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Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites

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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. The consequences of dysregulation in the balance of immunologic hyporesponsiveness, inflammation, and tissue repair can result in inflammatory responses associated with food allergy, inflammatory bowel disease, and cancer.

There has been intense interest in the mechanisms that underlie neighborhood- or tissue-specific regulatory responses that maintain immunologic homeostasis at mucosal sites. It is now recognized that epithelial cells (ECs) are a critical cell population in the initiation, regulation, and resolution of innate and adaptive immune responses at mucosal sites. ECs express a wide range of immune response genes including MHC class I and class II, co-stimulatory molecules, chemokines, cytokines, and prostaglandins. There has been particular interest in novel EC-derived cytokines including thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, and their ability to influence innate and adaptive immunity. Although diverse in amino acid sequence, structure, and patterns of expression, dysregulated production of these three EC-derived cytokines has been reported in multiple inflammatory disease states including inflammatory bowel disease, asthma and, atopic dermatitis. Rapidly emerging studies implicate TSLP, IL-25, and IL-33 as critical regulators of innate and adaptive immune responses associated with T_H2 cytokine-mediated inflammation at mucosal sites. Therefore, the focus of this review will be to highlight recent advances that have been made in the identification of the cellular sources and pathways that regulate expression of TSLP, IL-25, and IL-33. In addition, we will discuss the functional significance of this triad of EC-derived cytokines in influencing immunity, inflammation, and immuno-regulation in health and disease. Lastly, we will focus on recent evidence that suggests coordinated expression and cross-regulation between TSLP, IL-25, and IL-33 are critical proximal events in developing T_H2 cell-associated immune responses at mucosal barriers that may offer novel therapeutic targets in multiple inflammatory diseases.

1. Cellular sources and regulation of TSLP, IL-25, and IL-33

TSLP, IL-25, and IL-33 have all been implicated in promoting T_H2 cytokine responses *in vivo*. In order to 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*. This section will focus on the cellular sources of TSLP, IL-25, and IL-33 as well as the pathways that regulate their expression.

TSLP

While TSLP was initially identified and characterized as a novel cytokine produced by thymic stromal cells (1,2), it is predominantly expressed in epithelial cells of the skin (keratinocytes), lung (small airway epithelial cells), and intestine (intestinal epithelial cells) (2–5). Basal expression of TSLP mRNA in the steady state has been detected in epithelial cells of the tonsils, skin, lung, and intestine (3,5,6); however, the basal production of TSLP protein has only been reported in the lung, tonsils, and intestine (7–9). Structurally, TSLP resembles IL-7, consisting of a four-helix bundle cytokine. Despite poor amino acid sequence identity between murine and human TSLP they have similarity at the functional level (3).

Increased TSLP expression in ECs can be induced through exposure to viral, bacterial, or parasitic pathogens as well as ligation of Toll-like receptors (TLRs) (10–13). Exposure to proinflammatory cytokines such as IL-1 β and TNF- α , or to T_H2 cell-associated cytokines IL-4 and IL-13 alone or in combination has also been shown to induce the expression of TSLP (8, 11,14,15). Examination of the promoter regions for both human and murine TSLP revealed an upstream NF- κ B-binding site that is responsible for increased TSLP expression following exposure to IL-1 β and TNF- α (15) (Figure 1A). Interestingly, treatment of a lung epithelial cell line, MLE12, with IL-25 also resulted in increased TSLP expression (16), 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 can be upregulated by damage or trauma to ECs (12). 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 (17). These findings indicate a requirement for communication between immune cells and ECs for TSLP induction. Additionally, TSLP expression may be regulated through nuclear receptors as ablation of retinoid X receptors in keratinocytes results in increased TSLP expression in these cells (18). Further, treatment of normal keratinocytes with the nuclear receptor agonists vitamin D3 or low-calcemic analogs elevated TSLP production (19). Given the findings that TSLP can be induced by both exogenous stimuli (damage, infection, TLR ligation) and host-derived cytokines (Figure 1A), TSLP production by ECs and its regulation represent a critical pathway through which ECs and the immune system communicate.

IL-25

Despite the original report of restricted expression of IL-25 in T_H2 polarized CD4⁺ T cells (20), murine and human lung epithelial cell lines and isolated murine lung ECs have been reported to express IL-25 following exposure to allergens (16). IL-25, also known as IL-17E, is a member of the IL-17 cytokine family (reviewed in (21,22)). The first defined member of this cytokine family, IL-17A, was discovered through sequence homology to a Herpesvirus samirii open reading frame (23). Through subsequent analysis of genomic databases five additional members have been described designated IL-17B to IL-17F (20,24–27). While this family bears no close similarity to other known cytokines, the discovery of 30 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 (28).

Within the IL-17 cytokine family, individual members share the greatest sequence homology in the cysteine-rich C-terminus. In this structure, four to five conserved cysteines residues are believed to form a cysteine-knot, a structural motif common among several growth factors (22). All members are secreted as non-covalent homodimers, with the exception of IL-17F, which exists as a disulfide-bonded homodimer (29). Moreover, recent studies have demonstrated the production of IL-17A/F heterodimers by CD4⁺ T cells (30,31). The two most closely-related members, IL-17A and IL-17F, are 50% identical (21), and are the best characterized members of this family as they have been implicated in numerous inflammatory diseases (32–36). In contrast, IL-25 possesses the lowest degree of homology (17%) to IL-17A, does not share a common biological function (22), and instead has been implicated in T_H2 cytokine-mediated diseases including asthma and allergic airway inflammation (20,24,37, 38).

Although our understanding of the biology of IL-25 is increasing, how IL-25 is regulated is still poorly understood and nearly all attempts to characterize IL-25 expression *in vivo* have been at the mRNA level. Despite these limitations in detection, ECs, macrophages, microglia, and CD8⁺ and CD4⁺ T cells have all been reported to express IL-25 (16,39–41). In addition, exposure to allergens and air pollutants has been reported to increase IL-25 in lung ECs (16, 24,37,39). Airborne pathogens are not the only exogenous stimuli reported to increase IL-25 as infection with the helminth *Nippostrongylus brasiliensis* has also been shown to induce IL-25 expression (24) (Figure 1B). 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 the large intestine (41). 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 T_H2 cytokine responses by highlighting the induction of IL-25 expression following exposure to stimuli that elicit type 2 immune responses.

IL-33

Of the three cytokines that are the focus of this review, IL-33 is the most recently discovered. IL-33 was first described in 2005 and belongs to the IL-1 cytokine family (42). IL-33, in contrast to its other family members IL-1 and IL-18, has been shown to promote T_H2 cytokine responses (42). The distinct T_H2-cytokine associated functions of IL-33, in comparison to its other family members, are similar to the functional roles of IL-25 within the IL-17 cytokine family. 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.* (42) demonstrated that *in vitro* incubation of IL-33 with caspase-1 yielded an 18 kDa mature protein from a 30 kDa precursor; however, the involvement of caspase-1 in IL-33 maturation *in vivo* has not been established. 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 (43,44). Interestingly, IL-33 has also been ascribed transcriptional repressor properties based on nuclear localization to heterochromatin (45). Further characterization revealed that a N-terminal helix-turn-helix motif was responsible for IL-33 nuclear localization. Moreover, using a yeast two-hybrid system, it was shown that IL-33 repressed transcription in a manner consistent with IL-33 associating with heterochromatin

(45). 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.

