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Developmental Options and Functional Plasticity of Innate Lymphoid Cells

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Short title: ILC Differentiation and Plasticity

Abstract

Innate lymphoid cells (ILCs) are lineage- and antigen receptor-negative lymphocytes including natural killer (NK) cells and at least three distinguishable cell subsets (ILC1, ILC2, ILC3) that rapidly produce cytokines (IFN- γ , IL-5, IL-13, IL-17A, IL-22) upon activation. As such, ILCs can act as first-line defenders in the context of infection, inflammation and cancer. Due to the strong conservation between the expression of key transcription factors that can drive signature cytokine outputs in ILCs and differentiated helper T cells, it has been proposed that ILCs represent innate counterparts of the latter. Several distinct ILC precursors (ILCP) with pan-ILC (giving rise to all ILCs) or subset-restricted potentials have been described in both mouse and man. How and where these different ILCP give rise to more mature tissue-resident ILCs remains unclear. Recently, environmental signals have been shown to epigenetically influence canonical ILC differentiation pathways, generating substantial functional plasticity. These new results suggest that while ILC differentiation may be 'fixed' in principle, it remains 'flexible' in practice. A more comprehensive knowledge in the molecular mechanisms that regulate ILC development and effector functions may allow for therapeutic manipulation of ILCs for diverse disease conditions.

Introduction

ILC represent an expanding family of innate effector cells that have critical roles in the generation and maintenance of immunity, especially at mucosal surfaces. A standardized nomenclature has been adopted for describing the functionally different ILC subsets: group 1 ILC include NK and other ILC that produce IFN- γ and are dependent on T-bet for their development and function; group 2 ILC comprise ILC that produce type 2 cytokines (IL-4, IL-5, IL-9 and IL-13) and require Gata3 and ROR α ; group 3 ILC subtypes that produce IL-17 and/or IL-22 and utilize ROR γ t for their differentiation (reviewed in [1]). This nomenclature scheme clusters cells based primarily on functional outputs (cytokines) but also on the signature transcription factors (TF) that drive the expression of effector molecules. The TF-driven program that guides ILC differentiation parallels that of T helper (T_H) cell differentiation in a remarkable fashion, and as such, it has been proposed that ILCs represent ‘innate’ counterparts of adaptive T_H cells [2,3]. In this model, ILCs could provide an immediate source of cytokines required for pathogen control in the early phase of infection prior to the generation of antigen-specific T_H cells. Several reports have identified rapid cytokine production by ILCs during bacterial, fungal and viral infection that appear to play an important role in pathogen containment [4,5]. In this way, ILCs may serve a specific non-redundant role in immune defense, especially in the context of compromised adaptive immunity.

Since their discovery, ILCs occupy an ever increasing interest for the immunological community. Given their unique properties, clinicians have become aware that ILCs may have important roles in human disease, either as mediators of protection or as promoters of pathogenesis. Understanding the molecular and cellular mechanisms that regulate ILC development and differentiation, as well as the factors that condition ILC homeostasis, could lead to novel approaches that harness ILC functional capacities

in the clinic. In this review, we will discuss recent advances in our understanding of ILC development and function, in particular the mechanisms that control functional plasticity of tissue-resident ILCs.

A Generic Model of ILC Development (Figure 1)

ILC subsets that have been described share many common characteristics, including classical lymphoid morphology, an independence from the recombinase machinery, and a dependence on the transcriptional repressor ID2 and for γ_c cytokines in their generation from hematopoietic progenitors (reviewed in [6]). Innate and adaptive lymphocytes are derived from lymphoid-restricted progenitors in both mouse and humans; these populations possess precursor potential for T, B, NK cells and ILCs, although this property has not been rigorously demonstrated at the single cell level. ID2 is required for development of all known ILC subsets and NK cells (reviewed in [6]) whereas B and T cell development is permissive in its absence. As such, transcriptional regulation of *Id2* within lymphoid-restricted progenitors has been a focus of attention in order to help identify potential ILC precursors (ILCP). In the mouse, several groups used TF reporter mice in order to identify ILCP in the fetal liver or adult bone marrow: these included mice bearing GFP alleles in the *Id2*, *Zbtb16*, *Tcf7* or *Tox* loci [7,8,9**,10]. In every case, the characterized ILCP retained potential for NK and/or ILC1, ILC2 and ILC3 development but were not able to give rise to B, T or myeloid cells *in vitro* or after transfer *in vivo* [7,8,9**,10]. Interestingly, ILCP identified with *Id2* [7] or *Zbtb16* [8] reporters were not able to reconstitute the NK cell pool *in vivo*; in addition, PLZF-expressing ILCP were unable to generate CCR6⁺ ILC3 suggesting that at the population level, ILCP were heterogeneous and comprised both multipotent and more differentiated unipotent progenitors (Figure 1). Whether the ILCP identified by *Id2*,

