYs [20]. Thus, there is a growing need to identify novel vaccine and therapeutic approaches in defense against helminth infections.

### 1.1.2 Asthma, allergies and allergic disorders

Asthma and allergic diseases are among the major causes of illness and disability in the US for all ages. Major allergic diseases include asthma, allergic rhinitis, chronic rhinosinusitis, atopic dermatitis, and food allergy. A recent nationwide survey found that more than half (54 percent) of all US citizens test positive to one or more allergens, which is thought to be a predictor for the eventual development of subsequent or additional allergic conditions [30, 31]. In addition, the prevalence and severity of food allergies has increased in the past two decades. Reports from the US and Europe have shown approximately a two-fold increase in the number of children with peanut allergy alone while reports in the EU indicate the prevalence of food allergy is 7.5% in children and 2–4% in adults [32-34]. Further, asthma affects approximately 7% of adults and nearly 10% of children in the US [34] and since 1980 the incidence of asthma in the US has nearly tripled [35], with more than 23 million people affected with this disease. In Europe, the prevalence of children with asthma rose from less than 5% to 25% in 30 years [36].
Individuals with atopic dermatitis are predisposed to developing food allergies [37, 38] and atopic dermatitis is typically the first diagnosed allergic disorder in children. Moreover, atopic dermatitis and food allergies have also been linked to the development of asthma. This progression from atopic dermatitis to the development of food allergies, asthma and other allergic disorders is a phenomenon termed ‘the allergic march’. For instance, sensitivity to food allergens, including wheat, eggs, milk and peanuts is predictive of the development of asthma and these children are more likely to develop asthma or allergic inflammation [39]. Further, approximately one third of children with food allergy have asthma and children with asthma and food allergy are more likely to have fatal allergic reactions to food [38, 40]. Moreover, epidemiologic studies have linked obesity with the development and severity of asthma [41]. As the adverse impact of asthma and allergic diseases on public health continues to grow, there is an urgent need to develop novel approaches to treat and, eventually, prevent these diseases. Better understanding of the cellular and molecular events that regulate the development of Th2 cytokine responses will enable the scientific community to develop targeted therapeutics to prevent the onset of multiple inflammatory conditions.

1.2 CD4+ T helper cell subsets
The ability of the host to recognize and respond to distinct pathogens is a central part of mammalian host defense. Within the adaptive immune response, following infection or exposure to allergens, naïve CD4+ T helper (Th) cells can differentiate into one of various specialized effector cell subsets, including Th1, Th2 or Th17 cells as well as regulatory T cells (Treg), IL-22-producing T cells (Th22) and T follicular helper cells (Tfh). These subsets are defined by their unique expression of transcription factors, cytokine production profiles and effector functions. The development of CD4+ T helper
cell subsets is dependent upon three specific molecular signals: 1) T cell receptor (TCR) engagement, 2) co-stimulation and 3) cytokine signaling. Depending on the context of infection, these stimuli specify the appropriate T helper cell fate required to combat the invading pathogen [42, 43]. This section will discuss the molecular mechanisms and cytokine signals involved in the development of CD4⁺ Th1, Th2 and Th17 cell subsets, as well as their functions in immunity and inflammation.

1.2.1 T helper cell differentiation

Subsets of CD4⁺ T cells were first described in 1986 and their classification was based on differential effects on B cell responses. These subsets were termed Th1 and Th2 cells and were defined by their unique expression of the cytokines interferon (IFN)-γ and interleukin (IL)-4, respectively [44]. While, Th2 cell differentiation will be discussed in more detail in the following sections, subsequent studies demonstrated that Th1 cell differentiation requires the expression of the T-box binding transcription factor T-bet, as well as the IL-12/STAT4 signaling and IFN-γ/STAT1 signaling pathways [45-47]. Th1 cell differentiation results from IFN-γ/STAT1 signaling on TCR-activated T cells leading to increased expression of T-bet and the upregulation of IL-12Rβ2 [48]. This increased responsiveness to APC-derived IL-12 results in IFN-γ production from the CD4⁺ T cells and blocks their differentiation into Th2 cells by inhibiting IL-4 expression and GATA3 [49, 50]. Th17 cell differentiation is dependent on expression of the transcription factor RORγt and was originally thought to be dependent on the IL-23/STAT3 signaling pathway. However subsequent studies have identified that optimal Th17 cell differentiation occurs following stimulation with TGFβ or IL-1β combined with IL-6 [51-53], while IL-23 acts to sustain RORγt and IL-17A expression and Th17 cell survival.
Th1 and Th17 cell subsets are associated with protective immunity and/or inflammation and the use of in vivo infection models first demonstrated the biological significance and pathogen-specificity of these subsets. For instance, CD4+ Th1 cells mediate immunity to the intracellular protozoan parasites such as Leishmania major and Toxoplasma gondii [54, 55]. Th1 cell responses are characterized by expression of their signature cytokine IFN-γ and are required for immunity to intracellular pathogens such as viruses, bacteria and parasites. Th17 cells are characterized by their signature cytokine profile IL-17A, IL-17F, IL-21 and IL-22 and are associated with defense against certain extracellular bacteria and fungal pathogens [56-58]. In addition, recent studies have also demonstrated that Th17 cells are responsible for inflammation associated with multiple inflammatory and autoimmune diseases. For instance, Th17 cytokine responses are associated with experimental autoimmune encephalitis (EAE), inflammatory bowel disease (IBD), psoriasis and arthritis, diseases that were previously thought to be mediated by Th1 cytokine responses [8, 59]. These findings highlight that increased understanding of the molecular mechanisms that promote immunity and/or disease will benefit the development of more targeted and efficacious therapeutics.

1.2.2 Th2 cell differentiation and type 2 inflammation

CD4+ T helper 2 cells are characterized by their production of the effector cytokines IL-4, IL-5, IL-9 and IL-13. Th2 cell differentiation requires expression of the transcription factor GATA3, the master regulator of Th2 cell differentiation, and signaling through the IL-4/STAT6 pathway. IL-4/STAT6 signaling following TCR stimulation results in the upregulation of GATA3 and increased production of IL-4, IL-5 and IL-13, however, several additional transcription factors including NFAT, AP-1 and c-Maf can contribute to optimal/sustained IL-4 production [49, 60, 61]. Th2 cells are known to be required for
resistance to helminth parasites, but similar to Th17 cells, Th2 cells can also play dual roles in immunity: being either protective or pathologic. In the context of helminth infections, IL-4, IL-5 and IL-13 production from CD4⁺ T cells promotes expulsion of parasitic helminths through the induction of goblet cell hyperplasia, increased mucin production, increased smooth muscle contractility, alternative activation of macrophages (AAMacs), fibrosis, eosinophilia and mast cell degranulation. Collectively, these downstream effects of IL-4, IL-5 and IL-13 are termed ‘type 2 inflammation’. However, while the mechanisms involved in the initiation of innate immune responses following exposure to viral, bacterial or protozoan infections are well characterized [62-67], the innate immune pathways that promote Th2 cytokine responses in the context of allergy or helminth infection remain less well-characterized.

1.2.3 Helmiths as a model for CD4⁺ Th2 cell-dependent immunity

Many of the discoveries regarding the identification of effector functions and the regulation of Th2 cytokine responses have been made by studying murine models of helminth infections. For example, using the Schistosoma mansoni, Nippostrongylus brasiliensis and Trichuris muris infection models, investigators have demonstrated that Th2 cytokine responses and type 2 inflammation are responsible for mediating immunity and type 2 inflammation following helminth infections.

Studies in this thesis employ the Trichuris muris infection model to investigate the in vivo functional biology of IL-25-elicited innate cell populations (see Chapter 2). Trichuris, a soil transmitted helminth, is a natural pathogen of mice and being biologically similar to species that infect humans (Trichuris trichiura), provides a natural well-defined in vivo model for studying the development of Th2 cytokine responses [68]. Trichuris utilizes a
fecal/oral route of transmission whereby *Trichuris* eggs are ingested orally by the host and hatch in the distal small intestine. Larval parasites burrow into intestinal epithelial cells (IEC) that line the crypts of the cecum and proximal colon. Adult worms remain intimately associated with the IECs as they form syncytial tunnels and are in direct contact with IEC cytoplasm. The posterior end of the worms remains in the lumen to allow for mating and the deposition of eggs that propagate the *Trichuris* life-cycle [68].

Experimental infection with *Trichuris* provides a tractable model of CD4⁺ T cell-dependent immunity in the gut whereby resistance or susceptibility to *Trichuris* infection is determined by specific immune responses. For instance, susceptible mouse strains such as AKR mount a Th1 response characterized by production of IL-12 and IFN-γ and are unable to clear the parasites resulting in chronic infection. In contrast, resistant mice (B6 or BALB/c) develop a Th2 cell response (IL-4, IL-5, IL-9, and IL-13) and successfully expel parasites by day 21 post-infection [69, 70]. CD4⁺ T cells are required for immunity to *Trichuris*, as mice that lack T cells or depletion of CD4⁺ T cells in vivo results in an inability to expel parasites. Conversely, adoptive transfer of CD4⁺ T cells into severe combined immunodeficient mice (SCID) mice, which lack all B and T lymphocytes, resulted in the development of protective immunity during *Trichuris* infection [71]. These findings suggest that not only are CD4⁺ T cells alone necessary and sufficient to promote immunity to *Trichuris*, but that B cells are not required for immunity in this setting.

Subsequent studies analyzing the role of CD4⁺ T cells in infection have revealed that immunity to *Trichuris* is critically dependent on the generation of CD4⁺ Th2 cells (reviewed in [72-74]) and their production of IL-4, IL-5 and IL-13 [69, 71, 75, 76].
However, additional signaling pathways including MyD88, Notch and other cytokines play a role in the development of protective Th2 cytokine responses in the context of *Trichuris* infection. While MyD88 and TLR-4 signaling renders mice more susceptible to infection [77], blockage of Notch signaling pathways in T cells through the use of a dominant negative MAML protein demonstrated that this pathway is required for Th2 cell differentiation [78]. As a result, following infection with *Trichuris*, these mice exhibited increased production of IFN-γ and were susceptible to helminth infection [78], implicating roles for Notch signaling pathways in the development of protective Th2 cytokine responses. Collectively, *Trichuris* has provided a useful model to investigate the requirements of innate and adaptive immune components in the development of Th2 cytokine responses.

### 1.2.4 Epithelial cell-mediated responses to *Trichuris muris*

Due to the intimate association between adult *Trichuris* worms and IECs [68], IECs were suspected to be an important cell population in the initiation of protective Th2 cytokine responses following *Trichuris* infection. Indeed, *in vivo* manipulation of the epithelial cell-derived cytokines such as TSLP, IL-33 and IL-25 has emphasized the importance of IECs in resistance to infection [4, 79-81], and highlighted roles for TSLP, IL-33 and IL-25 in promoting Th2 cytokine responses (discussed in detail in Chapter 1.3-1.5). For instance, mice in which NFκB signaling was ablated only in intestinal epithelial cells (*IkkbΔIEC*) display decreased expression of TSLP [4]. As a result, these mice are unable to expel *Trichuris* parasites, as TSLP was shown to be critical in regulating the production of the pro-inflammatory cytokine IL-12p40 from DCs [4, 79]. Administration of IL-33 to susceptible mice was also demonstrated to promote immunity to *Trichuris* [80] by promoting Th2 cytokine responses and reducing parasite-specific Th1 responses.
While IL-33 is known to promote immunity it is currently not known whether IL-33 is necessary for immunity to helminth infections.

Similar to IL-33, administration of exogenous IL-25 could confer immunity to normally susceptible mice strains infected with *Trichuris* [81]. In addition, mice deficient in IL-25 signaling also display an impaired ability to expel *Trichuris* parasites as they were unable to mount protective Th2 cytokine responses compared to infected WT mice [81]. However, similar to TSLP, treatment of *Trichuris* infected IL-25-deficient mice with neutralizing antibodies against IL-12p40 or IFN-γ was able to restore immunity [81], indicating that IL-25 was not essential for the development of protective Th2 cell responses. Collectively, these data highlight the roles for TSLP, IL-33 and IL-25 in the development of immunity to helminth infections. While it is through the induction of Th2 cytokine responses and the concomitant type 2 inflammation, including increased production of IgE and IgG1, mastocytosis, alternatively activated macrophages, goblet cell hyperplasia, RELMβ and mucus production, and increased epithelial permeability and turnover [82-86] that ultimately result in expulsion of *Trichuris*, the cellular mechanisms by which TSLP, IL-33 and IL-25 promote these responses are not completely understood.

Despite being distinct cytokines and sharing little or no homology to one another, role for TSLP, IL-33 and IL-25 in the promoting Th2 cytokine-mediated responses are well documented. However, in order to fully understand how these cytokines initiate and support the development of these immune responses, it is critical to recognize both their cellular sources and targets *in vivo*. The following sections will focus on the functional biology, cellular sources and targets of TSLP, IL-33 and IL-25.
1.3 Functional biology of TSLP

TSLP has been shown to play a role in the regulation of pro-inflammatory cytokine production and resultant inflammation during helminth infections, allergic asthma, atopic dermatitis and chemically-induced colitis [4, 79, 87, 88]. Increased levels of TSLP were observed in human patients with atopic dermatitis [1] and in the airways of asthmatic patients, which correlated with disease severity [89]. Suggesting that TSLP is directly promoting Th2 cytokine responses, the targeted overexpression of TSLP in epithelial cells of the skin or lung in murine systems resulted in the development of severe Th2-cytokine mediated inflammation resembling atopic dermatitis or asthma, respectively [88, 90, 91]. Combined, these results establish a more causative relationship between TSLP and Th2 cytokine-mediated disease. Specifically, the increased expression of TSLP resulted in the development of CD4+ Th2 cells, inflammatory cell infiltration, eosinophil infiltration, increased IgE production and tissue damage [88, 90, 91]. The link between increased TSLP expression and disease has been further strengthened by the association of genetic TSLP polymorphisms in humans with the development of asthma [92] as well as the phenotype of transgenic mice with overproduction of TSLP [90]. In addition, mice deficient in the receptor for TSLP (Tslpr\textsuperscript{−/−}) were shown to be less susceptible to an ovalbumin (OVA)-induced model of allergic asthma. In this model, Tslpr\textsuperscript{−/−} animals failed to produce Th2 cell-associated cytokines and did not exhibit lung inflammation [87, 91], supporting a role for dysregulated TSLP production contributing to disease outcome.

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 indicate that
TSLP may play a role in the pathogenesis of other allergic diseases. For example, 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 [93]. Also, increased TSLP expression has been documented in the synovial fluid of human patients with rheumatoid arthritis [94]. Furthermore, genetic variants of TSLP and its receptor (TSLPR) correlate with the development of eosinophilic esophagitis and biopsies from patients suffering from eosinophilic esophagitis displayed increased expression of TSLP compared to control tissues [95].

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 intestinal ECs in influencing immune cell homeostasis and immunity to helminth parasites [4, 96, 97], however, TSLP does not appear to be required for immunity to some helminth infections, such as *S. mansoni*, *Heligmosomoides polygyrus* and *N. brasiliensis* [98, 99]. As discussed above, IEC-specific deletion of IKKβ resulted in susceptibility to the intestinal dwelling nematode *Trichuris* and was correlated to decreased expression of TSLP [4]. Despite being on a genetically resistant background, *Tslpr*Δ mice challenged with *Trichuris* develop a Th1 cytokine response characterized by high expression levels of IL-12p40 and IFN-γ, and were unable to clear infection at day 21 post-infection [4, 79]. The strong induction of TSLP mRNA upon infection [80] and its requirement for efficient early worm clearance [4], indicate that TSLP is a critical component of protective Th2 cytokine-mediated protective immune responses in the intestine.
1.3.1 Cellular sources and regulation of TSLP expression

While TSLP was initially identified and characterized as a novel cytokine produced by thymic stromal cells [100, 101], it is predominantly expressed in epithelial cells of the skin (keratinocytes), lung (small airway epithelial cells), and intestine (intestinal epithelial cells) [1, 101-103]. In the steady state, basal expression of TSLP mRNA and protein has been detected in epithelial cells of the tonsils, skin, lung, and intestine [4, 96, 102 2002, 104, 105].

Mast cells, basophils and ECs have all been reported to express TSLP. Cross-linking of the IgE receptor on mast cells results in elevated levels of TSLP mRNA [1] and basophils stimulated with papain upregulate TSLP mRNA and protein [106]. Increased TSLP expression in ECs can be induced through exposure to viral, bacterial, or parasitic pathogens as well as following ligation of Toll-like receptors (TLRs) [80, 107-109] and exposure to proinflammatory or Th2 cell-associated cytokines has also been shown to induce expression of TSLP [104, 107, 110, 111] (Fig. 1A). Interestingly, treatment of a lung epithelial cell line MLE12 with IL-25 also resulted in increased TSLP expression [112], suggesting a potential link between these two EC-derived cytokines (see Cross-regulation and interplay section below). Moreover, there is speculation that TSLP can influence wound healing since expression is upregulated by damage or trauma to ECs [108]. Although the molecular mediators and mechanisms of action remain unknown, recent work suggests that mast cells may also induce TSLP expression in ECs as mast cell-deficient mice fail to upregulate TSLP in a model of allergic rhinitis [93]. Given the findings that TSLP can be induced by both exogenous stimuli (damage, infection, TLR
ligation) and host-derived signals (Fig. 1A), TSLP production by ECs and its regulation represent a critical pathway through which ECs modulate immune responses.

### 1.3.2 Cellular targets and TSLP-TSLPR signaling

High affinity ligand binding of TSLP and subsequent signaling requires a heterodimeric receptor consisting of the IL-7Rα chain and a unique TSLPR chain that resembles the common cytokine receptor γ chain (γc) [103, 113]. 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 STAT5 in mice and humans and STAT3 in humans [114, 115] and appears to be JAK-independent, but may require a Tec kinase [102, 103, 114, 115] (Fig. 2A).

Initially, TSLPR expression was thought to be limited to immature B cells and myeloid cells such as monocytes and dendritic cells (DCs) [102, 103, 113], however, more recent work has demonstrated TSLPR expression on mast cells, basophils and CD4+ T cells [102, 106, 108, 116]. DCs and mast cells appear to express TSLPR constitutively [1, 106, 108], however, CD4+ T cells require TCR stimulation to induce receptor expression and become sensitive to TSLP treatment [102, 116, 117]. Additionally, human mast cells have been recently demonstrated to express the functional TSLPR complex and produce IL-5 and IL-13 as well as IL-6 and GM-CSF following exposure to TSLP [108].

More recent data has suggested that in vivo, TSLP selectively promotes the population expansion of basophils [11, 12]. These studies demonstrated that administration of recombinant TSLP or injection of a cDNA plasmid encoding TSLP resulted in increased frequencies of basophils in the blood and spleen [11, 12]. Studies performed by
Siracusa et al. also demonstrated that TSLP could promote peripheral basophilia in the absence of IL-3 signaling [12], a cytokine previously shown to regulate basophil development. Moreover, TSLP-elicited basophils expressed higher levels of T1/ST2 and produced more IL-4 and IL-6 in response to IL-3, IL-18 and IL-33 [12], indicating that TSLP-elicited basophils were functionally distinct from IL-3-elicited basophil populations. Collectively, while these reports identify multiple cellular targets for TSLP in vivo, they highlight that TSLP selectively induces basophil populations to influence Th2 cytokine responses.

1.4 Functional biology of IL-33

IL-33 was first described in 2005 and, along with IL-1 and IL-18, belongs to the IL-1 cytokine family [2]. IL-33, similar to its other family members IL-1 and IL-18, has been shown to promote Th2 cytokine responses [2, 80, 118-121]. Members of the IL-1 family are expressed as prodomains and only become functionally mature proteins following proteolytic processing by caspase-1. Consistent with other IL-1 family members, Schmitz et al. [2] demonstrated that in vitro incubation of IL-33 with caspase-1 yielded an 18 kDa mature protein from a 30 kDa precursor. IL-33, like other members of the IL-1 family, shares the IL-1/FGF β-trefoil fold structural motif consisting of 12 β-strands forming a single domain [122, 123]. Interestingly, IL-33 has also been ascribed transcriptional repressor properties based on nuclear localization to heterochromatin and using a yeast two-hybrid system, it was shown that IL-33 repressed transcription in a manner consistent with IL-33 associating with heterochromatin [124]. Despite this work, the functional significance of IL-33 nuclear localization and the role this phenomenon plays in the context of immunity or inflammation is not fully understood.
IL-33 is produced by ECs and has been implicated in the promotion of Th2 cytokine associated responses, although IL-33 has also been shown to inhibit proinflammatory cytokine responses. IL-33 treatment was found to reduce atherosclerotic plaque size, the number of infiltrating macrophages and T cells and production of IFN-γ [125]. In addition, IL-33 signaling has also been reported to regulate T. gondii-mediated inflammation [126] and has been linked to inflammatory bowel disease (IBD) [127] and lupus [128]. The link between IL-33 and Th2 cytokine production was formed from in vivo studies, in which administration of IL-33 to mice resulted in increased expression of IL-4, -5, and -13, elevated serum levels of IgE and IgA and additional hallmarks for type 2 inflammation including increased goblet cell hyperplasia, mucus secretion and eosinophilia [2].

While data suggest that IL-33 promotes Th2 responses in vivo, in the systems tested, IL-33 does not appear to be strictly required for the initiation of Th2 cytokine-mediated responses in vitro [129] or in vivo [130]. IL-33 expression is induced following infection with Trichuris [80], and administration of recombinant IL-33 early during infection conferred resistance to normally susceptible Trichuris-infected AKR mice [80], similar to the effects observed following early treatment with IL-25 [81], however, Hoshino et al. found no effect on the outcome of infection in mice deficient in IL-33 signaling [131]. Moreover, IL-33 treatment was insufficient to mediate expulsion of Trichuris in the absence of adaptive immunity highlighting the necessity of CD4+ T cells for resistance. Interestingly, IL-33 treatment increased the expression of TSLP and TSLPR mRNA in the colons of Trichuris-infected mice [80] demonstrating a potential association between two cytokines known to drive Th2 cytokine responses.
Increased expression of the IL-33 receptor (T1/ST2) has been reported in the serum of patients with asthma [132] and in mouse models of airway inflammation [133]. Further, two studies have demonstrated that neutralization of T1/ST2 resulted in the decreased levels of IL-4 and IL-5 in the bronchio-alveolar lavage fluid (BAL) and a reduction in eosinophil infiltration in an airway inflammation model [134, 135]. However, another report found no relationship between T1/ST2 expression and increased airway inflammation [131]. In support of the association between IL-33 and airway inflammation there was a recent report indicating that IL-33 induced airway hyperresponsiveness (AHR) associated with increased airway resistance and Th2 cytokine expression in the lungs [136].

Along with promoting protective Th2 cytokine responses, IL-33 can also have a pathologic role in vivo. Consistent with its ability to promote IL-13 production, IL-33 was found to be expressed in fibrotic liver tissue and promote collagen deposition [137], however, IL-33 was also expressed in the synovial joints of arthritis patients [138]. Moreover, blockade of T1/ST2 signaling attenuated inflammation observed in a mouse model of collagen-induced arthritis [138], highlighting a role for the IL-33 signaling pathway inflammatory diseases.

### 1.4.1 Cellular sources and regulation of IL-33 expression

Expression of IL-33 mRNA has been reported in tissues such as the CNS, lymph nodes, lung, skin, and colon, and in dendritic cells, macrophages, lung ECs and adipocytes [2, 41, 139], however, both helminth and influenza infections have been reported to increase production of IL-33 [80, 139]. Infection with *Trichuris* increased IL-33 mRNA with peak expression reported at day 3 post-infection compared to naïve controls [80]
(Fig. 1B). This infection-induced increase in IL-33 appears to be transient and temporal, suggesting a regulatory pathway to dampen its production following induction of immune responses. In addition, the H3N1-strain of influenza was also reported to increase IL-33 production from alveolar macrophages. Interestingly, while lung ECs were reported to produce IL-33 under homeostatic conditions, their production levels of IL-33 remained unchanged following flu infection [139].

### 1.4.2 Cellular targets and IL-33-T1/ST2 signaling

IL-33 signals through T1/ST2, a receptor closely related to IL-1R1 and IL-18Rα [140-142], but it does not bind IL-1 or IL-18. This receptor family is characterized by the presence of an intracellular Toll-IL-1R (TIR) domain [143, 144], and requires two components for effective signaling: a ligand binding chain and a second subunit, which mediates the downstream signaling events but does not physically interact with the ligand itself (reviewed in [145]) (Fig. 2B). Both a membrane form and soluble form of T1/ST2 exist *in vivo* [146, 147], however, the function of the soluble form of T1/ST2 is unclear. One potential role is to function as a decoy receptor, thus regulating constitutive IL-33 signaling *in vivo* (Fig. 2B). In its membrane form, T1/ST2 is present on Th2 polarized cells and mast cells independently of IL-4 [134, 148, 149]. IL-33 was found to associate with T1/ST2 [2], and receptor ligation resulted in activation of NF-κB [2]. IL-1R accessory protein (IL-1RαC) was subsequently identified as the second component of the functional IL-33R complex, as treatment with IL-33 did not induce a Th2 cytokine response in the absence of IL-1RαC [150]. Therefore, the IL-33 signaling receptor complex is comprised of T1/ST2 and IL-1RαC (Fig. 2B). Furthermore, MyD88 and TRAF6, as well as IL-1R associated kinase 4 (IRAK4), form a complex with IL-33
T1/ST2 is expressed by Th2 cells and mast cells. Incubation of Th2 cells with IL-33 increased production of IL-5 and IL-13 and reduced production of IFN-γ [2]. Additionally, mature mast cells and their precursors express T1/ST2 and treatment of human mast cells or CD34+ mast cell progenitors [108] with IL-33, but not IL-18, resulted in increased survival and cytokine production even in the absence of stem cell factor (SCF) [149]. Furthermore, Ho et al. demonstrated that murine mast cells responded to IL-33 in a MyD88-dependent manner [151]. Similar increases in cytokine production observed in basophils following treatment with IL-33 were also shown to require MyD88 [136, 152]. These studies suggest that MyD88 plays an important role in IL-33-mediated cytokine production in innate cell populations. Interestingly, inclusion of TSLP into IL-33-containing cultures could enhance cytokine production from mast cells [108], supporting a synergistic relationship between IL-33 and TSLP. Recent reports have also demonstrated that IL-33 stimulation can promote macrophages to adopt an alternative activation phenotype, characterized by expression of the mannose receptor and IL-4Rα [153] and demonstrated effects of IL-33 on basophils [12, 136, 154], eosinophils [155] and recently identified innate cell populations (discussed in Chapter 1.7), termed natural helper cells, nuocytes and innate type 2 helper cells [13-15].

