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Human natural killer cell development in secondary lymphoid tissues

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Abstract

For nearly a decade it has been appreciated that critical steps in human natural killer (NK) cell development likely occur outside of the bone marrow and potentially necessitate distinct microenvironments within extramedullary tissues. The latter include the liver and gravid uterus as well as secondary lymphoid tissues such as tonsils and lymph nodes. For as yet unknown reasons these tissues are naturally enriched with NK cell developmental intermediates (NKDI) that span a maturation continuum starting from an oligopotent CD34⁺CD45RA⁺ hematopoietic precursor cell to a cytolytic mature NK cell. Indeed despite the detection of NKDI within the aforementioned tissues, relatively little is known about how, why, and when these tissues may be most suited to support NK cell maturation and how this process fits in with other components of the human immune system. With the discovery of other innate lymphoid subsets whose immunophenotypes overlap with those of NKDI, there is also need to revisit and potentially re-characterize the basic immunophenotypes of the stages of the human NK cell developmental pathway *in vivo*. In this review, we provide an overview of human NK cell development in secondary lymphoid tissues and discuss the many questions that remain to be answered in this exciting field.

Introduction

The natural killer (NK) cell is the prototypic innate lymphoid cell (ILC) whose functional capabilities have evolved to provide direct antimicrobial and antitumor protection as well as indirect immunomodulation through the production of soluble chemokines and cytokines. NK cells are important if not necessary in a variety of clinical settings, and gaining a

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comprehensive understanding of how these cells are derived in humans can facilitate the development of targeted therapies to boost NK cell effector function for patients with cancer and/or immune deficiency, can allow us to better evaluate the downstream effects of therapeutic interventions, and can also provide insights into how NK cells undergo malignant transformation.

Though it was discovered more than twenty years ago that human NK cells can be derived *in vitro* from purified CD34⁺ hematopoietic precursor cells (HPC), the *in vivo* NK cell developmental pathway has remained somewhat of a mystery in relative comparison to the pathways for B cell and T cell development¹. For decades it was generally accepted that NK cells develop exclusively within the bone marrow (BM) similar to most other leukocyte populations other than T cells^{2,3}. BM ablation results in NK cell deficiency in mouse models, and human NK cells may be derived *in vitro* from BM-derived CD34⁺ HPC on BM-derived stroma or in BM stroma-derived cytokines⁴⁻⁹. Human BM has also recently been shown to contain CD34⁻ NK cell developmental intermediates (NKDI)¹⁰. Interestingly, NK cell precursors are normally detected in the circulation, and recent data indicate that specific CD34⁺ NK cell precursors are selectively enriched in extramedullary tissues where unique subsets of mature NK cells reside, suggesting that the latter may derive locally *in situ*¹¹. As the body has evolved to generate millions of NK cells that are self-tolerant yet capable of substantial tissue destruction and immunomodulation, the notion that NK cells may develop in multiple microenvironments carries important and interesting implications regarding how this process may be regulated *in vivo*.

Within the last ten years, numerous studies from multiple groups have demonstrated that human NKDI are naturally and selectively enriched in extramedullary tissues including the thymus, peripheral lymph nodes, gastrointestinal track, tonsils, gravid uterus, and liver. Interestingly, these NKDI are not only enriched in the aforementioned tissues, they may also provide some immune function *en route* to their full development into cytolytic NK cells. Here we provide an overview of NKDI and their development in human secondary lymphoid tissues (SLT).

Human natural killer cell developmental intermediates (NKDI) in SLT

To date there are no known surface antigens that are entirely specific to the human NK cell lineage. By convention, NK cells are minimally defined as lineage (Lin)⁻CD56⁺ lymphocytes, with “lineage” markers often including CD3 (for T cells), CD19 or CD20 (for B cells), and CD14 (for monocytes). Other pan-NK cell markers, such as NKp46, are typically selectively expressed on normal blood NK cells, but in humans even this marker is rarely expressed on some reactive or neoplastic T cells as well as non-NK ILCs in SLT¹²⁻¹⁵. In human peripheral blood, most NK cells show dim or moderate CD56 expression as well as bright coexpression of CD16¹⁶. These CD56^{dim} NK cells are thought to represent the most mature NK cell population in humans and are capable of robust natural cytotoxicity and target-induced cytokine production^{17,18}. In contrast, a small population of NK cells in the blood shows bright CD56 expression and low or undetectable coexpression of CD16. These CD56^{bright} NK cells show relatively higher capacity for *ex vivo* proliferation and monokine-induced cytokine stimulation yet relatively lower capacity for natural