Zbtb16, *Tcf7* and *Tox* expression represent distinct or overlapping subsets remains unclear, although a fraction of ID2⁺ ILCP express PLZF, some PLZF⁺ ILCP express *Tox* and TCF-1⁺ ILCP express *Tox*, *Nfil3* (a TF that activates *Id2*; [11]) and low levels of *Id2* suggesting that a fraction of ILCP express multiple TFs. It is not clear whether these multi-TF ILCP have multipotency.

The discovery of several distinct ILCP with different ILC potentials would be consistent with a model in which multipotent ILCP give rise to unipotent ILCP that have a more restricted cell fate potential (Figure 1). It is likely that environmental signals are involved in this transition and one likely regulator of this process involves the Notch pathway. Notch signals have been implicated in ILC2 and ILC3 subset development [12–14] and putative Notch targets (including *Tcf7* and *Bcl11b*) have been shown to be critical for normal development of ILC2 and ILC3 in mice [9,15**,16**,17]. Still, elucidating the transcriptional mechanisms that guide ILC differentiation remains a challenge. Single cell RNA sequencing provides the method of choice to understand the developmental relationships in complex precursor populations. This approach was recently applied to bone marrow lineage-CD127⁺a4b7⁺ cells (that are highly enriched in ILCP and ILC2; [7–9**,18]). The Liu lab delineated several related ILCP clusters as well as a developmental ILC2 trajectory [19**]. Within the ILCP populations, two clusters that apparently correspond to α LP progenitors [20] and to early innate lymphoid progenitors (EILP, [9**]) could be distinguished with dynamic *Hes1* and *Tcf7* expression suggestive of Notch signaling. Interestingly, one ILCP cluster was shown to express the inhibitory Ig superfamily member PD-1 and lineage-PD-1⁺ BM cells were shown to contain both unipotent and multipotent ILCP. Within more differentiated ILCP, a spike in *Bcl11b* expression was correlated with IL-25R expression and commitment to the ILC2

lineage. This report [19**] demonstrates the power of single cell RNA sequencing to advance our understanding of ILC developmental pathways.

The transcriptional control of ILC differentiation in mice involves more than 2 dozen identified trans-acting factors (reviewed in [6]). In contrast, our understanding of how TF condition human ILC development remains rudimentary. Multipotent lymphoid-restricted progenitors in humans with the CD34⁺CD38⁺CD127⁺CD45RA⁺ phenotype have been characterized, and include cells with T and B cell potential [21,22]. Committed NK precursors have been identified in human fetal liver, cord blood, adult bone marrow and tonsils [22], while ILC3-restricted precursors have been found in tonsils and intestinal lamina propria [23]; both of these unipotent ILCP also reside within the CD34⁺CD38⁺CD7⁺CD45RA⁺ compartment and failed to generate B or T cells *in vitro* or *in vivo* after transfer to immunodeficient hosts. Recently, the Caligiuri lab isolated a human multipotent ILCP from secondary lymphoid tissue that could give rise to NK cells *in vivo* as well as to all ILC subsets *in vitro* [24**]. Interestingly, this CD34⁺CD117⁺ cell population expressed the orphan nuclear receptor *RORC* suggesting a major role for this TF in human ILC development. Deciphering the transcriptional control of human ILC development remains a challenge, but large cohort studies [25**,26**] coupled with analysis of genomic variants (e.g.: SNPs) may provide clues to identify the key regulators of this process.

Developmental Options for NK cells and ILCs?

