Cytokine-Mediated Regulation of Innate Lymphoid Cell Plasticity in Gut Mucosal Immunity

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Mucosal barriers are active sites that encounter a bombardment of antigenic stimuli derived from both the commensal flora and a variety of pathogens, as well as from environmental insults. As such, the ability to mount appropriate innate immune responses is an important first line of defense that confers protection to the host. Central to innate immunity are innate lymphoid cells (ILCs), which were first described a decade ago, and represent a family of heterogeneous cells driven by specific transcription factors and exhibit distinct cytokine profiles that are shared with their CD4+ T-helper cell counterparts. ILCs are particularly enriched at mucosal surfaces, and the tissue microenvironment and cytokine milieu in which ILCs reside are critical factors that drive the behavior and overall function of these cells. In fact, ILCs situated at mucosal barriers must be able to temper their response to a constant exposure of environmental antigens, but also promptly react to pathogens or signals that are potentially harmful to the host. In this context, the ability of ILCs to readily transdifferentiate in response to their dynamic surroundings has become a vigorous area of research, and defining specific mechanism(s) of ILC plasticity is at the advent of discovery. This review will summarize what is currently known regarding the network of cytokines and regulatory elements that enable ILCs to readily transform, based on the range of diverse signals and signal gradients they encounter that lead to either protective or pathogenic function(s), with focus on the gut mucosal immune system.

Keywords: innate lymphoid cells (ILCs), cytokines, plasticity, gut mucosal immunity, innate immunity

INTRODUCTION

Innate lymphoid cells (ILCs) are a diverse family of developmentally-related immune cells that are heterogeneous in their tissue location, cytokine secretion, and effector functions. The term “ILC” has been widely used since 2010, with distinct subsets formally proposed in 2013, based on the transcription factors and specific cytokines regulating their development and function (1). Initially, three groups of ILCs were described, representing innate counterparts that functionally mirror...
CD4+ T-helper cell subsets. More recently, NK and lymphoid tissue inducer (LTi) cells have expanded this family, thereby representing five different ILC groups (2). Group 1 ILCs (ILC1s) express IFNγ and rely on the transcription factor, T-bet, but unlike closely-related NK cells, they do not depend on Eomesodermin (Eomes) and are, in general, non-cytotoxic; however, a phenotypic hybrid of ILC1s and NK cells also exists (3). ILC2s produce the Th2 cytokines, IL-4, IL-5, and IL-13, as well as IL-9 and amphiregulin, and are functionally influenced by the transcription factors, GATA3 and RORα. ILC3s utilize RORγt to drive production of IL-22, but also IL-17, and are distinguished into further subsets based on expression of the natural cytotoxicity receptors (NCRs), NKp46 and NKp44. LTi cells are also dependent on RORγt, but produce lymphotoxins and are critical for secondary lymphoid organ formation, including Peyer’s patches. Although organizing ILCs into these five subgroups provides a basic infrastructure to understand the ILC family in regards to development and function, it is also important to consider whether this diversity is discrete, or reflects a given subset’s adaptability to the changing tissue microenvironment, such as what occurs during disease pathogenesis, and for these cells to undergo transformation.

In this context, several significant questions remain unanswered regarding the mechanisms underlying ILC development, divergence, and differentiation. What kind of environmental cues and other regulatory factors are necessary to determine cell fate? How do these processes relate to that of the CD4+ T cell helper population? Which ILC subgroups are terminally-differentiated, or like their corresponding CD4+ T helper cells, can convert from one ILC type to another? Although investigation of ILC plasticity is still in its infancy, these questions are now being answered, particularly with the emergence of more advanced techniques, such as single-cell transcriptomics, which facilitates a better understanding of the complexity and heterogeneity of ILCs (4–7).

In general, ILC2s and ILC3s have the ability to transdifferentiate into ILC1s, which is a reversible process, and highly dependent on the cytokine milieu and tissue microenvironment in which these cells reside. Transformation of ILC2s to ILC3s has also been reported, while plasticity between “natural” and “inflammatory” ILC2s, as well as of NK cells to ILC1s, may represent transient stages or terminally-differentiated events during disease pathogenesis, respectively. The following sections will summarize current findings regarding ILC plasticity and what signals (e.g., cytokines) control these processes, with particular focus on the gut mucosal immune system. We will also discuss the more recently coined regulatory ILC (ILCreg) subset and its contribution to mucosal immunity.