While expression of IL-33 has been reported in tissues such as the CNS, lymph nodes, lung, skin, and colon, and in dendritic cells, macrophages, and in lung ECs (42), only one report has found increased expression of IL-33 as a result of an external stimulus. Infection with *Trichuris* increased IL-33 mRNA in colonic tissues with peak expression reported at day 3 post-infection compared to naive controls (10) (Figure 1C). 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. These findings suggest a role for IL-33 in initiating these responses. Once this response is established, IL-33 appears to be down-regulated and the immune response is maintained and propagated through other T_H2 cytokine promoting mediators, possibly including TSLP (see *Cross-regulation and interplay* section below).

Additional cellular sources of TSLP, IL-25, and IL-33—Although the current literature focuses on ECs as the major source of TSLP, IL-25, and IL-33 *in vivo*, others have reported additional cellular sources (Table 1). For example there is evidence that TSLP and IL-25 are produced by granulocytes. In contrast to constitutive production of TSLP by ECs, TSLP expression in innate cells requires stimulation. Cross-linking of the IgE receptor on mast cells results in elevated levels of TSLP mRNA (5) and basophils stimulated with papain upregulate TSLP mRNA and protein (46). Granulocytic cell populations have also been demonstrated to produce IL-25. Mast cell lines, as well as bone marrow-derived mast cells, following stimulation with PMA and a Ca²⁺ ionophore or IgE cross-linking express IL-25 at levels comparable to polarized T_H2 cells (47). Additionally, human eosinophils and basophils, two cell populations known to play a role in allergic inflammation, have also been shown to express IL-25 (48). Unlike TSLP or IL-33, IL-25 is expressed by T cells and antigen presenting cells (APC). 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 (41). Additionally, alveolar macrophages (39) and microglia cells, the main antigen-presenting cells within the central nervous system (CNS), express IL-25 (40). 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.

2. Cellular targets of TSLP, IL-25, and IL-33

Although TSLP, IL-25, and IL-33 are all produced by ECs and have all been implicated in the promotion of T_H2 cytokine associated responses, characterization of receptor distribution suggests that each cytokine has diverse target cell populations with multiple physiologic outcomes. This section will review recent evidence of defined patterns of cytokine receptor expression in addition to their utilization of distinct signaling pathways for gene regulation and function.

TSLP-TSLPR signaling

High affinity ligand binding of TSLP and subsequent signaling requires a heterodimeric receptor consisting of the IL-7R α chain and a unique TSLPR chain that resembles the common cytokine receptor γ chain (γ_c) (4,49). 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 (50,51). TSLP-mediated STAT5 activation appears to be JAK independent, however, partial abrogation of TSLP-mediated STAT5 phosphorylation was observed in cells expressing

a dominant negative form of Tec kinase indicating Tec family kinase involvement in TSLP signaling (3,4,50,51) (Figure 2A). These findings suggest unique signaling in the TSLPR pathway; however, the specific involvement of tyrosine kinases, their association with TSLPR, and the subsequent gene targets of TSLP-TSLPR interactions remain poorly defined.

Initially, based on mRNA and TSLP responsiveness, TSLPR expression was thought to be limited to immature B cells and myeloid cells such as monocytes and dendritic cells (DCs) (3,4,49). However, more recent work has highlighted a wider pattern of TSLPR expression as mast cells and human and murine CD4⁺ T cells are responsive to treatment with TSLP alone, or in conjunction with other cytokines (46,52–54) (Table 1). DCs and mast cells appear to express TSLPR constitutively (5,46,52), however CD4⁺ T cells require TCR stimulation to induce receptor expression and become sensitive to TSLP treatment (53–55).

Initial reports characterizing the function of TSLP on myeloid cell populations demonstrated that human monocytes and CD11c⁺ DC were sensitive to TSLP treatment *in vitro* and were potently stimulated to produce CCL17, a known ligand for CCR4, which is expressed on polarized T_H2 cells (4,5). Further, TSLP enhanced the spontaneous maturation of blood-derived human CD11c⁺ cells, increasing their expression of co-stimulatory molecules and their ability to promote naive CD4⁺ T cell proliferation (4,5). The putative link between TSLP and T_H2 cytokine responses was solidified when TSLP-treated DCs were demonstrated to drive IL-4, IL-5, and IL-13 production from naive CD4⁺ T cells upon co-culture (5).

Mechanistic analysis demonstrated that TSLP-treatment of DCs induced multiple changes in DC function to create a permissive environment for T_H2 cell differentiation. TSLP-treatment of human monocyte-derived DCs (mDCs) was shown to alter DC maturation such that the ability to upregulate MHC class II and the co-stimulatory molecules CD80 and CD86 was preserved while DC cytokine production was selectively altered, thus resulting in the ability to stimulate and skew T cell differentiation (5,7). TSLP-treated mDCs do not produce IL-12 and TSLP treatment of either mDCs or murine bone marrow-derived DCs (BMDCs) results in the inhibition of IL-12 production following bacterial stimulation or with TLR-ligands (6,7, 56). TSLP-treatment also induces the expression of OX40L, which promotes T_H2 cell differentiation in the absence of IL-12 (57). The presence of OX40L on TSLP-treated DCs also induces production of TNF- α in T_H2 cells (instead of IL-10), suggesting these cells may have altered functions compared to conventionally T_H2 polarized cells (57–59).

The ability of TSLP to promote T_H2 cytokine responses is not confined to its abilities to influence DC function, as it is also able act on mast cells and T cells. Human mast cells have been recently demonstrated to express the functional TSLPR complex and following exposure to TSLP to produce the T_H2 cytokines IL-5 and IL-13 as well as the proinflammatory cytokines IL-6 and GM-CSF (12). However, it is important to note that mast cells were unable to respond to TSLP alone and required IL-1 or TNF- α to induce cytokine expression (12) suggesting that a proinflammatory environment must be present for mast cells to be responsive to TSLP. Similarly, exposure to IL-33, an IL-1 family member, is also able to sensitize mast cells to TSLP stimulation (52). Additionally, *in vitro* studies demonstrated that following TCR stimulation human CD4⁺ T cells upregulate TSLPR expression and become sensitive to TSLP (54). TSLP treatment of stimulated murine CD4⁺ T cells *in vitro* also results in the expression of IL-4 (53). Collectively, these reports identify several cellular targets for TSLP *in vivo* and provide multiple mechanisms for TSLP to influence immune responses (Table 1). Interestingly, TSLP can alter the cellular programming, and thus the capacity of targeted cell populations by acting either *directly* on a population, as is the case for DC and T cells, or *in conjunction* with additional cytokine signals, as in the case of mast cells.

IL-25-IL-17Rb signaling

While TSLPR exhibits a broad distribution pattern, the receptor for IL-25 (IL-17Rb) is more restricted. IL-17Rb, also called IL-17Rh1, was identified from lung tissue and is a single-pass type I transmembrane protein, which possesses sequence homology to IL-17R (60). IL-17B was shown to bind to IL-17Rb; however, IL-25 binds IL-17Rb with a higher affinity than IL-17B (25). This ligation results in receptor 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 (61–63) (Figure 2B). IL-17Rb expression was detected in various tissues, including the lung and gastrointestinal tract and exists as both a membrane bound and soluble form (25,60). Stimulation of macrophages with TGF- β in the presence of IL-4 was able to robustly induce the expression of IL-17Rb, whereas TGF- β or IL-4 alone elicited only a mild increase in IL-17Rb (64). Additionally, airway smooth muscle cells (ASMC) have also been reported to express the receptor for IL-25 and this expression could be manipulated with the addition of IFN- γ or TNF α (65). Furthermore, inhibition of IKK β resulted in a marked reduction in the expression of IL-17Rb (65), implicating the canonical NF- κ B signaling pathway in the regulation of IL-17Rb. Moreover, increased levels of IL-17Rb were observed following systemic over-expression of IL-25 (66) suggesting that IL-25 amplifies cellular responsiveness to IL-25, a mechanism similarly used by TSLP and IL-33.

