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Diversity of human NK cell developmental pathways defined by single-cell analyses

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Abstract

Human natural killer (NK) and innate lymphoid cells (ILCs) include diverse specialized phenotypic and functional subsets that reflect their roles as innate immune effector cells present in tissue and circulation. In recent years, significant advances have been made in better defining their tissue resident phenotypes, developmental pathways, and phenotypic plasticity. Here we offer a brief review of new insights into human NK cell diversity specifically defined by next generation sequencing and single-cell transcriptomic studies and integrate these into our current models of human NK cell developmental trajectories and mature subsets. These studies highlight both a deeper understanding of innate lymphoid cell differentiation and homeostasis and underscore critical questions that remain outstanding in the field.

Keywords

natural killer cell; hematopoiesis; scRNA-Seq

Introduction

Human natural killer (NK) cells can be isolated from multiple tissues including peripheral blood (PB), liver, spleen, bone marrow, lung, lymph node, and uterus [1-3]. While it is becoming increasingly evident that many of these sites can host both tissue resident and transitory cells, the primary sites of NK cell development are most frequently defined as bone marrow and secondary lymphoid tissue (SLT) [4-8]. Bone marrow is the site of adult hematopoietic precursors that give rise to common lymphoid precursors from which innate lymphoid cells (ILCs) and NK cells are generated. The isolation of an early NK cell precursor and NK cell developmental intermediates from secondary lymphoid tissue suggests that an early precursor exits from the bone marrow and seeds SLT [6,8]. In addition to the earliest $CD34^{dim}$ $CD45RA^{+}$ integrin $\beta7^{bright}$ precursor that is found in PB and SLT

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Declaration of interests

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[8], more restricted ILC precursors are also found at low frequency in circulation [9-11]. While the identification and classification of innate lymphoid cell subsets have greatly increased our understanding of NK cell ontology [12,13], many outstanding questions remain about the trajectory and sites of human NK cell development.

Human NK cell development is frequently described as a linear model beginning with the earliest precursor (stage 1) and culminating in terminally mature $CD56^{dim}$ $CD16^{+}$ (stage 5) or $CD56^{dim}$ $CD16^{+}$ $CD57^{+}$ (stage 6) cells [14]. Briefly, stage 1 cells are defined as $CD34^{+}$ $CD117^{-}$ $CD94^{-}$ precursors, found in bone marrow and SLT, that are not responsive to IL-15 [15]. Stage 1 NK cells gain CD117 expression and become IL-15 responsive stage 2 cells, yet retain multilineage potential and can give rise to T cells or DC cells in addition to NK cells when cultured *ex vivo* [6]. Stage 3 cells are defined by loss of CD34 expression and commitment to the NK/ILC lineage, with CD56 expression marking divergence of conventional NK cells and ILC3 precursors and CD56 negative cells representing the emerging ILC2 population [10]. At stage 4, NK cells become $CD56^{bright}$ and start expressing NK cell markers and cytokines, and further commit to the conventional NK cell lineage as stage 4B NK cells can be defined by acquisition of NKp80 [16]. About 10% of NK cells in PB are stage 4B $CD56^{bright}$ cells, whereas terminally differentiated stages 5 and 6 NK cells are the predominant subsets found in PB where they comprise ~90% of NK cells [16-18]. Stages 4 to 6 NK cells are also present in tissue, such as lung, gut, and tonsil, where they express NK cell maturation markers similar to corresponding NK cell subsets in PB. However, despite phenotypic similarities between PB and tissue-resident NK cells, unique transcriptional signatures reveal the cellular environment as a source of NK cell heterogeneity within the same apparent developmental stage [5,19,20].

With the identification of ILC subsets and further dissection of the stages of human NK cell development, previous linear models of NK cell development have given rise to branching models that represent both lineage plasticity and dominant lineage trajectories. Single-cell transcriptomics studies have particularly provided important insight into the relationship between NK cell subsets, particularly in bone marrow, which is thought to be the primary site of human NK cell development, and PB, a common source of circulating mature NK cells. Similar studies have used single-cell approaches to describe the heterogeneity of ILC1, ILC2, ILC3, NK cells, and lymphoid tissue inducer (LTi) ILCs in different organs [2,13,20]. Since ILC subtypes and NK cells share many cell surface markers and exhibit lineage plasticity, it is important to delineate ILC and NK cell subsets when considering single cell datasets. Most notably, NK cells are the only cytotoxic ILC subset and can be distinguished by expression of molecules relating to effector function. In addition, the development of ILC subsets depends on distinct transcription factors; ILC1, ILC2, ILC3 require GATA3, whereas NK cell development depends on EOMES [21]. Recent reviews dissect such ILC diversity and their developmental landscape in relation to conventional NK cell subsets in detail [12,21,22]. Here, we will keep our focus on understanding the heterogeneity of conventional NK cells and their precursors.