**CYTOKINES THAT REGULATE PLASTICITY OF LTi AND ILC3s**

Plasticity within ILC subgroups was first observed in LTi cells, in which a gradient of RORγt expression is stabilized by IL-7 and the gut microbiome, while IL-12 and IL-15 accelerate its loss. Specifically, RORγt+ LTi cells produce IL-22 and are functionally protective, whereas RORγt− LTi cells secrete IFNγ and can induce colitis (8, 9). Similarly, ILC3s that are NKp46 CCR6−/low are able to differentiate into NKp46+ ILC3s, depending on upregulation of the prototypic ILC1 transcription factor, T-bet, which stimulates IFNγ and IL-22 production that are important for protection against *Salmonella* infection (10). In addition, Notch signaling mediated by T-bet guides the development of NKp46+ ILC3s (11). Interestingly, this upregulation of T-bet in a subpopulation of ILC3s is concurrent with loss of RORγt expression (8, 12).

How these “ex-ILC3s” are generated is of great interest and the focus of recent investigation. Although different mechanisms have been implicated, the tissue-specific microenvironment appears to play a critical role in skewing ILC3 identity, as the frequency of these subsets differs depending on their residence. Most abundant within the intestines are ILC3s, wherein CCR6+ ILC3s and/or LTi-like cells are most prominently found in cryptopatches, whereas NKp46+/−, CCR6−/low ILC3s are located within the lamina propria. Interestingly, Pearson et al. recently demonstrated that ILC3s can transdifferentiate into intestinal cryptopatches to the lamina propria, and this migration is dependent on GM-CSF (13). Another mechanism for ILC3 switching was revealed through the use of RORγt-GFP reporter mice (14), which have been instrumental for lineage-tracing experiments (15); these mice retain GFP expression in their RORγt+ cells, even after loss of RORγt expression. Studies show that within the ILC1 subset, some cells have the ability to produce IFNγ, a typical ILC1 cytokine, and still have traceable GFP expression, suggesting that they were likely once RORγt+ ILC3s (i.e., ex-ILC3s) (8–10, 16–18). Although ILC3s rely on IL-7 signaling for proper development and maintenance, ex-ILC3s downregulate CD127 and c-Kit, and are more responsive to IL-15; in contrast, all other ILC3 subsets are not entirely dependent on IL-15 signaling (18, 19). Furthermore, upon IL-23 stimulation, STAT4/T-bet-dependent regulation of NCR− ILC3s can promote IFNγ production and plasticity towards type 1 fate (20).

More recently, an intermediate population has been identified in human tonsils and small intestines, with characteristics of both ILC3s and intraepithelial CD103+ ILC1s (21). This population expresses CD103, CCR6, and CD300LF to different degrees, with scRNA-seq analysis providing evidence that ILC3s are able to differentiate in *vivo* into CD103+ ILC1s. Furthermore, Aioulos (encoded by IKZF3), a member of the Ikaros family of transcription factors, and expressed mainly by ILC1s and NK cells, is critical for skewing ILC3s into CD103+ and intraepithelial CD103+ ILC1s (21). The transcription factor, c-Maf, is also implicated in controlling the homeostatic balance of ILC3s by directly inhibiting T-bet, and therefore transdifferentiation to ILC1s (23, 24); in the absence of c-Maf, CD196+ ILC3s transdifferentiate to an ILC1 phenotype (23). Another factor, BCL6, regulates ILC3-to-ILC1 plasticity by repressing ILC3-promoting pathways, such as IL-23-induced signaling (24), resulting in reduced ILC1 frequency.

In humans, the transition from ILC3s to ILC1s is contingent on downstream signals directed by IL-12 that induce the
expression of T-bet (12). This is the case in inflamed intestinal tissues from Crohn’s disease patients, wherein increased ILC1s, at the expense of ILC3s, is observed, highlighting the prevalence of ILC3s during homeostasis, possibly by supporting T regulatory cell (Treg) activity via IL-2 (25), and ILC1s during (pathogenic) inflammatory events (26). Interestingly, differentiation from NKp46- to NKp46+ ILC3s, and then to NKp46-ex-ILC3s (i.e., “ILC1s”), modulated by low-to-high T-bet expression, is reversible both in vivo and in vitro, and is dependent on IL-23, IL-2, and IL-1β, and is further enhanced by retinoic acid (12). This ILC3-to-ILC1 polarization also depends on the presence of CD14+ and CD14- dendritic cells, wherein an increase in the former promotes ILC1 differentiation, and an increase in the latter, induces ILC3 skewing. These findings indicate that, as much as the cytokine milieu and activation of transcription factors can affect ILC subset composition, so can environmental signals from local immune cells. In other studies, fate-mapping experiments have established that NKp46+ROTY+ ILC3s can downregulate in vivo expression of NKp46, generating NKp46 ROY+ ILC3s (11, 27).