IL-25-IL-17Rb interactions have been shown to activate NF- κ B, STAT6, GATA-3, NFATc1, and JunB, as well as the MAPK and JNK pathways (16,25,48,67). Additionally, a role for TNF receptor-associated factor (TRAF)-6 as a downstream mediator of IL-25-IL-17Rb interactions has been demonstrated, as TRAF-6, but not other TRAF family members, was recruited to IL-17Rb following receptor ligation (Figure 2B). Moreover, IL-25-mediated activation of NF- κ B was dependent on TRAF-6. However, the phosphorylation of ERK1/2, p38, and JNK following treatment with IL-25 was independent of TRAF-6 (67,68), indicating that IL-17Rb ligation results in the activation of multiple intracellular signaling pathways. These findings are consistent with our own unpublished data where exposure of macrophages to IL-25 led to modest I κ B α degradation and the phosphorylation of ERK1/2 (unpublished data).

While ECs have been shown to be responsive to IL-25 (16), Cheung *et al.* (38) also demonstrated direct effects of IL-25 on eosinophils, increasing their survival and production of cytokines and adhesion proteins. Lung ECs, eosinophils, and ASMC (see above), represent three distinct cell lineages within the airway mucosa targeted by IL-25 illustrating the potential for multi-faceted effects of IL-25 during allergic airway inflammation. However, the effects of IL-25 are not restricted to cell populations residing in the airways, as additional responsive populations have 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 (20). Moreover, this NBNT cell population was capable of mediating the physiologic changes in the lung and intestine independent of T- and B-cells. Further characterization of this cell population excluded it as basophils, mast cells, or neutrophils (20,24). However, a subsequent report identified the IL-25-responsive cell population as a putative mast cell precursor (being c-kit⁺ and Fc ϵ R1⁻), which was induced independently of IL-4, -5, -9, and -13 (69). In addition to this mast cell progenitor population, CD4⁺ T cells also express IL-17Rb. This expression is increased on polarized T_H2 cells or T cells stimulated with TSLP-treated DCs (16,48), and substantially upregulated (over 1000-fold) on T_H2 memory cells (70). Moreover, this receptor expression was functional as IL-25 treatment resulted in increased the production of IL-4, -5, and -13. However, this cytokine production was dependent on IL-4 and STAT6 (16), 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.

IL-33-IL-33R signaling

IL-33 signals through the IL-1R-like molecule T1/ST2. ST2 is a receptor closely related to IL-1R1 and IL-18R α (71–73), 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 (74,75), 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 (76)) (Figure 2C). Similar to IL-17Rb, both a membrane form and soluble form of ST2 exist *in vivo* (77,78) as a result of alternative splicing. The function of the soluble forms of IL-17Rb and ST2 are unclear; however, one potential role is to function as a decoy receptor, thus regulating constitutive IL-33 and IL-25 signaling *in vivo* (Figure 2B and 2C). In its membrane form, ST2 is present on T_H2 polarized cells and mast cells independently of IL-4 (79–81). IL-33 was found to associate with ST2 (42), and receptor ligation resulted in activation of NF- κ B (42). IL-1R accessory protein (IL-1RAcP) was subsequently identified as the second component of the functional IL-33R complex, as treatment with IL-33 did not induce T_H2 cytokine production in the absence of IL-1RAcP (82). Therefore, the IL-33 receptor complex is comprised of ST2 and IL-1RAcP (Figure 2C). Furthermore, MyD88 and TRAF6, as well as IL-1R associated kinase 4 (IRAK4), form a complex with IL-33/ST2 to mediate further downstream signaling events (42). IL-33 stimulation has also been demonstrated to result in the phosphorylation of ERK1/2, p38, and I κ B α (Figure 2C) (42).

The finding that IL-33 binds and signals through ST2, which is expressed by T_H2 polarized cells and mast cells, suggested that IL-33 plays a role in augmenting T_H2 cytokine responses *in vivo*. Consistent with the observation that *in vivo* administration of IL-33 resulted in the induction of a T_H2 cytokine response (42), incubation of T_H2 polarized cells with IL-33 increased production of IL-5 and IL-13 and reduced the levels of IFN- γ (42). Additionally, mature mast cells and their precursors express ST2 and respond to IL-33. Treatment of human mast cells or CD34⁺ mast cell progenitors (52) with IL-33, but not IL-18, resulted in increased survival and cytokine production even in the absence of stem cell factor (SCF) (81), the growth factor that promotes mast cell survival and maturation. Furthermore, Ho *et al.* (83) demonstrated that murine mast cells responded to IL-33 in a MyD88-dependent manner. Similar increases in IL-4, -6, and -13 production were observed in basophils following treatment with IL-33 and were also shown to require MyD88 (84). 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 (52), again supporting a synergistic relationship between IL-33 and TSLP (See *Cross-regulation and interplay* section below). Although TSLP could augment the effects of IL-33 on mast cells, TSLP alone was not capable of inducing cytokine production indicating that IL-33 increases mast cell responsiveness to TSLP, potentially through upregulation of TSLPR. However, the effect of IL-33 on mast cells is limited to cytokine production as incubation of mast cells with IL-33 (and/or TSLP) failed to induce degranulation (52,83).

The increased IL-13 production from mast cells following stimulation with IL-33 (52,81,83) provides new insights into the *in vivo* biology of IL-33. In particular, Schmitz *et al.* (42) demonstrated that IL-33-mediated induction of a systemic T_H2 cytokine response was lost in the absence of IL-13. These data illustrate a mechanism in which tissue-localized mast cells respond to EC-derived IL-33 and increase their expression of IL-13 leading to the development of a T_H2 cytokine response. This does not preclude roles for other innate cell populations, as IL-33 influences the biology of basophils (84) and eosinophils (85) (see above). Moreover, the importance and necessity of IL-25 and/or TSLP in IL-33-driven induction of T_H2 cytokine response are critical questions to address. As it stands, it appears that IL-33 initiates these

responses, but whether they would be maintained or persist in the absence of IL-25 and/or TSLP remains unknown.

Collectively, these data illustrate that TSLP, IL-25, and IL-33 can target multiple cell lineages to initiate and/or influence immune responses (Table 1). 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 or additive cellular mechanisms to promote T_H2 cytokine-mediated immune responses at mucosal sites.