Collectively, these findings illustrate the ability of TSLP and IL-33 to promote Th2 cytokine responses and type 2 inflammation in vivo by targeting multiple cell populations, however, more recent data suggests that TSLP and IL-33 elicit distinct innate cell populations to influence Th2 cell differentiation. Moreover, the cellular mechanisms
used by TSLP and IL-33 to promote Th2 cytokine responses represent viable targets for therapeutics aimed at modulating Th2 cytokine-mediated inflammation associated with asthma, allergic disorders and helminth infections.

1.5 Functional biology of IL-25

IL-25 is a member of the IL-17 cytokine family (reviewed in [156, 157]), which contains six members that were discovered through analysis of genomic databases [3, 158-161], with IL-17A being the first discovered through sequence homology to a Herpesvirus samirii open reading frame [162]. While this family bears no close similarity to other known cytokines, the discovery of several homologues to IL-17 and two IL-17-receptor homologues within the *Strongylocentrotus purpuratus* (purple sea urchin) genome underscores the evolutionarily conserved nature of this cytokine family [163]. Within this cytokine family individual members share approximately 40-50% sequence identity although IL-25 possesses the lowest degree of homology to IL-17A at 17% [156, 157]. The greatest similarity between these cytokines is observed in a conserved cysteine-rich C-terminus, which forms a cysteine-knot structure and is related to a structural motif present several growth factors [156, 157]. All members exist as homodimers ranging in molecular weight from 20 to 35 kDa, however recent data suggests that IL-17A and IL-17F form heterodimers [164, 165].

Although originally described to promote type 2 inflammation, IL-25 has also been implicated in regulating intestinal inflammation during helminth infection as well as the pathogenesis observed during experimental autoimmune encephalomyelitis (EAE) [10, 81, 166]. Further supporting an immunoregulatory role for IL-25 are data that demonstrate that mice lacking commensal bacteria exhibit reduced expression of IL-25
in intestinal EC and exaggerated IL-23 and IL-17A levels, suggesting bacterial-derived signals may influence this immuno-regulatory pathway [167]. In addition, patients suffering from intestinal inflammation (i.e. Crohn’s disease or ulcerative colitis) express less IL-25 than control patients and display higher expression levels of IL-23 [168, 169]. Moreover, treatment of human CD14+ monocytes with IL-25 reduced their expression of pro-inflammatory cytokines and IL-25 could ameliorate disease in chemically-induced colitis models in mice [168]. Interestingly, however, there are no known associations between genetic polymorphisms in the IL-25 gene and the development of inflammatory diseases [170], despite the location of the IL-25 gene within an IBD susceptibility locus [170]. Nevertheless, combined, these data highlight a role for IL-25 in the regulation of pro-inflammatory immune responses in both mice and humans.

Similar to TSLP and IL-33, in addition to its immuno-regulatory properties, IL-25 has also been shown to promote the development of Th2 cytokine responses at mucosal sites through the induction of IL-4-producing innate immune cells [106, 171-175]. However, compared to TSLP and IL-33, the cellular mechanisms through which IL-25 mediates type 2 inflammation are less well characterized. For instance, TSLP treatment selectively elicits IL-4-producing basophils that expressed MHC class II and were capable of promoting Th2 cytokine responses [11, 12], IL-33 was shown to elicit eosinophils in the blood [11] and activate basophils [136] and natural helper cells (NHCs) [13]. In contrast, there are conflicting reports of IL-4-producing innate cell populations elicited by IL-25, among them a c-kit$$^\text{neg}$$ CD11b$$^+$$ Ly6C$$^+$$ monocyte-like cell population [176], CD4$$^+$$ NKT cells [171] and a NBNT c-kit$$^+$$ cell suggested to be a mast cell or mast cell precursor population [11, 16]. Thus, the innate cell populations responsible for promoting Th2 cytokine responses in response to IL-25 remain unclear.
This section will discuss the role of IL-25 in helminth infections and allergic disorders, its cellular sources and the cellular pathways targeted by IL-25 to initiate Th2 cytokine responses.

1.5.1 Role of IL-25 in immunity to helminth parasites and allergic disorders

IL-25 was originally reported to promote Th2 cytokine responses and type 2 inflammation using \textit{in vivo} administration of exogenous cytokine. Mice treated with IL-25 exhibited increased expression of IL-4, -5, and -13 in the lung, spleen, and liver, as well as elevated serum levels of IgE, increased goblet cell hyperplasia, mucus secretion and eosinophilia [3, 158]. Subsequent studies demonstrated that IL-25 is required for the development of a protective Th2 cytokine-mediated response and protective immunity following \textit{Trichuris} infection as mice deficient in IL-25 (\textit{Il17e}^{-/-}) fail to clear infection and display significantly reduced levels of IL-4 and IL-13 [81]. In the converse experiment, IL-25 treatment conferred resistance to genetically susceptible AKR mice. IL-25-treated AKR mice displayed increased Th2 cytokine production and elevated goblet cell numbers, resulting in efficient worm expulsion. Interestingly, SCID mice treated with IL-25 were unable to clear helminth infections and did not develop increased goblet cell number or increased mucus production indicating the requirement for adaptive immunity in IL-25-mediated expulsion of helminth infections [81]. However, \textit{Il17e}^{-/-} mice can develop a protective Th2 cytokine response upon blockade of endogenous Th1 cytokines, suggesting that IL-25 is not essential for the development Th2 cytokine responses [81]. In contrast to persistent infection of normally susceptible AKR mice, which results in minimal intestinal inflammation, \textit{Trichuris}-infected \textit{Il17e}^{-/-} mice developed a chronic infection characterized by development of severe intestinal inflammation and elevated expression of IFN-\gamma and IL-17A [81]. Consistent with a role
for IL-25 in protective immunity to helminth infections, IL-25 has also been shown to influence immunity to *N. brasiliensis*. Compared to WT mice, which expel parasites by day 10 post-infection, *N. brasiliensis*-infected *Il17e−/−* mice remain infected at this time point and display delayed expulsion of the parasites, taking until days 15-20 to clear infection. Moreover, treatment with IL-25 resulted in accelerated expulsion of *N. brasiliensis* infection, but this effect was dependent on Th2 cytokines (IL-4, -5, -9 or -13) [16].

As exogenous IL-25 elicited a granulocyte infiltration into the lungs, increased mucus production, and airway constriction, it was thus proposed that IL-25 played a role in the promotion of Th2 cytokine mediated allergic airway inflammation. This hypothesis was also supported by results showing that administration of exogenous IL-25, or forced over-expression of IL-25, resulted in elevated levels of IL-4, IL-5 and IL-13 which corresponded with increased antibody isotype switching and circulating eosinophil numbers [3, 177, 178]. Moreover, intravenous or intratracheal instillation of an adenoviral construct encoding IL-25 resulted in similar physiologic changes as intraperitoneal injection of recombinant protein and was correlated with increase expression of IL-5 and IL-13, as well as proteins involved in eosinophil recruitment and migration [3, 158].

Consistent with the initial characterization of IL-25, elevated expression of IL-25 and IL-17RB are observed in patients with chronic asthma and atopic dermatitis [179]. The link between IL-25 and Th2 cytokine-mediated inflammation was further supported by findings implicating IL-25 in murine models of allergic airway inflammation. Intranasal administration of IL-25 to mice results in the development of airway hyperreactivity
Additionally, in a model of sensitization and challenge with OVA plus alum to induce airway inflammation, Tamachi et al. [180] demonstrated that IL-25 mRNA expression was upregulated following OVA challenge. Critically, neutralization of IL-25 with a soluble IL-17RB fusion protein reduced eosinophil and CD4+ T cell recruitment into the lungs [180]. Neutralization of IL-25 also reduced levels of IL-5 and IL-13 in the BAL fluid and decreased goblet cell numbers. Subsequent studies using antibodies directed against murine IL-25 again demonstrated that neutralization of IL-25 reduced AHR [181]. While administration of anti-IL-25 mAb during the aerosolized OVA-challenge could ameliorate AHR, the effect was specific to the bronchio-constriction as no decrease in cytokine production, eosinophil recruitment or goblet cell numbers was observed [181]. Moreover, it was shown that IL-25 could induce AHR independent of IL-4, IL-5, IL-9 and IL-13 [181]. These findings suggest that IL-25 is playing a major role in the initiation and outcome of disease in this model. Further supporting a critical role for IL-25 in allergic airway inflammation, transgenic mice with lung-restricted expression of IL-25 exhibited exacerbated OVA-induced airway inflammation leading to increases in IL-4, IL-5 and IL-13 levels and increased cellular infiltrates in the BAL fluid [180]. It is important to note however that overexpression of IL-25 alone was not sufficient to mediate similar effects [180], indicating that IL-25 may not initiate immunological responses but may instead promote or sustain an ongoing response. Combined, these studies identify a role for IL-25 in mediating allergic inflammation in the lung.

While IL-25 is clearly involved in allergic airway inflammation, the mechanism whereby IL-25 elicits a Th2-mediated immune response remains unclear. CD4+ T cells have been proposed to mediate IL-25-induced type 2 inflammation [180], however, previous findings indicate that the effects of IL-25 are independent of T cells [3, 177, 178]. The
cell populations that IL-25 targets to promote Th2 cytokine responses will be discussed in more detail in Chapter 1.5.3 and will be the focus of this thesis.

1.5.2 Cellular sources and regulation of IL-25 expression

While IL-17A and IL-17F are associated with increased levels of IL-6 and TNFα, IL-25 has been implicated in promoting Th2 cell-mediated inflammation [3, 81, 158, 180, 182]. Consistent with these findings, exposure to allergens and air pollutants has been reported to increase IL-25 expression [112, 158, 180, 183], as has infection with the helminth *N. brasiliensis* [158] (Fig. 1C) or following viral infections [184]. Conversely, mice that lack commensal bacterial communities or mice treated with dextran sodium sulfate to induce colitis were found to have decreased expression levels of IL-25 in the colons [167, 185], indicating that commensal bacteria or epithelial barrier damage regulate IL-25 expression. While no induction of IL-25 message was observed in the *Trichuris* infection model, it was noted that compared to *Trichuris* resistant mice, susceptible mice exhibited decreased expression of IL-25 and its cognate receptor, IL-17RB, in colon [81]. While allergens and helminth infection have been shown to induce IL-25, the downstream factors responsible for regulating IL-25 expression remain poorly characterized. Analysis of the genomic sequence upstream of the IL-25 encoding region revealed putative STAT6, GATA-3, and NF-κB binding sites (unpublished data). However, the functional requirement of these transcription factors in the induction of IL-25 has not been investigated. Collectively, these studies support a role for IL-25 in the initiation of Th2 cytokine responses by highlighting the induction of IL-25 expression following exposure to stimuli that elicit type 2 immune responses.
Based on cDNA libraries, the original reports on IL-25 suggested restricted expression of IL-25 to Th2 polarized CD4⁺ T cells [3, 158]. However, subsequent studies identified that murine and human lung ECs express IL-25 following exposure to allergens or helminth infections [112, 186] and macrophages, microglia, and CD8⁺ and CD4⁺ T cells have all been reported to express IL-25 [81, 112, 166, 183].

Granulocytic cell populations have also been demonstrated to produce IL-25. Mast cell lines, as well as bone marrow-derived mast cells, express IL-25 at levels comparable to polarized Th2 cells. A limitation of these studies, however, was that they were only performed in *in vitro*-derived cell populations and were not able to measure IL-25 protein [187]. Moreover, these mast cell populations required chemical stimulation with PMA and a Ca²⁺ ionophore or IgE cross-linking to express IL-25 [187]. Human eosinophils and basophils, two cell populations known to play a role in allergic inflammation, have also been shown to express IL-25 [179], but again focused only at the mRNA level. Unlike TSLP or IL-33, T cells and antigen presenting cells (APC) have been reported to express IL-25. Utilizing a lacZ reporter system, CD4⁺ and CD8⁺ T cells were found to constitutively express IL-25 in the cecal patch, a lymphoid follicle similar to the human appendix [81]. The major caveat of these studies, however, was that they employed a reporter protein and relied on an enzymatic reaction to detect IL-25 protein levels. Additionally, alveolar macrophages [183] and microglia cells, the main antigen-presenting cells within the central nervous system (CNS), express IL-25 [166]. While various cell lineages are capable to producing TSLP, IL-25, and IL-33, their involvement and significance in augmenting immune responses remains unclear. Thus, while the contribution of these potential sources of TSLP, IL-25, and IL-33 warrants further
investigation, it is ECs, based on their location at the mucosal barrier, that represent a significant source of these potent cytokines.

### 1.5.3 Cellular targets and IL-25-IL-17RB signaling

IL-25 signals through IL-17RB, which is expressed in the lung and gastrointestinal tract and exists as both a membrane bound and soluble form [159, 188]. Initially it was thought that receptor ligation resulted in homodimerization and activation of signaling cascades, but the observation of shared receptor usage among IL-17 family members and the formation of heterodimERIC receptor complexes suggests more complex interactions [164, 189, 190] (Fig. 2C). In fact, subsequent studies identified that IL-25 signals through a heterodimeric receptor composed of IL-17RB and IL-17RA [191]. T cells, APC, epithelial cells and airway smooth muscle cells (ASMC) also express IL-17RB [3, 158, 159, 187, 192]. IL-25-IL-17RB interactions have been shown to activate NF-κB, STAT6, GATA-3, NFATc1, and JunB, as well as the MAPK and JNK pathways [112, 159, 179, 193]. Additionally, IL-17RB associates with TNF receptor-associated factor (TRAF) -6 (Fig. 2C). These findings are consistent with our own unpublished data as exposure of macrophages to IL-25 led to modest IκBα degradation and the phosphorylation of ERK1/2 (our own unpublished data).

The mechanism whereby IL-25 elicits a Th2-mediated immune response remains unclear. Previous findings indicating that the IL-25-mediated induction of type 2 inflammation is independent of T cells [3, 177, 178], however, CD4+ T cells express IL-17RB and this expression is reportedly increased on polarized Th2 cells or T cells stimulated with TSLP-treated DC [112, 179], and substantially upregulated (over 1000-fold) on Th2 memory cells [194]. Moreover, IL-25 treatment resulted in the production of
IL-4, IL-5, and IL-13 and was dependent on IL-4 and STAT6 [112], suggesting that either IL-17RB ligation results in STAT-6 phosphorylation or that IL-25-mediated effects are the result of increased IL-4 production. However, it is important to note fundamental differences in the respective conclusions drawn by these authors. Angkasekwinai et al. [112] conclude that IL-25 acts during in the initiation of Th2 cell polarization, whereas Wang et al. [179] postulate that IL-25 acts to sustain Th2 cell differentiation, leading to the development to Th2 memory cells. These conclusions are by no means mutually exclusive and need further examination to dissect the role of IL-25 in T cell differentiation and Th2 memory cell formation.

Lung ECs have been shown to be responsive to IL-25 [112] and studies by Cheung et al. suggests direct effects of IL-25 on human eosinophils [182], which have been shown to express the receptor for IL-25 [195]. Purified eosinophils treated with IL-25 displayed increased survival and selectively increased expression of ICAM-1, but down-regulated ICAM-3 and L-selectin. Another report has demonstrated that airway smooth muscle cells (ASMC) express the receptor for IL-25 [196] and although IL-25 did not have a dramatic effect on the production of extracellular matrix proteins by ASMC, this finding is consistent with previous data in which neutralization of IL-25 during airway challenge specifically decreased airway resistance [181]. Taken together, these data indicate that IL-25 signaling in ASMC results in increased bronchio-constriction.

1.5.4 Undefined targets of IL-25

Additional IL-25-responsive populations have also been reported. In the original description of IL-25, a non-B/non-T cell (NBNT) population produced IL-13 in response to systemic IL-25 administration [3]. Moreover, this NBNT cell population was capable of
mediating the physiologic changes in the lung and intestine independent of lymphocytes. Further characterization of this cell population excluded it as basophils, mast cells, or neutrophils [3, 158]. However, a subsequent report identified the IL-25-responsive cell population as a putative mast cell precursor (being c-kit⁺ and FcεR1⁻), which was induced independently of IL-4, IL-5, IL-9, and IL-13 [16]. In addition to this mast cell progenitor population, in a subsequent study which employed a model of chronic airway hyper-responsiveness, IL-25 was found to elicit a c-kit⁻⁰⁺ CD11b⁺ Ly6C⁺ monocyte-like cell population [176]. This CD11b⁺ Ly6C⁺ cell population was found to be a source of IL-4 in the lungs following chronic allergen exposure [176]. In another model of airway hypersensitivity reaction (AHR), IL-25 was shown to promote IL-4 production in CD4⁺ NKT cells. This subset of NKT cells preferentially expressed IL-17RB and was critical to the development of AHR, as adoptive transfer of this cell population into NKT cell-deficient mice successfully reconstituted AHR [171]. In addition, three independent laboratories identified c-kit⁺ innate cell populations (discussed in Chapter 1.7), termed natural helper cells, nuocytes and innate type 2 helper cells [13-15], that respond to IL-25 and promote type 2 inflammation.

Collectively, these data illustrate that IL-25 can target multiple cell lineages to initiate and/or influence immune responses (Table 1), however, the identity of the predominant cellular target of TSLP, IL-33 and IL-25 remains unclear. In addition, the contribution of these various cellular targets to the development of immune responses initiated by IL-25 are poorly defined and warrant further investigation. The cellular mechanisms through which IL-25 promotes CD4⁺ Th2 cytokine responses and type 2 inflammation is the focus of this thesis and will be tested in Chapters 2 and 3. Moreover, while EC-derived TSLP, IL-25, and IL-33 have unique responsive cell populations, they also share
common cellular targets indicating that they could potentially act through common cellular mechanisms to promote and/or amplify the magnitude of a developing Th2 cytokine-associated inflammation at mucosal sites.

1.6 EC-derived cytokines: Cross-regulation and interplay

EC-derived TSLP, IL-25, and IL-33 each license Th2 cytokine responses yet appear to do so through distinct mechanisms. However, the coordinated expression of this triad of EC-derived cytokines, coupled with cross-regulation of expression of ligands and receptors, suggests that a complex interplay exists between TSLP, IL-33 and IL-25. Moreover, recent data suggests that TSLP, IL-33 and IL-25 may not function independently of one another. For instance, both IL-25 [112] and IL-33 [80] can induce TSLP mRNA in ECs (Fig. 3) suggesting that IL-25 and IL-33 signal upstream of TSLP. IL-33 treatment induced increased levels of TSLPR in the colons of *Trichuris*-infected mice [80], and IL-33 can also sensitize mast cells to TSLP stimulation [108], suggesting regulation of TSLP-TSLPR signaling by IL-33. Additionally, TSLP, IL-25, and IL-33 are each able to upregulate IL-17RB [150, 177, 179] indicating that sensitization to IL-25 expression is an important and common effect of each EC-derived cytokine.

Beyond the ability of TSLP, IL-25 and IL-33 to regulate expression and to augment cellular responsiveness to each other, this triad also targets similar innate and adaptive cell populations (Table 1 and Fig. 3). TSLP and IL-25 both signal and influence antigen presenting cells such as macrophages and DCs [1, 96, 192, 197] to promote permissive conditions for Th2 cell differentiation. TSLP and IL-25 are also both able to directly influence naïve CD4+ T cell populations. TSLP can induce GATA-3 and IL-4 in naïve CD4+ T cells [102] while IL-25 induces the production of IL-4, IL-5 and IL-13 [112].
Differentiated Th2 cells are also sensitive to IL-33, which increases their secretion of IL-5 and IL-13 [2].

TSLP, IL-25, and IL-33 also directly influence the biology of innate cell populations including mast cells, eosinophils, and basophils. TSLP, in conjunction with IL-33 induces mast cell production of IL-4, IL-5, IL-13, and production of Th2 cell attracting chemokines [108], while both IL-25 and IL-33 have been reported to modulate eosinophil biology they induce different downstream events. IL-25 acts to increase the survival of eosinophils as well as the production of chemokines [195, 198] and IL-33 induces degranulation and superoxide production [155].

While evidence exists to support a degree of cross-regulation between these EC-derived cytokines, the complexities of the relationship(s) between these cytokines has not yet been clearly defined. Future analysis of the cross-regulation that operates between these EC-derived cytokines is necessary to determine biological significance of these interactions, however the ability of TSLP and IL-33 to influence the biology of IL-25-elicited innate cell populations will be tested in Chapter 4. The emerging understanding of these inter-relationships suggests that combined targeting or overexpression of TSLP, IL-33 and IL-25 may offer a novel and highly effective therapeutic approach to modulate the onset, progression or severity of Th2 cytokine-mediated inflammation associated with asthma, allergic disorders, and helminth infections at mucosal sites.

1.7 Extramedullary hematopoiesis

Hematopoietic stem cells (HSCs) are a rare long-lived cell population with the capacity for multi-lineage differentiation and self-renewal. HSCs give rise to a series of
progenitors that gradually lose their lineage differentiation and self-renewal potential. HSCs and other progenitors are responsible for replenishing cells of both the innate and adaptive immune system [199]. For example, HSC populations can be fractionated into both long-term and short-term populations. Both populations possess multi-potent lineage potential but are designated so based on their ability to sustain reconstitution in multiple sequential hosts. Further, multi-potent progenitors (MPPs) still maintain the ability to generate all known hematopoietic lineages, but have lost their capacity for self-renewal. MPPs subsequently give rise to myeloid progenitors (MPs) and early lymphoid progenitors (ELPs). MPs give rise to either megakaryocyte and erythrocyte progenitors (MEPs) or granulocyte and macrophage progenitors (GMPs) [200], while ELPs yield T, B and NK cells give rise to pro-lymphocytes or common lymphoid progenitors (CLPs) that can then become pre-B cells [201, 202]. Progression from HSCs and commitment to a specific lineage differentiation pathway is regulated by various growth and differentiation factors.

HSCs and other progenitors are primarily bone marrow-resident, however, HSCs are not entirely restricted to the bone marrow. HSC populations have been found to constantly recirculate between the bone marrow and blood [203, 204] and blood collected from adult mice contains a small, but stable population of functional HSCs [205, 206]. Moreover, HSCs have been recovered from extramedullary sites, such as the muscle, liver and spleen [204, 207, 208] and mobilization of HSCs into the periphery thus allowing for collection and transplantation through the administration of exogenous growth factors has long been employed as a treatment strategy against leukemia or following chemotherapy [209-214]. Thus, HSCs represent a highly migratory cell population, however, why HSCs traffic through the blood and peripheral tissues and the
biological significance of these circulating cells play remains less well understood. Studies have suggested that circulating HSCs may be acting in a role of immunosurveillance. Nagai et al. demonstrated that HSC populations expressed Toll-like receptors (TLRs) and were able to respond to and differentiation in response to stimulation with TLR-ligands [215]. Further, HSCs recovered from the thoracic duct, which originated in the bone marrow, were found to traffic to and differentiate in sites of TLR-ligand challenge in secondary hosts [216]. Not only are HSC populations capable of differentiating in response to pathogen-derived signals, but progenitor populations themselves have been observed to possess effector functions. For instance, CD34+ progenitor cells derived from asthmatic patients have been shown to produce IL-5 and IL-13 and have been reported to Th2-associated cytokines following stimulation with TSLP and IL-33 [217].

Data in Chapter 3 demonstrates that IL-25 elicits a multi-potent progenitor type 2 (MPPtype2) cell population and along with the recent identification of other previously unrecognized innate cell populations, natural helper cells (NHCs), nuocytes, and innate type 2 helper (Ih2) cells (discussed in Chapter 4) has provided new insights into our understanding of the cellular mechanisms that lead to the development of CD4+ Th2 cell-dependent immunity and/or inflammation at mucosal sites [13-15, 17]. Briefly, NHCs were found in fat-associated lymphoid clusters (FALC) in the mesentery and in fatty deposits in the peritoneal cavity and surrounding the kidneys [13]. Nuocytes and Ih2 cells were found to be an IL-13-producing cell population that could promote the development of protective Th2 cytokine responses following infection with *N. brasiliensis* [14, 15].
MPP\textsuperscript{type2} cells, nuocytes, Ih2 cells and NHCs all express c-kit, but lack expression of known hematopoietic cell lineage markers [13-15, 17]. These populations all respond to IL-25 and/or IL-33 and could promote CD4\textsuperscript{+} Th2 cell-dependent immunity and/or inflammation [13-15, 17]. Based on these recent findings, a fundamental question to be addressed is whether NHCs, MPP\textsuperscript{type2} cells, nuocytes or Ih2 cells are the same cell population, related cell lineages, or represent four distinct and unrelated cell lineages. The relationships between MPP\textsuperscript{type2} cells, NHCs, nuocytes and Ih2 cells will be discussed in Chapters 4 and 5. Further, how the epithelial-cell derived cytokines TSLP and IL-33 regulate the functions of these distinct cell populations have not been examined. In Chapter 4, IL-25-elicited MPP\textsuperscript{type2} cells were found to possess lymphoid potential, in addition to the myeloid potential of IL-25-elicited MPP\textsuperscript{type2} cells described in Chapter 3. Together, reports of circulating ‘sentinel’ HSCs and Th2-cytokine associated multi-potent progenitors and other ILC subsets in mice and humans suggests a mechanism of extramedullary hematopoiesis in the elicitation and propagation of immune response whereby HSCs, and potentially other precursor cells, are able to recognize and respond to pathogen-derived signals and directly contribute to protective or pathologic immune responses. Therefore, extramedullary hematopoiesis may represent an ancient evolutionarily conserved pathway to initiate a very localized and rapid immune response, which arose prior to the compartmentalization of hematopoiesis to the bone marrow niche.