Redefining hematopoiesis with single-cell sequencing

Hematopoiesis is classically described as a tree-like model where HSCs unidirectionally give rise to more lineage-restricted progenitors leading to terminal differentiation [23]. This model is commonly supported by experiments in which specific subpopulations of blood cells are isolated with antibodies and differentiated to show their limited lineage potential. Recent scRNA-seq studies have revealed a more continuous transcriptional differentiation landscape that lacks classically defined precursor stages (Fig. 1) [24-28], (reviewed in [29]). The differences between more discrete surface marker expression patterns and transcriptome-wide measures that define heterogeneous intermediates suggest that cells undergo much more subtle and complex transcriptional calculation that precedes a surface marker phenotype. Single-cell sequencing technologies enable us to study such lineage priming at high resolution in addition to defining cellular heterogeneity and trajectories of development that may not be inferred by lineage potential experiments.

With this in mind, we can consider what such experiments tell us about the developmental trajectory of human NK cells. Several studies have performed scRNA-seq to interrogate hematopoiesis using bone marrow cells from a single time point. Due to the relatively fast turnover and abundance of precursors in the bone marrow, most steps from progenitor to mature blood cells can be captured without sequential sample collection and aligned along pseudotime [27,28]. However, as shown in the bone marrow human cell atlas data [27], T cells and NK cells appear disconnected from the bone marrow landscape that identifies developmental lineages from the HSCs. Such discontinuity signifies that due to the spatial separation of the developing T cells in the thymus and NK cells presumably in SLT or other tissues, intermediate populations are extremely rare in the bone marrow, which presents a unique challenge in mapping their developmental landscape. Whether this discontinuity supports extramedullary sites of NK cell development as the dominant sites in adults, or is a technical feature of not sequencing enough cells to define intermediate populations, is unclear.

Capturing NK cell developmental subsets

Murine NK cells are thought to predominantly develop in the bone marrow (reviewed in [30]). The trajectory of murine NK cell development has been captured by isolating $CD3e^{-}$ $CD122^{+}$cells, thought to contain both immature and mature NK cells, and performing scRNA-seq [31]. This approach identified five NK developmental subsets: one immature, one mature, and three transitional NK cell groups, and further defined mTORC1 and mTORC2 signaling as key determinants of NK cell maturation dictating movement between immature and transitional stages of development [31].

As human NK cells have been shown to develop in other sites besides bone marrow, fully understanding human NK cell development requires sequencing of multiple organs. Crinier et al. [32] performed scRNA-seq on NK cells from the spleen to enable comparison between mouse and human splenic NK cells. The earliest ($CD56^{neg}$) NK cell precursors would not have been included in this study, as $CD56^{+}$ cells from the human spleen were sequenced. Unbiased analysis demonstrated that the cells isolated and analyzed

were primarily mature NK cell subsets analogous to $CD56^{bright}$ and $CD56^{dim}$ subsets [32]. However, as most NK cells detected by flow cytometry in spleen are stages 4 and 5 ($CD56^{bright}$ and $CD56^{dim}$) [4], it is not surprising that earlier stages of NK cell development were not identified. Similarly, a comparison of ILC subsets from human tonsil included $CD45^{+}Lin^{-}CD56^{+}CD127^{-}NKG2A^{+}CD16^{-}$ NK cells, which enabled the single-cell transcriptomic analysis of NK cells, but not early NK cell precursors [13]. Bulk RNA-seq data of early NK precursors from tonsil and blood have outlined developmental trajectories using phenotypically sorted subsets (Figure 1) [10,32]. However, scRNA-seq in this space is still required to populate this landscape further and delineate the developmental process.

While the trajectory of mature human NK cell development has not yet been captured in high resolution, a recent study has defined embryonic human NK cell trajectories within the fetal liver and bone marrow without discontinuity [33]. Using scRNA-seq, the authors show a smooth trajectory from hematopoietic stem cells and multipotent progenitors to NK cells and other hematopoietic cell types. Interestingly, while NK cells were identified, neither T cells nor ILCs were found in fetal bone marrow, validating the observation that T cell development is occurring in the thymus and further suggesting that fetal ILC development may similarly be occurring at other tissue sites. The differentiation trajectory analysis defined highly proliferative lymphoid-myeloid progenitors (LMPs) expressing genes that include *IGLL1*, *HMGB2*, and *CD79B* that were positioned upstream of mature NK cells and B cells, monocytes, and plasmacytoid dendritic cells. This study demonstrates how data from multiple donors and organs can be integrated to generate a comprehensive view of NK cell development. Collecting enough NK cells and progenitors from SLT without enrichment or FACS sorting presents a significant challenge due to their low frequency [34]. For example, a scRNA-seq experiment from mouse spleen with minimal selection defined 230 NK cells from 9,552 sequenced cells [35]. Therefore, strategic gating with developmental markers and pooling samples from multiple donors, as was done to interrogate fetal bone marrow and liver, may be necessary to overcome this difficulty but would likely provide a deeper understanding of the trajectory of human NK cell development in adult tissue.