3. EC-derived cytokines: Functions in the development of the immune system

While TSLP, IL-25, and IL-33 are each capable of influencing immune responses in adult animals, only TSLP, with its distinct similarities to IL-7, has been demonstrated to influence immune cell development under circumstances of dysregulated expression. Mice deficient in either IL-25 (41) or the IL-33 receptor Tl/ST2 (86,87) breed and develop normally and have no detectable defects in either lymphoid or myeloid cell development. Mice with a germline deletion of the TSLPR experience no developmental defects in any immune cell compartment (88), however further studies have revealed situations in which TSLP can modulate lymphoid development and alter lymphoid populations *in vivo*. Initially described as a factor able to support and promote the development of the NAG8/7 B cell line *in vitro*, TSLP was characterized as a B lymphocyte growth factor (1,2). Subsequent *in vitro* studies demonstrated that B cell precursors must differentiate to the large pre-B stage and express the pre-B cell receptor in order to respond to TSLP treatment (89,90). However, subsequent *in vivo* studies utilizing TSLPR^{-/-} mice demonstrated no role for the TSLP pathway in B lymphopoiesis. TSLPR^{-/-} mice were indistinguishable from littermate control animals and had normal populations of B cells in adult animals (88). Despite unaltered B cell development in the absence of TSLP responsiveness, systemic overexpression of TSLP through varied methodologies *in vivo* results in altered composition and frequency of B cell populations (91–93). For example, targeted overexpression of TSLP in the skin increased circulating levels of TSLP that resulted in enhanced numbers of immature and naive B cells in the periphery, loss of splenic marginal zone B cells, and increased numbers of peritoneal B-1b B cells (91). B cell development was also impacted in a TSLP-dependent manner in murine models studying the importance of Notch signaling in maintaining epidermal integrity. Demehri *et al.* demonstrated that successive loss of Notch signaling specifically in the epidermis results in increased serum levels of TSLP, and that the increase in TSLP production was responsible for the lethal B-lymphoproliferative disorder observed in these mice (94). While there was no evidence suggesting a direct regulation of TSLP by Notch in epithelial cells, it appears that the inability to form fully differentiated keratinocytes capable of barrier formation results in the overexpression of TSLP (94). Similar to the skin transgenic model, these mice with heightened TSLP exhibited greatly expanded pre and immature B cell compartments in the periphery (94). Taken together, these data suggest that while TSLP is dispensable for B lymphopoiesis under normal conditions, under conditions of excess it can influence B cell development *in vivo*.

Despite the similarity of TSLP to IL-7 and the TSLPR chain to the γ_c , the TSLP-TSLPR pathway does not seem to be strictly required for T cell development. Examination of TSLPR^{-/-} mice revealed normal T cell lymphopoiesis and normal T cell populations in adult mice (88). However, the increased lymphoid defect seen in γ_c /TSLPR double deficient over γ_c single deficient mice, and the ability of transgenically overexpressed TSLP to restore lymphoid development in IL-7 deficient mice, indicates that TSLP maintains the ability to support or promote T cell lymphopoiesis (55,95). *In vitro*, TSLP was demonstrated to increase thymopoiesis in murine fetal thymic organ culture (96). Further, TSLP appears to influence the proliferative capacity of CD4⁺ T cells as addition of TSLP to TCR stimulated CD4⁺ T cells

in vitro increased cell division (55). Additionally, CD4⁺ T cells from TSLPR^{-/-} mice expand less efficiently than WT CD4⁺ T cells in irradiated hosts (55). Thus, similar to B cell development, TSLP is not critical for T lymphopoiesis under normal conditions but alterations in the availability of TSLP may affect T cell development or optimal T cell expansion under certain circumstances.

Thymic expression of TSLP has been demonstrated to function in DC activation and T regulatory cell (T reg) development. Expression of TSLP within the epithelial cells of the Hassal's corpuscles region of the human thymic medulla correlates with DC activation and the subsequent positive selection of high-affinity self-reactive Tregs (97). Although mice lack the Hassal's corpuscle structure within the thymus, there is evidence to support a potential role for murine TSLP in the direct promotion of Treg development. Supplementation of TSLP within fetal thymic organ cultures increased expression of FoxP3 and culture of thymocytes with TSLP promoted the differentiation of CD4⁺CD25⁺FoxP3⁺ cells (98,99). Recent work has also suggested that TSLP treated murine DCs may induce Treg development (100). Despite these findings, TSLPR^{-/-} mice have normal numbers of circulating CD4⁺CD25⁺FoxP3⁺ Tregs indicating that TSLP is not essential for the development of natural Tregs. While not required for Treg development in an intact animal, studies conducted using a chimeric mouse model where developing T cells are deprived of IL-7 and TSLP signaling pathways demonstrated a defect in Treg development in the thymus (101). However, using the same model system, TSLP did not appear to play a role in Treg homeostasis as it was not required for mature Treg cell survival in the periphery (101). Further, lamina propria DCs isolated from TSLPR^{-/-} mice did not exhibit any defect in mediating FoxP3⁺ Treg conversion *in vitro* suggesting that TSLP is also not required for adaptive Treg development in the periphery (102).

Taken as a whole, these findings suggest that TSLP is not essential for B or T cell lymphopoiesis or the development/selection of FoxP3⁺CD4⁺ T cells *in vivo*. However, TSLP is able to modulate these processes in cases of increased or altered availability.

4. Functional biology of EC-derived TSLP, IL-25, and IL-33

TSLP, IL-25, and IL-33 are distinct cytokines sharing little to no homology to one another or to their respective family members. They are also diverse in their cellular source, structure, and in the cell populations in which they target. Despite these overwhelming differences, TSLP, IL-25, and IL-33 are all produced by ECs and share a common functionality in the promotion of T_H2 cytokine-mediated responses *in vivo*. Thus, their involvement in T_H2 responses underscores their importance in the development of both protective T_H2 cytokine responses in the context of helminth infections and pathologic responses in cases of allergic inflammation (103,104). Beyond these functions, TSLP, IL-25, and IL-33 have been demonstrated to exhibit immuno-regulatory properties implicating these EC-derived cytokines as important modulators of mucosal immune responses in general.

Protective roles during helminth infection

TSLP—Although TSLP expression in the skin and the lung has been linked to pathologic T_H2 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 (6,7,105). IEC-specific deletion of IKK β resulted in susceptibility to the intestinal dwelling nematode *Trichuris* and was correlated to decreased expression of TSLP (6). Resistance or susceptibility to *Trichuris* infection is determined by specific immune responses. Susceptible mice strains such as AKR mount a T_H1 cytokine response and are unable to clear the parasites resulting in chronic infection. In contrast, resistant mice (B6 or BALB/c) develop a T_H2 cytokine response and successfully expel parasites (106,107). Despite being on a genetically

resistant background, TSLPR^{-/-} mice challenged with *Trichuris* failed to clear worms at day 21 post-infection (6). The strong induction of TSLP mRNA upon infection (10), and its requirement for efficient early worm clearance (6), indicate that TSLP is a critical component of protective T_H2 cytokine-mediated protective immune responses in the intestine.

IL-25—Similar to TSLP, IL-25 is required for the development of a protective T_H2 cytokine-mediated response and protective immunity following *Trichuris* infection as IL-25^{-/-} mice fail to clear infection and display significantly reduced levels of IL-4 and IL-13 (41). In the converse experiment, IL-25 treatment conferred resistance to genetically susceptible AKR mice. IL-25-treated AKR mice displayed increased T_H2 cytokine production and elevated goblet cell numbers, resulting in efficient worm expulsion (41). 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 (41). However, IL-25^{-/-} mice can develop a protective T_H2 cytokine response upon blockade of endogenous T_H1 cytokines, suggesting that IL-25 is not essential for the development T_H2 cytokine responses (41).

In contrast to persistent infection of normally susceptible AKR mice, which results in minimal intestinal inflammation, *Trichuris*-infected IL-25^{-/-} mice developed a chronic infection characterized by development of severe intestinal inflammation. Furthermore, the observed inflammation was correlated with elevated production of IFN- γ and IL-17A mRNA in the intestinal mucosa and mesenteric LN compared to WT controls despite intact expression of IL-10. Although these studies require further investigation, this finding indicates that the mechanism whereby IL-25 limits IL-17A-mediated intestinal inflammation is independent of the classical IL-10/T regulatory cell pathway. Additionally, these findings suggest that IL-25 may play a dual role in both the development of T_H2 cytokine-dependent immunity and in limiting intestinal inflammation (see *Putative immuno-regulatory roles* section below) (41).

