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Re(de)fining innate lymphocyte lineages in the face of cancer

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Abstract

Innate lymphocytes play critical roles in maintaining tissue homeostasis and integrity of the host at steady state and during pathogenic insults. The successive identification of new innate lymphocyte subsets has revealed an incredible diversity within the family. While this heterogeneous population can be grouped based on their cytotoxic potential into exclusively cytokine-producing helpers and cytolytic killers, the exact developmental relationships between the subsets are not fully understood. The former group is enriched at mucosal surfaces, whereas innate lymphocytes with cytotoxic potential can be identified in a wider array of tissues, including tumors. Although their cytotoxicity suggests an antitumor role, the nature of tumor-elicited innate lymphocyte responses has only begun to be investigated and the identities of participating subsets still remain contentious. In this review, we provide a brief overview of innate lymphocyte biology, review the current knowledge on their ontogeny, and discuss their roles in tumor immunosurveillance.

Introduction

The vertebrate immune system has evolved to mount distinct responses tailored to different pathogenic insults. Intracellular pathogens, such as viruses and certain bacteria, and cellular transformation events endanger the integrity of host cells. Type 1 responses characterized by pro-inflammatory cytokine production and cytotoxicity protect from such challenges by limiting the growth of and directly killing infected or stressed cells. Extracellular threats, including some bacteria, fungi, and parasites are often found at barrier sites and compete with the host for nutrients. Type 2 and 3 responses combat such challenges by mobilizing and coordinating multiple types of immune cells through cytokine production, cell-cell communication and recruitment.

Bidirectional communication between innate and adaptive immune cells initiates and orchestrates tailored immune responses. T lymphocytes, in particular, have been well characterized for their differentiation plasticity. Whereas $CD8^+$ T cells mediate direct killing of target host cells, $CD4^+$ T cells coordinate immune responses by differentiating into discrete subsets that produce distinct signature cytokines. Other lymphocytes that lack rearranged antigen-specific receptors have been identified and mirror T cells in their functional responses and differentiation requirements (1). These innate lymphocytes include the prototypic member, natural killer (NK) cells, and a diverse group of innate lymphoid cells (ILCs). NK cells are circulatory lymphocytes that directly kill target cells through the

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release of granzymes and perforin, thereby considered a counterpart to $CD8^+$ T cells (2). In contrast, ILCs are rare in circulation or secondary lymphoid organs but exist in nonlymphoid tissues as tissue-resident cells (3). Based on their transcription factor–driven and functional identities, ILCs are grouped into three classes, ILC1, ILC2, and ILC3, that mirror the helper T cell subsets (4).

Characteristics of innate lymphocytes

ILC1s and NK cells fall into the same group: type 1 innate lymphocytes. Both are characterized by the expression of the transcription factor T-bet; response to pro-inflammatory cytokines, interleukin (IL)-12 and IL18; and production of type 1 cytokines, including interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) (5). In addition, both NK cells and ILC1s express an array of activating and inhibitory receptors that detect alterations in the identity and expression of major histocompatibility class I molecules (MHCI) (6, 7). Through MHCI, intracellular information can be surveyed by the immune system. Viruses that downregulate MHCI to evade detection by $CD8^+$ T cells render the infected host cells susceptible to killing by NK cells (6).

Cellular stress induces the expression of certain MHCI-like molecules that activate NK-cell activity (6). To what extent these NK receptors regulate ILC1 functions has not been extensively studied. In contrast to NK cells, which recirculate, ILC1s are tissue resident. Although the barrier functions of ILCs have been primarily studied at mucosal surfaces, nonmucosal organs also contain sizable ILC populations. These cells are important for tissue homeostasis, and dysregulation of their activities can result in immunopathology, such as kidney ischemia and obesity (8, 9).

Type 2 innate lymphoid cells (ILC2s) require the transcription factor *Gata3* and *Rora* for development and the production of effector cytokines, IL5, 9, and 13 (10–12). ILC2s promote type-2 responses, which are important for eliminating helminth infections. Dysregulation of ILC2s can cause allergic inflammation, which are implicated in asthma, skin, and lung diseases (13).