1.8 Outline of Thesis

This thesis will explore the cellular mechanisms through which IL-25 promotes CD4\textsuperscript{+} Th2 cytokine responses, with an emphasis on previously unrecognized innate cell populations that influence the development of CD4\textsuperscript{+} Th2 cell differentiation. Chapter 2 will address whether IL-25 promotes Th2 cytokine responses through mast cell-
dependent mechanisms. The IL-25-mediated initiation of Th2 cytokine responses through the induction of a multi-potent progenitor cell population will be investigated in **Chapter 3**. Lastly, **Chapter 4** will examine the relationships between the recently described cell populations, MPP_{type2} cells, natural helper cells, nuocytes and innate helper type 2 cells, and the effects of TSLP and/or IL-33 stimulation on these cell populations.
Figure 1. Epithelial cells as a source of TSLP, IL-33 and IL-25 \textit{in vivo}.

Figure 1. TSLP expression is induced following helminth infection, tissue damage, and exposure to Th2 and pro-inflammatory cytokines (A). To date only \textit{Trichuris} infection has been shown to upregulate expression of IL-33 in intestinal ECs (B). \textit{Nippostrongylus}-infection and exposure to common allergens upregulates expression of IL-25 (C). EC, epithelial cell; IL, interleukin; TSLP, thymic stromal lymphopoietin.
Figure 2. Binding of TSLP to its receptor complex results in the phosphorylation of STAT-5 and STAT-3, although the involvement of a specific JAK has not been elucidated (A). TRAF-6 and MyD88 are both required for intact IL-33 signaling. Additionally, IL-33 can activate both the MAPK and JNK pathways (B). IL-25-mediated activation of NF-κB is dependent on TRAF-6, whereas activation of the MAPK pathway does not require TRAF-6 (C). STAT, signal transducers and activators of transcription; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; TRAF, TNF receptor associated factor.
Figure 3. Cross-regulation between TSLP, IL-33 and IL-25.

Figure 3. Target cell populations are indicated including a summary of the effects of each of these cytokines on that cell lineage.
Table 1. Summary of cellular sources, stimuli that induce expression and cellular targets of TSLP, IL-25, and IL-33.

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<th>Cellular sources</th>
<th>Inducing stimuli</th>
<th>Target cell populations</th>
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<td><strong>TSLP</strong></td>
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<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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<td>Intestinal ECs</td>
<td>Helminth parasites</td>
<td>Mast cells</td>
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<td>Keratinocytes</td>
<td>IL-1, TNF-α</td>
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<td>Mast cells</td>
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<td>Microglia</td>
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Chapter 2

IL-25 promotes Th2 cytokine responses through the induction of a lineage\(^\text{neg}\) innate c-kit\(^{+}\) cell population

2.1 Abstract

CD4\(^{+}\) T helper (Th) 2 cells secrete interleukin (IL)-4, IL-5 and IL-13 and are required for immunity to gastrointestinal helminth infections. However, Th2 cells also promote chronic inflammation associated with asthma and allergic disorders. The non-hematopoietic cell-derived cytokines thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 have been implicated in inducing Th2 cell-dependent inflammation at mucosal sites [1-4], but how these cytokines influence innate immune responses remains poorly defined. In this Chapter, we show that IL-25, a member of the IL-17 cytokine family, promotes Th2 cytokine responses that correlates with the accumulation of a non-B/non-T cell (NBNT) c-kit\(^{+}\) cell population in the gut-associated lymphoid tissue (GALT). The NBNT c-kit\(^{+}\) cell population was distinct from known innate cell populations and lacked markers associated with basophils, eosinophils and mast cells. Further, the induction of this cell population required IL-17RB and IL-17RA, but did not require signaling through SCF or IL-3, cytokines required for mast cell differentiation. In addition, adoptive transfer of IL-25-elicited c-kit\(^{+}\) cells into normally susceptible *Trichurus*-infected *Il17e\(^{-/-}\*) mice conferred protective immunity to helminth infection, suggesting that IL-25 promotes Th2 cytokine responses at mucosal sites through the induction of this novel c-kit\(^{+}\) cell population.

2.2 Introduction

There has been intense interest in the mechanisms that promote and/or regulate
adaptive immune responses at mucosal sites. However, in the steady-state these immune cell populations remain hyporesponsive to a vast array of innocuous environmental stimuli to which they are simultaneously exposed. Based on their localization to mucosal surfaces, epithelial cells (ECs) represent the first line of defense between host tissues and external environment. As such, ECs play a pivotal role in the detection of invading pathogens and are a critical cell population in the initiation, regulation, and resolution of innate and adaptive immune responses at mucosal sites. Not only do ECs express a wide range of immune response genes including MHC class I and class II, co-stimulatory molecules, and chemokines, but they also secrete numerous cytokines, including TSLP, IL-33 and IL-25, that promote the development of Th2 cytokine responses. Although diverse in amino acid sequence, structure, and patterns of expression, rapidly emerging studies implicate TSLP, IL-33 and IL-25 as critical regulators of Th2 cytokine-mediated inflammation through the induction of distinct innate cell populations. Consistently, dysregulated production of these three EC-derived cytokines has been reported in multiple inflammatory disease states including inflammatory bowel disease, asthma and atopic dermatitis.

As discussed in Chapter 1.4, epithelial cell-derived TSLP, IL-33 and IL-25 have been reported to promote the development and regulation of Th2 cytokine responses at mucosal sites through the induction of IL-4-producing innate immune cells [106, 171-175]. Data from our laboratory demonstrated that TSLP treatment selectively elicits IL-4-producing basophils in the blood. Moreover, TSLP-elicited basophils expressed MHC class II and were capable of promoting Th2 cytokine responses, either through direct interactions with CD4⁺ T cells or in cooperation with dendritic cells. IL-33 elicits eosinophils in the blood [11], but can also activate basophils [136] and natural helper
cells (NHCs) [13]. In contrast, there are conflicting reports of IL-4-producing innate cell populations elicited by IL-25. Initial reports of IL-25 described the induction of a NBNT innate cell population that arose independently of the adaptive immune system. In subsequent studies employing a model of chronic airway hyper-responsiveness, IL-25-producing eosinophils were found to elicit a c-kit$^{neg}$ CD11b$^+$ Ly6C$^+$ monocyte-like cell population [176]. This CD11b$^+$ Ly6C$^+$ cell population was found to be a source of IL-4 in the lungs following chronic allergen exposure [176]. In another model of airway hypersensitivity reaction (AHR), IL-25 was shown to promote IL-4 production in CD4$^+$ NKT cells. This subset of NKT cells preferentially expressed IL-17RB and was critical to the development of AHR, as adoptive transfer of this cell population into NKT cell-deficient mice successfully reconstituted AHR. Other studies identified that IL-25 promotes the population expansion of a NBNT c-kit$^+$ cell population [11, 16, 171]. Based on c-kit expression, this IL-25-elicited NBNT cell population was suggested to be a mast cell or mast cell precursor population [11, 16], however this cell population lacked expression of FcεRIα, a common marker of mature mast cells. Thus, the innate cell populations responsible for promoting Th2 cytokine responses in response to IL-25 remain unclear.

In this Chapter, we show that administration of IL-25 results in the accumulation of a NBNT c-kit$^+$ cell population in the gut-associated lymphoid tissue (GALT) and correlated with the development of Th2 cytokine responses. The NBNT c-kit$^+$ cell population was distinct from known innate cell populations, as it lacked markers associated with basophils, eosinophils and mast cells. Further, the induction of this cell population required IL-17RB and IL-17RA, but did not require the cytokines SCF or IL-3, which are required for mast cell differentiation. In addition, adoptive transfer of IL-25-elicited c-kit$^+$
cells into normally susceptible *Trichuris*-infected *Il17e*<sup>-/-</sup> mice conferred protective immunity to helminth infection, indicating that IL-25 promotes Th2 cytokine responses through the induction of this previously unrecognized c-kit<sup>+</sup> cell population.

### 2.3 Methods

#### 2.3.1 Mice and administration of exogenous cytokine

Balb/c, C57BL/6 and W<sup>th</sup> mice were obtained from the Jackson Laboratory and IL-4/eGFP reporter mice were obtained from M. Mohrs (Trudeau Institute). *Il17e*<sup>-/-</sup> mice were provided by R.A. Kastelein (Schering-Plough Biopharma). *Il17ra*<sup>-/-</sup> and *Il17rb*<sup>-/-</sup> mice were provided by J.E. Tocker and A.L. Budelsky (Amgen). *IL-3Rβc*<sup>-/-</sup> mice were provided by Taku Kambayashi (UPenn). Animals were bred and housed in specific pathogen-free conditions at the University of Pennsylvania. All experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the guidelines of the IACUC of the University of Pennsylvania. All mice used were 4-12 weeks of age. Mice were treated intraperitoneally with PBS or recombinant IL-25 (0.4 µg; R&D Systems) daily for 4 days.

#### 2.3.2 Flow cytometry

Mesenteric lymph nodes (MLN) from IL-4/eGFP reporter mice were separated from the mesentery, homogenized by passing through a 70 µm nylon mesh filter and stained with anti-mouse fluorochrome-conjugated monoclonal antibodies against CD3ε, CD4, CD8, TCRβ, TCRγδ, B220, CD19, CD11b, CD11c, MHC class II, Gr-1, NK1.1, FcεRIα, c-kit, CD45.2, CD49b and CCR3 (eBioscience and BD Bioscience). Peritoneal exudate cells ('peritoneum') were collected using peritoneal lavage with injection of 10 mL PBS and aspirated using the same syringe. Mesentery was processed as previously described.
[13]. Cells were run on a BD FACSCanto II using DiVa software (BD Bioscience) and analyzed with FlowJo software (Version 8.7.1; Tree Star, Inc.).

2.3.3 Mast cell differentiation assays

Femurs, tibias and spleens were harvested from WT C57Bl/6 mice. The heads of the bones were removed and the bone marrow was flushed with media from the bones using a syringe and needle. Marrow and spleens were homogenized by passing the tissue through a 70 µm filter. An aliquot of the homogenized cells was stained with fluorescent-labeled antibodies against CD4, CD8, CD19, CD11b, c-kit and FcεRIα (eBioscience) to assess for ‘ex vivo’ (input) levels of mast cells. 1x10^6 cells were cultured in the presence of SCF (50 ng mL^-1; R&D systems), IL-3 (10 ng mL^-1; R&D systems) and/or IL-25 (100 ng mL^-1; R&D systems). Following in vitro culture, resultant progeny were assessed for expression of CD4, CD8, CD19, CD11b, c-kit and FcεRIα (eBioscience) by flow cytometry as described above.

2.3.4 Ovalbumin (OVA) immunization, helminth infections and adoptive transfer

3-5 x10^6 CFSE-labeled OVA-specific CD45.2 CD4+ T cells were transferred i.v. into CD45.1 congenic recipient mice and 24 hours later immunized i.p. with 100 µg OVA emulsified in IFA, with one cohort receiving 5x10^4 IL-25-elicited c-kit+ cells intraperitoneally (i.p.). IL-25-elicited c-kit+ cell populations were sorted using a FACSaria (BD Bioscience). Proliferation of OVA-specific CD4+ T cells in the spleen was assessed two days post-immunization and cytokine production was measured by ELISA. *Trichuris muris* infections were performed as previously described [81]. Briefly, mice were infected with 250 embryonated *Trichuris* eggs via oral gavage. *Trichuris*-infected
II17e−/− mice were left untreated or given 5x10⁴ IL-25-elicited c-kit+ cells i.v. at day 10 post-infection. Worm counts were performed at day 20 post-infection. MLN cells were collected at necropsy, plated in medium alone or polyclonally stimulated with 1 µg ml⁻¹ each of αCD3 and αCD28 (eBioscience). After 48 hours, cell-free supernatants were assessed for cytokine production by sandwich ELISA (eBioscience). Trichuris-specific IgG1 antibody titers were analyzed by ELISA as described previously [81]. Total serum IgE was measured using the OptEIA IgE ELISA kit according to the manufacturer's instructions (BD Biosciences). For Nippostrongylus brasiliensis infections, WT mice were infected s.c. with 500 infective third-stage larvae (L3) (provided by Joseph Urban, USDA) and treated with 0.5 mg/day of anti-IL-25 or control IgG (from J.E. Tocker and A.L. Budelsky; Amgen) on days 0, 2, 4, 6 and 8. MLN cells from infected mice were assessed at day 10 post-infection for the induction of c-kit+ cells.

2.3.5 Histology and cytospin preparation
Colon sections were fixed in 4% (vol/vol) paraformaldehyde and embedded in paraffin wax. 4 µm sections were stained with Periodic acid-Schiff/Alcian blue.

2.3.6 Real-time PCR
RNA from colonic tissues of mice was isolated by TRizol extraction (Invitrogen) or collected from sorted cell populations using RNeasy Mini kit (Qiagen). Whole tissues were homogenized with a tissue homogenizer (TissueLyzer; Qiagen) and cDNA was prepared with SuperScript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR analysis used commercial QuantiTect primer sets for Il4, Il5, and Il13 (Qiagen) and SYBR Green chemistry (Applied Biosystems). All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Target genes were normalized for
endogenous β-actin levels and relative quantification of samples were compared to controls.

2.3.7 Statistical analysis

Results are shown as means ± s.e.m for individual animals. Statistical significance was determined by Student’s t-test. Results were considered significant at P < 0.05.

2.4 Results

2.4.1 IL-25 promotes type 2 inflammation in vivo through the induction of a c-kit+ cell population in the GALT

To address which innate cell populations express IL-4 following administration of IL-25, we employed IL-4/eGFP reporter mice [218] in which an enhanced green fluorescent protein (eGFP) coding region has been inserted behind the fourth exon of the IL-4 gene. This system allows for the tracking, identification and characterization of cells expressing eGFP as a marker for the capacity to express IL-4. Following administration of IL-25 to IL-4/eGFP mice, a 1.8-fold increase in the total cell numbers was observed in the MLN, with equivalent increases in the total numbers of CD4+ and CD8+ T cells, CD19+ B cells and NBNT CD11b+ macrophages (Fig. 4A). In contrast, IL-25-treatment resulted in a 56-fold increase in a NBNT c-kit+ cell population (Fig. 4B), indicating that the NBNT c-kit+ cells exhibited the greatest relative population expansion following IL-25 administration, and consistent with previous reports. The IL-25-mediated population expansion of the NBNT c-kit+ cells was associated with the development of Th2 cytokine-dependent inflammation characterized by increased expression of Il4, Il5 and Il13 mRNA in the large intestine (Fig. 5A), elevated levels of serum IgE (Fig. 5B) and increased mucin production in the large intestine (Fig. 5C), a finding consistent with previous reports [3].
Administration of IL-25 to IL-4/eGFP reporter mice increased the frequency of c-kit⁺ cells in all compartments of the GALT examined including the MLN (4% versus 28% of NBNT cells) (Fig. 6A), the Peyer's patches (11% versus 54%) (Fig. 6B) and cecal patch (10% versus 36%) (Fig. 6C). However, IL-25 did not elicit this population in the spleen or bone marrow (data not shown), suggesting that IL-25-responsive cells may be located in the GALT. Further, analysis of the IL-25-elicited c-kit⁺ cells in the GALT revealed two distinct cell populations distinguished by expression of IL-4/eGFP (Fig. 6A-C, right panels; GFP⁺, blue gates; GFP⁻, green gates), indicating that the IL-25-elicited c-kit⁺ cells are a heterogeneous population.

### 2.4.2 Induction of the c-kit⁺ cell population is dependent on IL-25/IL-25R signaling pathway

Previous studies have shown that infection with the gastrointestinal helminth parasite *Nippostrongylus brasiliensis* results in increased Th2 cell responses that are associated with the population expansion of macrophage, basophil and mast cell populations [173, 174, 219]. Further, increased expression of IL-25 and increased frequencies of a c-kit⁺ cell population have been reported following exposure to *N. brasiliensis* [16, 158]. Therefore to test whether *N. brasiliensis* infection results in increased frequencies of c-kit⁺ cells and whether this induction is dependent on IL-25, mice were infected with 500 infective stage L3 larvae and treated with either isotype control antibody or anti-IL-25 monoclonal antibody and assessed for the induction of c-kit⁺ cells in the MLNs at d10 post-infection. Similar to administration of exogenous IL-25, increased frequencies and absolute numbers of a NBNT c-kit⁺ cell population were observed in the MLN of WT mice following infection with *Nippostrongylus* (Fig. 6D and E). Further, the total cell number of *Nippostrongylus*-induced c-kit⁺ cells was reduced following administration of
anti-IL-25 monoclonal antibody (WT infected + control IgG, 58981 \(\pm\) 4975; WT infected + anti-IL-25 mAb, 26109 \(\pm\) 3039), indicating that IL-25 signaling was necessary for the induction of the c-kit\(^+\) cell population following helminth infection.

Initial reports identified IL-17RB as the receptor for IL-25; however, more recent data have suggested that IL-25 signals through the heterodimeric receptor composed of IL-17RA and IL-17RB subunits [191]. In addition, both IL-17A and IL-17F signal though IL-17RA, and IL-17A and IL-17F have been found to heterodimerize [164, 165]. Therefore, to determine whether IL-17RB, IL-17RA and/or IL-17A are required for the IL-25-mediated induction of the c-kit\(^+\) cells and type 2 inflammation, WT, \(Il17rb^{--}\), \(Il17ra^{--}\), and \(Il17a^{--}\) mice were treated with exogenous IL-25 and assessed for the induction of type 2 inflammation. Administration of IL-25 to \(Il17a^{--}\) mice resulted in increased frequencies of c-kit\(^+\) cells (data not shown), however, both \(Il17rb^{--}\) and \(Il17ra^{--}\) mice failed to exhibit IL-25-elicited population expansion of the c-kit\(^+\) cells (Fig. 7A) or the development of IL-13 and mucin responses (Fig. 7B and C), indicating that both IL-17RB and IL-17RA, but not IL-17A, are required for the IL-25-mediated induction of the c-kit\(^+\) cell population.

### 2.4.3 IL-25-induced c-kit\(^+\) cells do not represent NKT cell, basophil, eosinophil or mast cell populations

We sought to determine whether IL-25-elicited c-kit\(^+\) cells expressed surface markers associated with other known immune cell lineages. IL-25-elicited c-kit\(^+\) GFP\(^{neg}\) or c-kit\(^+\) GFP\(^{+}\) cells lacked expression of CD4 (Fig. 8A), indicating that they were distinct from the IL-4-producing CD4\(^+\) NKT cell populations described in AHR [171]. Moreover, neither the c-kit\(^+\) GFP\(^{neg}\) cells nor c-kit\(^+\) GFP\(^{+}\) cells expressed other surface markers expressed on CD4\(^+\) lymphocytes (CD3\(\epsilon\), TCR\(\beta\), TCR\(\gamma\delta\), CD69 and CD62L) (Fig. 8A). Further, the
IL-25-elicited c-kit+ populations did not express CD19 (a marker for B lymphocytes) (Fig. 8B) or markers associated with basophil, eosinophil or mast cell lineages (CD49b, CCR3 and FcεRIα, respectively) (Fig. 8C). Taken together, these data indicate that IL-25-elicited c-kit+ cells are not T- or B-lymphocytes, NKT cells, basophils or eosinophils.

Analysis of the IL-25-elicited cells revealed that in comparison to c-kit+ mast cells, this cell population exhibited intermediate expression of c-kit (Fig. 9A). This finding, combined with the lack of surface expression of FcεRIα, indicates that the IL-25-elicited c-kit+ cells are distinct from mature mast cell populations and that IL-25 may promote the population expansion of this c-kit+ cells population independently of the factors that regulate mast cell differentiation in vivo, stem cell factor (SCF) and IL-3. To test whether the c-kit+ cell population could arise in response to IL-25 in the absence of the factors that regulate mast cell biology in vivo, mast cell deficient mice, W-sash (Wsh) and IL-3Rβc−/−, were utilized [220, 221]. Wsh mice carry a spontaneously generated loss-of-function mutation in the white spotting (W) locus leading to impaired c-kit expression and decreased responsiveness to SCF. This impaired expression ultimately results in a deficiency in multiple cell lineages including both connective tissue and mucosal mast cells [222]. IL-3Rβc−/− mice lack both the common β chain (βc), shared by IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and a redundant β subunit of the IL-3 receptor (βIL-3) found only in mice, but not humans. Thus, these mice are fully deficient in IL-3 signaling, and as a result lack mucosal mast cell populations, but not connective tissue mast cells.

WT mice, Wsh and IL-3Rβc−/− mice were treated daily with IL-25 for 4 days. Delivery of IL-25 elicited increased frequencies of c-kit+ cells in Wsh and IL-3Rβc−/− mice (Fig. 9B).
and induced equivalent expression of *Il13* mRNA and mucin responses in WT, W<sup>th</sup> and IL-3Rβ<sup>c−</sup> mice (Fig. 9C and D) compared to untreated controls. Taken together, these findings demonstrate that IL-3 and SCF signaling are not required for IL-25 to induce this c-kit<sup>+</sup> cell population and support the hypothesis that IL-25 acts independently of classical mast cell populations to promote Th2 cytokine responses *in vivo*.

While SCF and IL-3 were not required for IL-25-mediated induction of the c-kit<sup>+</sup> cells *in vivo*, it did not rule out the possibility that IL-25 played a role in mast cell development. Therefore, we sought to investigate the role of IL-25/IL-17RB interactions in mast cell development. Bone marrow or spleen from WT mice were harvested, homogenized and cultured *in vitro* in the presence of SCF and IL-3 in the presence of absence of IL-25. Following 8 days *in vitro*, mast cell differentiation was assessed by flow cytometry. At day 0, no mast cells (c-kit<sup>+</sup> FcεRIα<sup>+</sup>) were observed in either the bone marrow or spleen from WT mice (Fig. 10A and B, “ex vivo”). As expected, following 8 days *in vitro*, c-kit<sup>+</sup> FcεRIα<sup>+</sup> mast cell populations were observed from bone marrow and spleen cultured with SCF and IL-3 (Fig. 10A and B). Interestingly, IL-25 alone did not result in the differentiation of mast cells (data not shown), nor did the inclusion of IL-25 affect mast cell differentiation in the presence of SCF and IL-3 (Fig. 10A and B), suggesting that IL-25 is neither sufficient for nor plays a role in mast cell development. Interestingly, repeated exposure to IL-25 alone or in combination with SCF and IL-3 *in vitro* did not preferentially increase the frequency of c-kit<sup>+</sup> FcεRIα<sup>+</sup> cells in bone marrow or spleen cultures (Fig. 10A and B), indicating that an additional factor(s) was necessary for IL-25 to drive the induction of the c-kit<sup>+</sup> population *in vivo*. Collectively, these data support the hypothesis that IL-25 acts independently of mast cells to elicit the c-kit<sup>+</sup> cells and associated type 2 inflammation.
2.4.4 IL-25-elicited c-kit+ cells promote Th2 cytokine-dependent responses in vivo.

To address the role IL-25-elicited c-kit+ cells might play in the development of Th2 cytokine responses in vivo, a well-established model was adopted in which OVA immunization induces an antigen-specific Th2 cytokine response [223-226]. This model provides a system whereby the effects of IL-25-elicited c-kit+ cells on the kinetics or magnitude of Th2 cytokine responses can be tested. First, CFSE-labeled OVA-specific TCR transgenic T cells were transferred alone or in combination with IL-25-elicited c-kit+ cells into naïve congeneric recipients. Both cohorts were subsequently immunized i.p. with OVA/IFA and T cell proliferation and cytokine responses were measured 48 hours post-immunization. OVA-specific CD4+ T cells began to proliferate (Fig. 11A, left panel) and accumulated at the site of immunization (Fig. 11B). However, in mice that also received IL-25-elicited c-kit+ cells, OVA-specific CD4+ T cell proliferation was augmented (Fig. 11A, right panel) and there was a significant increase in the accumulation of antigen-specific cells at the site of immunization (Fig. 11B). Further, MLN cells isolated from mice receiving both OVA-specific CD4+ T cell and IL-25-elicited c-kit+ cells secreted elevated levels of IL-13 compared to those cells isolated from mice that received OVA-specific CD4+ T cells alone (Fig. 11C). These data indicate that IL-25-elicited c-kit+ cells could promote antigen-specific Th2 cytokine responses in vivo and suggest that the presence of these cells is able to influence the kinetics of a developing Th2 cytokine response.

In a second approach, we utilized the Trichuris infection model to investigate whether IL-25-elicited c-kit+ cells could promote protective immunity in the context of a helminth infection. As discussed above (see section 1.2.3), Trichuris infection provides a model
to study the development of CD4+ Th2 cytokine responses and IL-25 is required for the development of a protective immunity following Trichuris infection as Trichuris-infected Il17e−/− mice display significantly elevated levels of IFN-γ, reduced levels of IL-4 and IL-13 and are unable to clear infection. Moreover, these mice develop a chronic infection characterized by development of severe intestinal inflammation and elevated production of IFN-γ and IL-17A in the intestinal mucosa and MLN. Therefore, to test whether IL-25-elicited c-kit+ cells could promote Th2 cytokine responses in vivo, WT or Il17e−/− mice were infected with Trichuris, with one Trichuris-infected Il17e−/− cohort receiving IL-25-elicited c-kit+ cells, and immunity was assessed at d20 post-infection. As expected, Trichuris-infected Il17e−/− mice produced low levels of Th2 cytokines (Fig. 11D), reduced parasite-specific IgG1 (Fig. 11E), impaired mucin responses (Fig. 11F) and were susceptible to infection (Fig. 11G). Adoptive transfer of IL-25-elicited c-kit+ cells from WT mice into Trichuris-infected Il17e−/− mice resulted in elevated production of Trichuris-specific IL-4, IL-5 and IL-13 in the MLN (Fig. 11D), elevated serum parasite-specific IgG1 (Fig. 11E), increased mucin responses (Fig. 11F) and host protective immunity (Fig. 11G). Taken together, these data indicate that IL-25-elicited c-kit+ cells could promote protective immunity to helminth infection in vivo.