IL-25 also plays a protective role in another model of helminth infection as *Nippostrongylus*-infected IL-25^{-/-} mice display delayed expulsion of the parasites. Conversely, treatment of mice with IL-25 resulted in accelerated expulsion of *Nippostrongylus* infection but this effect was dependent on at least one classical T_H2 cytokine (IL-4, -5, -9 or -13) (69). The proposed mechanism for the observed delay in the absence of IL-25 was that IL-25 was inducing the expansion of a NBNT cell population to express IL-4 and IL-13, as treatment with IL-25 resulted in increased frequencies of this cell population in the mesenteric lymph nodes. Additionally, using IL-4/eGFP reporter mice, expression of GFP in the c-kit⁺ cell population preceded that of CD4⁺ T cells (69). Further characterization of this cell population, based on surface marker expression and effector capacity, revealed it to be a putative mast cell precursor (69) (see above). However, IL-25-mediated induction of immune responses through innate cell mechanisms remains contentious in light of the fact that in the context of helminth infections, innate cell populations are not sufficient for protective immunity.

Two recent reports have provided an alternative T cell-dependent mechanism for IL-25-mediated induction of protective immunity (16,48). This mechanism is founded on the observed effects of IL-25 on CD4⁺ T cells and is consistent with the involvement of IL-25 and T cells in the clearance of helminth infections. However, it is important to note fundamental differences in the respective conclusions drawn by these authors. Angkasekwina *et al.* (16) conclude that IL-25 acts during in the initiation of T_H2 cell polarization, whereas Wang *et al.* (48) postulate that IL-25 acts to sustain T_H2 cell differentiation, leading to the development to T_H2 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 T_H2 memory cell formation. Moreover, these findings do not preclude a role for IL-25 in directly modulating the biology of innate cells.

IL-33—As mentioned previously, IL-33 expression is induced following infection with *Trichuris* (10). Considering previous findings that IL-33 is involved in the promotion of T_H2 cytokine response and the observed peak expression early during infection, this indicates that IL-33 is acting during the initiation of these responses. Despite these observations, Hoshino *et al.* found no effect on the outcome of *Nippostrongylus*-infection in mice deficient in IL-33 signaling (86). However, in contrast to results using *Nippostrongylus*, administration of recombinant IL-33 to *Trichuris*-infected AKR mice from day -1 to 20 post-infection conferred resistance to susceptible AKR mice (41), similar to the effects observed following early treatment with IL-25 (41). Not only were these mice successful at expelling worms, but expulsion was associated with the development of protective T_H2 cytokine response with increased expression of IL-4, -9, and -13, elevated goblet cell numbers, and increased levels of serum IgE (10). Conversely, late administration of IL-33 (day 35 to 49 post-infection), a time point in which a T_H1 cytokine response is fully established, failed to induce protection, despite increased production of IL-4, -9, and -13. 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 (10). Interestingly, IL-33 treatment increased the expression of TSLP and TSLPR mRNA in the colons of *Trichuris*-infected mice (10) demonstrating a potential association between two cytokines known to drive T_H2 cytokine responses (see *Cross-regulation and interplay* section below).

Further supporting a role for IL-33 in the development of T_H2-cytokine responses is work using IL-33R deficient mice. Utilizing ST2^{-/-} mice, which lack the extracellular domain common to both the soluble and membrane-bound form of ST2, Townsend and colleagues (87) observed a defect in the induction of a T_H2-cytokine response *in vivo* following intravenous injection of *Schistosoma mansoni* eggs despite no observable defect in the ability of cells from ST2 null mice to differentiate into T_H1 or T_H2 polarized cells *in vitro*. This infection model is characterized by elevated expression of T_H2 cytokines, with T-cell, eosinophil, and macrophage recruitment leading to granuloma formation around eggs trapped in the lungs (108). While injection of *S. mansoni* eggs in WT mice resulted in the development of granulomas in the lungs, mice deficient in ST2 displayed decreased granuloma formation and T_H2 cytokine production. However, if mice were presensitized with an intraperitoneal injection of *S. mansoni* eggs followed by i.v. challenge, ST2^{-/-} mice displayed T_H2 cytokine induction and granuloma formation equivalent to WT counterparts (87).

Taken together, these findings suggest that while no single member of this triad of EC-derived cytokines is the ‘silver bullet’ required for the development of protective T_H2 cytokine responses *in vivo*, TSLP, IL-25, and IL-33 all act to promote optimal T_H2 cytokine responses at mucosal sites.

Roles in promoting pathologic inflammation

The prevalence of T_H2 cytokine-mediated allergy and inflammation continues to rise. Recent reports by the CDC estimate that within the United States 54% of the population suffers from at least one allergy and 20 million people are affected by asthma alone (109,110). Additionally it is estimated that 10–20% of children and 1–3% of adults in industrialized countries will present with cases of atopic dermatitis (111). The incidence of food allergy is also increasing as it is estimated that one in five children in North America will experience some form of allergy (112). While there have been great advances in understanding the etiologies and pathogenesis of these conditions, much work remains to be done, and the discovery of the involvement of TSLP, IL-25, and to a lesser extent IL-33 in these diseases adds to our understanding of disease initiation, progression, and regulation.

TSLP—TSLP was first associated with dysregulated T_H2 cytokine production in the human disease of atopic dermatitis as skin biopsies from atopic dermatitis lesions showed greatly enhanced levels of TSLP compared to non-lesion biopsies (5). Heightened TSLP expression was also detected in the airways of asthmatic patients and correlated with disease severity (113). A more causative relationship between TSLP and T_H2 cytokine-mediated disease was established with transgenic over-expression models. Targeted overexpression of TSLP in ECs of the skin or lung results in the development of severe T_H2 cytokine-mediated inflammation resembling atopic dermatitis and asthma respectively (18,114,115). In both tissues the increased expression of TSLP resulted in the development of CD4⁺ T_H2 cells, inflammatory cell infiltration, eosinophil infiltration, increased IgE production, and damage to the tissues. The T_H2 cells that developed were highly polarized, producing high levels of IL-4 with little to no IFN- γ production being detected (18,114,115). Further solidifying the connection between dysregulated TSLP production and disease, TSLPR^{-/-} mice were shown to be less susceptible to an OVA-induced model of allergic asthma, as TSLPR^{-/-} animals failed to produce T_H2 cell-associated cytokines and did not exhibit lung inflammation (115,116).

Although tissue-specific overexpression of TSLP in the lung resulted in increased levels of circulating total IgE, the inflammatory response was largely restricted to the lung. Activated, cytokine producing CD4⁺ T cells isolated from this site expressed the homing marker CCR4. Furthermore, while CD4⁺ IL-4⁺ T cells were detectable in the lung and draining lymph nodes, few cytokine positive cells were detected in peripheral lymph nodes (115). The localized inflammation suggests that TSLP influences immune cells in the immediate microenvironment. However, examination of either the K5-TSLP or K14-TSLP transgenic mice, which overexpress TSLP in epidermal keratinocytes, does reveal systemic alterations in the immune response. These mice display increased serum levels of IL-5 and eosinophils circulating in the blood (18), IL-4-producing CD4⁺ T cells in non-draining lymph nodes (115), and develop skin-localized inflammation. These findings do not fit with a model of localized action of TSLP and suggest that tissue-specific alterations in TSLP expression may have systemic effects. However, characterization of both the K5-TSLP and K14-TSLP transgenic mice found that the transgene was able to induce high levels of TSLP to the extent that TSLP becomes detectable in the serum and could thus act systemically (91,95). Therefore it remains possible that at physiological levels of expression, TSLP only acts on cells locally and must rely on other cells or mediators to influence systemic responses.