Type 3 innate lymphoid cells (ILC3s) express the transcription factor RORγt (encoded by *Rorc*) and produce IL22 and IL17a (14). ILC3s are implicated in the maintenance of intestinal homeostasis by responding to tissue damage and alterations in microbiota, but can also play a pathogenic role in colitis (15). The lymphoid tissue inducer (LTi) cells are the prototypic member of the ILC3 family and are critical for lymphoid organogensis early in life through the expression of lymphotoxin (16). ILC2s and ILC3s are enriched at mucosal tissues, such as the intestines and lungs, whereas ILC1s are found in a wider range of tissues. Together, ILCs maintain tissue homeostasis and promote immune responses against pathogenic insults.

Innate lymphocyte development

Of the three classes of ILCs, ILC2s are the most homogeneous, and have an identified committed progenitor in the bone marrow (17–19). ILC3s, although more heterogeneous than ILC2s, share a core signature gene expression program driven by RORγt (encoded by

Rorc) (7). A common progenitor for all ILCs has been identified as expressing both the transcription factor PLZF and the surface marker PD1 (18, 20). This population has lost LTi potential, suggesting that, although expressing RORγt, LTis are a distinct lineage of ILCs from ILC3s.

The ontogeny of group 1 innate lymphocytes, which include NK cells and ILC1s, is still highly contentious. Both cell types share many features that underlie their classification into group 1 innate lymphocytes. Although extensive gene-expression profiling revealed only a small set of genes whose expression pattern can distinguish between NK cells and ILC1s (7), fate mapping and adoptive transfer experiments conclusively demonstrated that NK cells and ILC1s are distinct lineages of lymphocytes (17, 20). Although both are derived from the common lymphoid progenitor (CLP) and downstream early innate lymphocyte progenitor (EILP) (21), ILC1s develop from common helper innate lymphoid progenitors (CHILPs) (17), which have lost the potential to differentiate into NK cells. The identity of the NK cell–restricted progenitor in mouse is still unknown (Fig. 1). Although NK progenitors (NKPs), including the pre-NKP and the refined NKPs (rNKPs) can readily differentiate into $NK1.1^{+}NKp46^{+}$-expressing cells, whether this population still retains ILC1 potential is unclear.

Parabiosis experiments suggest that ILCs are tissue resident and seed the tissues early in life without much input from the circulation later (3). Although it remains possible that differentiated ILCs self-renew to maintain its population, it is also likely that multipotent ILC progenitors seed the tissue and differentiate to replenish the ILC compartment as the organism develops. The latter possibility has been implicated in humans. A tissue-residing innate lymphoid cell progenitor (ILCP) has been identified that can give rise to cytotoxic innate lymphocytes and all ILCs *in vitro* and when adoptively transferred into lymphopoenic mice (22). These ILCPs can also be found in circulation (22), although to what extent the circulating ILCPs contribute to the maintenance of the ILC compartment is unclear. The mouse counterpart of the circulating ILCP in human has yet to be identified, but the ILC2 pool can be moderately replenished from circulation in response to *N. Brasiliensis* infection. It remains unclear whether these newly recruited ILC2s developed from differentiated or multipotent progenitors from, if at all, the bone marrow (3).

Tissue-resident cytotoxic innate lymphocytes

The tissue-resident innate lymphocytes are a heterogeneous population that can be further delineated by the expression of CD127 (the IL7 receptor). $CD127^{hi}$ ILCs, found in the small intestine lamina propria are noncytotoxic cytokine producers (7, 17), whereas the $CD127^{lo/-}$ innate lymphocytes, residing in the liver, salivary gland, mammary gland, and small intestine epithelium express granzyme B as well type 1 cytokines to various degrees (23–27) (Fig. 1). Fate mapping experiments using *Rorc*-Cre revealed that a substantial fraction of $CD127^{hi}$ ILC1s in the small intestine lamina propria are ex-RORγt-expressers, or ex-ILC3s (17). Conversely, $ROR\gamma t^{-}$ ILC1s can upregulate *Rorc* and produce ILC3-signature cytokines when cultured under polarizing conditions as shown *ex vivo* with human ILCs or under adoptive transfer settings in mice (21, 28, 29). Thus the functional plasticity of ILC1/3s is reminiscent of the T_H1/T_H17 interconversion in the gut and during autoimmune

neuroinflammation. Notably, among the *Rorc*-Cre fate-mapped ILCs in the small intestine lamina propria, the vast majority are in fact CD127^{hi}, indicating that high CD127 expression marks a subset of noncytotoxic cytokine-producing ILC1s that can interconvert with ILC3s (17) (Fig. 1). These CD127^{hi} ILC1s with exclusively helper functions are hereafter designated ILC1h.