As mentioned earlier, T-bet itself can direct the development of NKp46+ ILC3s, which is mediated through Notch signaling (10). Confounding this finding, however, Notch is also reported to regulate plasticity within ILC3 subsets by controlling the fate of NKp46+ ILC3s (28). Furthermore, interconversion within the ILC3 subset occurs in response to TGFβ (29). Transcripts for the two subunits of the TGFβ receptor, TGFβ receptor I and II, can be detected in ILC3s (30). Deletion of TGFβ receptor II leads to expansion of NKp46+ ILC3s, indicating that TGFβ impairs the development of NKp46+ ILC3s. Additionally, TGFβ antagonizes Notch signaling, implying that the ILC3 phenotype depends on fine-tuning the divergent effects of both TGFβ and Notch signaling that may be required to preserve homeostasis, in vivo (29).

Taken together, these studies indicate that ILC3s exhibit bidirectional differentiation that can be modulated by T-bet and RORγ gradients within the ILC3 lineage (Figure 1A). Are ex-ILC3s generated from NCR+ ILC3s, and are they all dependent on Notch and TGF-β signaling? How does T-bet and RORγ detect the extent of inflammation and deliver equivalent immune responses? To determine how these transcription factors readily impose various ILC3 effector programs, it is imperative to recognize the underlying molecular mechanisms for ILC3 plasticity. How this balance is maintained could reflect blunted inflammatory responses (i.e., in presence of commensal flora during homeostasis), while being poised to mount a vigorous immune reaction when challenge or insult occurs, particularly at mucosal barriers. Various ILC3 subsets may be actively modulated by the temporal degree of inflammation that then directs T-bet and RORγ expression.

**CYTOKINES THAT REGULATE PLASTICITY OF ILC2S**

ILC2s have also been reported to alter their functional and transcriptional programs. ILC2s are capable of converting to IFNγ-producing ILC1s in both mice and humans (31-33). Specifically, ILC2s derived from human blood proliferate in vitro in the presence of IL-2 and IL-7, upregulate T-bet, and secrete IFNγ via IL-12 (34). Other studies show that, in the presence of IL-1β, human ILC2s not only express T-bet, but also the IL-12 receptor subunits, IL-12Rβ1 and IL-12Rβ2, enabling ILC2s to respond to IL-12 (32). Interestingly, patients with defects in IL-12Rβ1 suffer from a syndrome referred to as Mendelian susceptibility to mycobacterial disease; these individuals are not capable of generating ILC2s that potentially can convert to ILC1s (34). IL-12 is also important in inducing genomic modifications in the IFNγ locus, allowing for IFNγ secretion; these IFNγ-expressing “ex-ILC2s” are also able to secrete IL-13 (32). Similarly, Bal et al. showed in an inflammatory environment (i.e., lung tissues of patients with chronic obstructive pulmonary disease) that ILC2s can convert into IFNγ-producing ILC1s by exposure to either combination IL-1β and IL-12, IL-33, or TSLP (thymic stromal lymphopoietin) and IL-12 (31). After adoptive transfer into humanized mice, these ILC2s downregulate chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTH2) and c-Kit expression, which are typical markers for ILC2s (31). These CRTH2 Kit+ ex-ILC2s also express increased Tbx21 (T-bet) compared to CRTH2 Kit+ ILC2s. In humans, two subsets of CRTH2+ ILC2s were identified in peripheral blood, based on CD117 expression: CD117+ ILC2s, indicating mature ILC2s, and CD117+ ILC2s, showing some features of ILC3s, such as RORγ expression (35, 36). Upon IL-33 stimulation, CD117+ ILC2s produce Th2 cytokines, whereas IL1β and IL-23 induce these cells to produce IL-17 (35, 36) and CCR6, which is also expressed by IL-17-producing ILC3s (37). ILC2s have also been reported to display phenotypic plasticity in response to infectious agents, such as influenza virus, respiratory syncytial virus, Staphylococcus aureus, and interestingly, cigarette smoke (33). GATA3 expression is downregulated in ILC2s in response to exposure to these agents, with a subsequent increase in IL-12Rβ2, IL-18Rα, and T-bet. This effect was confirmed in vivo by adoptive transfer of ILC2s from ST2/IL-33R reporter mice into recipients lacking T cells and ILCs (Rag2+/Il2rg− mice) that were then infected with influenza. Although donor ILC2s downregulate GATA3 and upregulate IL-18Rα and IL-12Rβ2, they do not express T-bet; however, upon stimulation with IL-12 and IL-18, a portion of these ex-ILC2s are capable of IFNγ secretion, suggesting skewing towards ILC1s (33).