Given the ability of TSLP to influence DC responses *in vitro* (4–7), TSLP may target DCs to modulate localized immune responses. Either skin or lung resident DCs could become conditioned or activated by locally produced TSLP, migrate to the draining lymph node, initiate T_H2 cell differentiation, and imprint on the T cells the proper receptors to return to sites of inflammation. The expression of TSLPR on activated CD4⁺ T cells (53,54) also raises the possibility that TSLP could act directly on infiltrating CD4⁺ T cells to promote T_H2 cell development. These possibilities are not mutually exclusive however, and the ability of DCs to produce the T_H2 cell attracting chemokines, CCL17 and CCL22 (4,5), may also act to help initiate and/or promote local T_H2 cytokine responses. However, as DC responses *in vivo* have not been examined directly in any of the existing literature and the TSLPR^{-/-} mice lack receptor expression on all cells, it remains uncertain as to which cells TSLP is acting on *in vivo* to initiate T_H2-cytokine mediated inflammation.

Additionally, while the infiltration of CD4⁺ T_H2 cells was observed in both transgenic models, there is evidence to suggest an innate cell component is involved. In the K5-TSLP skin transgenic model, the contribution of T cells to the inflammatory phenotype was determined by generating K5-TSLP transgenic mice that were also T cell deficient. In the absence of T cells, immune pathology still developed and inflammatory cell infiltration was observed (114). Given the more recent work highlighting the ability of TSLP, in the context of TNF- α ,

IL-1, and IL-33, to induce mast cell cytokine production (12,52) it is possible that innate cells play a dynamic role in either initiating or perpetuating the inflammation caused by TSLP overexpression.

The link between increased TSLP expression and disease has also been strengthened by the phenotype of transgenic mice whose gene alterations lead to the dysregulated overproduction of TSLP. Mice lacking the nuclear hormone receptor retinoid X receptor (RXR) isotypes RXR- α and RXR- β expression specifically in keratinocytes have significantly increased production of TSLP and develop a T_H2 cytokine-mediated chronic dermatitis. This dermatitis mirrors human atopic dermatitis and the inflammation seen in the K14 TSLP mice (18). Further, application of topical vitamin D3 and other agonists for the RXR- α and RXR- β signaling pathways lead to increased TSLP expression and in turn severe chronic dermatitis (19).

While reports of TSLP-induced T_H2 cytokine-mediated diseases have been largely restricted to the skin and the lung, there are new reports emerging that TSLP may play a role in the pathogenesis of other allergic diseases. Increased TSLP expression has been reported in the nasal epithelium in a mouse model of allergic rhinitis and neutralization of TSLP using a monoclonal anti-TSLP antibody inhibited the development of disease (17). Also, increased TSLP expression has been documented in the synovial fluid of arthritic joints of human patients with rheumatoid arthritis (117).

IL-25—As treatment with 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 T_H2 cytokine mediated allergic airway inflammation. This hypothesis was also supported by results showing that administration of exogenous IL-25 or forced overexpression of IL-25 resulted in elevated expression of IL-4, -5 and -13, which corresponded with increased antibody isotype switching and circulating eosinophil numbers (20,66,118). 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 increased expression of IL-5 and IL-13, as well as proteins involved in eosinophil recruitment and migration (20,24).

Consistent with the initial characterization of IL-25, elevated expression of IL-25 and IL-17Rb were observed in tissues from patients with chronic asthma and atopic dermatitis (48). The link between IL-25 and T_H2 cytokine-mediated inflammation was further supported by findings implicating IL-25 in murine models of allergic airway inflammation. In a model of sensitization and challenge with ovalbumin plus alum to induce airway inflammation, Tamachi *et al.* (37) 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. Depletion of IL-25 also reduced levels of IL-5 and IL-13 in the BAL fluid and decreased goblet cell numbers (37). Further, intranasal administration of IL-25 to mice results in the development of airway hyperreactivity (AHR) (24). Moreover, it was shown that IL-25 could induce AHR independent of IL-4, -5, -9, and -13 (119). 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, -5, and -13 levels and increased cellular infiltrate in the BAL fluid (37). It is important to note, however, that overexpression of IL-25 alone was not able to mediate similar effects (37), indicating that IL-25 may not initiate immunological responses, but may instead promote or sustain an ongoing response. These studies conclusively support the notion that IL-25 can exacerbate inflammation in the lung, however, whether IL-25 plays a primary or auxiliary role in the development of allergic airway inflammation remains to be determined.

While IL-25 is clearly involved in allergic airway inflammation, the mechanism whereby IL-25 elicits a T_H2 -mediated immune response remains unclear. Despite previous findings indicating that the effects of IL-25 are independent of T cells (20,66,118), Tamachi *et al.* (37) suggest a role for $CD4^+$ T cells and STAT6, as they observed a decrease in both AHR and cytokine production following depletion of $CD4^+$ T cells in OVA-challenged IL-25 transgenic mice. An alternative mechanism proposed by Cheung *et al.* (38) suggests direct effects of IL-25 on human eosinophils, which have been shown to express the receptor for IL-25 (68). Purified eosinophils treated with IL-25 displayed increased survival and selectively increased expression of ICAM-1, but down-regulated ICAM-3 and L-selectin (38). While cells of innate and adaptive immunity have been the focus of the efforts to characterize the cellular targets of IL-25 (see above), another report has demonstrated that ASMCs express the receptor for IL-25 (65). Although IL-25 did not have a dramatic effect on the production of extracellular matrix proteins by ASMCs, this finding is consistent with previous data in which neutralization of IL-25 during airway challenge specifically decreased airway resistance (119), indicating that IL-25 signaling in ASMCs results in increased bronchio-constriction. However, none of these hypotheses are mutually exclusive and collectively they illustrate the possibility that IL-25 may act on numerous cell lineages, including eosinophils, $CD4^+$ T cells, and ASMCs to enhance cell recruitment, survival, and cytokine production at mucosal sites.

IL-33—The putative link between IL-33 and T_H2 cytokine production was formed as *in vivo* administration of IL-33 in mice resulted in increased expression of IL-4, -5, and -13 in the lung, spleen, and liver, as well as elevated serum levels of IgE and IgA. The induction of a T_H2 cytokine response by IL-33 was also correlated with increased goblet cell hyperplasia, mucus secretion, and eosinophilia (42). While the data suggest that IL-33 promotes T_H2 response *in vivo*, IL-33 does not appear to be strictly required for the initiation of T_H2 cytokine-mediated responses *in vitro* (87) or *in vivo* (86).

Despite the observed increases in ST2 expression in the serum of patients with asthma (120) and in mouse models of asthma (121), the involvement of IL-33/ST2 signaling in airway inflammation is less defined. Two studies have demonstrated that antibody-mediated neutralization of ST2 (or treatment with an ST2-IgG fusion protein) resulted in the decreased levels of IL-4 and IL-5 in the BAL and a reduction in eosinophil infiltration following OVA sensitization and aerosolized challenge (79,122). Moreover, the specificity of this inhibition was observed to be on T_H2 effector cells as neutralization of ST2 in recipient mice decreased airway resistance and production of T_H2 cytokines (122). This specificity ruled out any bystander effects from ST2 blockade on mast cells, another cell population shown to express ST2, suggesting that IL-33 enhances airway inflammation by specifically modulating the biology of T_H2 effector cells. Further supporting the association between IL-33 and airway inflammation was a recent report demonstrating that IL-33 induced AHR and this was associated with increased airway resistance and T_H2 cytokine expression in the lungs (84). However, using $ST2^{-/-}$ mice Hoshino *et al.* found no relationship between ST2 expression and increased airway inflammation (86). Thus, in light of these conflicting reports, additional studies are required to determine the contribution of IL-33 signaling in the development of airway inflammation.