2.5 Discussion
Multiple distinct cell lineages, including airway smooth muscle cells, ECs and T cells, are reported to be responsive to IL-25 [112, 182], illustrating the potential for multi-faceted effects of IL-25 in vivo. However, in the original report of IL-25 it was noted that administration of exogenous IL-25 resulted in the induction of an IL-13-producing cell population that lacked expression of markers for B- and T- cells. Moreover, this cell population was capable of mediating physiologic changes independent of T- and B-cells,
the first indication that IL-25 acted through an innate cell population. However, further characterization of this cell population determined that it did not represent a basophil, mast cell or neutrophil [3, 158]. Subsequent studies identified various innate cell populations that were induced by IL-25 and could promote Th2 cytokine responses involved in allergic inflammation or immunity to helminth infection.

For instance, employing a model of chronic airway hyper-responsiveness, a c-kit<sup>neg</sup> CD11b<sup>+</sup> Ly6C<sup>+</sup> monocyte-like cell population was found to be responsive to IL-25 and to be the source of IL-4 in the lungs following exposure to allergens [176]. In another model, AHR, CD4<sup>+</sup> NKT cells were shown to preferentially express IL-17RB, produce IL-4 in response to IL-25 and to be critical to the development of AHR. Yet another study identified that IL-25 promotes the population expansion of an undefined NBNT c-kit<sup>+</sup> cell population [11, 16, 171], which was induced independently of IL-4, -5, -9, and -13 [16]. Based on c-kit expression, this IL-25-elicited NBNT cell population was proposed to be a mast cell or mast cell precursor population [11, 16]; however this cell population lacked expression of FcεRIα, a common marker of mature mast cells. Thus, the innate cell populations responsible for promoting Th2 cytokine responses following IL-25 stimulation remain unclear.

Here we show that administration of IL-25 resulted in the induction of a c-kit<sup>+</sup> cell population, which did not express markers for T cells, B cells or markers associated with basophil, eosinophil or mast cell lineages. These data were consistent with previous reports indicating that IL-25 acted through an unidentified innate cell population. The finding that IL-25 elicited a c-kit<sup>+</sup> cell population was consistent with the cell population identified by Fallon <i>et al</i>. [16]. Both cell populations expressed c-kit but lacked
expression of FcεRIα and as a result, also lacked surface bound IgE. Moreover, based on c-kit expression, the IL-25-elicited c-kit+ cells were distinct from the IL-25-responsive CD11b+ Ly6C+ monocyte-like cell population identified in chronic allergen exposure [176]. Further, this c-kit+ cell population lacked expression of CD4, indicating that these cells were not the IL-4-producing CD4+ NKT cell populations described in AHR [171]. In addition, the IL-25-elicited c-kit+ cell population was initially hypothesized to represent a mast cell or mast cell precursor population. However, we showed that IL-25 was capable of inducing this cell population in Wsh and IL-3R-deficient mice, indicating that IL-25 elicits this cell population independently of SCF or IL-3, factors that regulate mast cell differentiation. These data indicate that IL-25-elicited c-kit+ cells do not represent classical mast cells.

One possible explanation for the differences observed between these multiple reports could be in the timing at which IL-25-responsive innate cell populations are investigated. Of note, the CD11b+ Ly6C+ monocyte-like cell population was identified using model of chronic allergen exposure. In this model, the mice were sensitized and challenged with allergens over the course of 4 weeks [176], compared to the 4 days of IL-25 administration used in our studies. Therefore, the timing at which the IL-25-responsive innate cell populations are investigated may influence the innate cell populations identified. Thus, these disparate results may indicate that at different timepoints, IL-25 may act on different cell populations. Alternatively, these data may indicate that the initial IL-25-elicited cells undergo differentiation to become different cell lineages at later timepoints.
In addition, interestingly, the IL-25-elicited c-kit$^+$ cell population displayed heterogeneity in its expression of IL-4/eGFP, with approximately 50% being GFP$^{\text{neg}}$ and 50% being GFP$^+$, suggesting that the IL-25-elicited c-kit$^+$ population might be composed of distinct cell populations or may represent the same cell populations in differential activation states. The functional significance of the heterogeneity in IL-4/eGFP expression will be discussed in Chapter 3. Collectively, data presented in Chapter 2 indicate that the IL-25-elicited c-kit$^+$ cells are not T- or B-lymphocytes, NKT cells, basophils, eosinophils or mast cells, and suggest that IL-25-elicited c-kit$^+$ cells represent a previously unrecognized innate cell population.
Figure 4. IL-25 preferentially elicits a non-B/non-T c-kit+ cell population.

Figure 4. **A, B**, Mesenteric lymph node (MLN) cell numbers from control or IL-25-treated IL-4/eGFP reporter mice. Numbers of total MLN cells or of T cells (CD4+ or CD8+), B cells (CD19+) and macrophages (CD11b+ MHC class II+) (**A**) and total numbers of non-B non-T (NBNT) c-kit+ cells (**B**). *, *P* < 0.05. Error bars indicate s.e.m. Data in **A-B** are representative of more than five independent experiments (control, n=16; IL-25-treated, n=34).
Figure 5. IL-25 promotes type 2 inflammation in vivo.

A-C, IL-4/eGFP reporter mice were treated daily with recombinant IL-25 for 4 days. Real-time PCR analysis of \( \text{Il4} \), \( \text{Il5} \) and \( \text{Il13} \) mRNA expression from proximal colon sections of control or IL-25-treated IL-4/eGFP reporter mice (A). Total serum IgE levels measured by ELISA (B). Periodic acid-Schiff/Alcian blue staining of proximal colon sections from control or IL-25-treated IL-4/eGFP reporter mice (C). Scale bar, 50 μm. Error bars indicate s.e.m. Data in A-C are representative of at least two independent experiments (control, n=4; IL-25-treated, n=7).
Figure 6. IL-25 elicits c-kit+ cells in the GALT that display heterogeneous expression of IL-4/eGFP.

A-C, Frequency of c-kit+ GFPneg or c-kit+ GFP+ cells in the NBNT cell compartment from the MLNs (A), Peyer’s patches (PP) (B) and cecal patch (CP) (C) was assessed by flow cytometry. D, E, Induction of the c-kit+ cell population in WT Balb/c mice following infection with N. brasiliensis. Frequency of the c-kit+ cell population (D) and total numbers (E) in the MLN of control (Crtl) or infected (INF) mice. Plots shown are gated on live, CD4neg CD8neg CD11bneg CD11cneg and B220neg cells or as indicated. *, P < 0.05. Error bars indicate s.e.m. Data in A-C are representative of more than five independent experiments (control, n=16; IL-25-treated, n=34). Data in D-E are representative of at least two independent experiments (Crtl, n=2; INF, n=6).
Figure 7. IL-17RB and IL-17RA are required for IL-25-mediated induction of the c-kit+ cell population and Th2 cytokine responses.

Figure 7. A-C, C57BL/6 (WT), Il17rb−/−, and Il17ra−/− mice were treated daily with recombinant IL-25 for 4 days. MLN cells were analyzed for c-kit and FcεRI expression (A). Plots shown are gated to exclude CD4, CD8, B220, CD11b and CD11c positive cells. Real-time PCR analysis of Il13 mRNA expression (B). Periodic acid-Schiff/Alcian blue staining of proximal colon sections from control or IL-25-treated mice (C). Scale bar, 50 μm. Error bars indicate s.e.m. Not detected, N.D. Data in A-C are representative of at least two independent experiments (control, n=2; IL-25-treated, n=6).
Figure 8. IL-25-induced c-kit⁺ cells do not represent NKT cell, basophil, eosinophil or mast cell populations.
Figure 8. **A-C**, Expression of surface markers on c-kit$^+$ GFP$^{\text{neg}}$ (blue line) or c-kit$^+$ GFP$^+$ (green line) cells from IL-25-treated mice analyzed by flow cytometry. Shaded histograms indicate the positive control for each respective surface marker (**A**, gated on CD4$^+$ T cells expressing each respective marker; **B**, gated on B cells; **C**, CD49b, gated on CD49$^+$ FcεRI$^+$ basophils; CCR3, gated on c-kit$^{\text{neg}}$ CCR3$^+$ eosinophils; FcεRI, gated on c-kit$^+$ FcεRI$^+$ mast cells. IL-25-elicited c-kit$^+$ cells in (**A** and **B**) were FACS-purified prior to surface staining. Positive controls in (**A** and **B**) were derived from whole splenocytes stained ex vivo. Cells in (**C**) were surface stained ex vivo. Plots shown in (**C**) are gated on live CD4$^{\text{neg}}$ CD8$^{\text{neg}}$ B220$^{\text{neg}}$ CD11b$^{\text{neg}}$ and CD11c$^{\text{neg}}$ cells. Data are representative of at least two independent experiments.
Figure 9. IL-25-induced c-kit$^+$ cells do not represent a classical mast cell population.

A

Figure 9. Expression of c-kit by mature mast cells (from peritoneum, red line), NBNT c-kit$^+$ cells (from MLN, green line) and B220$^+$ cells (from MLN, shaded line) from IL-25-treated IL-4/eGFP reporter mice (A). B-D, Recombinant IL-25 was administered daily to C57BL/6 (WT) or W$^{sh}$ mice for 4 days. MLN cells were analyzed for c-kit and FcεRI surface expression by flow cytometry (B). Plots shown are gated to exclude CD4, CD8, B220, CD11b, and CD11c positive cells or as indicated. Numbers are frequencies of c-kit$^+$ FcεRI$^{neg}$ cells of total NBNT population. Real time-PCR analysis of Il13 mRNA (C). Periodic acid-Schiff/Alcian blue staining of proximal colon sections from control or IL-25-treated mice (D). Scale bar, 100 μm. Error bars indicate s.e.m. Data in (A) are representative of 10 independent experiments (control, n=16; IL-25-treated, n=34). Data in B-D are representative of at least two independent experiments (control, n=2; IL-25-treated, n=6-8).
Figure 10. IL-25/IL-17RB signaling does not influence the differentiation of c-kit⁺ FcεRI⁺ mast cell populations.

Figure 10. Bone marrow cells from WT or Il17rb⁻/⁻ mice were cultured in the presence of SCF and IL-3 with or without the addition of recombinant IL-25 for 8 days were analyzed for c-kit and FcεRI surface expression by flow cytometry. Plots shown are gated to exclude CD4, CD8, B220, CD11b and CD11c positive cells. Numbers indicate frequencies of total NBNT population. Data are representative of at least two independent experiments.
Figure 11. IL-25-elicited c-kit⁺ cells promote Th2 cytokine-dependent responses in vivo.
Figure 11. **A-C**, CFSE-labeled CD45.2 CD4⁺ OTII cells were adoptively transferred i.v. into CD45.1 congenic recipients and mice were subsequently immunized i.p. with OVA/IFA in the presence or absence of transferred IL-25-elicited c-kit⁺ cells. Proliferation of donor CD45.2 CD4⁺ OTII cells isolated from the spleens of recipient mice receiving T cells alone (red shaded histogram) or T cells and OVA/IFA immunization (black histograms) was measured by flow cytometry (**A**). Frequency of CD4⁺ OTII cells per total CD4⁺ T cells isolated from the peritoneum (**B**). IL-13 production from αCD3/αCD28-stimulated MLN cells was measured by ELISA (**C**). Data in **A-C** are representative of two independent experiments (n=7). *, P < 0.05, **, P < 0.01. **D-G**, Il17e⁻/⁻ mice were infected with *Trichuris* (INF) or were infected and received IL-25-elicited c-kit⁺ cells (INF + c-kit⁺). Cytokine production by *Trichuris* antigen-stimulated MLN cells (**D**), *Trichuris*-specific serum IgG1 antibody titers (**E**), Periodic acid-Schiff/Alcian Blue staining of proximal colon sections of naïve or infected mice plus or minus c-kit⁺ cells (**F**), and number of worms from *Trichuris*-infected mice (**G**) were assessed at day 20 post-infection. Scar bar, 50 µm.
Chapter 3

IL-25-elicited c-kit$^+$ cells represent a previously unrecognized multi-potent progenitor cell population

3.1 Abstract

Previous data demonstrated that administration of IL-25 resulted in the population expansion of NBNT c-kit$^+$ cell population that was distinct from all known innate cell populations, including basophils, eosinophils and mast cells. Further, IL-25-elicited c-kit$^+$ cells were able to promote Th2 cytokine responses and confer protective immunity to helminth infection. However, the identity of this innate cell population and how it promoted CD4$^+$ Th2 cell responses remained unknown. Hematopoietic stems cells (HSCs) and multi-potent progenitors (MPPs) are characterized by their lack of expression of known lineage markers and expression of c-kit and have been implicated in immunosurveillance. Peripheral HSCs express Toll-like receptors (TLRs) and are capable of differentiating upon stimulation with microbial products. In this Chapter, the characterization of the IL-25-elicited Lin$^{neg}$ c-kit$^+$ cells present in the periphery revealed that they are MPP-like cell population. IL-25-elicited Lin$^{neg}$ c-kit$^+$ cells expressed Sca1 and CD34, but lacked expression of CD150, a phenotype consistent with BM-resident MPPs. Further, IL-25-elicited c-kit$^+$ GFP$^{neg}$ cells isolated from IL-4/eGFP reporter mice exhibited multi-potent capacity, giving rise to cells of monocyte/macrophage and granulocyte lineages both in vitro and in vivo. In addition, progeny derived from IL-25-elicited c-kit$^+$ GFP$^{neg}$ cells were competent antigen presenting cells and could promote CD4$^+$ Th2 cell differentiation. Therefore, IL-25-elicited c-kit$^+$ GFP$^{neg}$ cells were termed MPP$^{type2}$ cells.
3.2 Introduction

While c-kit is commonly recognized as a marker for mature mast cells, many other cell populations express c-kit, particularly during hematopoiesis. Among these are hematopoietic stem cells (HSCs). HSCs are a rare long-lived cell population found in the bone marrow and are responsible for replenishing cells of both the innate and adaptive immune system [199]. HSCs give rise to a series of progenitors that gradually lose their lineage differentiation potential and self-renewal, which are separated into 3 classes, (i) long-term and (ii) short-term HSCs and (iii) multi-potent progenitors (MPP), all of which are Lineage\textsuperscript{neg} and Sca1\textsuperscript{+} c-kit\textsuperscript{+}. This division is based on the capacity for self-renewal and sustained ability for reconstitution of multiple hematopoietic lineages. Both long-term and short-term HSC populations possess multi-potent lineage potential but are designated so based on their ability to sustain reconstitution in multiple sequential hosts, while MPPs have lost their capacity for self-renewal but still maintain the ability to generate all known lineages. Phenotypically, these populations are classified based on expression of Sca1, c-kit, CD34 and CD150, where long-term HSC are CD34\textsuperscript{neg} CD150\textsuperscript{+}, short-term HSC are CD34\textsuperscript{+} CD150\textsuperscript{+} and MPP are CD34\textsuperscript{+} CD150\textsuperscript{neg} [227]. Progression from HSCs and commitment to a specific lineage differentiation pathway is regulated by various growth and differentiation factors. MPPs subsequently give rise to myeloid progenitors (MPs) and early lymphoid progenitors (ELPs). MPs give rise to either megakaryocyte and erythrocyte progenitors (MEPs) or granulocyte and macrophage progenitors (GMPs) [200], while ELPs yield T, B and NK cells give rise to pro-lymphocytes or common lymphoid progenitors (CLPs) that can then become pre-B cells [201, 202].
HSCs and other progenitors are primarily bone marrow-resident, however, HSCs are not entirely restricted to the bone marrow and in fact represent a highly migratory cell population. HSC populations constantly recirculate between the bone marrow and blood [203, 204] and stable, albeit small populations of HSCs can be collected from the blood or lymphatics of adult mice [205, 228]. Moreover, HSCs have been recovered from extramedullary sites, such as the muscle [207], liver [208] and spleen [204], however why HSCs traffic within the body and the biological significance of circulating HSCs remains poorly understood. Several studies have suggested that circulating HSCs may be acting in a role of immunosurveillance, as HSC populations have been reported to express Toll-like receptors (TLRs) and were able to respond to and differentiate in response to stimulation with TLR-ligands [215]. Further, HSCs recovered from the thoracic duct, which originated in the bone marrow, were found to traffic to and differentiate in sites of TLR-ligand challenge in secondary hosts [216]. Together, these reports suggest that HSCs, and potentially other precursor cells, are able to recognize and respond to pathogen-derived signals and thus initiate rapid local immune responses. However, whether IL-25 influences the population expansion and/or differentiation of peripheral HSC populations and whether these cells can influence CD4+ Th2 cell responses has not been examined.

In this Chapter, we demonstrate that the IL-25-elicited c-kit+ cells display a surface phenotype consistent with bone marrow-resident MPPs as they lack expression of lineage markers, but expressed Sca1 and CD34. Surprisingly, the frequencies and surface marker expression of MPPs, short-term and long-term HSCs in the BM were unchanged following administration of IL-25. Moreover, analysis of proliferation as measured by BrdU incorporation revealed that only c-kit+ cells in the periphery and not in
the bone marrow were proliferating, suggesting that IL-25 promoted the expansion of an MPP-like cell population in the periphery. IL-25-elicited c-kit\(^+\) GFP\(^+\) cells were found to differentiate into mast cells, but interestingly, the c-kit\(^+\) GFP\(^{neg}\) cells exhibited multi-potent capacity, giving rise to cells of monocyte/macrophage and granulocyte lineages both in vitro and in vivo. In addition, progeny derived from IL-25-elicited c-kit\(^+\) GFP\(^{neg}\) cells, but not c-kit\(^+\) GFP\(^+\) cells, were competent antigen presenting cells and could promote CD4\(^+\) Th2 cell differentiation. Thus, the IL-25-elicited c-kit\(^+\) GFP\(^{neg}\) cells were termed MPP\(^{type2}\) cells. These data indicate that IL-25 promotes Th2 cytokine responses through the induction of a multi-potent progenitor cell population.

3.3 Methods

3.3.1 Mice, exogenous cytokines and BrdU labeling

Balb/c and C57BL/6 mice were obtained from Jackson Laboratory and IL-4/eGFP reporter mice were obtained from M. Mohrs (Trudeau Institute). Ly5.2/Cr (CD45.1) congenic mice were obtained from NCI. Animals were bred and housed in specific pathogen-free conditions at the University of Pennsylvania. All experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the guidelines of the IACUC of the University of Pennsylvania. All mice used were 4-12 weeks of age. Mice were treated intraperitoneally with PBS or recombinant IL-25 (0.4 \(\mu\)g; R&D Systems) daily for 4 days. For in vivo BrdU labeling, Mice were injected i.p. with 1 mg of BrdU (Sigma-Aldrich) on day 1 and day 3 of IL-25-treatment. BrdU incorporation was analyzed using an APC-BrdU kit as recommended by the manufacturer (BD Pharmingen).

3.3.2 Flow cytometry, cell sorting and cytospin preparations
MLN from IL-4/eGFP reporter mice were separated from the mesentery, homogenized by passing through a 70 μm nylon mesh filter and stained with anti-mouse fluorochrome-conjugated monoclonal antibodies against CD3ε, CD4, CD8, TCRβ, TCRγδ, B220, CD19, CD11b, CD11c, MHC class II, Gr-1, NK1.1, Ter119, FcεRIα, c-kit, Sca1, CD150, CD34, CD45.2 (eBioscience and BD Bioscience). Peritoneal exudate cells ('peritoneum') were collected using peritoneal lavage with injection of 10 mL PBS and aspirated using the same syringe. Mesentery was processed as previously described[13]. Cells were run on a BD FACSCanto II using DiVa software (BD Bioscience) and analyzed with FlowJo software (Version 8.7.1; Tree Star, Inc.). IL-25-elicited c-kit+ (GFP+ or GFPneg) cell populations were sorted using a FACSARia (BD Bioscience) and for in vitro differentiation studies were incubated in the presence of SCF (50 ng mL⁻¹; R&D systems) and IL-3 (10 ng mL⁻¹; R&D systems). Following in vitro culture, progeny were assessed for expression of CD11b, MHC class II, CD115 (M-CSFR), Ly6C, FcεRIα, and c-kit (eBioscience) by flow cytometry as described above. Sort-purified IL-25-elicited c-kit+ (GFP+ or GFPneg) cell populations or their resultant progeny were subjected to cytospin (Cytospin 3, Thermo Fisher Scientific) and stained by Diff-quick for analysis of cellular morphology. For in vivo differentiation assays, 3x10⁴ CD45.2 IL-25-elicited c-kit+ GFPneg cells were FACS-purified and transferred i.p. into CD45.1 congenic recipient mice. Recipient mice were treated 4 times with 1 μg each of SCF and IL-3 and differentiation of donor cells was assessed on day 6 post-transfer by flow cytometry as described above.

3.3.3 HSC differentiation assays

FACS-purified cell populations were plated onto semi-confluent OP9 stromal cells (ATCC # CRL-2749), as previously described [229]. Monolayers were irradiated (3000
rad) prior to co-culture with FACS-purified populations. Cells were incubated in the presence of SCF (50 ng mL\(^{-1}\); R&D systems) and IL-3 (10 ng mL\(^{-1}\); R&D systems).

### 3.3.4 CD4\(^+\) T cell co-cultures

Naïve OVA-specific CD4\(^+\) T cells were isolated from DO11.10 mice as previously described [11]. T cells were stained with fluorochrome-conjugated monoclonal antibodies against CD4, CD62L, and CD44 (eBioscience), re-suspended in 2% FBS in HBSS with 2 mM EDTA (Gibco) with DAPI (1 \(\mu\)g mL\(^{-1}\); Molecular Probes) and naïve T cells sorted based on live cells (DAPI\(^{\text{neg}}\)) CD4\(^+\) CD62L\(^\text{hi}\) CD44\(^\text{lo}\) using a FACSARia (BD Bioscience). T cells were labeled with CFSE (Molecular Probes) and co-cultured with \textit{in vitro} derived progeny from sorted IL-25-elicited c-kit\(^+\) (GFP\(^+\) or GFP\(^{\text{neg}}\)) cell populations. Culture were either untreated (media) or cultured in the presence of OVA peptide (1 \(\mu\)g mL\(^{-1}\)) with or without blocking antibodies against MHC class II (5 \(\mu\)g mL\(^{-1}\); M5/114; eBioscience) or IL-4R\(\alpha\) (5 \(\mu\)g mL\(^{-1}\; mIL4R-M1; BD Bioscience) in complete medium (DMEM Iscove’s with 10% (vol/vol) heat-inactivated FBS, 2 mM glutamine, 100 U mL\(^{-1}\) of penicillin, 100 \(\mu\)g mL\(^{-1}\) of streptomycin, 25 mM HEPES and 50 \(\mu\)M \(\beta\)-mercaptoethanol). Cell-free supernatants were assessed for cytokine production by standard sandwich ELISA (eBioscience) following 4h stimulation with PMA (50 ng mL\(^{-1}\)) and ionomycin (750 ng mL\(^{-1}\)) (Sigma Aldrich).

### 3.3.5 Real-time PCR

RNA from colonic tissues of mice was isolated by TRizol extraction (Invitrogen) or collected from sorted cell populations using RNeasy Mini kit (Qiagen). Whole tissues were homogenized with a tissue homogenizer (TissueLyzer; Qiagen) and cDNA was prepared with SuperScript Reverse Transcriptase (Invitrogen). Quantitative real-time
PCR analysis used commercial QuantiTect primer sets for \( \text{Il4}, \text{Il5}, \text{Il13}, \text{Gata3}, \text{Maf}, \text{Junb}, \text{Stat6} \) and \( \text{Il1rl1} \) (Qiagen) and SYBR Green chemistry (Applied Biosystems). All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Target genes were normalized for endogenous \( \beta \)-actin levels and relative quantification of samples were compared to controls.

### 3.3.6 Statistical analysis

Results are shown as means ± s.e.m for individual animals. Statistical significance was determined by Student's t-test. Results were considered significant at \( P < 0.05 \).

### 3.4 Results

#### 3.4.1 IL-25-elicited c-kit\(^*\) cells display a MPP-like surface phenotype, but IL-25 does not affect HSC populations in the bone marrow

C-kit, the receptor for stem cell factor (SCF), is commonly recognized as a marker for mature mast cells, however, HSCs and MPP are also characterized by their expression of c-kit. Therefore to determine whether IL-25-elicited c-kit\(^*\) cells represent HSCs, we examined expression of progenitor cell-associated cell surface markers on IL-25-elicited c-kit\(^*\) cells. Consistent with an HSC-like phenotype [227, 230], IL-25-elicited c-kit\(^*\) GFP\(^{\text{neg}}\) and c-kit\(^*\) GFP\(^{\text{pos}}\) populations were found to lack expression of known lineages markers including CD3\(\varepsilon\), CD8\(\alpha\), CD8\(\beta\), TCR\(\alpha\beta\), TCR\(\gamma\delta\), B220, CD19, CD11b, CD11c, NK1.1, Gr-1, and Ter119, and were thus defined as Lin\(^{\text{neg/lo}}\) (Fig. 12). HSC populations can be divided into three classes based on expression of Sca1, CD34 and CD150, where long-term HSCs are CD150\(^{\text{neg}}\) CD34\(^{\text{neg}}\), short-term HSCs are CD150\(^{\text{pos}}\) CD34\(^{\text{neg}}\) and MPPs are CD150\(^{\text{neg}}\) CD34\(^{\text{neg}}\) [227]. Therefore, to more specifically characterize the IL-25-elicited c-kit\(^*\) cells, these cells were assessed for expression of Sca1, CD150 and CD34.
The majority of the IL-25-elicited c-kit^+ GFP^neg and c-kit^+ GFP^+ cells expressed Sca1, were CD150^neg, and exhibited heterogeneous expression of CD34 (Fig. 13A-C). Therefore, the IL-25-elicited cell populations in the GALT exhibited a surface phenotype most consistent with an MPP-like cell population.