Interestingly, similar to ILC3-to-ILC1 conversion, ILC2-to-ILC1 transdifferentiation is also reversible. Although the mechanism for this process is not entirely clear, eosinophil-derived IL-4 appears to prevent IL12-mediated ILC2 differentiation to ILC1 in an inflammatory milieu, such as within nasal polyps of patients with chronic rhinosinusitis (31). Furthermore, expression of the receptors for IL-1β, IL-12, IL-18, and IL-33 influence ILC2 expansion and T-bet induction, facilitating ILC2 conversion towards an ILC1 phenotype (31-33).

TGFβ from pulmonary epithelial cells has been observed to promote allergic immune responses by expanding IL-13-secreting...
ILC2s (30). IL-33 induces proliferation of ILC2s and stimulates TGFβ secretion in lung airways to activate ILC2 function and migration (30). The possibility therefore exists that TGFβ, in line with other inflammatory mediators, acts as a modulator of ILC2 and ILC3 fate. In fact, it has been proposed that murine ILC2s can be categorized into two subsets: a transient “inflammatory” subpopulation whose fate and function is dependent on the transcription factor, BATF, and expresses more IL-25R (thereby responding preferentially to IL-25), and a tissue resident “natural” ST2+ ILC2 subpopulation induced by IL-33 (38, 39). Inflammatory (i)ILC2s are not responsive to IL-33, but can convert into natural (n)ILC2s, both in vivo and in vitro, and express low quantities of RORγt and upon stimulation, IL-17 and IL-13, indicating plasticity between iILC2s and ILC3-like cells (38).

Although this phenomenon has not been recapitulated in humans, human clones of ILC2s and ILC3s that secrete both IL-22 and IL-13 have been characterized (40, 41). Similarly, ILC2s...
can respond to inflammation in skin and lungs by transdifferentiating into IL-17-producing ILC3s; specifically, ILC2s co-cultured with dermal cells and the fungus, Candida albicans, produce IL-17 and acquire a phenotype similar to that of NKp44+ ILC3s (35). Finally, patients with more severe non-allergic asthma, with blunted Th2 responses, display both IL-5- and IL-13-producing ILC2s, and IL-17-producing ILC3s and ILC2s (42).

Together, these studies provide support for the existence of ILC2 phenotypic flexibility (Figure 1B). Future studies will benefit from experiments that can trace genetic lineages to clarify issues related to ILC2 plasticity. Discriminating between in vivo expansion of already low numbers of ILC subsets vs. transdifferentiation is challenging. Identifying extracellular influences and why lineage flexibility is necessary, will facilitate knowledge as to how pathogenic responses can be controlled and skewed toward protective function(s), leading to potential therapeutic interventions (8, 43).