Mapping diversity of mature NK cell subsets with novel transcriptional signatures

The diversity of NK cells at the protein level has been demonstrated at a single-cell level with high-dimensional flow cytometry and cytometry by time-of-flight (CyTOF) [3,36]. Single-cell transcriptomics provides a way of further dissecting the cells with transcriptome-wide measures and gaining a more comprehensive view of the identified subpopulations. Single-cell transcriptomics as applied to NK cell biology has been most frequently used to better understand the identity and origins of mature NK cell subsets. Crinier et al. generated scRNA-seq datasets from spleen, blood, and bone marrow NK cells to better define and understand the structure and heterogeneities of NK cell subsets [32,37]. In both humans and mice, NK cells most prominently clustered according to their tissue of origin, consistent with other studies using bulk RNA-Seq showing that phenotypically equivalent subsets from tissue and blood have unique transcriptional profiles [5,32]. Unbiased analysis revealed two clusters of human NK cells in the blood, hNK_Bl1, and hNK_Bl2, corresponding to

known subsets of more cytotoxic CD56^{dim} and cytokine producing CD56^{bright} populations, respectively [5,32]. Spleen and bone marrow NK cells also showed cell clusters that aligned with CD56^{dim} and CD56^{bright} cells, but more importantly, showed additional subpopulations of relatively smaller sizes. The characteristics and functionality of the bone marrow subsets have been debated, but the clustering of subgroups is robust [37-39]. Gene ontology of the differentially expressed genes indicates that the differences between these NK cell subtypes are likely to be biologically meaningful.

The massively parallel and unbiased nature of scRNA-seq also enables discovering new transcriptional signatures within subpopulations, which can provide a robust way to identify CD56^{+} NK cells from other datasets with mixed cell populations. Crinier et al [32] used correlation with NKp46 expression to define a consensus NK cell gene signature with 13 genes. The NK core gene list from humans contains activating receptors (*CD160,KLRC3* [NKG2E], *CD244* [2B4], *KLRF1* [NKp80]), the inhibitory receptor *KLRC1* [NKG2A], antimicrobial proteins (*GNLY*), cytolytic proteins (*PRF1*), inflammatory cytokines (*XCL2*), and transcription factors, recapitulating the functional characteristics of NK cells. The consensus list for mouse NK cells has genes from similar categories; however, only two of the genes are shared between humans and mice (*IL18RAP, PRF1*). Such differences emphasize how human and mouse immune systems have divergently evolved to adapt to their environment while conserving the biological identity of NK cells.

Expression patterns of well-characterized genes are often used to define NK cell subpopulations. For example, *IL7R*, *SELL*, *GZMK*, and *KLRC1* are commonly used to identify CD56^{bright} NK cells [40,41]. In addition to known marker genes, clustering analysis reports new transcriptional signatures for each subpopulation. In particular, Yang et al. [41] identified CD44 and XCL1 as novel markers for CD56^{bright} NK cells, and Smith et al. [40] discovered a shared signature between CD56^{bright} and terminally differentiated NK cells; CD69, DUSP1, FOS, and JUN. Single-cell sequencing also provides an opportunity to identify smaller subpopulations that were impossible to detect in bulk data processing. Two studies have sequenced a greater number of blood NK cells after enriching them from PBMCs (3061 cells, [41]; 8462 cells, [40]) and further dissected this population into as many as seven clusters. In addition to CD56^{bright} cells, both studies dissect CD56^{dim} populations into active, mature, and terminally differentiated populations. Smith et al., with more than 8000 cells, describe additional minor populations, including type1 INF responding cells, cytokine-induced memory like (CIML) cells, and a novel NK cell population with decreased expression of ribosomal genes [40]. Smith et al. sequenced the greatest number of cells and included a cytokine-induced activation condition that increased the dimensionality of the data. With an *in silico* down-sampling experiment, they demonstrate that processing a lower number of cells results in a smaller number of clusters where the minor cell populations get absorbed into larger populations. This result highlights the importance of depth of the sequencing and breadth of the sample collection in discovering rare novel subsets with confidence.