To investigate whether IL-25 was acting to increase proliferation of progenitor populations in the bone marrow or in the periphery, WT mice were treated daily with IL-25 for 4 days and injected with bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) on days 1 and 3. BrdU is a synthetic nucleotide analog that is incorporated into newly replicated DNA during the S phase of the cell cycle. Surprisingly, treatment with IL-25 did not increase the frequencies of c-kit^+ cells in the bone marrow, nor did IL-25 treatment result in increased BrdU incorporation by the c-kit^+ population (Fig. 14A). In contrast, administration of IL-25 resulted in increased frequencies of c-kit^+ cells at peripheral sites, including the MLN (Fig. 14B) and increased frequencies of c-kit^+ cells that incorporated BrdU compared to c-kit^+ cells from naïve mice (Fig. 14B). Thus, these findings demonstrate that IL-25 results in increased proliferation of cells only in the periphery.

One potential caveat, however, this that these data cannot rule out the possibility of increased egress of proliferating (BrdU^+) cells from the bone marrow. Therefore, to determine whether IL-25 influenced the frequencies of HSC populations in the bone marrow or their surface phenotype we analyzed these populations in control and IL-25-treated mice. We observed no change in the frequencies of total HSCs (defined by Lineage^neg Sca1^+ c-kit^+) following treatment with IL-25 (6.5% in controls compared to 7.8% in IL-25-treated) (Fig. 15A, left panels). Further, no changes were seen in any of
the 3 HSC populations based on CD34 and CD150 expression (Fig. 15A, right panels), indicating that IL-25 does not influence the frequencies of bone marrow-resident HSC populations in vivo. However, as stated previously, the IL-25-elicited cells display an intermediate expression of c-kit (see Fig. 9A). Therefore, we assessed the c-kit<sup>int</sup> cells in the bone marrow of control and IL-25-treated animals (Fig. 15A and Fig. 15B) for surface expression of CD34 and CD150. As previously observed, IL-25-treatment did not result in increased frequencies of the c-kit<sup>int</sup> cells in the bone marrow (Fig. 15B) and only a small proportion of the c-kit<sup>int</sup> cells in control mice expressed either CD34 or CD150 (Fig. 15B), indicating that c-kit<sup>int</sup> cells in the bone marrow do not represent any of the known HSC populations. These data suggest that IL-25 does not alter HSC populations in the bone marrow. This finding, combined the previous findings that administration of IL-25 results in the accumulation of a proliferative, MPP-like cell population in the GALT suggests that IL-25 acts in the periphery to elicit the population expansion of a c-kit<sup>+</sup> MPP-like cell population.

### 3.4.2 IL-25-elicited c-kit<sup>+</sup> cells exhibit multi-potent capacity and differentiate into myeloid and/or granulocyte populations

To assess the capacity of the IL-25-induced c-kit<sup>+</sup> MPP-like cell population to exhibit multi-potent potential, c-kit<sup>+</sup> GFP<sup>neg</sup> or c-kit<sup>+</sup> GFP<sup>+</sup> cells from IL-25-treated IL-4/eGFP reporter mice were sorted and cultured in vitro in the presence of SCF and IL-3 (Fig. 16A-F). Cytospin preparations were performed from FACS-purified populations revealed that both c-kit<sup>+</sup> GFP<sup>neg</sup> and c-kit<sup>+</sup> GFP<sup>+</sup> populations shared a similar cell morphology of a small, agranular cell with a single, non-segmented nucleus (Fig. 16D and F, ‘input’) which is consistent with previous reports [16]. As a positive control for the differentiation of macrophages and mast cell, unfractionated bone marrow from naïve mice was used.
Following exposure to SCF and IL-3, un-fractionated bone marrow cells differentiated into a CD11b+ macrophage-like population (Fig. 16A, orange gate) and a CD11bneg granulocyte population (Fig. 16A, black gate) that could be separated into c-kit+ FcεRI+ mast cells (Fig. 1A, blue gate) and c-kitneg FcεRI+ basophils (Fig. 16A, green gate) as determined by surface marker expression and cell morphology (Fig. 16B). Sorted IL-25-elicited c-kit+ GFP+ cells failed to give rise to CD11b+ progeny but did yield a CD11bneg c-kit+ FcεRI+ population (Fig. 16C, blue gate). Consistent with flow cytometric analysis, the progeny of c-kit+ GFP+ cells were morphologically similar to mast cells (Fig. 16D), suggesting that this cell population is a precursor to mast cells. Interestingly, IL-25-elicited c-kit+ GFPneg cells were distinguished from the c-kit+ GFP+ population by their ability to give rise to multiple cell lineages including CD11b+ macrophages (Fig. 16E, orange gate), CD11bneg c-kit+ FcεRI+ mast cells (Fig. 16E, blue gate) and CD11bneg c-kitneg FcεRI+ basophils (Fig. 16E, green gate). The multi-potent potential of the IL-25-elicited c-kit+ GFPneg cell population was confirmed by examination of the cell morphology of the resultant progeny, which displayed characteristics of macrophages, mast cells or basophils (Fig. 16F), including cell populations with non-segmented nuclei and vacuolar cytoplasm (macrophages) as well as agranular cell populations with non-segmented (mast cells) or segmented nuclei (basophils).

To further assess the myeloid lineage potential of the IL-25-induced c-kit+ GFPneg population, we adopted both a liquid culture system and a co-culture system in which FACS-purified cells were plated onto a monolayer of OP9 stromal cells in the presence of cytokines and/or growth factors. While stromal cell contact is not always necessary for differentiation, as is the case for mast cells [231], the OP9 stromal cell co-culture
system provides the cell-cell interactions necessary for the myeloid and lymphoid differentiation of HSCs to be examined [232-234]. This system has been used extensively to investigate the development of HSCs into fully differentiated cell populations [235-238]. Purified IL-25-elicited c-kit+ GFP<sup>neg</sup> and c-kit+ GFP<sup>+</sup> populations were cultured onto a semiconfluent monolayer of OP9 stromal cells in presence of SCF and IL-3 and assessed for myeloid differentiation at day 8. As previously observed, IL-25-elicited c-kit+ GFP<sup>neg</sup> cells seeded into liquid cultures containing SCF and IL-3 differentiated into CD11b<sup>+</sup> cells (orange histograms) (Fig. 17A). Moreover, the majority of these cells co-expressed additional markers for myeloid lineages including M-CSFR, Ly6C and MHC II (Fig. 17A). Consistent with liquid cultures, IL-25-elicited c-kit+ GFP<sup>neg</sup> cells seeded onto OP9 stromal cells generated CD11b<sup>+</sup> progeny cells that were also M-CSFR<sup>+</sup>, Ly6C<sup>+</sup> and MHC II<sup>+</sup> (Fig. 17B). Thus, these results support the hypothesis that the IL-25-elicited c-kit+ GFP<sup>neg</sup> cells represent a cell population with myeloid differentiation potential.

To determine whether the IL-25-elicited c-kit+ GFP<sup>neg</sup> cell population could exhibit multi-potent potential <i>in vivo</i>, IL-25-elicited c-kit+ GFP<sup>neg</sup> cells were isolated from WT CD45.2 donor mice and adoptively transferred into naïve CD45.1 congenic recipients. Six days following transfer, c-kit+ GFP<sup>neg</sup> donor cells were found to have differentiated into CD11b<sup>+</sup> cells as well as CD11b<sup>neg</sup> c-kit<sup>+</sup> FcεRI<sup>+</sup> cells (Fig. 18). These results support the hypothesis that IL-25-elicited c-kit+ GFP<sup>neg</sup> cells represent a cell population that exhibits multi-potent potential, capable of giving rise to cells of the monocyte/macrophage and granulocyte lineages both <i>in vitro</i> and <i>in vivo</i>.
3.4.3 Progeny from IL-25-elicited c-kit+ GFP^neg cells promote Th2 cell differentiation in a MHC class II-dependent manner

The ability of the IL-25-elicited c-kit^+ GFP^neg cells to differentiate into CD11b^+ MHC II^+ cells led to the hypothesis that these cells were capable of presenting antigen and driving Th2 cell differentiation of naïve CD4^+ T cells. Therefore, to examine the functional capacity of progeny derived from c-kit^+ GFP^neg- or c-kit^+ GFP^+ cells, the IL-25-elicited c-kit^+ cell populations were sorted (Fig. 19A, left panel) and cultured in vitro in the presence of SCF and IL-3 for 8 days. The majority of the progeny derived from c-kit^+ GFP^+ cells were IL-4/eGFP^+ but MHC class II^neg, while the c-kit^+ GFP^neg-derived progeny contained both IL-4/eGFP^+ and MHC class II^+ cell populations (Fig. 19A, right panels), indicating that progeny derived from c-kit^+ GFP^neg cells, collectively, may influence T cell proliferation and/or differentiation. To test this, the c-kit^+ GFP^neg- or c-kit^+ GFP^+-derived progeny were pulsed with OVA and co-cultured with CFSE-labeled OVA-specific TCR transgenic CD4^+ T cells. When co-cultured with the progeny from either c-kit^+ GFP^+ or c-kit^+ GFP^neg cells in medium alone, T cells exhibited minimal proliferation (1% CFSE^dim, Fig. 19B and C, shaded histograms). In addition, when cultured with c-kit^+ GFP^+-derived progeny cells in the presence of OVA peptide, no T cell proliferation was observed (Fig. 19B, black histogram) and no IL-4 or IL-13 protein was detected in culture supernatants (data not shown), indicating that progeny derived from IL-25-elicited c-kit^+ GFP^+ cells alone cannot promote Th2 cell differentiation.

In contrast, following co-culture with OVA peptide and progeny derived from c-kit^+ GFP^neg cells, 42% of CD4^+ T cells were CFSE^dim (Fig. 19C, black histogram) and the observed increased T cell proliferation was associated with increased production of IL-4 and IL-13 (Fig. 19D), indicating that IL-25-elicited c-kit^+ GFP^neg cells could promote Th2 cell differentiation. Further, the ability of c-kit^+ GFP^neg-derived progeny to influence Th2
cell differentiation was MHC class II-dependent (Fig. 19C and D), as inclusion of a blocking antibody to MHC class II resulted in decreased proliferation and cytokine production. Further, inclusion of anti-IL-4Rα mAb did not affect T cell proliferation in the presence of OVA peptide (Fig. 19C), but resulted in decreased production of IL-4 and IL-13 (Fig. 19D), indicating that both MHC class II and IL-4R signaling are required for the c-kit+ GFPneg-derived progeny to promote Th2 cell differentiation. No IFN-γ was detected in any culture conditions (data not shown), suggesting that IL-25-elicited c-kit+ cells selectively promote Th2 cytokine responses. These results indicate that IL-25-elicited c-kit+ cells contain a population of progenitors with multi-potent capacity whose progeny act to promote CD4+ Th2 cell differentiation. Thus, this cell population has been termed MPPtype2 cells.

3.5 Discussion

The inability to characterize the IL-25-elicited c-kit+ cells as any of the known innate cell lineages raised fundamental questions in regards to the identity of this cell population. C-kit expression is commonly associated with mature mast cells, however, hematopoietic stem cells (HSCs) in the bone marrow also express c-kit, as well as being Linneg and Sca1+. HSCs are multi-potent cells and give rise to cells of both the innate and adaptive immune system. HSCs have been shown to traffic within the body and it has been suggested that circulating HSCs may be acting in a role of immunosurveillance, as HSC populations have been reported to express Toll-like receptors (TLRs) and were able to respond to and differentiate in response to stimulation with TLR-ligands [215, 216]. Together, these reports suggest that HSCs, as well as other precursor cells, are able to recognize and respond to pathogen-derived signals and thus initiate rapid, local immune responses. However, whether IL-25-elicited c-kit+
cells represent HSCs or other progenitors, both in surface phenotype or multi-potent potential, and whether these cells can influence CD4+ Th2 cell responses has not been examined.

Here we demonstrate that the IL-25 elicited a c-kit+ cell population in the periphery that display a surface phenotype consistent with bone marrow-resident MPPs (being Linneg Sca1+ CD34+). Surprisingly, the frequencies of bone marrow-resident MPP and HSC populations were unchanged following administration of IL-25, suggesting that IL-25 promoted the expansion of an MPP-like cell population in the periphery. The IL-25-elicited c-kit+ GFP+ cells were found to differentiate into mast cells, a finding consistent with the hypothesis first proposed by Fallon et al. [16]. Additionally, the c-kit+ GFPneg cells exhibited multi-potent capacity, giving rise to cells of monocyte/macrophage and granulocyte lineages both in vitro and in vivo. In addition, progeny derived from IL-25-elicited c-kit+ GFPneg cells, but not c-kit+ GFP+ cells, were competent antigen presenting cells and could promote CD4+ Th2 cell differentiation. Thus, the IL-25-elicited c-kit+ GFPneg cells were termed MPPtype2 cells. Collectively, these data illustrate that IL-25-elicited c-kitint cells are not terminally differentiated cells, but instead represent cell populations that possess multi-potent progenitor properties and are capable of promoting Th2 cytokine responses.

Interestingly, while IL-25-elicited c-kit+ GFPneg cells possess multi-potent potential, it appears that these cells require a secondary stimulus in order to commence their differentiation, as in the presence of media or IL-25 alone, these cells fail to undergo differentiation. This finding suggests that IL-25 is acting to expand these populations in vivo, and it is only in the context of an appropriate stimulus that this population can then
differentiate. While the dependence on a secondary stimulation at first appears to be a limitation of this cell, the implication of this suggests that the IL-25-elicited MPP\textsuperscript{type2} cell population may display plasticity in its differentiation potential. For instance, in the presence of SCF and IL-3, MPP\textsuperscript{type2} cells were found to differentiate into monocytes and granulocytes. However, it could be that in the presence of alternative cytokines that differential progeny could arise. This implies that given the local cytokine milieu present, IL-25-elicited MPP\textsuperscript{type2} cells could ‘tailor’ their response appropriately to specify the appropriate immune response.

Nevertheless, the finding that IL-25-elicited MPP\textsuperscript{type2} cells possess multi-potent potential (Fig. 20), coupled with reports that peripheral HSCs express TLRs and can respond to microbial stimulation [215, 216], implicate extramedullary hematopoiesis as a previously unrecognized mechanism to initiate the development of rapid and local type 2 immune responses and/or to sustain effector cell populations at mucosal sites required for the elimination of pathogens from host tissues. Moreover, the identification of effector functions in progenitor populations from helminth-infected individuals and asthmatic patients [217, 239] further supports this hypothesis. Given the evolutionarily conserved nature of the IL-17 cytokine family [163], the innate cell populations targeted by IL-25 may represent ancient evolutionary conserved pathways for the generation of effector cell populations that preceded the development of the adaptive immune response or compartmentalization of hematopoiesis to the bone marrow. In support of this hypothesis, another member of the IL-17 cytokine family, IL-17A, has been shown to influence granulopoiesis [240], and emerging evidence supports the presence of other ‘primitive’ innate cell populations, including lymphoid tissue-inducer cells, NK22 cells and innate lymphoid cell populations [241-244], which appear to be innate sources of Th1-
and Th17-associated cytokines that may be involved in immunity to extracellular bacteria and the promotion of intestinal inflammation. Collectively, the data presented here provide the first link between the IL-17 cytokine family, extramedullary hematopoiesis and the development of type 2 inflammation.
Figure 12. IL-25-elicited c-kit$^+$ cells are Lin$^{neg}$.

Figure 12. Expression of combined lineage markers (CD3ε, CD8α, CD8β, TCRβ, TCRγδ, B220, CD19, CD11b, CD11c, Gr-1, NK1.1 and Ter119) by c-kit$^{int}$ GFP$^{neg}$ (blue line) and c-kit$^{int}$ GFP$^{+}$ (green line) cells from mesenteric lymph nodes of IL-25-treated IL-4/eGFP reporter mice. Plot shown is gated on live cells. Data are representative of two independent experiments. (n=4-6 per group).
Figure 13. IL-25-elicited c-kit\(^+\) cells display a surface phenotype consistent with multi-potent progenitor cells.

**Figure 13.**

**A, B**, Frequency of c-kit\(^+\) cell population from MLNs of control (**A**) or IL-25-treated (**B**) IL-4/eGFP reporter mice. **C**, Expression of HSC markers by c-kit\(^+\) GFP\(^\text{neg}\) (**blue histograms**) or c-kit\(^+\) GFP\(^+\) (**green histograms**) cells from IL-25-treated IL-4/eGFP reporter mice. Number (**italics**) indicates the mean fluorescent intensity (MFI) of each respective marker. Plots shown are gated on live, lineage\(^\text{neg}\) cells (CD3\(\varepsilon\), CD8\(\alpha\), CD8\(\beta\), TCR\(\beta\), TCR\(\gamma\delta\), B220, CD19, CD11b, CD11c, Gr-1, NK1.1 and Ter119) or as indicated. Data in **A-C** are representative of two independent experiments (control, n=4; IL-25-treated, n=7).
Figure 14. Administration of recombinant IL-25 results in increased BrdU⁺ c-kit⁺ cells in the MLNs but not in the bone marrow.
Figure 14. A, B, C57BL/6 (WT) mice were treated daily with IL-25 for 4 days and injected with the bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) on days 1 and 3. Bone marrow (A) or mesenteric LN (B) cells were analyzed for BrdU incorporation in the NBNT cell compartment. Plots shown are gated to exclude CD4, CD8, B220, CD11b, and CD11c positive cells or as indicated. Data in A and B are representative of at least two independent experiments.
Figure 15. IL-25 does not affect the frequencies of bone marrow resident hematopoietic stem cells.
Figure 15. **A, B**, Expression of HSC markers by HSC or c-kit<sup>int</sup> cell populations in the bone marrow of control (**A**) or IL-25-treated (**B**) IL-4/eGFP reporter mice was analyzed by flow cytometry. Plots shown are gated on live lineage<sup>neg</sup> cells (CD3ε, CD8α, CD8β, TCRβ, TCRγδ, B220, CD19, CD11b, CD11c, Gr-1, NK1.1 and Ter119) or as indicated. Data are representative of at least two independent experiments. (control, n=4; IL-25-treated, n=6).
Figure 16. IL-25-elicited c-kit$^+$ cells exhibit multi-potent capacity and differentiate into myeloid and/or granulocyte populations.
Figure 16. **A, C, E**, Flow cytometric analysis of myeloid and granulocyte differentiation of un-fractionated bone marrow from control mice (A) or FACS-purified live, lineage\(^{neg}\) c-kit\(^+\) GFP\(^+\) (C) or c-kit\(^+\) GFP\(^{neg}\) (D) cells from IL-25-treated IL-4/eGFP reporter mice following *in vitro* culture in SCF (50 ng mL\(^{-1}\)) and IL-3 (10 ng mL\(^{-1}\)). **B, D, F**, Cytospin preparation of progeny from cultures seeded with un-fractionated bone marrow (B), IL-25-elicited c-kit\(^+\) GFP\(^+\) cells (D) or IL-25-elicited c-kit\(^+\) GFP\(^{neg}\) cells (F) following culture *in vitro* in the presence of SCF and IL-3. Cells stained with Diff-quick. Scale bar, 20 μm. Data in **A-F** are representative of at least three independent experiments.
Figure 17. IL-25-elicited c-kit+ GFPneg cells possess monocyte/macrophage potential.

**A**

IL-25-elicited

![Flow cytometry plots showing c-kit+ GFPneg cell populations cultured in liquid culture (A) or co-cultured on OP9 stromal cells (B) in the presence of SCF (50 ng mL⁻¹) and IL-3 (10 ng mL⁻¹). Cultures were assessed for surface expression of monocyte/macrophage markers at day 8 by flow cytometry. Plots shown are gated on live cells or as indicated. Numbers indicate the percentage of gated region of total population. Data in A and B are representative of at least two independent experiments.](image)

**B**

IL-25-elicited

![Flow cytometry plots showing c-kit+ GFPneg cell populations cultured in liquid culture (A) or co-cultured on OP9 stromal cells (B) in the presence of SCF (50 ng mL⁻¹) and IL-3 (10 ng mL⁻¹). Cultures were assessed for surface expression of monocyte/macrophage markers at day 8 by flow cytometry. Plots shown are gated on live cells or as indicated. Numbers indicate the percentage of gated region of total population. Data in A and B are representative of at least two independent experiments.](image)
Figure 18. IL-25-elicited c-kit$^+$ GFP$^{\text{neg}}$ cells display multi-potent potential in vivo.

Figure 18. CD45.1 congeneric recipients were left untreated or given 2.5-3 x10$^4$ c-kit$^+$ GFP$^{\text{neg}}$ cells i.p. from IL-25-treated CD45.2 mice. Recipient mice were treated 4 times with 1 µg each of SCF and IL-3 and differentiation of donor cells isolated from the peritoneal cavity was assessed on day 6 post-transfer. Plots shown are gated on live cells or as indicated. Data are representative of at least two independent experiments. (n=4).
Figure 19. Progeny from IL-25-elicited c-kit⁺ GFPneg cells promote Th2 cell differentiation in a MHC class II-dependent manner.
Figure 19. **A**, FACS-purified IL-25-elicited c-kit\(^+\) cells were cultured for 8 days in SCF and IL-3 and the resultant progeny from c-kit\(^+\) GFP\(^+\) (**green line**) or c-kit\(^+\) GFP\(^{neg}\) (**blue line, shaded**) cells were assessed for expression of IL-4/eGFP and MHC class II. Plots are gated on total live population. Results in **(A)** are representative of three independent experiments. **B, C**, CFSE-dilution by OVA-specific CD4\(^+\) CD62L\(^{hi}\) CD44\(^{lo}\) T cells following 4 day co-culture with SCF and IL-3-derived progeny from FACS-purified IL-25-elicited c-kit\(^+\) GFP\(^+\) (**B**) or c-kit\(^+\) GFP\(^{neg}\) (**C**) cells in the presence of OVA peptide with or without addition of monoclonal antibodies against MHC class II or IL-4R\(\alpha\). Cells were analyzed by flow cytometry for surface expression of CD4 and CFSE dilution. Plots are gated on CD4\(^+\) T cells. **D**, IL-4 and IL-13 protein in cell-free supernatants from (**C**) was assayed by ELISA. Results in **B-D** are representative of at least two independent experiments.
Figure 20. Schematic of the mechanism whereby IL-25 promotes Th2 cytokine responses in vivo. Mucosal epithelial cells have been shown to express TSLP, IL-33, and IL-25. While TSLP and IL-33 have been shown to elicit basophils or eosinophils, respectively, IL-25 elicits c-kit+ GFP+ and c-kit+ GFPneg cell populations. While the c-kit+ GFP+ cells give rise to mast cells, the c-kit+ GFPneg cell population possesses multi-potent capacity, being able to differentiate into monocyte/macrophages, mast cells and basophils that collectively promote CD4+ Th2 cell differentiation.
Chapter 4

MPP\textsuperscript{type2} cells are functionally distinct from, but can differentiate into natural helper cells

4.1 Abstract

The recent identification of previously unrecognized innate cell populations, termed multi-potent progenitor type 2 (MPP\textsuperscript{type2}) cells, natural helper cells (NHCs), nuocytes, and innate type 2 helper (Ih2) cells has provided new insights into our understanding of the cellular mechanisms that lead to the development of CD4\textsuperscript{+} Th2 cell-dependent immunity and/or inflammation at mucosal sites \cite{13-15, 17}. All four cell populations are activated by IL-25 and/or IL-33 and capable of promoting Th2 cytokine responses. Based on these recent findings, a fundamental question to be addressed is whether NHCs, MPP\textsuperscript{type2} cells, nuocytes or Ih2 cells are the same cell population, related cell lineages, or represent four distinct and unrelated cell lineages. In this chapter, we show that IL-25-elicited MPP\textsuperscript{type2} cells are phenotypically distinct from NHCs/nuocytes, based on expression of T1/ST2, IL-7R\textalpha, and CD90. In addition, administration of IL-25 or IL-33 to IL-4/eGFP reporter mice elicited distinct innate cell populations in the mesenteric lymph nodes. While both IL-25 and IL-33 elicited increased frequencies of c-kit\textsuperscript{+} cells, a finding consistent with published data \cite{14-17}, IL-33-treatment resulted in the induction of a higher frequency of T1/ST2\textsuperscript{+} c-kit\textsuperscript{+} cells, a phenotype most consistent with a nuocyte population. In contrast, the vast majority of IL-25-elicited c-kit\textsuperscript{+} cells lacked expression of T1/ST2, a surface phenotype most consistent with MPP\textsuperscript{type2} cells. In addition, IL-25-elicited MPP\textsuperscript{type2} cells and nuocytes both expressed the receptor for TSLP (TSLPR) and upon stimulation with TSLP and IL-33, IL-25-elicited MPP\textsuperscript{type2} cells differentiated into nuocytes, while nuocytes did not display multi-potent potential. Together, these data
indicate that MPP\textsuperscript{type2} cells are distinct from and could represent a progenitor to NHC populations.