In the liver, the CD127$^{lo/-}$ type 1 innate lymphocytes can be further subdivided based on the expression of two integrin molecules, CD49a and CD49b. The tissue-resident CD49a^{+} population expresses substantially more granzymes than the circulating CD49b^{+} conventional NK cells (7). These CD49a^{+}CD127$^{lo/-}$ type 1 innate lymphocytes have been formerly named tissue resident NK (trNK) (25). However, recent evidence points to their closer relationship to ILC1s than NK, based on their developmental requirements. Genetic studies showed that NK cells strictly require the transcription factor *Nfil3* and *Eomes* for development and maintenance, whereas CD127^{hi} ILC1s are unaffected by *Eomes* deficiency (25, 30). The liver-resident CD49a^{+}CD127$^{lo/-}$ innate lymphocytes develop independently of *Nfil3* and *Eomes*, supporting their closer lineage relationship to ILCs than NK cells (25, 30). In addition, CD49a^{+}CD127$^{lo/-}$ innate lymphocytes appear to share a similar progenitor with all other ILCs, collectively named CHILP (17). A fraction of CHILP, highly expresses PLZF and PD1 and retains potential for all CD127^{hi} ILCs, but not LTis. These cells can also give rise to liver-resident innate lymphocytes at a much higher frequency than giving rise to conventional NK cells in both adoptive transfer settings and during the steady state (18, 20). Although by this criteria, the vast majority of liver CD49a^{+} innate lymphocytes are ILC1s, whether circulating NK cells can also upregulate CD49a, downregulate *Eomes*, and acquire a tissue-resident program is still unknown. Before such a possibility is ruled out, for instance, with cell-fate tracing experiments, the term “trNK” is better kept to distinguish this NK-derived population from CHILP-derived PLZFCre-fate mapped ILC1s (Fig. 1).

All CD127^{hi} helper ILCs require the transcription factor *Gata3* for development (31). However, liver ILC1s, commonly deemed as the prototypical helper ILC1s, are unaffected by *Gata3* deficiency (25). A striking parallel is seen during thymocyte development, in which deletion of *Gata3* at the double-positive stage results in the specific loss of CD4^{+}, but not CD8^{+}, mature thymocytes (32, 33), suggesting that the *Gata3*-dependent specification of the helper program may be a common theme in lymphocyte development. Although the exact developmental stage at which *Gata3* acts to specify helper ILC differentiation is still unknown, current data suggest a dichotomy of tissue-resident type 1 innate lymphocytes into *Gata3*-dependent exclusive cytokine producers, or helper CD127^{hi} ILC1s (ILC1hs) as typified in the intestine, and *Gata3*-independent cytotoxic killer CD127$^{lo/-}$ ILC1s (ILC1ks), first identified in the liver. Although both develop from the PLZF^{+} fraction of CHILP, it remains possible that the PLZF^{+} population is heterogeneous and contains distinct progenitors for ILC1hs and ILC1ks. Alternatively, the two types of ILC1s may share a common progenitor and the fate choice may diverge later in a *Gata3*-dependent manner. It is also possible that a yet to be identified non-NK, non-helper ILC progenitor downstream of EILP specifically gives rise to ILC1ks. Whether ILC1hs can gain cytotoxic potential and contribute to the ILC1k pool in the periphery is unclear either (Fig. 1). Human intestinal CD127^{hi}, but not CD103^{+}CD127$^{lo/-}$, ILC1s can differentiate into ILC3s (29), suggesting

against the possibility of peripheral conversion from ILC1h to ILC1k. Whether the lack of plasticity is a general feature for mouse ILC1hs and ILC1ks needs more rigorous testing.