Several reports have demonstrated that NK cells and other ILCs (ILC1, ILC2, ILC3) have distinct developmental requirements as well as homeostatic properties. For example, NK cells do not require *Gata3* for their development [27], whereas other ILCs do [18,28–32]. As mentioned above, ID2⁺ and PLZF⁺ ILCP have the potential to generate

ILC1-3 but apparently not NK cells [7,8] suggesting a cellular basis for this dichotomy. Lastly, NK cells strongly express the TF *Eomes* (EOMES) that in concert with *Tbx21* (encoding T-BET) regulates generation of cytoplasmic granules rich in perforin, granzymes and other mediators that allow NK cells to lyse susceptible target cells [33]. As similar granules are absent from other ILCs, these findings have bolstered the notion that NK cells represent innate versions of CD8⁺ CTL, whereas other ILCs are more similar to innate versions of T_H cells. While this model remains to be validated, the observations suggest that specialization within the NK and ILC system will have biological consequences. Interestingly, a recent report from the Rudensky lab using parabiosis in mice demonstrated that unlike NK cells that freely circulate throughout the body, ILC1-3 are primarily tissue-resident cells that do not redistribute systemically [34**]. As such, ILCs have properties in common with tissue-resident memory T cells (T_{RM}; reviewed in [35]).

NK and ILC1 share surface phenotypes (NKp46, NKG2D), many functional properties (IFN- γ , TNF) but demonstrate important differences (CD127, MHC class I-specific inhibitory receptors); this topic has been the subject of an excellent recent report (reviewed in [36]). Initial studies in mice identified two distinct hepatic NK cell subsets differing in TRAIL and CD49b expression [37]; these were later recognized to represent classical EOMES⁺ NK cells (CD49b⁺) and tissue-resident CD49b⁻ EOMES⁻ ILC1 [38]. Similar tissue-resident NK cells have been also identified in thymus, intestine, skin, uterus and salivary glands (reviewed in [36,39]). While proposed to represent distinct lineages, more recent evidence from the Reiner lab suggests that NK may be generated from ILC1 following upregulation of *Eomes* [40*]. Ectopic *Eomes* expression was sufficient to convert T-BET⁺ ILC1 into CD49b⁺ NK cells that bore CD11b, KLRG1 and a restricted Ly49 receptor repertoire in both lymphoid and non-lymphoid tissues [40*].

Whether EOMES-directed conversion of ILC1 to NK cells resulted in altered tissue residency properties was not reported, but these observations suggest that ILC1 <-> NK cell plasticity may occur via EOMES modulation. It is interesting to note that unusual subsets of human liver NK cells with an EOMES⁺T-BET⁻ phenotype have recently been reported [41*,42*].

TF expression is dynamically regulated in T_{RM} cells under the influence of environmental factors, which promotes tissue residency of these effector T cells subsets [43**,44**]. TGFβ also plays an important role in the generation of T_{RM}, although the mechanism of action remained poorly defined. Recently, the Colonna lab showed that TGFβ could promote the differentiation of tissue-resident ILC1 in the salivary gland [45**]. The amount of TGFβ present in the salivary gland increased with age, and directed the balance between NK cells and ILC1 associated features. Genetic ablation of TGFβR in NKp46⁺ cells resulted in a strong decrease in CD49a⁺ ILC1 in the salivary glands; this effect operated at several levels. TGFβ signaling actively promoted CD49a expression but also up-regulated levels of CD69 and CD103 that are linked to tissue-residency [45**]. Interestingly, the TGFβ effect operated through repression of *Eomes*, allowing salivary gland ILC1 to maintain an intermediate mixed phenotype (CD49a⁺TRAIL⁺CD127⁻T-BET⁺EOMES^{lo}) that shared features with both classical NK cells and ILC1. Together, this work [45**] stresses the importance of tissue-dependent environmental signals in shaping NK cells and ILC1 development, phenotype and function.

Several TFs have been shown to be essential for ILC2 development, including the signature TF *Gata3*, but also *Rora*, *Tcf7*, *Gfi1* and *Bcl11b* [9,15,16,30,46,47]. As mentioned above, *Tcf7* and *Bcl11b* are considered as Notch-dependent targets, which can help explain the strong impact of Notch signals in promoting ILC2 development in