4.2 Introduction

Along with the identification of MPP\textsuperscript{type2} cells, three other independent laboratories recently identified similar previously unrecognized innate cell populations that respond to IL-25 and/or IL-33 and could promote CD4\textsuperscript{+} Th2 cell-dependent immunity and/or inflammation [13-15, 17] (summarized in Table 2). These cell populations are termed natural helper cells (NHCs), nuocytes, or innate type 2 helper (Ih2) cells. NHCs were found in fat-associated lymphoid clusters (FALC) in the mesentery and in fatty deposits in the peritoneal cavity and surrounding the kidneys. Within the FALC, this cell population was found in close proximity to T cells and B cells and was capable of promoting the proliferation of B1 B cells and production of IgA [13]. In a second study [14] it was demonstrated that treatment of IL-13/eGFP reporter mice with either exogenous IL-33 or IL-25 increased the frequency of an IL-13/eGFP\textsuperscript{+} cell population in the MLN and spleen. This cell population is termed ‘nuocytes’ after the 13\textsuperscript{th} letter of the Greek alphabet, \textit{nu}, and could promote the development of protective Th2 cytokine responses following infection with a related helminth parasite, \textit{Nippostrongylus brasiliensis}. Lastly, Ih2 cells were identified using the Rosa-floxed-stop-YFP system [15] as an IL-13-producing innate cell population that was expanded in frequency in response to IL-25, IL-33 or following \textit{N. brasiliensis} infection. Ih2 cells promoted increased eosinophil numbers and immunity to helminth infection.

MPP\textsuperscript{type2} cells, nuocytes, Ih2 cells and NHCs all express c-kit, but lack expression of hematopoietic cell lineage markers including lineage-associated markers for T cells, B
cells, macrophages, dendritic cells, NK cells, lymphoid tissue inducer (LTi) cells or neutrophils. Further, these cells did not co-express FcεRIα, CD49b or CCR3 (markers for mature mast cells, basophils and eosinophils, respectively) [13-15, 17], indicating these novel cell populations were distinct from other known cell lineages. In addition, IL-25- and/or IL-33-responsive MPP<sup>type2</sup> cells, NHCs, nuocytes, and Ih2 cells displayed the common functionality of promoting the development of type 2 inflammation [13-15, 17]. For instance, adoptive transfer of IL-25-elicited MPP<sup>type2</sup> cells into normally susceptible *Trichuris muris*-infected *Il17e<sup>-/-</sup>* mice conferred protective immunity as evidenced by increased production of Th2 cytokines, which correlated with reduced numbers of parasites. Similarly, nuocytes, Ih2 cells, and NHCs were found to produce high levels of the Th2-associated cytokines IL-5 and IL-13 and adoptive transfer of either population was associated with increased type 2 inflammation and/or development of immunity to helminth infection.

Interestingly, however, further analysis of these cell populations revealed that while nuocytes and NHCs expressed T1/ST2 (a subunit of the IL-33 receptor), IL-7R<sub>α</sub> and CD90, IL-25-elicited MPP<sup>type2</sup> cells lacked expression of these surface markers. In addition, only IL-25-elicited MPP<sup>type2</sup> cells were found to possess multi-potent potential being able to generate progeny of myeloid and granulocyte lineages, suggesting that MPP<sup>type2</sup> cells represent a distinct cell population. The similarities and differences between MPP<sup>type2</sup> cells, NHCs, nuocytes and Ih2 cells in surface phenotype and functionality (see Table 2) provoke fundamental questions about the relationships between these newly described innate cell populations. For instance, whether MPP<sup>type2</sup> cells, NHCs, nuocytes or Ih2 cells represent the same cell population, but represent distinct stages of activation or represent four distinct unrelated cell lineages remains
poorly defined. Further, how distinct epithelial-derived cytokines, including TSLP, IL-33 and IL-25 regulate the relationships and functions of these distinct cell populations have not been examined.

In this chapter, IL-25-elicited MPP\textsubscript{type2} cells are shown to be phenotypically and functionally distinct from NHCs based on expression of T1/ST2, IL-7R\(\alpha\), and CD90 and their transcription factor expression profile. In addition, administration of IL-25 or IL-33 to IL-4/eGFP reporter mice elicited distinct innate cell populations in the mesenteric lymph nodes. While both IL-25 and IL-33 elicited increased frequencies of c-Kit\(^+\) cells, a finding consistent with published data [14-17], IL-33-treated resulted in the induction of a higher frequency of T1/ST2\(^+\) c-Kit\(^+\) cells, a phenotype most consistent with a nuocyte population. In contrast, the vast majority of IL-25-elicited c-Kit\(^+\) cells lacked expression of T1/ST2, a surface phenotype most consistent with MPP\textsubscript{type2} cells. In addition, IL-25-elicited MPP\textsubscript{type2} cells and nuocytes both expressed the receptor for TSLP (TSLPR) and upon stimulation with TSLP and IL-33, IL-25-elicited MPP\textsubscript{type2} cells differentiated into nuocytes, while nuocytes did not display multi-potent potential. Combined these data indicate the MPP\textsubscript{type2} cells are distinct from and represent direct progenitors to NHC populations and that they may represent a progenitor for NHCs.

4.3 Methods

4.3.1 Mice, administration of exogenous cytokine, antibody blockade

Balb/c and C57BL/6 mice were obtained from Jackson Laboratory, C57BL/6 Ly5.2/Cr (CD45.1) mice were obtained from NCI and IL-4/eGFP reporter mice were obtained from M. Mohrs (Trudeau Institute). Il33\(-/-\) mice were provided by D. E. Smith (Amgen). Animals were bred and housed under specific pathogen-free conditions at the University
all experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the guidelines of the IACUC of the University of Pennsylvania. All mice used were 4-12 weeks of age. Mice were treated intraperitoneally with PBS, recombinant IL-25 or IL-33 (0.4 µg; R&D Systems) daily for 4 days. For antibody-mediated blockade, mice were treated with 0.25 mg/day of anti-T1/ST2, anti-IL-25 or control IgG (from D. E. Smith and A. L. Budelsky, Amgen) on days -3, -1, 0, 2 and 4.

4.3.2 Flow cytometry and cell sorting
MLN from IL-4/eGFP reporter mice were separated from the mesentery, homogenized by passing through a 70 µm nylon mesh filter and stained with anti-mouse fluorochrome-conjugated monoclonal antibodies against CD3ɛ, CD4, CD8, TCRβ, CD19, CD11b, CD11c, NK1.1, FcɛRIα, c-kit, Sca1, CD127 (IL-7Rα), TSLPR, CD90 and CD45.2 (eBioscience and BD Bioscience). T1/ST2 staining was performed using T1/ST2 biotinylated mAb (MD Biosciences) and eFluor450-conjugated streptavidin (eBioscience). Peritoneal exudate cells (‘peritoneum’) were collected using peritoneal lavage with injection of 10 mL PBS and aspirated using the same syringe. Cells were run on a BD LSR II using DiVa software (BD Bioscience) and analyzed with FlowJo software (Version 8.7.1; Tree Star, Inc.). For cell sorting, MLNs and PECs from IL-25-treated IL-4/eGFP reporter mice were isolated stained with DAPI (1 µg mL⁻¹; Molecular Probes) as described above and Live (DAPIneg) IL-25-elicited c-kitint (GFP* or GFPneg) cell populations were sorted using a FACSaria (BD Bioscience).

4.3.3 In vitro differentiation assays
FACS-purified cell populations were seeded into 96-well flat bottom TC plates (BD Falcon) and incubated in the presence of SCF (50 ng mL\(^{-1}\); R&D systems), IL-3 (10 ng mL\(^{-1}\); R&D systems) TSLP (100 ng mL\(^{-1}\); R&D systems) and/or IL-33 (50 ng mL\(^{-1}\); R&D systems) for eight days. Cytokines and culture media were replenished at days three and six post-culture. Following *in vitro* culture, progeny were assessed for expression of IL-4/eGFP, CD11b, Fc\(\varepsilon R\alpha\), and c-kit, T1/ST2, CD127 (IL-7R\(\alpha\)) and CD90 by flow cytometry as described above. Cell-free supernatants were assessed for IL-4, IL-5 and IL-13 cytokine production by standard sandwich ELISA (eBioscience).

### 4.3.4 *In vivo* adoptive transfers

CD45.1 congenic recipient mice were treated via hydrodynamic tail vein injection with a TSLP-encoding cDNA plasmid, as described previously. Seven days following cDNA injection, \(2 \times 10^4\) FACS-purified IL-25-elicited MPP\(^{type2}\) cells were transferred i.p. into CD45.1 congenic recipient. Recipient mice were treated 4 times with 0.4 \(\mu g\) of recombinant IL-33 and differentiation of CD45.2 donor cells was assessed on day 5 post-transfer.

### 4.3.5 Real-time PCR

RNA from colonic tissues of mice was isolated from sorted cell populations using RNeasy Mini kit (Qiagen) and cDNA was prepared with SuperScript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR analysis used commercial QuantiTect primer sets for *Il4*, *Il5*, *Il13*, *Gata3*, *Maf*, *Junb*, *Stat6* and *Il1rl1* (Qiagen) and SYBR Green chemistry (Applied Biosystems). All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Target genes were normalized for
endogenous β-actin levels and relative quantification of samples were compared to controls.

4.4 Results

4.4.1 IL-25-elicited MPP$^{\text{type2}}$ cells are distinct from natural helper cell/nuocyte populations.

Natural helper cells (NHCs) and nuocytes are a newly described innate cell population that are defined by their expression of c-kit, T1/ST2, IL-7R$\alpha$, CD90 and CD44 [13, 14]. Both populations were responsive to IL-25 and IL-33, suggesting that these two populations may be identical, but distinguished by their anatomical localization. While NHCs were isolated from lymphoid clusters in the mesentery surrounding the MLNs and intestines or the fatty omentum in the peritoneal cavity [13], nuocytes were found in the MLN and spleen [14]. Analysis of the c-kit$^+$ cells elicited by IL-25 revealed that the majority of the IL-25-elicited MPP$^{\text{type2}}$ cells (c-kit$^+$ GFP$^{\text{neg}}$) were T1/ST2$^{\text{neg/lo}}$ and IL-7R$\alpha^{\text{neg}}$, and expressed little or no mRNA encoding Gata3, Junb, Maf, Stat6 and Il1rl1 (Fig. 21B), all of which are expressed by NHCs. Combined, these data suggest that IL-25-elicited MPP$^{\text{type2}}$ cells are distinct from NHCs.

To test whether administration of IL-25 results in increased frequencies of c-kit$^+$ cells or NHCs in the peritoneum or mesentery, mice were treated with IL-25 and cells from these compartments were analyzed for the presence of c-kit$^+$ cells. Delivery of IL-25 resulted in increased frequencies of c-kit$^+$ cells in the peritoneum (4% versus 17%) (Fig. 22A) and mesentery (6% versus 20%) (Fig. 22B). Although IL-25 treatment resulted in increased cellularity in the mesentery, no changes were observed in the frequency of c-kit$^+$ IL-7R$\alpha^+$
T1/ST2+ NHCs or in the expression of CD44 or CD90 on NHCs (Fig. 22C), indicating that IL-25-elicited MPPtype2 cells are distinct from NHCs.

MPPtype2 cells were previously reported to possess multi-potent potential and could give rise to multiple cell lineages, while NHCs and nuocytes were reported to be a terminally differentiated cell population [13, 14]. To test this, IL-25-elicited MPPtype2 cells or nuocytes were sort-purified (Fig. 23A,C), cultured in the presence of SCF and IL-3 and assessed for differentiation into multiple lineages by flow cytometry. Following 8 days in vitro, cultures seeded with MPPtype2 cells contained CD11b+ macrophages as well as CD11b- c-kit+ FcεRI+ mast cells, but did not yield T1/ST2+ IL-7R+ nuocytes (Fig. 23B). However, analysis of the cultures seeded with NHC/nuocytes revealed that this cell population could not differentiate into macrophages or mast cells, but retained expression of T1/ST2 (Fig. 23D), indicating that NHCs/nuocytes are a terminally differentiated cell population and are unable to give rise to other cell lineages. Combined with the differences observed in surface phenotype, the finding that MPPtype2 cells possess multi-potent potential further supports the hypothesis that MPPtype2 cells are distinct from NHCs/nuocytes.

4.4.2 IL-25 and IL-33 differentially elicit distinct populations of nuocytes and MPPtype2 cells in the MLNs

While previous reports have shown that both IL-25 and IL-33 can expand c-kit+ cell populations capable of promoting Th2 cytokine responses, whether the composition of the IL-25- versus IL-33-elicited c-kit+ cells is identical is not known. To directly test whether IL-25 and IL-33 elicit distinct or similar c-kit+ cell populations in the MLNs, the expression of T1/ST2, Sca1, IL-7Rα, CD90 and IL-4/eGFP was characterized on c-kit+ cells from the MLNs of IL-25- or IL-33-treated IL-4/eGFP reporter mice. As previously
shown [14-17], compared to naïve mice, administration of IL-25 or IL-33 resulted in increased frequencies of c-kit+ cells in the MLNs compared to untreated controls (Fig. 24A). Analysis of the IL-25- or IL-33-elicited c-kit+ cells revealed that 7% of the IL-25-elicited c-kit+ cells were T1/ST2+, while administration of IL-33 resulted in increased frequencies of T1/ST2+ c-kit+ cells (26% of c-kit+ cells compared to 7%) (Fig. 24A). However, T1/ST2+ c-kit+ cells (black histograms) from either IL-25- or IL-33-treated mice displayed a phenotype consistent with nuocytes as both populations expressed Sca1, IL-7Rα, CD90 and IL-4/eGFP (Fig. 24B and data not shown). This finding is consistent with published studies demonstrating redundancy in IL-25 and IL-33 to induce nuocyte cell populations.

Critically, the majority (92%) of the IL-25-elicited c-kit+ cells in the MLN lacked expression of T1/ST2, and analysis of this cell population (gray shaded histograms) for expression of Sca1, IL-7Rα, CD90 and IL-4/eGFP revealed that the T1/ST2neg c-kit+ cells expressed Sca1, but exhibited little to no expression of IL-7Rα, CD90 or IL-4/eGFP (Fig. 24B and data not shown), a phenotype consistent with MPPtype2 cells. Collectively, these data indicate that IL-33 more potently induces nuocytes, and the majority of IL-25-elicited c-kit+ cells represent MPPtype2 cells, suggesting that while redundancy in induced cell populations may exist, IL-25 and IL-33 elicit distinct populations of c-kit+ cells.

IL-25 and IL-33 were shown to act redundantly and independently to elicit nuocyte cell populations [14]. To test the requirement for the IL-33-signaling pathway in the IL-25-mediated induction of c-kit+ cells, IL-4/eGFP reporter mice were treated daily with either an isotype control (IgG) or a neutralizing monoclonal antibody against T1/ST2 beginning at day -2 and continuing throughout the course of IL-25-treatment. Consistent with
previous results, IL-25-treated mice displayed increased frequencies of c-kit$^+$ cells (14%) in the MLN compared to untreated control mice (2%) (Fig. 25A) and blockade of IL-33 signaling did not affect the IL-25-mediated induction of c-kit$^+$ cells (Fig. 25A). In addition, while the frequency of T1/ST2$^+$ c-kit$^+$ cells was reduced by treatment with the anti-T1/ST2 antibody (from 17% to 8%), the expression of IL-7R$\alpha$, CD90 or IL-4/eGFP on the T1/ST2$^{neg}$ c-kit$^+$ (MPP$^{type2}$) cells (gray shaded histograms) and T1/ST2$^+$ c-kit$^+$ cells (nuocytes) (black histograms) was not affected, compared to control treated mice (Fig. 25B). In addition, administration of IL-25 to WT C57Bl/6 or Il33$^{-/-}$ mice resulted in the induction of c-kit$^+$ cells at similar frequencies in the MLN (Fig. 26A). Further, the frequencies of MPP$^{type2}$ cells or nuocytes or their respective expression of IL-7R$\alpha$ and CD90 remained unchanged in the absence of IL-33 (Fig. 26B and C). Taken together, these data indicate that IL-25 acts independently of the IL-33/T1/ST2 signaling pathway to promote the induction of MPP$^{type2}$ cells.

Next, to address whether the IL-25 signaling pathway was necessary for the IL-33-mediated induction of NHC/nuocytes, IL-4/eGFP reporter mice were treated daily with either an isotype control (IgG) or a neutralizing monoclonal antibody against IL-25 beginning at day -2 and continuing throughout the course of IL-33-treatment regimen. Again, consistent with previous results, IL-33-treated mice displayed increased frequencies of c-kit$^+$ cells (19%) in the MLN compared to naïve mice (5%) (Fig. 27A) and the T1/ST2$^+$ c-kit$^+$ cells (black histograms) were IL-7R$\alpha^+$, CD90$^+$ and IL-4/eGFP$^+$ consistent with a NHC/nuocyte surface phenotype (Fig. 27B). Blockade of IL-25 signaling resulted in increased frequencies of c-kit$^+$ cells (Fig. 27A) and elevated frequencies of T1/ST2$^+$ c-kit$^+$ cells (Fig. 27A). However, neutralization of IL-25 did not affect the expression levels of IL-7R$\alpha$, CD90 or IL-4/eGFP on the T1/ST2$^+$ c-kit$^+$ cells.
(black histograms) (Fig. 27B). Combined, these findings indicate IL-25 and IL-33 act independently to elicit MPP\textsuperscript{type2} cells or NHCs/nuocytes, respectively.

4.4.3 IL-25-elicited MPP\textsuperscript{type2} cells have the capacity to differentiate into a NHC/nuocyte-like cell population

Previous reports have identified potential interactions between TSLP, IL-25 and IL-33 (see Chapter 1.6). For example, IL-25 or IL-33 can induce TSLP expression in epithelial cells and increase expression of a subunit of the TSLP receptor (TSLPR). Additionally, TSLP, IL-25, and IL-33 are each able to upregulate IL-17RB (a subunit of the IL-25 receptor). These observations suggest the existence of complex interactions between TSLP, IL-25 and IL-33 and indicate possible mechanisms whereby Th2 cytokine responses initiated by TSLP, IL-25 or IL-33 can be amplified by the other EC-derived cytokines. The finding that IL-25 and IL-33 act redundantly to elicit nuocytes and that these cells express the receptors for both IL-25 and IL-33 further support the hypothesis of cross-regulation between these signaling pathways. To address whether TSLP might influence the IL-25-elicited c-kit\textsuperscript{+} cells, the expression of TSLPR on IL-25-elicited MPP\textsuperscript{type2} cells (T1/ST2\textsuperscript{neg} c-kit\textsuperscript{+}) and nuocytes (T1/ST2\textsuperscript{+} c-kit\textsuperscript{+}) was characterized. IL-25-elicited MPP\textsuperscript{type2} cells (thin black histogram) and nuocytes (bold black histogram) isolated from the MLNs expressed TSLPR (Fig. 28A and B). This finding indicates that IL-25-elicited MPP\textsuperscript{type2} cells and nuocytes express TSLPR and suggests that TSLP may influence the proliferation, survival, cytokine production or differentiation of these cells.

TSLP is an IL-7-like cytokine that was initially described as a B and T lymphocyte growth factor and was shown to promote B and T lymphopoiesis [102]. In addition, IL-33 has been shown to influence the development and/or cytokine production of NHCs and nuocytes [13, 14]. The ability of TSLP and IL-33 to promote lymphopoiesis and nuocyte
development, respectively, combined with the TSLPR and T1/ST2 expression observed on IL-25-elicited MPP\textsuperscript{type2} cells and nuocytes suggested that TSLP and/or IL-33 might influence the population expansion, proliferation or differentiation of MPP\textsuperscript{type2} cells and nuocytes. To test this, IL-4/eGFP\textsuperscript{neg} T1/ST2\textsuperscript{neg} MPP\textsuperscript{type2} cells were sort-purified from IL-25-treated IL-4/eGFP reporter mice (Fig. 29A) and cultured in the presence of IL-33 alone, TSLP alone, IL-33 plus TSLP, or in combination with SCF, IL-3, IL-33 and TSLP. Following eight days \textit{in vitro}, the resultant progeny were assessed for surface marker expression by flow cytometry. When cultured in the presence of IL-33 alone, MPP\textsuperscript{type2} cells yielded a small but identifiable CD11b\textsuperscript{+} macrophage-like cell population, but did not give rise to a CD11b\textsuperscript{neg} c-kit\textsuperscript{+} Fc\varepsilon RI\textsuperscript{+} mast cell population or a CD11b\textsuperscript{neg} T1/ST2\textsuperscript{+} CD90\textsuperscript{+} nuocyte cell population (Fig. 29B). Culturing MPP\textsuperscript{type2} cells in the presence of TSLP alone resulted in increased cell recovery compared to stimulation with IL-33 alone and the majority of these cells were CD11b\textsuperscript{neg} T1/ST2\textsuperscript{+} with a proportion of them expressing CD90, a phenotype consistent with nuocytes (Fig. 29B). These data demonstrate that TSLP signaling renders MPP\textsuperscript{type2} cells more responsive to IL-33 signaling and that MPP\textsuperscript{type2} cells may represent a progenitor of NHC/nuocytes.

To test the combined effect of TSLP and IL-33 on MPP\textsuperscript{type2} cell differentiation, sort-purified MPP\textsuperscript{type2} cells (c-kit\textsuperscript{+} T1/ST2\textsuperscript{neg} GFP\textsuperscript{neg}) were cultured in TSLP and IL-33 and assessed for nuocyte differentiation. The inclusion of IL-33 with TSLP increased cell survival/recovery compared to TSLP alone and resulted in the induction of a T1/ST2\textsuperscript{+} IL-7R\alpha\textsuperscript{+} nuocyte-like cell population (Fig. 29B). Importantly, when cultured in the presence of SCF, IL-3, IL-33 and TSLP, the multi-potent potential of MPP\textsuperscript{type2} cells was restored as progeny consistent with macrophage, mast cell, and nuocyte cell populations are identified (Fig. 29B). These findings suggest that following IL-25-mediated induction of
MPP_{type2} cells, this cell population is capable of differentiating into a multiple of cell lineages based on the subsequent stimuli encountered. In addition, when cultured in the presence of SCF, IL-3, IL-33 and TSLP, sort-purified IL-25-elicited nuocytes (c-kit^+ IL-4/eGFP^+ T1/ST2^+) (Fig. 30A) retained expression of T1/ST2 but did not yield a CD11b^+ macrophage-like cell population or a CD11b^{neg} c-kit^+ FceRI^+ mast cell population (Fig. 30B), indicating that this cell population is not multi-potent. Combined these findings indicate that TSLP and IL-33 stimulation together results in MPP_{type2} cell differentiation into nuocyte cell populations.

To determine whether the MPP_{type2} cells differentiate into nuocytes in vivo, MPP_{type2} cells were isolated from IL-25-treated CD45.2 donor mice and adoptively transferred into TSLP-cDNA injected CD45.1 congenic recipients, which were treated daily i.p. with IL-33 from d0-4 (Fig. 31A). Four days following transfer, CD45.2 donor cells recovered from the peritoneal cavity-draining lymph nodes were found to have differentiated into CD11b^+ cells as well as CD11b^{neg} T1/ST2^+ IL-7Rα^+ nuocyte cells (Fig. 31B). Moreover, CD45.2 donor cells isolated from the Peyer's patches exclusively differentiated into CD11b^{neg} T1/ST2^+ IL-7Rα^+ nuocytes (Fig. 31C). These results support the hypothesis that IL-25-elicited MPP_{type2} cells represent a multi-potent progenitor cell population capable of generating multiple cell lineages, including nuocytes, both in vitro and in vivo.

4.5 Discussion

The recent identification of MPP_{type2} cells, NHCs, nuocytes and Ih2 cells has altered our understanding of the cellular mechanisms that promote CD4^+ Th2 cell-dependent immunity and/or inflammation at mucosal sites. However, the similarities between MPP_{type2} cells, NHCs, nuocytes and Ih2 cells have lead recent reports to conclude that
MPP\textsuperscript{type2} cells, nuocytes and NHCs are the same cell population [139, 245, 246]. MPP\textsuperscript{type2} cells, NHCs, nuocytes and Ih2 cells all express c-kit, but lack expression of surface markers for any known cell lineage [13-15, 17], indicating that these cell populations were previously unrecognized innate cell populations. In addition, all four cell populations displayed the common functionality of promoting the development of type 2 inflammation, yet key differences have raised fundamental questions about whether MPP\textsuperscript{type2} cells, NHCs, nuocytes and Ih2 cells are the same cell lineage, related lineages or represent four distinct and unrelated cell lineages.

Data presented in this chapter demonstrate that, based on expression of T1/ST2, IL-7R\textalpha, and CD90, IL-25-elicited MPP\textsuperscript{type2} cells are phenotypically distinct from NHCs/nuocytes. Moreover, IL-25-elicited MPP\textsuperscript{type2} cells, but not nuocytes, possess multi-potent potential, further distinguishing MPP\textsuperscript{type2} cells from NHC/nuocytes. In addition, IL-25-elicited MPP\textsuperscript{type2} cells expressed the receptor for TSLP (TSLPR) and upon stimulation with TSLP in combination with IL-33, IL-25-elicited MPP\textsuperscript{type2} cells differentiated into T1/ST2\textsuperscript{+} nuocytes, indicating that MPP\textsuperscript{type2} cells represent direct progenitors of NHC/nuocytes. Combined, these data not only indicate that MPP\textsuperscript{type2} cells are distinct from NHC/nuocytes, but also demonstrate that MPP\textsuperscript{type2} cells may represent a progenitor of NHC/nuocyte cell populations.