Tumor-infiltrating innate lymphocytes

Tumors present a challenge to the immune system, because tolerance mechanisms constitutively curb immune responses against self. Effective antitumor immune responses require the immune system to detect and destroy self-derived cancer cells. Although high mutation loads in tumors generate immunogenic neoepitopes rendering transformed cells susceptible to recognition and destruction by the adaptive immune system, not all tumors carry such mutations (34, 35). Early stage pre-cancerous lesions in particular may not harbor many mutations, making their detection by the adaptive immune system difficult. Tumors with loss-of-function mutations in antigen presentation pathways are invisible to conventional cytotoxic T cells, even if they harbor neoepitopes (36). Thus, the adaptive immune system may miss early transformation events, and neoepitope-dependent antitumor responses by adaptive lymphocytes are ineffective against tumors with low somatic mutations. Indeed, in a spontaneous oncogene-driven breast tumor model, $CD8^+$ T cells fail to restrain early tumor growth. Rather, innate lymphocytes appear to be essential for early tumor immunosurveillance (26). In other tumor models, depletion of $NK1.1^+$ cells accelerated tumor growth (37–39), further supporting an antitumor role of group 1 innate lymphocytes. Thus, group 1 innate lymphocytes utilize a nonredundant immunosurveillance mechanism.

Although $NK1.1^+$ innate lymphocytes in the tumors are implicated as the major contributors to antitumor immunity, the ontogeny of these cells is still unresolved. In the PyMT-driven breast tumor model, all $NK1.1^+$ innate lymphocytes are $CD127^–$, and can be further divided into two populations, based on the expression of the two integrins, CD49a and CD49b. The $CD49a^–CD49b^+$ subset among the $NK1.1^+$ innate lymphocytes are conventional NK cells as they recirculate and are strictly dependent on *Nfil3* (26). In contrast, the $CD49a^+CD49b^{+/–}$ $NK1.1^+$ innate lymphocytes are tissue-resident and only moderately reduced in *Nfil3*-deficient mice (26). Furthermore, the core ILC1 gene expression signature defined by liver-resident $CD49a^+$ $NK1.1^+$ innate lymphocytes is also shared by intratumoral $CD49a^+$ $NK1.1^+$ innate lymphocytes (26). These data collectively suggest that tumor-infiltrating $CD49a^+$ $NK1.1^+$ innate lymphocytes resemble the $CD49a^+$ $NK1.1^+$ cytotoxic innate lymphocytes in the liver and salivary glands, thus designated ILC1-like cells. Whether these tumor-infiltrating ILC1-like cells are also *Gata3*-independent, like the liver ILC1ks, remains to be tested. Although both NK cells and ILC1-like cells are cytotoxic, ILC1-like cells, but not NK cells, acquire the integrin CD103 whose ligand, E-cadherin, is abundantly expressed by tumor cells (26). CD103 assists in granule positioning and release during tumor cell lysis (40). These results suggest that although both tumor-infiltrating NK cells and ILC1-like cells can kill transformed targets, they mediate cancer immunosurveillance through distinct mechanisms.

In the PyMT-driven breast tumor model, IL15-deficient animals showed accelerated tumor growth at a time point earlier than mice lacking $CD8^+$ T cells (26), suggesting a contribution from $NK1.1^+$ lymphocytes, but not $CD8^+$ T cells for restraining early tumor growth.

Notably, mice deficient for *Nfil3*, despite lacking conventional NK cells, did not show accelerated tumor growth, implying a dominant role of ILC1-like cells, rather than conventional NK cells, in mediating early tumor immunosurveillance (26). This indirect evidence would be aided by more genetic tools that specifically target NK cells or ILC1-like cells, to finely tease out their relative contribution to antitumor immunity.

Beside oncogene-driven cancer models, type 1 innate lymphocytes are also reported in transplantable tumor models (41–43). A population of $CD127^{-}CD49a^{+}$ $NK1.1^{+}$ innate lymphocytes was found in tumor explants and carcinogen-induced sarcoma. The majority of them co-express CD49b and *Eomes*, phenotypically resembling the ILC1-like cells found in breast tumors and salivary gland (41). The study proposed that these tumor-infiltrating and liver $CD49a^{+}NK1.1^{+}$ innate lymphocytes are converted from circulating conventional NK cells by tissue-derived signals, such as IL15 and TGFβ, based on adoptive transfer experiments (41). However, adoptive transfer experiments of CHILP already definitively demonstrated that conventional NK cells and all $CD127^{+}$ ILCs, as well as liver ILC1-like cells, develop from distinct progenitors, making NK to ILC conversion at the steady-state an unlikely event (17, 20).