5. Putative immuno-regulatory roles for EC-derived cytokines

TSLP, IL-25, and IL-33 play important roles in the initiation and promotion of both protective and pathologic T_H2 cytokine responses. In addition, it has become increasingly clear that these cytokines have functionality that extends beyond T_H2 cytokine-associated biology. For example, they each exhibit immuno-modulatory or immuno-regulatory functions depending on the tissue site and manner of expression, further highlighting their ability to influence multiple aspects of the immune response.

TSLP

Constitutive TSLP expression in the intestine has been implicated in helping to maintain the hyporesponsiveness of intestinal immune cells. It was found that IEC-derived TSLP was critical for the conditioning of DCs to inhibit IL-12 production and to promote T_H2 cell differentiation (7). Consistent with this, DCs isolated from the GALT of TSLPR^{-/-} mice exhibited elevated expression of IL-12/23p40 under steady state conditions (6). TSLP may also play a role in modulating infection-induced inflammation following infection with *Trichuris*, as TSLPR^{-/-} mice developed more severe infection-induced inflammation and elevated production of IFN- γ compared to WT control animals (6). Further, human patients suffering from Crohn's disease, a T_H1 cytokine-mediated intestinal inflammatory disorder, displayed reduced or undetected levels of TSLP mRNA in lesional biopsies compared to healthy tissue (7). Taken together these data suggest that in the intestine TSLP expression may be important for limiting proinflammatory cytokine production under both homeostatic conditions and following infection.

Although the anti-inflammatory capabilities of TSLP have not been directly assessed in previous *in vivo* studies in the skin and lung, the data available is consistent with a potential regulatory role for TSLP. For instance, TSLPR^{-/-} mice treated in an OVA-induced allergic asthma model exhibited significantly increased IL-12 mRNA levels in the lungs compared to WT animals (116). Additionally, transgenic overexpression of TSLP in the skin decreased CD4⁺ IFN- γ ⁺ T cells in transgenic mice in comparison to normal littermate controls (114).

As described above, TSLP may also play an immuno-regulatory role by influencing Treg populations. Although intestinal-derived DCs lacking TSLPR expression were still capable of inducing peripheral Treg conversion *in vitro* (102), TSLP treatment of DCs isolated from the bone marrow of NOD mice was able to increase the frequency of CD4⁺CD25⁺FoxP3⁺ T cells upon co-culture (123). As retinoic acid is an important mediator of peripheral conversion, and IEC-derived TSLP can be induced through modulation of retinoid X receptor expression (18, 19), it remains possible that TSLP may be involved in the peripheral conversion of Tregs at mucosal sites. Thus, further work must be conducted to fully understand the ability of TSLP-treated DCs to modulate peripheral Treg cell populations. In addition, a potential role for TSLP in directly influencing peripheral CD4⁺ T cells to convert into Tregs has not yet been evaluated.

Despite evidence supporting an anti-inflammatory/regulatory role for TSLP in mucosal sites in general, it remains possible that the anti-inflammatory functions of TSLP documented in the intestine are unique to the intestinal microenvironment. While the skin and the lung are also exposed to the external environment, the intestine is unique in its size, antigen load, and diversity of associated commensal microorganisms (124). Given the heightened level of stimulation in the intestine, it is possible that TSLP has developed unique regulatory functions to maintain intestinal immune homeostasis. More research in skin and lung models of inflammation and infection will be required to assess the ability of TSLP to regulate proinflammatory cytokine production at these sites.

IL-25

IL-25 may play an immuno-regulatory role in both mucosal and non-mucosal immune responses. At mucosal sites, IL-25 may act to regulate the production of IL-17A in the intestine following infection. As discussed previously (see *Protective roles during helminth infection* above) *Trichuris*-infected IL-25^{-/-} mice exhibit severe infection-induced inflammation accompanied by increased expression of IFN- γ and IL-17A (41). IL-25 has also been implicated in the pathogenesis of experimental autoimmune encephalomyelitis (EAE). While EAE is not localized to any mucosal surface and is driven by a T_H17 cytokine-mediated immune response, EAE disease progression induces IL-25 expression (40,125). Mice deficient in IL-25 display

accelerated disease onset and an increase in the severity of disease (40), suggesting that IL-25 may act to inhibit EAE-mediated inflammation. Accelerated disease progression was associated with increased frequencies of CD4⁺ IL-17A⁺ T cells, which were found to be pathologic as treatment of IL-25^{-/-} mice with a neutralizing antibody against IL-17A reduced the severity of disease (40,125). The ability of IL-25 to limit inflammation in the CNS was independent of IFN- γ , but was dependent on intact IL-13/IL-4R α signaling, indicating that IL-25 inhibited the development of T_H17 cells by specifically promoting T_H2 differentiation. Further supporting an immuno-regulatory role for IL-25 is our recent data, which identify a link between commensal bacteria, IL-25 expression, and T_H17 cell populations. Mice lacking commensal bacteria exhibit reduced expression of IL-25 in intestinal ECs and exaggerated IL-23 and IL-17A levels, suggesting that bacterial-derived signals may influence this immuno-regulatory pathway (Zaph, in press).

Collectively, these findings implicate a novel anti-inflammatory role for IL-25 as a key factor in the attenuation of IL-17-mediated inflammation. Analysis of the molecular mechanisms of IL-25-mediated regulation will benefit the development of targeted therapeutics for autoimmune diseases in which IL-17 has been implicated in being the causal agent of disease.

IL-33

IL-33 mediated-inhibition of proinflammatory cytokine responses has been reported in atherosclerosis as IL-33 treatment results in reduced atherosclerotic plaque size. Atherosclerosis is an inflammatory disease of the vascular endothelial cells mediated by T_H1 cells producing high levels of IFN- γ (126). Schmitz *et al.* (42) demonstrated IL-33 expression was present in vascular smooth muscle cells as well as in endothelial cells. Treatment of ApoE^{-/-} mice on a high-fat diet with IL-33 led to a reduction in lesion size and, concomitantly, in the number of infiltrating macrophages and T cells. This decrease in inflammation was associated with decreased production of IFN- γ , increased production of IL-5 and IL-13, and elevated levels of serum IgG1, IgE, and IgA (127). Consistent with IL-33 reducing atherosclerosis, neutralization of ST2 resulted in the exacerbation of atherosclerotic plaque formation in ApoE^{-/-} mice with increased levels of IFN- γ (127).

While EC-derived TSLP, IL-25, and IL-33 each directly promote T_H2 cytokine responses, they also maintain the ability to modulate other immune responses. Increases in TSLP, IL-25, and IL-33 are associated with decreases in either IFN- γ and/or IL-17A production as well as decreased inflammation. While TSLP appears to directly regulate DC-derived IL-12/23p40 production (5–7), the mechanism of IL-25 and IL-33 in down-regulating IFN- γ and IL-17A production appears to be indirect and through the promotion of T_H2 cytokine production. However, the data collected to date does not rule out the possibility that each cytokine may act either directly or indirectly to decrease proinflammatory cytokine production. Therefore, more work is needed to fully understand the immuno-regulatory properties of TSLP, IL-25 and IL-33.

6. EC-derived cytokines: Cross-regulation and interplay

EC-derived TSLP, IL-25, and IL-33 each contribute to immuno-regulation and the promotion of T_H2 cytokine responses in their own distinct ways, however, the partial overlap of inducing stimuli and target cell populations indicates cross-regulation and interactions between these cytokines may occur. 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.