MPP\textsuperscript{type2} cells, NHCs, nuocytes and Ih2 cells are all responsive to IL-25 and/or IL-33, and published reports have demonstrated that both IL-25 and IL-33 can similarly elicit c-kit\textsuperscript{+} cell populations [13-17], but whether IL-25- versus IL-33-elicited cells are the same or unique is not known. While administration of either IL-25 or IL-33 resulted in the induction of T1/ST2\textsuperscript{+} c-kit\textsuperscript{+} nuocyte cells, administration of IL-33 resulted in a higher
frequency of nuocytes, compared to treatment with IL-25. Moreover, compared to IL-25-induced nuocytes, IL-33-elicited nuocytes exhibited higher expression levels of IL-7Rα, CD90 and IL-4/eGFP, a potential indication of a more mature and/or activated phenotype. Surprisingly, the majority of IL-25-elicited c-kit⁺ cells lacked expression of T1/ST2, IL-7Rα, and CD90, a surface phenotype most consistent with MPPtype2 cells. Combined, these data identify differences between IL-25- and IL-33-elicited innate lymphoid cell populations and further indicate that while redundancy exists between IL-25 and IL-33 in terms of eliciting similar innate cell populations, these two cytokines may predominantly act through distinct cellular mechanisms to promote Th2 cytokine responses at mucosal sites. Although IL-25 and IL-33 may act independently of one another, there is evidence for cross-talk between these pathways. The finding that IL-25-elicited MPPtype2 cells can differentiate into nuocytes in the presence of TSLP and IL-33, but not with either TSLP or IL-33 alone, suggests that TSLP and IL-33 synergize to promote nuocyte differentiation. This observation also suggests complex interactions between these three epithelial cell-derived cytokines and demonstrates cross-regulation between IL-25, IL-33 and TSLP. Taken together, these observations highlight the need for more investigation into the identity, function and lineage relationships between MPPtype2 cells, nuocytes, NHCs and Ih2 cells both in the steady state and in the context of disease as well as the effects of IL-25, IL-33 and TSLP on all of these cell populations.

While the identification of MPPtype2 cells, nuocytes, NHCs and Ih2 cells provides new insights into understanding the cellular mechanisms through which CD4⁺ Th2 cell-dependent cytokine and associated effector responses at mucosal sites are initiated and regulated, these innate lymphoid cell populations may play broader roles at mucosal
sites. Indeed, recent reports have described roles for NHCs/nuocytes in models of airway hyperresponsiveness, during flu infection and in wound healing responses in the lung [139, 247]. Moreover, the existence of NHCs/nuocytes has been described in humans [248, 249], however human MPP\textsuperscript{type2} cells have yet to be identified. Despite these advances, the impact of MPP\textsuperscript{type2} cells, nuocytes, NHCs and Ih2 cells in human disease and the respective roles these cell populations play in other Th2 cytokine-associated diseases such as asthma and allergy has yet to be determined. Thus, understanding the early innate pathways involved in the induction of Th2-associated immune responses may help establish novel therapeutic approaches for the treatment of helminth infections and allergic diseases in humans.
**Table 2. Comparison of MPP\textsuperscript{type2} cells, nuocytes, Ih2 cells and NHCs.**

<table>
<thead>
<tr>
<th>Anatomical Location</th>
<th>MPP\textsuperscript{type2} Cells</th>
<th>Nuocytes</th>
<th>Innate type2 Helper Cells</th>
<th>Natural Helper Cells</th>
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<tbody>
<tr>
<td>mLN &amp; GALT</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes (Sca1\textsuperscript{med})</td>
<td>Yes</td>
</tr>
<tr>
<td>mLN &amp; spleen</td>
<td>Yes</td>
<td>Yes</td>
<td>Unknown</td>
<td>Yes</td>
</tr>
<tr>
<td>mLN, spleen &amp; liver</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<table>
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<tr>
<th>Surface Phenotype</th>
<th>Lin-</th>
<th>C-KIT+</th>
<th>Sca1\textsuperscript{+}</th>
<th>interleukin infection</th>
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<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Unknown</td>
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<td>Nn</td>
<td>No</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>T1/ST2+</td>
<td>CD90.2+</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>CD44+</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<th>Method of Induction</th>
<th>IL-25 Tx &amp; interleukin infection</th>
<th>IL-25-or IL-33-Tx &amp; interleukin infection</th>
<th>IL-25-or IL-33-Tx &amp; interleukin infection</th>
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<td>Predilection</td>
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<th>Physiologic Significance</th>
<th>IFN-γ, IL-6, IL6 mRNA</th>
<th>secrete IL5, IL-13</th>
<th>secrete IL5, IL-13</th>
<th>secrete IL5, IL-13</th>
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<tbody>
<tr>
<td></td>
<td>† goblet cell mucin</td>
<td>† goblet cell mucin</td>
<td>† eosinophils</td>
<td>† goblet cell mucin</td>
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</tbody>
</table>

<table>
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<tr>
<th>Anti- Helminth</th>
<th>Yes</th>
<th>Yes</th>
<th>Yes</th>
<th>Unknown</th>
</tr>
</thead>
</table>

|------------|--------------------------|--------------------------|--------------------------|--------------------------|

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Figure 21. IL-25-elicited c-kit$^+$ cells are distinct from natural helper cells.

Expression of surface markers on c-kit$^+$ GFP$^{\text{neg}}$ (blue line) or c-kit$^+$ GFP$^+$ (green line) cells from IL-25-treated mice analyzed by flow cytometry. Shaded histograms indicate the positive control for each respective surface marker. IL-7R$\alpha$, gated on CD4$^+$ T cells; T1/ST2, gated on c-kit$^+$ FcεRI$^+$ mast cells). Cells in (A) were surface stained ex vivo. Gata3, Junb, Maf, Stat6 and Il1rl1 mRNA expression by naïve CD4$^+$ T cells (N), IL-4/eGFP$^+$ CD4$^+$ T cells from helminth-infected mice (Th2) or IL-25-elicited c-kit$^+$ GFP$^{\text{neg}}$ or c-kit$^+$ GFP$^+$ cells was measured by real-time PCR (B). Data are representative of at least two independent experiments.
Figure 22. IL-25 elicits a c-kit$^+$ cell population in the mesentery, but does not affect natural helper cell populations.

**A** WT mice were treated with IL-25 daily for 4 days and induction of c-kit$^+$ cells in the peritoneum (A) and mesentery (B) of control or IL-25-treated mice was assessed by flow cytometry. Plots shown are gated on live CD3ε$^{neg}$ CD4$^{neg}$ CD19$^{neg}$ CD11b$^{neg}$ cell populations and in (A) gated to exclude FcεR1κ$^+$ mast cells. Frequencies of natural helper cells in the mesentery of control or IL-25-treated mice were assessed by flow cytometry (C). Plots shown are gated on live CD3ε$^{neg}$ CD4$^{neg}$ CD19$^{neg}$ CD11b$^{neg}$. Data in A and B are representative of at least two independent experiments. (control, n=7; IL-25-treated, n=7). Data in (C) are representative of two control-treated and three IL-25-treated mice.
Figure 23. IL-25-elicited MPP\textsuperscript{type2} cells, but not nuocytes, possess multi-potent potential.

A

B

C

D

Figure 23. A, C, FACS-purification gating of IL-25-elicited MPP\textsuperscript{type2} cells (lineage\textsuperscript{neg} T1/ST2\textsuperscript{neg} c-kit\textsuperscript{+} GFP\textsuperscript{neg}) (A) or nuocytes (lineage\textsuperscript{neg} T1/ST2\textsuperscript{+} c-kit\textsuperscript{+} GFP\textsuperscript{+}) (C) from IL-25-treated IL-4/eGFP reporter mice. Plots shown are gated on live, lineage\textsuperscript{neg} cells (CD3\textepsilon, CD8\alpha, CD19, CD11b, CD11c, Gr-1).

B, D, Flow cytometric analysis of myeloid, granulocyte and nuocyte differentiation of FACS-purified live, MPP\textsuperscript{type2} cells (lineage\textsuperscript{neg} T1/ST2\textsuperscript{neg} c-kit\textsuperscript{+} GFP\textsuperscript{neg}) (B) or nuocytes (lineage\textsuperscript{neg} T1/ST2\textsuperscript{+} c-kit\textsuperscript{+} GFP\textsuperscript{+}) (D) from IL-25-treated IL-4/eGFP reporter mice following \textit{in vitro} culture in SCF (50 ng mL\textsuperscript{-1}) and IL-3 (10 ng mL\textsuperscript{-1}). Data in A-D are representative of three independent experiments.
Figure 24. IL-25 and IL-33 differentially elicit nuocytes and MPP^type2 cells in the MLNs.

A

![Scatter plots showing frequency of c-kit^+ cells in the NBNT cell compartment from the mesenteric lymph node (MLN) of control, IL-25- or IL-33-treated IL-4/eGFP reporter mice and their expression of T1/ST2 was assessed by flow cytometry.](A)

B

![Histograms showing IL-7Rα, CD90.2 (Thy1.2) and IL-4/eGFP expression on T1/ST2^+ c-kit^+ cells (black histograms) or T1/ST2^neg c-kit^+ cells (gray shaded histograms) from IL-25- or IL-33-treated mice.](B)

Figure 24. **A, B**, Frequency of c-kit^+ cells in the NBNT cell compartment from the mesenteric lymph node (MLN) of control, IL-25- or IL-33-treated IL-4/eGFP reporter mice and their expression of T1/ST2 was assessed by flow cytometry (**A**). Plots are gated on live, lineage^neg^ (CD4, CD8, CD11b, CD11c, and CD19) cells. IL-7Rα, CD90.2 (Thy1.2) and IL-4/eGFP expression on T1/ST2^+ c-kit^+ cells (black histograms) or T1/ST2^neg^ c-kit^+ cells (gray shaded histograms) from IL-25- or IL-33-treated mice (**B**). Data in **A** and **B** are representative of 3 independent experiments (control, n=6; IL-25-treated, n=9; IL-33-treated, n=9).
Figure 25. IL-33 signaling is not required for the IL-25-mediated induction of MPP^type2 cell or nuocyte cell populations.

Figure 25. Frequency of c-kit^+ cells and their expression of T1/ST2 in the NBNT cell compartment from the mesenteric lymph node (mLN) of control or IL-25-treated IL-4/eGFP reporter mice treated with either isotype (IgG) or anti-T1/ST2 monoclonal blocking antibodies (A) was assessed by flow cytometry. Plots are gated on live, lineage^neg (CD4, CD8, CD11b, CD11c, and CD19) cells. IL-7R^α, CD90.2 (Thy1.2) and IL-4/eGFP expression on T1/ST2^+ c-kit^+ cells (black histograms) or T1/ST2^neg c-kit^+ cells (gray shaded histograms) from isotype- or anti-T1/ST2-treated IL-25-treated mice (B). Data in A and B are representative of 3 independent experiments (control, n=6; IL-25-treated, n=9; anti-T1/ST2 IL-25-treated, n=9).
Figure 26. IL-25-elicits c-kit$^{\text{int}}$ cell populations independent of IL-33.

Figure 26. Frequency of c-kit$^+$ cells in the NBNT cell compartment of MLN of control or IL-25-treated WT and $\text{Il33}^{-/-}$ mice (A). The expression of T1/ST2 on c-kit$^+$ cells from IL-25-treated WT or $\text{Il33}^{-/-}$ mice (B). Expression of IL-7R$\alpha$ and CD90.2 (Thy1.2) on T1/ST2$^+$ c-kit$^+$ cells (black histograms) or T1/ST2$^{\text{neg}}$ c-kit$^+$ cells (gray shaded histograms) from MLN (C). (control, n=4; IL-25-treated WT, n=6; IL-25-treated $\text{Il33}^{-/-}$, n=9).
Figure 27. IL-25 signaling is not required for the IL-33-mediated induction of c-kit+ nuocyte population.

Figure 27. Frequency of c-kit+ cells and their expression of T1/ST2 in the NBNT cell compartment from the MLN of control or IL-33-treated IL-4/eGFP reporter mice treated with either isotype (IgG) or anti-IL-25 monoclonal antibodies (A) was assessed by flow cytometry. Plots are gated on live, lineage<sup>neg</sup> (CD4, CD8, CD11b, CD11c, and CD19) cells. IL-7Rα, CD90.2 (Thy1.2) and IL-4/eGFP expression on T1/ST2<sup>+</sup> c-kit+ cells (black histograms) or T1/ST2<sup>neg</sup> c-kit+ cells (gray shaded histograms) from isotype- or anti-IL-25-treated IL-33-treated mice (B). Data in A and B are representative of 3 independent experiments.
Figure 28. IL-25-elicited MPP\textsuperscript{type2} cells and nuocytes express the receptor for TSLP.

Figure 28. TSLPR\textalpha expression on IL-25-elicited MPP\textsuperscript{type2} cells or nuocytes in the MLN. Frequency of c-kit\textsuperscript{+} cells in the NBNT cell compartment from the MLN (A) of control or IL-25-treated IL-4/eGFP reporter mice. Expression of T1/ST2 on IL-25-elicited c-kit\textsuperscript{+} cells was assessed by flow cytometry (B). TSLPR\textalpha expression on T1/ST2\textsuperscript{+} c-kit\textsuperscript{+} cells (black histograms) or T1/ST2\textsuperscript{neg} c-kit\textsuperscript{+} cells (dashed histograms) from MLN (B). Plots are gated on live, lineage\textsuperscript{neg} (CD4, CD8, CD11b, CD11c, and CD19) cells or as indicated. Shaded histograms in b,d represent TSLPR\textalpha expression on bone marrow cells from TSLPR-deficient mice. Data in A and B are representative of 2 independent experiments.
Figure 29. IL-25-elicited MPP\textsuperscript{type2} cells represent a direct progenitor population of nuocytes.
Figure 29. FACS-purification gating of IL-25-elicited MPP$^{\text{type2}}$ cells (lineage$^{\text{neg}}$ T1/ST2$^{\text{neg}}$ c-kit$^{+}$ GFP$^{\text{neg}}$) from IL-25-treated IL-4/eGFP reporter mice (A). Flow cytometric analysis of myeloid, granulocyte and nuocyte differentiation of FACS-purified live, MPP$^{\text{type2}}$ cells (lineage$^{\text{neg}}$ T1/ST2$^{\text{neg}}$ c-kit$^{+}$ GFP$^{\text{neg}}$) following \textit{in vitro} culture in IL-33 alone (50 ng mL$^{-1}$), TSLP alone (1 µg mL$^{-1}$), IL-33 and TSLP or IL-33 and TSLP in combination with SCF and IL-3 (B). Data in A and B are representative of three independent experiments.
Figure 30. IL-25-elicited nuocytes do not display multi-potent potential.

Figure 30. FACS-purification gating of IL-25-elicited nuocytes (lineage\textsuperscript{neg} T1/ST2\textsuperscript{+} c-kit\textsuperscript{+} GFP\textsuperscript{+}) from IL-25-treated IL-4/eGFP reporter mice (A). Flow cytometric analysis of myeloid, granulocyte and nuocyte differentiation of FACS-purified live, nuocytes following \textit{in vitro} culture in IL-33 (50 ng mL\textsuperscript{-1}) and TSLP (1 µg mL\textsuperscript{-1}) in combination with SCF (50 ng mL\textsuperscript{-1}) and IL-3 (10 ng mL\textsuperscript{-1}) (B). Data in A and B are representative of two independent experiments.
Figure 31. IL-25-elicited MPP\textsuperscript{type2} cells differentiate into T1/ST2\textsuperscript{+} IL-7R\textalpha\textsuperscript{+} nuocytes \textit{in vivo}.

\textbf{A}

CD45.1 recipients $\xrightarrow{\text{TSLP-cDNA (i.v.)}}$ IL-25-elicited CD45.2 MPP\textsuperscript{type2} cells $\xrightarrow{\text{assess CD45.2 donor cell differentiation}}$

\textbf{B}

Untreated CD45.1 $\xrightarrow{\text{MPP\textsuperscript{type2} cells}}$ CD45.1

\textbf{C}

Untreated CD45.1 $\xrightarrow{\text{MPP\textsuperscript{type2} cells}}$ CD45.1

Figure 31. \textbf{A-C}, CD45.1 congenic recipients were injected with a TSLP cDNA encoding-plasmid via hydrodynamic tail vein injection. At day 7 post-cDNA injection, mice were given 2-3 x10\textsuperscript{4} MPP\textsuperscript{type2} cells (c-kit\textsuperscript{int} GFP\textsuperscript{neg}) i.p. from IL-25-treated CD45.2 mice. Recipient mice were then treated daily for 4 days with 0.5 \textmu g IL-33 (A) and differentiation of donor cells isolated from the MLN (B) and PP (C) was assessed on day 6 post-transfer. Plots shown are gated on live cells or as indicated. Data are representative of two independent experiments. (n=4).
Chapter 5

Summary, Discussion and Future Directions

5.1 Characterization of the innate immune cell populations elicited by IL-25

CD4+ T helper (Th) 2 cytokine responses are responsible for the development of allergic diseases, including asthma and allergy, and are critical for protective immunity against helminth infections [72, 250-255]. Given the increasing prevalence of allergic disorders in industrialized countries, and the estimated two billion individuals worldwide that are infected with soil-transmitted helminth parasites [256-260], a more comprehensive understanding of how Th2 cytokine responses are initiated and regulated could benefit the design of new therapeutic strategies to prevent or treat these conditions. While the mechanisms involved in the initiation of innate immune responses following exposure to viral, bacterial or protozoan infections are well characterized [62-67], the innate immune pathways that promote Th2 cytokine responses in the context of allergy or helminth infection remain less well defined. Previous in vitro studies have determined that cognate interactions via major histocompatibility complex (MHC) class II, T cell receptors (TCR) and interleukin (IL) -4 are required for optimal differentiation of naïve T cells into Th2 cells [251, 261, 262]. Consistent with these data, dendritic cells (DCs), which lack the ability to produce IL-4, in most circumstances appear to be insufficient to promote Th2 cell differentiation in vivo [11, 106, 263, 264]. Historically, IL-4 producing innate cell populations, such as basophils, eosinophils and mast cells were considered late-phase effector cell populations, however, more recent studies have challenged this concept through the demonstration that these innate cells can provide an early source of IL-4 and influence the development of optimal Th2 cell responses. These observations have provoked interest in the early cellular and molecular events that regulate the
development and activation of IL-4-producing innate cell populations. Based on the findings that the epithelial cell-derived cytokines TSLP, IL-33 and IL-25 are known to promote Th2 cytokine responses through the induction of distinct IL-4-, IL-5- and IL-13-secreting innate cell populations [11, 14, 16, 17] (discussed in Chapter 1.4.1-1.4.3), the goal of this thesis was to interrogate and define the cellular mechanisms through which IL-25 promotes Th2 cytokine responses.

The results presented in this thesis identify a previously unrecognized multi-potent progenitor (MPP) cell population and provide new insights into the cellular mechanisms through which IL-25 initiates and regulates CD4+ Th2 cell-dependent immune responses at mucosal sites. In Chapters 2 and 3, IL-25 was found to promote the accumulation of a NBNT c-kit+ cell population that lacked expression of known lineage markers. This cell population displayed a surface phenotype consistent with bone marrow-resident MPP cells and possessed the capacity to differentiate into myeloid and granulocyte lineages that could initiate CD4+ Th2 cell differentiation, and was therefore termed MPPtype2 cells. In Chapter 4, MPPtype2 cells were found to be distinct from other recently identified c-kit+ innate cell populations (NHCs, nuocytes and Ih2 cells). In addition, MPPtype2 cells were capable of differentiating into NHCs/nuocytes following stimulation with TSLP and IL-33, indicating that MPPtype2 cells possess both lymphoid and myeloid potential.

Despite several reports identifying IL-25-responsive innate cell populations [3, 16, 158, 171, 176], the cellular mechanisms through which IL-25 promoted Th2 cytokine responses remained poorly characterized. In Chapter 2, IL-4/eGFP reporter mice were employed to identify IL-4-competent innate cell populations elicited by IL-25. While administration of IL-25 resulted in a significant increase in total cell numbers in the
MLNs, T cell, B cell, DC and macrophage populations were only increased 1.5-2 fold. In contrast, IL-25 was shown to predominantly elicit a NBNT c-kit⁺ cell population, which displayed a 56-fold increase compared to control treated mice. In addition, this cell population also lacked expression of lineage-specific surface markers for basophil, mast cell or eosinophil populations. Of all the innate cell populations reported, data from the studies presented here are most consistent with the NBNT c-kit⁺ cell populations identified by Fort et al. and Fallon et al. that was elicited following administration of exogenous IL-25 [3, 16]. This IL-25-elicited NBNT c-kit⁺ cell population lacked expression of FcεRIα and surface bound IgE, but was presumed to be a mast cell or mast cell precursor population [11, 16]. However, data presented in this thesis demonstrate that IL-25 was capable of eliciting this NBNT c-kit⁺ cell population and Th2 cytokine responses in two mast cell deficient mouse strains (Wsh or IL-3R-deficient mice) (see Fig. 9). These findings suggested that IL-25-elicited c-kit⁺ cells were not mast cells and that IL-25 could promote Th2 cytokine responses independently of mast cells.

The IL-25 elicited a c-kit⁺ cell population identified in this thesis displayed a surface phenotype consistent with bone marrow-resident MPPs, being Lin⁻ Sca1⁺ CD34⁺ but lacking expression of CD150, suggesting that IL-25 promoted the expansion of an MPP-like cell population in the periphery. The IL-25-elicited c-kit⁺ GFP⁺ cells were found to differentiate into mast cells, while, the c-kit⁺ GFP⁻ cells exhibited multi-potent capacity, giving rise to cells of monocyte/macrophage and granulocyte lineages both in vitro and in vivo. In addition, progeny derived from IL-25-elicited c-kit⁺ GFP⁻ cells were competent antigen presenting cells and could promote CD4⁺ Th2 cell differentiation. Collectively, data presented in Chapters 2 and 3 indicate that the IL-25-elicited MPP² cells are not T- or B-lymphocytes, NKT cells, basophils, eosinophils or mast cells, and
suggests that they represent a previously unrecognized innate cell population through which IL-25 promotes CD4+ Th2 cell differentiation.

5.2 Relationship(s) between the MPP\textsuperscript{type2} cells and the other previously unrecognized innate cell populations

When MPP\textsuperscript{type2} cells were identified, three other independent laboratories had identified previously unrecognized innate cell populations that are regulated by epithelial cell-derived cytokines and promote CD4+ Th2 cytokine responses. The identification of NHCs, nuocytes and Ih2 cells has provided new insights into the cellular and molecular mechanisms that initiate and regulate Th2 cytokine-dependent (type 2) inflammation at mucosal sites [13-15]. However, these recent findings provoke fundamental questions regarding the relationships between MPP\textsuperscript{type2} cells, NHCs, nuocytes and Ih2 cells and whether these are the same cell population described by four independent laboratories, are potential precursors of one another or represent four distinct and unrelated cell populations.

At the time MPP\textsuperscript{type2} cells, NHCs, nuocytes and Ih2 cells were identified, the interrelationships between these innate cell populations was not known. While all these cell populations shared a common functionality and phenotype based on cell morphology and surface marker expression (being Lin− c-kit+ Sca1+), suggesting that these four populations may be the same cell population, subsequent flow cytometric analyses revealed differences in expression of some surface molecules. For instance, the majority of IL-25-elicited MPP\textsuperscript{type2} cells in the MLNs did not express T1/ST2 (a subunit of the IL-33 receptor) or IL-7Rα and were also negative for expression of CD90 (Thy1), CD25 and CD44. In contrast, NHCs, nuocytes and Ih2 cells differed from MPP\textsuperscript{type2} cells in that they expressed T1/ST2 and IL-7Rα, CD90 and CD44 (summarized in Table 2).
These differences in surface marker expression may indicate that MPP_{type2} cells are less differentiated than nuocytes, NHCs and Ih2 cells. Expression of specific transcription factors is also associated with lineage commitment. In support of this hypothesis, while all four cell populations displayed a surface phenotype most commonly associated with hematopoietic stem cells (Lin⁻ c-kit⁺ Sca1⁺) [227, 230], MPP_{type2} cells expressed little or no mRNA encoding GATA3, c-Maf, STAT6 and JunB in comparison to CD4⁺ Th2 cells, whereas NHCs and Ih2 cells expressed Maf (c-Maf), Gata3, Junb and Stat6 as well as Id2 and low levels of Tbx21 (T-bet). Furthermore, only MPP_{type2} cells displayed multi-potent potential, being able to yield progeny cells of the myelomonocyte or granulocyte cell lineages (see Fig. 15). Therefore, based on expression of surface markers and transcription factors, and their multi-potent potential, MPP_{type2} cells appear to be distinct from NHCs, nuocytes and Ih2 cells, a potential indication of a distinct developmental and/or differentiation state of the MPP_{type2} cell population. However it is not known whether MPP_{type2} cells are progenitors of NHCs, nuocytes or Ih2 cells. One hypothesis is that MPP_{type2} cells cannot differentiate into NHCs, nuocytes or Ih2 cells and that IL-25 and IL-33 act to expand multiple distinct cell populations (Fig. 32A). This would be an intriguing finding considering the similarities between these four cell populations as it would suggest that either MPP_{type2} cells are restricted in their capacity to generate specific cell lineages or that NHCs, nuocytes and Ih2 cells develop from progenitor populations unrelated to MPP_{type2} cells.

An alternative possibility is that IL-25-elicited MPP_{type2} cells can differentiate into NHCs, nuocytes and Ih2 cells along with macrophages, basophils and mast cells (Fig. 32B). This hypothesis was tested in Chapter 4 and, indeed MPP_{type2} cells, under certain conditions, differentiated into a T1/ST2⁺ IL-7Rα⁺ NHCs/nuocyte-like cell population. This
is a somewhat surprising observation considering the proposed lymphoid lineage of NHCs/nuocytes. This finding not only suggests that MPP\textsuperscript{type2} cells possess myeloid and granulocyte potential (macrophage, basophils and mast cells) but can also give rise to cell populations of lymphoid lineages and highlights the multi-potent potential of MPP\textsuperscript{type2} cells. This point will be discussed further below (see Chapter 5.3). While MPP\textsuperscript{type2} cells possess the capacity to differentiate into NHC/nuocytes, it is possible that MPP\textsuperscript{type2} cells are not the only source of NHC/nuocytes. However, whether BM-resident progenitors including HSCs, MPP and common lymphoid progenitors (CLPs) can differentiate into NHC/nuocytes and the cytokines or other factors that regulate this process is not known.

The similarity in surface marker expression on NHCs, nuocytes and lh2 cells also raises questions about the relationship between these cell populations with most striking difference between them being their respective anatomical localization. It is possible that nuocytes and NHCs are distinct, however it is also possible that the same cell population was described independently. If the latter is correct, one possible explanation of their different localization is that adipose tissues may serve as reservoirs for NHCs and following stimulation with IL-25 or IL-33 NHCs residing in the FALC mobilize and migrate into peripheral tissues such as the MLN and spleen, thereby becoming nuocytes. However, if NHCs and nuocytes are distinct cell populations, one question becomes whether NHCs are confined to the FALC or other adipose-associated tissues and whether these cells are expanded following helminth infection or administration of IL-25 or IL-33.