The apparent discrepancy between the report by Gao, *et al* and the current paradigm highlights an outstanding problem in the field, namely, the lack of lineage-defining markers for NK cells and ILC1s. *Eomes* expression is widely accepted as a convenient marker to distinguish NK cells from ILC1-like cells in the liver, but such a distinction is difficult to draw when one looks beyond the liver. In the salivary gland and PyMT-driven tumors, both $CD49a^{+}$ non-NK ILC1-like cells and NK cells express *Eomes*. Hence, *Eomes* expression alone does not exclusively define the NK lineage. *Eomes* expression can be suppressed by TGFβ signals (23), so ILC1s can not be reliably defined by the lack of *Eomes* expression. Taken together, these data present an alternative interpretation of the results by Gao, *et al*: NK cells adopt ILC1-like phenotypes rather than trans-differentiate into ILC1s under certain conditions, particularly during lymphopenia with a rich cytokine environment. Indeed, active IL15 and TGFβ signaling promote CD49a and CD103 expression by NK cells (23, 41). Although these findings failed to resolve the lineage relationships between NK cells and ILC1-like cells, they nevertheless raise the possibility that the tissue-resident $CD49a^{+}$ ILC1-like population in breast tumor models may be heterogeneous with some contribution from conventional NK cells. Further fate-mapping experiments are needed to address this hypothesis.

Whether innate lymphocytes assume a pro- or anticancer function varies between tumor models. In patients with high-grade serous cancer, the presence of $CD56^{+}$ type 1 innate lymphocytes is associated with suppressed T cell responses, implying an immunomodulatory role (44). Altered expression or mutations in NK activating receptors on type 1 innate lymphocytes have also been reported to correlate with increased metastasis and progressive tumor development (45–47), although whether defective innate lymphocyte responses are the causes of tumor progression remains to be tested. In mouse models, although conventional NK cells are dispensable for constraining PyMT-driven tumor growth, they exhibited some antitumor effects in transplantable or carcinogen-induced cancer models. This discrepancy is likely caused by the natural distribution of resident ILC1-like

cells in breast tissues. Their proximity to transformation events and increased cytotoxic potential allows them to survey the epithelium more efficiently than do NK cells. In contrast, tumor explants acutely disrupt the tissue structure and their aggressive growth may not allow enough time for a resident ILC population to co-develop. Thus, this paucity of surveillance by cytotoxic lymphocytes necessitates strong NK cell responses to mediate early control of tumor growth in transplantable tumor models. Carcinogen-induced sarcomas are enriched for neoepitopes and elicit a strong adaptive immune response. NK cells may in this case mediate antibody-dependent cytotoxicity through their Fc receptors binding to antibodies deposited on tumor cells.

Concluding remarks

As one of the latest additions to the lymphocyte family, the discovery of ILCs provided valuable insights into how lymphocytes evolved, and at the same time prompted a re-evaluation of lymphocyte-mediated immune responses. Although cytotoxic innate lymphocytes have been implicated in antitumor immunity, the precise ontogeny of these cells is still unresolved. Although identification of a committed ILC progenitor excludes the interconversion between NK cells and $CD127^+$ ILC1hs or liver ILC1ks, direct evidence that ILC1-like cells in other tissues represent a distinct lineage from NK cells is still lacking. Although it is comforting that various human tumors elicited cytotoxic innate lymphocyte responses similar to those reported in murine models, the precise origin of these CD56-expressing cells is all the more confusing. Given the important yet controversial role of cytotoxic innate lymphocytes in tumors, understanding the ontogeny of these cells may allow for novel therapeutic opportunities aimed at manipulating their activity to restrain tumor growth.