**CYTOKINES THAT REGULATE PLASTICITY OF ILC1S AND NK CELLS**

Previous sections of this review have highlighted interconversion of ILC3s and ILC2s into ILC1s, and vice-versa. This section will examine ILC plasticity of the closely-related subgroups, NK cells and ILC1s. Although both subsets possess identical cell surface markers (NKp46, NKG2D), parallel transcriptional programming, and similar cytokine profiles (IFNγ, TNF), they are now considered distinct populations. For instance, NK cells developmentally do not need GATA3 (44), but some ILC1s do (45–48). The proposed cellular basis of this divergence is due to the transcription factors, inhibitor of DNA binding protein-2 (ID2) and promyelocytic leukemia zinc finger protein (PLZF), which are expressed in ILC precursors, but not in NK cells (18, 49). Recent studies, however, using polychromic reporter mice, show that ILC precursors actually have considerable NK precursor activity, challenging the existing paradigm that considers ILC1 and NK cells as two different subsets (50, 51). Moreover, conversion of NK cells into ILC1s is observed in mice with non-alcoholic fatty liver disease, and likely mediated, in part, by TGFβ (59). Gao et al., recently identified NK cells that convert into intermediate ILC1s (intILC1s), also via TGFβ (58). Surprisingly, even though it is well-established that SMAD4 promotes signaling by TGFβ family members, and TGFβ encourages differentiation towards the ILC1 lineage, Cortez, et al. demonstrated that SMAD4 deficiency in ILC1s does not affect their differentiation, but instead converts SMAD4-deficient NK cells into ILC1-like cells (59). This suggests that SMAD4 acts as a negative regulator of TGFβ signaling in NK cells, and inhibits typical TGFβ imprinting that is characteristic of ILC1s. More importantly, these intILC1s possess gene expression profiles that are intermediate between ILC1s and NK cells, and are incapable of limiting tumor burden or viral load. Whether these ILC1-like cells can actually revert back to NK cells is uncertain, but these studies reveal a mechanism by which the ILC continuum is controlled by a rheostat that includes a cytokine milieu driven by the local tissue microenvironment (Figure 1C).

**NEW ILC ON THE BLOCK: ILCREGS**

Like Tregs, ILCs are reported to have a corresponding population, aptly-called ILCREGs (60). Wang, et al. identified ILCREGs in mouse and human intestines that are induced upon inflammatory stimuli, such as DSS, anti-CD40 antibody, as well as Salmonella typhimurium and Citrobacter rodentium infection (60). ILCREGs purportedly originate from common helper ILC precursors (CHILP), and not ILC precursors, and express the transcription factors, ID3 and Sox4. Although ILCREGs do not express CD4 or Foxp3, they possess a gene identity distinct from other ILC subsets and Tregs. Importantly, ILCREGs produce IL-10 and TGFβ, and suppress activation of ILC1s and ILC3s, but not ILC2s, in an IL-10-dependent manner. TGFβ is necessary for maintenance of ILCREGs and autocrine TGFβ is required for its expansion during intestinal inflammation. The role of ILCREGs in colorectal cancer (CRC) show that these cells transdifferentiate from ILC3s during CRC progression via TGFβ, indicating potential pro-tumorigenic function during ILC3-to-ILCReg plasticity (61). Furthermore, retinoic acid is reported to induce transdifferentiation of ILC2s into IL-10-producing ILCREGs during airway inflammation (62), while ILC2s provide a predominant and inducible source of IL-10 in the GI tract (63). The existence of this novel ILC subset is, however, controversial and the topic of current investigation that elicits several open-ended questions. How do the transcription factors, ID3 and Sox4, synergize to modulate ILCReg development? Since ILCREGS constitutively express Tgfb1, Tgfb2, Il2rb, and Il2rg, can they be stimulated directly by IL-2 and TGFβ? Why are ILC2s unresponsive to the effects of IL-10-producing ILCREGS? It is interesting to note that a subset of IL-10-secreting ILC2s, detected in murine lungs after IL-33 treatment or papain stimulation, associates with reduced lung eosinophilia (64). Comparable to ILCREGS, these IL-10+ ILC2s also express the anti-inflammatory gene, Retnla, thereby suggesting anti-inflammatory properties, yet warrants further investigation in other pathogenic inflammatory states.
CONCLUSIONS AND FUTURE DIRECTIONS

ILCs are particularly enriched at mucosal surfaces, and studies over the last decade highlight various functions that are important at the intestinal barrier (65), both in maintaining gut homeostasis, but also during chronic inflammation. ILC plasticity allows adaptability in response to changes in the local tissue microenvironment that are critical to appropriately respond to pathogenic challenge, without the need for de novo ILC generation and recruitment. Investigation into the precise mechanisms that control plasticity of specific ILC subsets, particularly at mucosal surfaces, is in its infancy and will aid in further understanding disease pathogenesis and designing targeted therapies in the future.

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CS and K-AB contributed equally to researching, writing of initial drafts, and assembling manuscript. TP conceptualized, edited, and assembled the final submitted manuscript. All authors contributed to the article and approved the submitted version.

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