cytotoxicity in comparison to the CD56^{dim} NK subset¹⁹. A recent study also shows that peripheral CD56^{dim} NK cells produce low levels of IFN- γ earlier than CD56^{bright} NK cells²⁰. Whereas CD56^{bright} NK cells normally only constitute up to 10% of the circulating NK cell pool in healthy individuals, they are naturally enriched and comprise the major mature NK cell population in SLT where they likely provide immunomodulatory cytokines in the setting of infection²¹. Interestingly, multiple lines of evidence suggest that CD56^{bright} NK cells not only represent a functionally distinct lymphocyte population, they are also the direct precursors to CD56^{dim} NK cells: 1) the CD56^{bright} NK subset is the major NK cell population that is derived early *in vitro* when CD34⁺ HPC are cultured in NK development supportive conditions, whereas CD56^{dim} NK cells develop later over time; 2) likewise, CD56^{bright} NK cells appear to accumulate earlier in the blood following bone marrow or stem cell transplantation, whereas CD56^{dim} NK cells accumulate at later time points; 3) CD56^{bright} NK cells are relatively more abundant in fetal and post-natal blood in contrast to adult peripheral blood; 4) CD56^{bright} NK cells have longer telomeres compared to CD56^{dim} NK cells, suggesting that CD56^{dim} NK cells have undergone more rounds of proliferation; 5) NK cells with immunophenotypic and functional features intermediate between those of the CD56^{bright} and CD56^{dim} subsets have been described in adult human blood; and 6) purified CD56^{bright} NK cells can develop into CD56^{dim} NK cells in *in vitro* and *in vivo* experimental systems^{22–28}. These data provide substantial evidence for a linear relationship between the two major blood NK cell subsets; however, the possibility that CD56^{bright} and CD56^{dim} NK cells represent distinct terminal maturation pathways in the NK cell lineage (akin to CD4⁺ and CD8⁺ T cells) has not been formally disproven. Moreover, it should be noted that purified CD56^{dim} NK cells that are activated *in vitro* can adopt a CD56^{bright}-like immunophenotype with loss of CD16 and upregulation of CD56 suggesting that at least some CD56^{bright} cells may be activated NK cells^{29, 30}. This transition of CD56^{dim} phenotype to CD56^{bright} phenotype may also occur *in vivo*³¹. As discussed below, *ex vivo* analyses of NKDI in SLT directly link the CD56^{bright} NK cell population to less mature developmental populations, whereas there is as yet no known direct link between these populations and the CD56^{dim} NK cell subset. Therefore, the current prevailing view is that CD56^{bright} NK cells are the direct precursors to CD56^{dim} NK cells in a linear maturation model.

As mentioned above, *in vitro*-derived NK cells are predominantly CD56^{bright}, at least during the first few weeks of cultures and especially in the absence of stromal feeder cells^{11, 32–34}. Although multiple growth factors likely facilitate the development of NK cells, interleukin-15 (IL-15), which is produced by mesenchymal stromal cells as well as hematopoietic cells such as dendritic cells, is considered to be the most important NK cell homeostatic cytokine known to date. IL-15 promotes the proliferation and survival of mature NK cells and is also capable of inducing the differentiation and maturation of CD34⁺ HPC into CD56^{bright} NK cells in stroma-free medium^{35–37}. IL-15 is typically provided in soluble form *in vitro*, yet *in vivo* it is presented *in trans* as a membrane bound ligand in association with the IL-15 receptor alpha chain (IL-15R α)^{38, 39}. In order to respond to IL-15, recipient cells must express the common gamma chain (CD132) as well as the IL-2R α (CD122)^{40, 41}. As such, human NK cell precursors have been traditionally defined as CD34⁺CD122⁺ cells. Such cells have been detected by flow cytometry in *in vitro* NK cell

development systems and rarely *ex vivo*^{32, 42, 43}; however, the staining is weak and not reliable, and therefore other surrogate markers are necessary to identify human NK cell precursors. Through a systematic analysis of freshly isolated and subsequently cultured BM-derived and PB-derived CD34⁺ HPC populations, we previously determined that human IL-15-responsive NK cell precursors are enriched within a small yet distinct HPC subset: CD34⁺CD45RA⁺CD117⁺integrin β_7 ⁺. These cells comprise a minute fraction of the total BM-derived CD34⁺ population (<1%), whereas they are moderately relatively increased in the circulation (up to 5–10% of total PB CD34⁺ HPC in adults). In contrast, in SLT these cells are highly enriched, often representing up to half of total SLT-derived CD34⁺ HPC (Figure 1). This is likely due to specific trafficking of BM-derived NK cell precursors to SLT and entry via high endothelial venules at least in part mediated by distinctively high expression of L-selectin (CD62L) on circulating CD34⁺CD45RA⁺CD117⁺integrin β_7 ⁺ cells¹¹.