vitro from early lymphoid progenitors [9,14] How these different TFs guide ILC2 differentiation is not completely understood. Some TFs (e.g.: *Gata3*, *Bcl11b*, *Gfi1*) promote ILC2 reactivity by activating expression of essential cytokine receptors (IL-25R, IL-33R) that allow ILC2 to sense ‘alarmins’ within inflamed tissues and by enhancing type 2 cytokine (IL-4, IL-5, IL-13) production ([15,47]; reviewed in [48]). Recently, several reports have shown *Bcl11b* and *Gfi1* additionally operate in ILC2 via active repression of alternative ILC fates and functions. *Bcl11b*-deficient ILC2 lose *Gata3* expression and up-regulate *Rorc* [15]; this results in a shift towards an ILC3-like phenotype and function. Similarly, *Gfi1*-deficient ILC2 de-repress IL-17 production [47]. How these TFs achieve ILC3-specific repression is not clear, although it was shown that *Bcl11b* could directly silence *Ahr* expression in ILC2s [15]. Interestingly, the lysine methyltransferase *G9a* was recently identified as an important epigenetic modifier required for ILC2 development [49**]. Zaph and colleagues showed that mice with hematopoietic ablation of *G9a* had a severe reduction of ILC2 in bone marrow, lymph nodes, lung and adipose tissue. Interestingly, *G9a*-deficient ILC2 de-repressed ILC3-associated target genes, resulting in increased IL-17A production [49**]. Taken together, these studies demonstrate that TFs guide lineage-specific ILC differentiation through a combination of activating and reciprocal repressive mechanisms. Manipulation of these TF pathways in mature ILCs may provide a novel means to selectively regulate effector functions in the context of inflammation and chronic infection.

NK cell and ILC function: fixed in principle, plastic in practice

Earlier studies of T helper cells demonstrated that effector functions were not fixed but rather could be modulated by extrinsic signals such as cytokines and other growth factors (reviewed in [50]). This ‘plasticity’ was apparent for several T_H cell

subsets, including T_H17 and T_H2 cells that could produce IFN- γ after stimulation with cytokines such as IL-12 and IL-23 (reviewed in [51]). Such 'inflammatory' IL-17⁺IFN- γ ⁺ T cells have been proposed as major mediators of auto-immune disease, suggesting that deregulated T cell plasticity could be pathogenic.

In a similar fashion, several ILC subsets have been shown to have 'plastic' properties whereby environmental signals can modulate TF profiles and cytokine outputs. Initial studies in mice identified that *Rorc* expression in certain intestinal ILC3 subsets could be down-regulated generating 'ex-ILC3' that up-regulate T-BET expression thereby adopting an ILC1-like phenotype with IFN- γ production capacity [52]. This ILC3 \rightarrow ILC1 plasticity was promoted by extrinsic signals (IL-12, IL-18) and prevented by IL-7; similar studies using human ILC3 suggested that acquisition of IFN- γ secretion could be facilitated by DC- and monocyte-derived cytokines including IL-12 [52,53]. Curiously, an ILC1 \rightarrow ILC3 plasticity was also demonstrable in the human and mouse systems that was promoted by IL-1 β and IL-23 [54]. Taken together, these studies clearly identify functional plasticity of human and mouse ILC3 subsets similar to that previously observed with T_H17 \rightarrow T_H1 cells.

Recent studies using *Ncr1*-directed Cre expression revealed an unexpected phenotypic plasticity of NKp46 expression in mucosa-associated Ncr1⁺ ILC3 subsets [55*,56*]. An unusual subset of NKp46⁻ ILC3 with a previous history of NKp46 expression ('fate-mapped' NKp46 ILC3) could be identified that showed enhanced functional capacities (IL-22, GM-CSF production) and surface markers indicating distinct homing properties (CD49a, CCR6). Concerning the mechanism of NKp46 modulation, evidence was obtained that TGF β and Notch signals are involved [55*,56*]. Interestingly, perturbations in commensal communities failed to alter the proportions of 'fate-mapped' NKp46 ILC3 [55*]. Along with the work of Colonna [45**], these studies

[55*,56*] demonstrate the potent capacity of unique environmental signals (e.g. TGF β) to coordinate several distinct ILC subsets within specific tissues.

Recently plasticity has also been reported for human and mouse ILC2. Cytokine (IL-33, IL-1 β) expanded ILC2 could up-regulate T-BET expression and IFN- γ production capacity in an IL-12-IL-12R-dependent fashion. In some reports, plastic ILC2 maintained GATA3 and IL-13 producing capacity (ILC2/1), while in other studies plastic ILC2 extensively down-regulate GATA3 expression thereby resemble ILC1 [57**,58**,59**,60**]. Interestingly, IL-4 was able to prevent IFN- γ production in ILC2. IL-13+IFN- γ + plastic ILC could be detected in the inflamed gut of Crohn's disease patients [60**], while patients with severe COPD, lung ILC2 were diminished in number and ILC1 were augmented in a disease-dependent fashion [57**,58**]. Mouse studies using ST2-GFP reporter mice, showed that lung-resident ILC2 significantly down-regulated *Gata3* and type 2 cytokine production, becoming ILC1 in response to COPD-associated stimuli, including influenza virus infection, bacteria (*Staphylococcus aureus*) and cigarette smoke model [58**]. Together, these patient studies support the notion that human and mouse ILC2 can adopt an ILC1-like phenotype in inflamed tissues.