Additionally, are nuocytes or other innate cell populations restricted from entering the FALC and, if so, by what mechanism? These are important questions that will need to
be addressed in order to obtain a more comprehensive understanding of the lineage relationship between MPP<sup>type2</sup> cells, nuocytes, Ih2 cells and NHCs. One potential indication that these cell populations are distinct is that Ih2 cells were not observed in fat-associated lymphoid clusters (FALC) [15]. In addition, NHCs were found in FALC in the mesentery and in fatty deposits in the peritoneal cavity and surrounding the kidneys and it has been hypothesized that adipose tissue serves as a reservoir for HSCs that are then able to mobilize into the periphery [265, 266]. However, NHCs and Ih2 cells were not increased in frequency in the mesentery following administration of IL-25 or IL-33. In light of the phenotypic similarities between NHCs and nuocytes, this observation raises the possibility that NHCs represent a ‘quiescent’ IL-25- and/or IL-33-responsive cell population resident in FALC that then enters the periphery following stimulation, thus becoming a ‘nuocyte’.

IL-25 and IL-33 were reported to induce nuocytes at similar frequencies [14], however whether IL-25- and IL-33-elicited nuocytes exhibited a similar surface phenotype was not examined. In Chapter 4, it was shown that IL-33 induces a significantly higher frequency of T1/ST2<sup>+</sup> c-kit<sup>+</sup> nuocytes in the MLN compared to IL-25. In addition, we saw similar effects in the caudal LNs, indicating that IL-33 elicits a more substantial nuocyte population in the periphery compared to IL-25. Moreover, compared to IL-25-elicited nuocytes, IL-33-induced nuocyte populations expressed higher levels of CD90 and IL-4/eGFP suggesting that IL-33-elicited nuocytes exhibited a more differentiated/activated surface phenotype. Whether this apparent difference in activation status correlates to cytokine production or biological function remains to be seen, however this observation highlights potential differences in IL-25- versus IL-33-elicited innate cell populations.
In addition, the ability of MPP\textsuperscript{type2} cells, NHCs, nuocytes and Ih2 cells to promote the development of Th2 cytokine- and associated effector responses \textit{in vivo} raises fundamental questions regarding the interactions of these cell populations with other hematopoietic and non-hematopoietic cell populations necessary to elicit type 2 inflammation. For example, MPP\textsuperscript{type2} cells, nuocytes, Ih2 cells and NHCs are all elicited in the absence of the adaptive immune system and can promote type 2 inflammation in this setting [13-17], indicating that these populations can act on non-hematopoietic cell lineages and independently of T cells and B cells. However, regarding MPP\textsuperscript{type2} cells and NHCs, current data suggest that they are capable of influencing lymphocyte function [13, 17]. For instance, progeny derived from MPP\textsuperscript{type2} cells can interact with T cells and NHCs can promote B cell proliferation and antibody production. Whether these interactions are required for their ability to promote type 2 inflammation and whether nuocytes and Ih2 cells are able to interact with the adaptive immune system in the mucosa or periphery has not yet been examined.

5.3 MPP\textsuperscript{type2} cells and extramedullary hematopoiesis

Hematopoietic stem cells (HSCs) are a rare long-lived cell population with the capacity for multi-lineage differentiation and self-renewal and progression from HSCs and commitment to a specific lineage differentiation pathway is regulated by various growth and differentiation factors, such as SCF, IL-7 and Notch signaling pathways. HSC populations constantly recirculate throughout peripheral tissues [203-205, 207, 208, 228, 267] and mobilization of HSCs into the periphery through the administration of exogenous growth factors has been a common strategy used in the treatment of leukemia or following chemotherapy [209-214]. Recent reports have hypothesized that HSCs are not simply passively trafficking through peripheral tissues but may be acting in
a role of immunosurveillance as they are able to recognize microbial products and differentiate into effector cell populations [215, 216]. These reports suggest that HSCs, and potentially other precursor cells, are able to recognize and respond to pathogen-derived signals and through extramedullary hematopoiesis can initiate a very localized and rapid immune response. The identification of MPP\textsuperscript{type2} cells as a previously unrecognized multi-potent progenitor suggests that extramedullary hematopoiesis is involved in licensing Th2 cytokine responses. However, whether this cell population represents a homogeneous population of individual cells capable of differentiating into multiple lineages or a heterogeneous population of lineage-committed precursors is still unclear.

One possible scenario is that the MPP\textsuperscript{type2} cell population consists of a homogeneous pool of multi-potent cells whereby each individual cell is capable of giving rise to both myelomonocytic and granulocyte cell lineages (Fig. 33A). An alternative model is that MPP\textsuperscript{type2} cells represent a heterogeneous population of cells comprised of multiple distinct progenitors, each only capable of differentiating into either monocyte and macrophage lineages or granulocyte lineages (Fig. 33B). Yet another scenario could exist, which is a combination of the first two models, where MPP\textsuperscript{type2} cells are a heterogeneous mixture but contains both single lineage-restricted progenitor cells as well as individual multi-potent progenitor cells. Further single cell analyses of the IL-25-elicited MPP\textsuperscript{type2} cells will be necessary to determine the homogeneity of this cell population, however flow cytometric analysis has provided a possible indication that MPP\textsuperscript{type2} cells are a heterogeneous population. Examination of the IL-25-elicited c-kit\textsuperscript{+} cells revealed that approximately 25% of MPP\textsuperscript{type2} cells (c-kit\textsuperscript{+} GFP\textsuperscript{neg}) were Ly6C\textsuperscript{+}, but no Ly6C expression was observed in the c-kit\textsuperscript{+} GFP\textsuperscript{*} fraction (Fig. 34A and B). This is
an intriguing finding given the myeloid potential of the MPP\textsuperscript{type2} cell population and the known expression of Ly6C on developing monocytes in the periphery [268]. Thus, this Ly6C\textsuperscript{+} fraction of MPP\textsuperscript{type2} cells may represent myeloid lineage-restricted progenitors, while the Ly6C\textsuperscript{neg} cell may only differentiate into granulocyte lineages. Additional experiments will be necessary to test the hypothesis that MPP\textsuperscript{type2} cells represent a heterogeneous population comprised of multiple lineage-restricted progenitors.

Another report identified an IL-25-responsive CD11b\textsuperscript{+} Ly6C\textsuperscript{+} monocyte-like cell population in a model of chronic airway hyper-responsiveness [176]. However, this cell population lacked expression of c-kit. One possible explanation for this difference is the site at which these cells are identified. While we observed increased frequencies of c-kit\textsuperscript{+} cells in the lungs following administration of IL-25, chronic allergen exposure in the lungs may result in the induction of unique innate cell populations. For example, CD4\textsuperscript{+} NKT cell populations have also been described in the lungs following allergen exposure and have been shown to produce IL-4 in response to IL-25 [171], suggesting that in the lungs IL-25 may elicit or act through various innate cell populations. Another possible explanation for the differences observed is in the timing at which IL-25-responsive innate cell populations are investigated. A chronic model of allergen exposure, in which mice are sensitized and challenged with allergens over the course of 4 weeks, was used to identify the CD11b\textsuperscript{+} Ly6C\textsuperscript{+} monocyte-like cell population. These disparate results may indicate that at different timepoints (acute versus chronic) and in distinct tissues, IL-25 may act on different cell populations. Alternatively, these data may indicate that the initial IL-25-elicited cells undergo differentiation to become different cell lineages at later timepoints. Consistent with this, data presented in this thesis demonstrate that IL-25-elicited MPP\textsuperscript{type2} cells have the capacity to give rise to cells of myeloid lineages (see Fig. 134).
15) and, moreover, IL-25-elicited MPP\textsuperscript{type2} cells cultured in the presence of SCF and IL-3 differentiate into CD11b\(^+\) M-CSFR\(^+\) monocyte/macrophage-like cell populations that co-express Ly6C\(^+\) (see Fig. 16). Therefore, it is possible that during chronic allergic inflammation IL-25-elicited MPP\textsuperscript{type2} cells differentiate into monocyte/macrophage populations, however whether MPP\textsuperscript{type2} cells and IL-25-responsive CD11b\(^+\) Ly6C\(^+\) monocyte populations are the same cell population or share a direct lineage relationship and the role of MPP\textsuperscript{type2} cells in allergic disorders still needs to be examined.

In addition, how distinct epithelial-derived cytokines regulate the relationships and functions of MPP\textsuperscript{type2} cells is still not well defined. IL-33 (and IL-7) promote the proliferation and/or cytokine production of NHCs and nuocytes [13, 14], yet as one would expect, when cultured in the presence of IL-33 alone, MPP\textsuperscript{type2} cells, which lack T1/ST2, do not survive or differentiate in culture. However, IL-25-elicited MPP\textsuperscript{type2} cells do express TSLPR (a subunit of the receptor for TSLP). This finding, combined with the known homology of TSLP to IL-7 and the association between TSLP and B and T lymphopoiesis (discussed in Chapter 1.5) indicated that TSLP may direct the lymphoid differentiation of MPP\textsuperscript{type2} cells. And while culturing MPP\textsuperscript{type2} cells in the presence of TSLP alone did not result in nuocyte differentiation, it did result in increased cell recovery and increased expression of T1/ST2 on the resultant progeny. This finding suggests that TSLP signaling promotes MPP\textsuperscript{type2} cell survival/proliferation and indicates that TSLP in synergy with IL-33, may influence the proliferation, survival, cytokine production or differentiation of IL-25-elicited MPP\textsuperscript{type2} cells.

When cultured in the presence of TSLP and IL-33, increased cell survival/recovery along with the generation of a T1/ST2\(^+\) IL-7R\(\alpha\)\(^+\) nuocyte-like cell population was observed from
cultures initially seeded with MPP$_{\text{type2}}$ cells, indicating that MPP$_{\text{type2}}$ cells possess lymphoid potential. Interestingly, this synergistic effect between TSLP and IL-33 has been reported previously in mast cells [108], as both IL-33 and TSLP stimulation are required for mast cell activation [108, 269]. Of note, MPP$_{\text{type2}}$ cells did not differentiate into nuocytes in the presence of SCF and IL-3, while TSLP and IL-33 stimulation did not result in the development of macrophage, mast cell or basophil populations. Importantly, however, the multi-potent potential of MPP$_{\text{type2}}$ cells can be confirmed, as culturing MPP$_{\text{type2}}$ cells in a cocktail of cytokines including SCF, IL-3, IL-33 and TSLP resulted in the generation of progeny consistent with macrophages, mast cells and nuocyte cell populations. As discussed previously, IL-25 appears to be expanding MPP$_{\text{type2}}$ cells in vivo and not influencing their lineage commitments. The implication of this observation is that, similar to HSCs, IL-25-elicited MPP$_{\text{type2}}$ cells possess some level of plasticity in their differentiation potential, which is regulated by secondary stimuli such as epithelial cell-derived cytokines or other growth factors. This implies that depending on the context of infection, the subsequent local cytokine milieu can shape the effector cell populations derived from MPP$_{\text{type2}}$ cells through extramedullary hematopoiesis and thus immediately ‘adapting’ the immune response to the specific pathogen encountered. While these data demonstrate the ability of MPP$_{\text{type2}}$ cells to differentiate into multiple cell lineages, including both myeloid and lymphoid, the biological significance of the plasticity of MPP$_{\text{type2}}$ cells and their propensity to differentiate into a specific cell lineages is still unclear and warrants further investigation.

The ability of TSLP and IL-33 to synergize and act on the IL-25-elicited MPP$_{\text{type2}}$ cells also suggests the existence of complex interactions between TSLP, IL-25 and IL-33 and indicate possible mechanisms whereby TSLP, IL-25 and IL-33 can amplify Th2 cytokine
responses initiated by each other. In support of this hypothesis, previous reports have demonstrated that both IL-25 and IL-33 can induce TSLP mRNA and IL-33 treatment induced increased levels of TSLPR [80, 112], while TSLP, IL-25 and IL-33 are each able to upregulate IL-17RB [150, 177, 179] (see Fig. 3), indicating that sensitization to each of these cytokines is able to increase the production of and responsiveness to the other two cytokines. Collectively, the coordinated expression of this triad of EC-derived cytokines, coupled with cross-regulation of expression of ligands and receptors, suggests that a complex interplay exists between TSLP, IL-33 and IL-25. And while the complexities of the relationships between these cytokines has not yet been clearly defined, increasing our understanding of the interactions between TSLP, IL-33 and IL-25 may offer new, highly effective therapeutic approaches to modulate the onset, progression or severity of Th2 cytokine-mediated inflammation associated with asthma, allergic disorders, and helminth infections.

Another question raised by the observed multi-potent potential of MPP\textsuperscript{type2} cells, is the lineage potential of MPP\textsuperscript{type2} cells found in naïve mice. While MPP\textsuperscript{type2} cells are present in naïve animals, only the lineage-potential of IL-25-elicited MPP\textsuperscript{type2} cells was examined in the current studies. It is not known whether MPP\textsuperscript{type2} cells isolated from naïve mice share similar lineage differentiation potential observed in IL-25-elicited MPP\textsuperscript{type2} cells. It is possible that IL-25 does not impart any lineage restriction on MPP\textsuperscript{type2} cells but rather serves as an activating signal to promote the population expansion of these cells and that other cytokines influence their differentiation. Alternatively, MPP\textsuperscript{type2} cells from naïve mice might possess a broader repertoire in terms of lineage differentiation and IL-25 may restrict MPP\textsuperscript{type2} cells to myelomonocytic and granulocyte lineages. Additional
studies are necessary in order to address the lineage potential of MPP\textsuperscript{type2} cells from naïve mice and the effects of IL-25 on the lineage potential of MPP\textsuperscript{type2} cells.

5.4 Subsets of innate lymphoid cell populations

Given the evolutionarily conserved nature of the IL-17 cytokine family [163] as well as common signaling pathways shared between IL-33 and the IL-1/TNF superfamily and Toll-like receptors [2, 143, 145], the innate cell populations targeted by IL-25 and IL-33 may represent evolutionary conserved pathways for the generation of effector cell populations that preceded the development of the adaptive immune response or compartmentalization of hematopoiesis to the bone marrow. In support of this hypothesis, another member of the IL-17 cytokine family, IL-17A, has been shown to influence granulopoiesis [240]. Moreover, similar to MPP\textsuperscript{type2} cells, NHCs and nuocytes, emerging evidence supports the presence of other populations of innate cells that serve both protective and pathologic roles in host immunity. Among these is a population of cells termed innate lymphoid cells (ILCs). ILCs represent a group of related, ROR\textsuperscript{γt}-dependent innate cell populations that exhibit a very similar surface marker expression, cytokine production and biological significance, including lymphoid tissue-inducer (LTi) cells, ILC17 cells, NKp46\textsuperscript{+} cells and NK22.

These ILCs all arise from a common Id2-dependent precursor cell population and depend on the common γ-chain signaling pathway, particularly IL-7 [13, 270, 271]. Based on these findings, ILCs were hypothesized to be of a lymphoid lineage and, indeed, a recent report has demonstrated that adult ILC populations arise from a subset of ROR\textsuperscript{γt\textsuperscript{neg}} common lymphoid progenitors (CLPs) [272]. Consistent with the known requirement of Notch signaling in lymphoid development, bone marrow-derived CLPs
were dependent on the Notch2 signaling pathway to differentiation into RORγt+ ILCs [272]. ILCs produce IL-17 and/or IL-22 and are involved in lymphoid tissue formation, immunity to infectious microorganisms and in tissue remodeling. In particular, LTi cells are characterized as Linneg c-kit+ IL-7Rα+, but can also express CD4 and CD90 and induce the formation of lymph nodes during embryogenesis [273-275]. LTi cells persist after birth and are important for immunity to extracellular bacterial infections and tissue remodeling [276-278]. IL-17-producing ILCs express RORγt and T-bet, but are distinguished from LTi cells by their lack of expression of CD4 and c-kit. In contrast to other ILC populations, which appear to be protective in the context of bacterial infections, ILC17 cells have been shown to promote IL-23-driven intestinal inflammation [244]. NKp46+ cells and NK22 cells are another ILC subset that has been termed ‘ILC22’. These cells are similar to LTi and NK cells in that they express c-kit and IL-7Rα, but also express NKp44 or NKp46 [241, 242, 277, 279, 280]. Again, similar to LTi cell, recent reports have demonstrated that IL-22-producing NKp46+ cells or NK22 cells mediate protective immune responses to the extracellular bacterium *Citrobacter rodentium* [242, 281].

Collectively, the recent identification of ILCs along with MPPtype2 cells, NHC and nuocytes combined with their respective association with Th17 and Th2 cytokine responses, suggests that similar to T helper cells, functionally heterogeneous populations of innate cell populations exist. If one classifies all innate cell populations as ‘ILCs’, it is apparent that distinct subsets exist. Within this concept of ILC subsets, Spits and Di Santo have proposed that natural killer (NK) cells, which produce IFN-γ, can be thought of as ‘ILC1’ cells, whereas based on their production of IL-5 and IL-13 and ability to promote type 2 inflammation, MPPtype2 cells, NHCs and nuocytes, can be classified as
Further, RORγt+ ILCs, which appear to be innate sources of Th17-associated cytokines, represent the innate cell population analogous to Th17 cells. No ‘regulatory’ ILC population has been formally described, however ILC populations have been demonstrated to limit influenza-mediated inflammation and play a role in tissue repair response [247]. The remarkable analogy of ILC subsets to T helper cell subsets is further highlighted by the transcription factor- and cytokine-regulated differentiation programs of ILC subsets. NK cells and ILC17 cells arise from a common Id2-dependent precursor, however of the ‘ILC2’ cell populations, only NHCs have been shown to require Id2. However, subsequent differentiation and development into specific ILC subsets appears to be regulated by different cytokines and transcription factors. For instance, in the case of NK cells (‘ILC1’), IL-15 (and to a lesser extent IL-7) promotes NK cell proliferation and cytokine production. Further, as is true for Th1 cell, Tbet also appears to be important for NK cell development and cytokine production. Alternatively, IL-7 and IL-23 along with RORγt seem to regulate the development of ‘ILC17’ cells. Although IL-25 and IL-33 have been shown to induce ‘ILC2’ populations, no transcription factor has been identified that regulates the differentiation these cell populations. Potential candidates include GATA3, however, previous reports have indicated that other classic Th2-associated innate cell populations, such as basophils, eosinophils and mast cells do not require GATA3 expression for their development or production of IL-4 [172]. Interestingly, within the ‘ILC2’ populations, MPP<sup>type2</sup> cells appear to give rise to NHCs and nuocytes, which are more effector-like cell populations, suggesting that MPP<sup>type2</sup> cells may not be under the same transcription regulations as NHCs or nuocytes. Thus, whether MPP<sup>type2</sup> cells are Id2-dependent and whether they share similar transcription factor-regulated development/differentiation profiles with NHCs/nuocytes still needs to be investigated.
The similarities in function between MPP\textsuperscript{type2} cells, NHCs and nuocytes within ‘ILC2’ populations and similar shared functions among the cell populations within ‘ILC17’ subset suggest that these are redundant populations. However, this apparent redundancy within these ILC subsets may in fact point to specificity. This hypothesis is particularly evident in the ‘ILC17’ subset, where multiple cell populations with similar cytokine expression profiles can either be protective or mediate intestinal inflammation. Moreover, while IL-25 and IL-33 both elicit cell populations that promote Th2 cytokine responses, data presented in Chapter 4 show that IL-33 preferentially elicits nuocytes. Thus, the elicitation of multiple yet distinct innate cell populations may represent mechanisms to generate specificity prior to the adaptive immune response. However, whether distinct ILC subsets are involved in different disease models or are elicited in response to specific pathogens has not been determined. Another interesting hypothesis is that similar to T helper cell subsets, ILC subsets may negatively regulate one another. For instance, the development of ‘ILC2’ subsets may actively hinder the development or survival of ‘ILC17’ subsets. Recent data has suggested that IL-25 can dampen LTi responses \textit{in vivo} [283]. Although this was attributed to an indirect effect on LTi cells, it is possible that IL-25 also inhibits ‘ILC17’ development by promoting ‘ILC2’ subsets. Whether these ILC subsets antagonize one another and are mutually exclusive in the steady-state or in the context of disease remains to be tested, but this presents an attractive phenomenon to target in the context of Th17- or Th2-associated diseases in humans.

Further, the identification of MPP\textsuperscript{type2} cells, nuocytes and NHCs in murine systems has posed the challenge to investigators to identify homologous cell populations in human
patients. Recent reports have identified a Lin^{neg} CD56^{neg} CD161^{+} CRTH2^{+} cell population that is derived from CD34^{+} progenitor cells and expresses IL-5 and IL-13 in humans [248, 249]. In addition, under certain circumstances Lin^{neg} c-kit^{+} IL-7R\alpha^{+} human lymphoid tissue-inducer (LTi) –like cells are able to produce IL-5 and IL-13 [284]. These results could simply be due to differences among human and murine ILCs or they could suggest lineage relationships and/or plasticity between human ILC subsets. Despite these differences, several reports have identified progenitor cell populations in helminth-infected individuals and in asthmatic patients that exhibit effector functions [217, 239]. These reports, while mostly correlative, support the potential for progenitors and extramedullary hematopoiesis as a mechanism to initiate the development of rapid and local immune responses and/or to sustain effector cell populations required for the elimination of pathogens from host tissues. While the existence of MPP^{type2} cells, nuocytes or NHC populations in humans and their impact on other Th2 cytokine-associated diseases such as asthma and allergy has yet to be fully elucidated, understanding the early innate pathways involved in the induction of Th2-associated immune responses may help establish novel therapeutic approaches for the treatment of helminth infections and allergic diseases in humans.

5.5 Concluding Remarks

The results presented in this thesis provide new insights into understanding the cellular mechanisms through which IL-25 initiates and regulates CD4^{+} Th2 cell-dependent cytokine responses at mucosal sites. The data presented in Chapter 2 demonstrate that IL-25 promotes Th2 cytokine responses through the induction of a previously unrecognized c-kit^{+} innate cell population. Further, these data indicate that, despite previous reports, this cell population is not a mast cell and IL-25 elicits this population
and subsequent Th2 cytokine responses independently of SCF and IL-3 signaling pathways. In Chapter 3, we identified the c-kit+ cell population as a multi-potent progenitor cell population with the capacity to differentiate into myeloid and granulocyte lineages and contribute to the induction of CD4+ Th2 cell differentiation through extramedullary hematopoiesis. As such, these cells were termed MPP<sup>type2</sup> cells. In Chapter 4, we demonstrated that MPP<sup>type2</sup> cells are distinct from other recently identified c-kit+ innate cell populations (NHCs and nuocytes). In addition, we showed that MPP<sup>type2</sup> cells represent direct progenitors of NHCs/nuocytes, and that TSLP and IL-33 synergize to promote this differentiation event, identifying cross-regulatory pathways between IL-25, TSLP and IL-33. Collectively, the data presented here identify possible mechanisms whereby innate cell populations recognize and respond to extracellular pathogens or allergens and are able to provide both antigen presentation and/or a source of IL-4, IL-5 or IL-13 thereby establishing conditions permissive for the development of CD4+ Th2 cells and type 2 inflammation. Further, these data contribute to emerging literature describing specialized innate cell populations that appear to provide distinct and specific immune responses prior to the development of the adaptive immune system. Understanding these early innate pathways involved in the induction of Th2 cytokine-associated immune responses may help establish novel therapeutic approaches for the treatment of helminth infections and allergic diseases in humans.
Figure 32. The identification of MPP\textsuperscript{type2} cells, nuocytes, Ih2 cells and NHCs raises new questions about the lineage relationships between these previously unrecognized innate cell populations. It is unclear whether MPP\textsuperscript{type2} cells are progenitors of NHCs, nuocytes and Ih2 cells. One potential outcome is that MPP\textsuperscript{type2} cells, in addition to macrophages, basophils and mast cells, give rise to nuocytes, Ih2 cells or NHCs (A). A second possibility is that IL-25-elicited MPP\textsuperscript{type2} cells are not progenitors of NHCs, nuocytes or Ih2 cells and these four cell types are unrelated cell populations (B).
Figure 33. Do MPP\textsuperscript{type2} cells represent a homogeneous or heterogeneous multi-potent progenitor cell population?

Figure 33. The multi-potent potential observed in IL-25-elicited MPP\textsuperscript{type2} cell populations raises the possibility that this is a homogeneous cell population in which each single individual cell possesses the capacity to generate multiple lineages (A). Alternatively, MPP\textsuperscript{type2} cells may be a heterogeneous population composed of distinct precursors that are committed to either myeloid or granulocyte lineages (B). Importantly, however, within this hypothesis, the possibility for the presence of a single multi-potent progenitor still exists.
Figure 34. MPP\textsuperscript{type2} cells display heterogeneous expression of Ly6C.

Frequency of c-kit\textsuperscript{+} cells in the NBNT cell compartment from the mesenteric lymph node (MLN) of IL-25-treated IL-4/eGFP reporter mice. c-kit\textsuperscript{+} GFP\textsuperscript{+} and c-kit\textsuperscript{+} GFP\textsuperscript{neg} cells were assessed for expression of Ly6C. Plots are gated on live, lineage\textsuperscript{neg} (CD4, CD8, CD11b, CD11c, and CD19) c-kit\textsuperscript{+} cells or as indicated. Data are representative of at least 3 independent experiments.


142. Dale, M. and M.J. Nicklin, Interleukin-1 receptor cluster: gene organization of IL1R2, IL1R1, IL1RL2 (IL-1Rrp2), IL1RL1 (T1/ST2), and IL18R1 (IL-1Rrp) on human chromosome 2q. Genomics, 1999. 57(1): p. 177-9.