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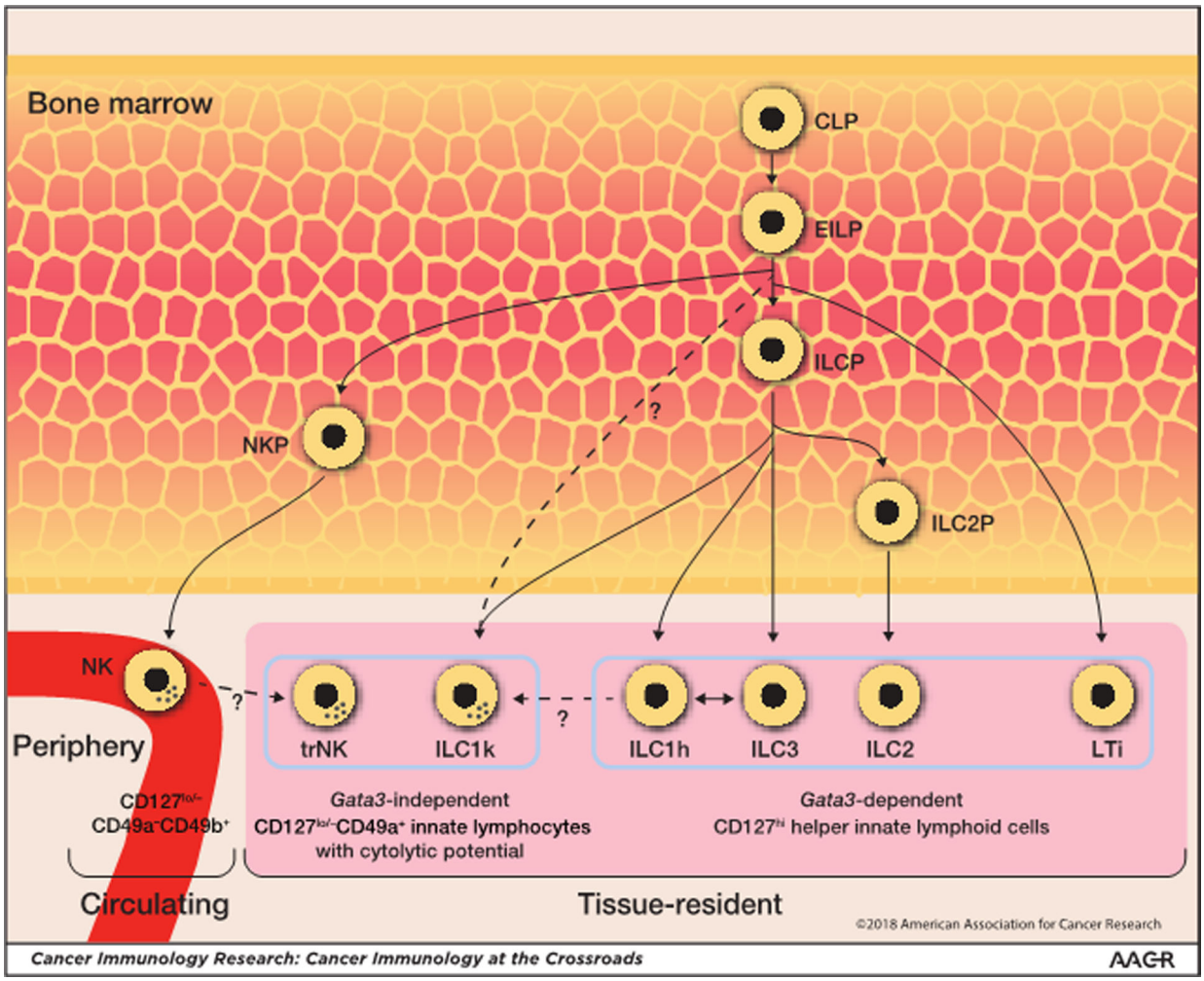

Figure 1. Ontogeny of innate lymphocytes.

The common lymphoid progenitor (CLP) gives rise to all lymphocyte lineages. Downstream of CLP, the early innate lymphocyte progenitor (EILP) can develop into all innate lymphocyte lineages but not T or B cells. Circulating NK cells develop from a yet to be identified NK-restricted progenitor (NKP) whereas tissue-resident innate lymphocytes are derived from $PLZF^{+}$ innate lymphoid cell progenitors (ILCP), which do not have NK cell potential. The $PLZF^{+}$ population contains multipotent progenitors for all $CD127^{hi}$ helper ILCs. There also exists a group of tissue-resident $CD127^{lo/-}$ innate lymphocytes with cytolytic potential, although their lineage identity is not fully understood. This population of innate lymphocytes may be heterogeneous, receiving inputs from both the ILC lineage, as demonstrated for the ILC1ks in the liver, and tissue-resident NK (trNK) cells, via *in situ* differentiation from circulating mature NK cells. Alternatively, a yet to be identified progenitor downstream of EILPs may exist that specifically gives rise to ILC1ks, but not helper ILCs or NK cells. In the periphery, it also remains unknown whether ILC1hs can acquire cytotoxic potential.