ILC 'regulomes'

The molecular underpinnings that allow segregation of ILCs into three groups are poorly understood. Recent technological advances allow the analysis of epigenetic and transcriptomic features of ILCs using extremely small cell numbers [61**,62**] or even single-cells [63**]. As such, 'regulomes' (comprehensive chromatin landscapes fashioned by epigenetic modifications and regulating gene expression) and transcriptomes have been characterized for both human and mouse ILC subsets [61**,62**,63**] that are clearly distinct from adaptive T and B lymphocytes. Interestingly, ILC

regulomes appear to be established very early in the developmental process: compared to hematopoietic stem cells and common lymphoid progenitors, the signature TF and cytokine loci associated with mature ILC subsets are already ‘poised’ well before ILC differentiation is complete. This contrasts with T_H cells where characteristic cytokine signatures are only observed after antigen-specific T cell expansion. This property of ‘poised’ chromatin may provide a molecular explanation for the rapidity by which ILC are able to respond to stimulation compared to naïve T cells.

These epigenetic approaches also highlight how the tissue microenvironment allows “decoding” of the regulome into the transcriptome. When comparing chromatin landscapes, cell subsets cluster in a predictable fashion: hematopoietic precursors (HSC, MPP, CLP) clustered together, whereas more differentiated lymphocytes segregate based on activation states and effector phenotypes. Interestingly, this was not the case when hierarchical clustering was performed on transcriptomes; in that case, cell populations primarily clustered based on whether they derived from similar tissue environments (ILC subsets in the gut clustered differently from those present in lymphoid organs) [62**]. These results indicate that the regulome only provides a framework of transcriptional potentials that is transformed under the influence of micro-environmental cues into the appropriate gene expression profile.

Amit and co-workers discovered remarkable intestinal ILC complexity using unbiased massive parallel single-cell RNA-seq (MARS-seq) approaches that overcome the limitations of bulk population analyses [63**]. While the three main ILC groups were clearly identified, subset diversity within groups was extensive (identifying ILC1a-d subsets, ILC2a-d subsets, ILC3a-e subsets) as well as several novel subsets (ILCXa, ILCXb) that failed to be categorized using the current nomenclature [63**]. The impact of microbial communities on intestinal regulomes and transcriptomes was investigated

by comparing normal mice to antibiotic-treated and germ-free mice. Interestingly, changes in bacterial loads within the gut lead to remarkable modifications of ILC subset distributions and specifically to de-repression of ILC3 properties within ILC1 and ILC2 subsets. Furthermore, ILC regulomes were also apparently under the influence of commensal derived signals that help promote ILC1- as well as ILC2-specific programs in the small intestine [63**].

Concluding remarks

ILCs continue to fascinate both basic immunologists and translational researchers due to their unique properties in immunity and their therapeutic potential in disease. Recent advances in ILC biology have shed light on the developmental pathways that allow these effector cells to emerge during lymphopoiesis and the mechanisms that regulate their potent cytokine production capacities. As ILCs show the ability to adapt to changing tissue environments, their use as stable immunomodulators in disease may pose challenges. Nevertheless, a better understanding of the signals that restrict ILC plasticity may allow for approaches that harness the potential of these novel immune subsets.

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Figure Legend

Figure 1. Generic model for NK/ILC cell development and plasticity

ILC differentiation proceeds in a stepwise fashion from hematopoietic stem cells (HSC) via lympho-myeloid-primed progenitors (LMPP) and common lymphoid progenitors (CLP) that are restricted to the lymphoid lineage. Further commitment to the ILC lineage generates multipotent ILC precursors (ILCP) including a lymphoid precursors (aLP), early ILC precursors (EILP), common 'helper' ILC precursors (CHILP), and ILCP expressing PLZF or PD-1. Subsequent specification further restricts unipotent ILCP that can only give rise to ILC1, ILC2, ILC3 or NK cells. Mature ILCs show substantial plasticity that is influenced by tissue signals, including Notch ligands and cytokines.

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