Moving beyond the transcriptome

Recent studies have explored epigenetic mechanisms that shape NK cell heterogeneity by pairing sorted bulk RNA-seq with ChIP-seq or ATAC-seq [42,43]. These studies demonstrate that NK subpopulations are transcriptionally and epigenetically distinct and further identify the transcription factor Bcl11b, or an axis of transcription factors (TCF1-LEF1-MYC axis), that shape NK cell subpopulations [42,43]. Epigenetic surveys at the single-cell level are becoming available to show another aspect of such observations. Ranzoni et al. [33] performed scRNA-seq and scATAC-seq on fetal human liver and bone marrow cells. The authors discovered further heterogeneity within transcriptionally homogenous HSCs and multipotent progenitors. Specifically, ATAC-seq data revealed that HSCs/MPPs could have different activity levels of transcription factors that prime cells at promoters before lineage commitment can be transcriptionally detected. This study demonstrates how single-cell multi-omics studies can reveal a new level of heterogeneity within hierarchical epigenetic mechanisms that precedes transcriptional readout and adds further complexity to our re-designed model of hematopoiesis. Further, these multi-omics studies highlight how bulk sequencing, with its depth, can be used to detect and reveal the subtle differences between known subpopulations, and single-cell sequencing, with its breadth, can complement to reveal further heterogeneity within a cell population.

Conclusions

Hematopoiesis and immune cell development are processes of making cells of ever-increasing heterogeneity. With scRNA-seq, we have started to perceive the variety of cell types, developmental intermediates, and other aspects that were not previously described. As other single-cell sequencing technologies mature, we are gaining further insight into the transcriptomic and epigenetic complexity of developing NK cells. We now must move beyond observations to gain a better understanding of the functional significance of this complexity. In addition, as human NK cells undergo trafficking during development, multiple organs must be analyzed to map the developmental landscape. Finally, the spatial distribution of NK cells and their precursors in tissue, and their local cell-cell interactions, must be investigated to fully understand the complexity and road map of developing NK cells.

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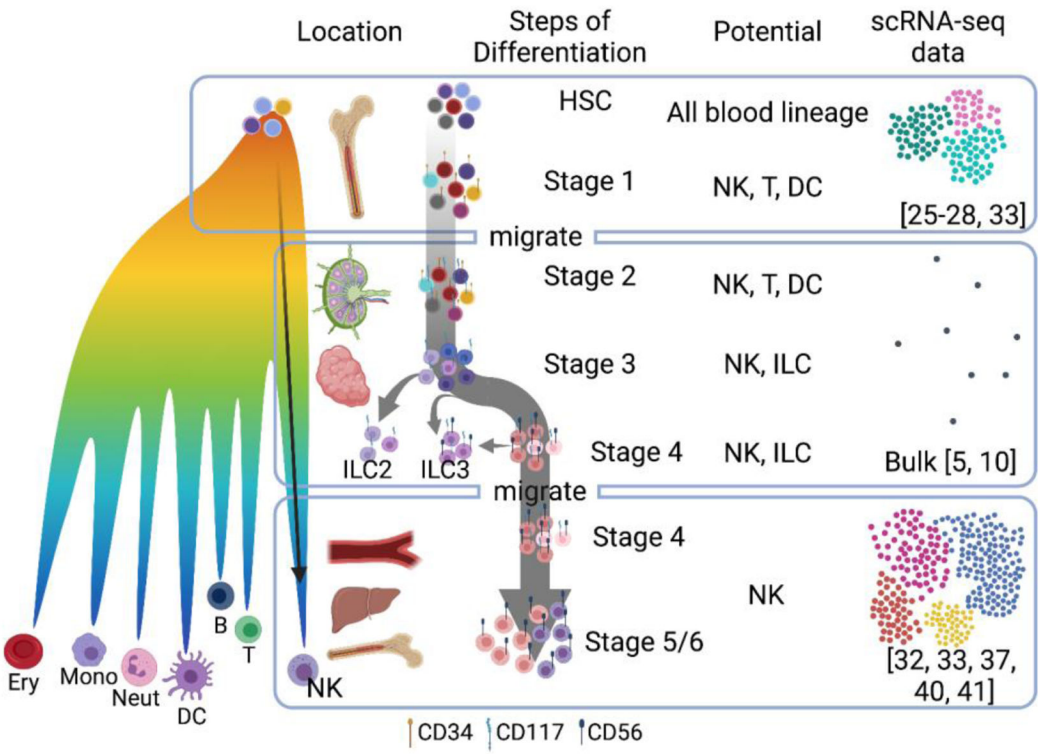

Figure 1. scRNA-seq reveals the developmental landscape of NK cells.

Left: a revised model of hematopoiesis with continuous cell states. Right: our current knowledge of NK cell differentiation steps including references for human datasets discussed in the text.