In terms of direct regulation of expression, both IL-25 (16) and IL-33 (10) can induce TSLP mRNA in ECs (Figure 3) suggesting that IL-25 and IL-33 are upstream of TSLP. This is

additionally supported by the ability of IL-33 to sensitize mast cells to TSLP (52). While TSLP is unable to induce mast cell activation on its own (12), IL-33 treatment of mast cells induces the production of T_H2 cytokines and chemokines, and sensitizes mast cells to further stimulation with TSLP (52). Examination of induced receptor expression also provides insight into the cross-regulation of these cytokines. IL-25 and IL-33 each upregulate their cognate receptor complexes (66,82) (Figure 3), indicating a positive feedback loop to amplify responsiveness to TSLP, IL-25, and IL-33. Exogenous IL-33 treatment induced increased levels of TSLPR in the colons of *Trichuris*-infected mice (10), again suggesting regulation of TSLP-TSLPR signaling by IL-33. Additionally, TSLP, IL-25, and IL-33 are each able to upregulate IL-17Rb (48,66,82) indicating that sensitization to IL-25 expression is an important and common effect of these EC-derived cytokines. Based on these data, it appears that neither IL-25 nor TSLP regulate the production of IL-33 or IL-33 receptor complexes and suggests that IL-33 sits atop the EC-derived cytokine hierarchy that promotes T_H2 cytokine responses. 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 Figure 3). TSLP and IL-25 both signal and influence antigen presenting cells such as macrophages and DCs. TSLP-treatment of DC induces DC maturation (increased levels of costimulatory molecules, MHC class II) (5), expression of OX40L (57), secretion of T_H2 cell-attracting chemokines (5), and decreased production of IL-12/23p40 (5,7) which in turn creates permissive conditions for naive CD4⁺ T cells to differentiate into T_H2 cells (3). IL-25 is also capable of signaling in macrophage and DC populations (64), however, the outcome of this signaling remains unclear. TSLP and IL-25 are also both able to directly influence naive CD4⁺ T cell populations. TSLP can induce GATA-3 and IL-4 in naive CD4⁺ T cells (53) while IL-25 induces the production of IL-4, IL-5, and IL-13 (16). While TSLP has not been found to act on T_H2 effector or T_H2 memory cells, interaction of T_H2 cells with TSLP-treated DCs sensitizes these cells to stimulation with IL-25 (48). IL-25 treatment of T_H2 cells that have been stimulated with TSLP-treated DCs sustains GATA-3, c-MAF, and JunB expression independent of IL-4 (48). Differentiated T_H2 cells are also sensitive to IL-33, which increases their secretion of IL-5 and IL-13 (42).

TSLP, IL-25, and IL-33 also directly influence the biology of innate cell populations including mast cells, eosinophils, and basophils. TSLP, either in conjunction with IL-1, TNF α , or IL-33 induces mast cell production of IL-4, IL-5, IL-13, and production of T_H2 cell attracting chemokines (12,52). IL-25 alone is able to induce the expansion of a NBNT ckit⁺ cell population *in vivo*. Additionally, this presumed mast cell progenitor population expresses IL-4, IL-5, and IL-13 in response to IL-25 (69). IL-33 alone is capable of acting on mast cells to induce expression of IL-4, IL-5, and IL-13 as well as increasing survival, chemokine production, and sensitization to TSLP treatment (52). Moreover, IL-33, but not TSLP or IL-25, elicits increased cytokine production from basophils (84).

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 (68,128), while IL-33 acts to induce degranulation and superoxide production (85). Although not an immune cell population, IL-25 is also able to target airway smooth muscle cells (65). While the mechanism and function of this remains poorly defined, IL-25 appears to increase the contractility of these cells (Figure 3). The degree of cross-regulation and interplay becomes more complex when the downstream mediators activated by TSLP, IL-25 and IL-33 are considered. In particular, each of these cytokines is able to either directly or indirectly induce the production of IL-4 from one of more cell populations. This IL-4 then acts in a positive feedback loop on CD4⁺ T cells to induce and sustain IL-4 secretion by CD4⁺ T cells. The elevated levels of IL-4 may also act to induce EC production of TSLP, further amplifying an ongoing T_H2 cytokine response. Additionally, TSLP, IL-25, and IL-33 induce the expression of chemokines that attract multiple cell types that are responsive to TSLP,

IL-25 and IL-33 and can act to induce further expression of TSLP, IL-25, and IL-33. Future analysis of the cross-regulation that operates between these EC-derived cytokines is necessary.

7. Concluding remarks

Based on their location at the interface between host tissues and external environment it is apparent that ECs, in part, through their expression of TSLP, IL-25, and IL-33, represent a critical cell population in the initiation of T_H2 cytokine responses at mucosal sites. Recent data supports the roles for these EC-derived cytokines in the licensing of these responses in protective, pathologic and immuno-regulatory contexts. Moreover, additional evidence suggests that TSLP, IL-25, and IL-33 may not function independently of one another. Rather, 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-25, and IL-33. The emerging understanding of these inter-relationships suggests that combined targeting or overexpression of TSLP, IL-25, and IL-33 may offer a novel and highly effective therapeutic approach to modulate the onset, progression or severity of T_H2 cytokine-mediated inflammation associated with asthma, atopic disorders, and helminth infections at mucosal sites.

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Figure 1. Epithelial cells as a source of TSLP, IL-25, and IL-33 *in vivo*

TSLP expression is induced following helminth infection, tissue damage, and exposure to T_H2 and pro-inflammatory cytokines (A). *Nippostrongylus*-infection and exposure to common allergens upregulates expression of IL-25 (B). To date only *Trichuris* infection has been shown to upregulated expression of IL-33 in intestinal ECs (C). EC, epithelial cell; IL, interleukin; TSLP, thymic stromal lymphopoietin.

Figure 2. Downstream signaling pathways activated following receptor ligation by TSLP, IL-25, and IL-33

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). IL-25-mediated activation of NF- κ B is dependent on TRAF-6, whereas activation of the MAPK pathway does not require TRAF-6 (B). TRAF-6 and MyD88 are both required for intact IL-33 signaling. Additionally, IL-33 can activate both the MAPK and JNK pathways (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-25, and IL-33

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.

	Cellular sources	Ref.	Inducing stimuli	Ref.	Target cell populations	Ref.
TSLP	Thymic stromal cells Tonsillar crypt EC Intestinal ECs Keratinocytes Mast cells Basophils	1,2 3,5,11,12,15 5 7,13 5,14 5 46	TLR ligands Viruses Bacteria Helminth parasites IL-1, TNF- α IL-4, IL-13 IL-25 IL-33 Tissue damage FcE cross linking Papain	11-13 11 7,13 10 8,11,14,14 16 10 12 5,46 46	Monocytes Dendritic cells CD4 ⁺ T cells Mast cells B cells	4,5,49 4-7,9 53-55 12,52 1,2,49,91-95
IL-25	Epithelial cells Macrophages Microglia T cells Mast cells Basophils Eosinophils	16 39 40 20,41 47 48 48	Allergens Helminth parasites FcE cross linking	16,24 24 47	Epithelial cells Macrophages TH2 T cells Eosinophils NBNT ckit ⁺ cells Smooth muscle cells	16 64 16,50 38 69 65
IL-33	Epithelial cells Macrophages Dendritic cells	42	Helminth parasites	10	T cells Mast cells Basophils Eosinophils	42 52,81,83 84 85