Given the natural and selective enrichment of CD34⁺ NK cell precursors and their progeny, CD56^{bright} NK cells, within the T cell rich regions of SLT, we hypothesized that human NK cells develop in these tissues and that other NKDI could be identified there. In support of this hypothesis, we identified five populations in human SLT that appear to represent distinct stages of an NK cell maturation continuum *in situ*. These populations, which all show lymphoid morphology, may be referred to as stage 1 – stage 5 cells and may be minimally identified by direct *ex vivo* flow cytometry immunophenotyping using a combination of antibodies against CD34, CD117, CD94, and CD16 among Lin[−] events (Figure 2)^{33, 44}. Stages 3–5 have also been recapitulated *in vitro* following culture of CD34⁺ cord blood HPC on murine fetal liver stromal cells⁴⁵. Freshly purified stage 1 cells (CD34⁺CD117[−]CD94[−]CD16[−]) and stage 2 cells (CD34⁺CD117⁺CD94[−]CD16[−]) are both CD45RA⁺ and can develop into mature NK cells *in vitro*. However, stage 1 cells do not express CD122 and do not respond to IL-15 *ex vivo*, whereas stage 2 cells do possess these attributes and represent the IL-15-responsive CD34⁺CD45RA⁺CD117⁺integrin β_7 ⁺ NK cell precursor discussed earlier. Interestingly, both populations retain non-NK cell lineage potential *ex vivo* and can develop into dendritic cells and T cells under supportive *in vitro* conditions⁴⁴. Whether or not these cells truly give rise to NK cells and/or these other populations *in vivo* is not known, but it is noteworthy that an extrathymic pathway of human T cell development was recently described in pediatric tonsils, and this pathway appears to branch off from the SLT NK cell developmental pathway at the stage 1 population⁴⁶. We have also observed that some CD34⁺CD117[−] cells in SLT express blood dendritic cell antigen 2 (BDCA-2) and/or CD11c, suggesting that divergent pathways of dendritic cell development may also occur in these tissues (A.G.F. and M.A.C., unpublished observations).

The transition from stage 2 to stage 3 is marked by the loss of CD34 expression as well as that of other antigens including HLA-DR and integrin β_7 ⁴⁴. In addition, stage 3 cells do not retain T or dendritic cell developmental potential *ex vivo*. Although stage 3 cells express relatively low amounts of CD122 as well as variable expression of some NK cell surface markers and functional receptors, they do not express MHC-binding inhibitory receptors, including CD94/NKG2A and killer immunoglobulin-like receptors (KIR), which are

characteristically expressed on mature NK cells. Likewise, stage 3 cells also do not possess the two hallmark functions of mature NK cells: the ability to produce interferon gamma (IFN- γ) and the ability to mediate perforin-dependent natural cytotoxicity⁴⁴. Therefore, stage 3 cells were originally described as committed immature NK cells^{33, 44}. Interestingly, following their discovery in SLT, stage 3 cells, or at least a fraction of this population (Lin⁻CD34⁻CD117⁺CD94⁻CD16⁻) were shown to possess unique *ex vivo* functional capabilities including the ability to produce high amounts of IL-22 and the ability to upregulate cell adhesion molecules on mesenchymal cells in co-cultures^{47–49}. Based on these and other data discussed below, at least a portion of the originally described stage 3 population is now theorized to represent a distinct ILC population (ILC3) according to some authorities^{50–52}. Nonetheless, as CD34⁺ and CD94⁺ are mutually exclusive populations in human tissues (and in human NK cell development experimental cultures), the requirement for NK cells to pass through a CD34⁻CD94⁻ stage 3 intermediate has not been disproven. In addition to the aforementioned immunophenotype, human stage 3 NK cell precursors may be identified by their coexpression of NKRP1A (CD161), which was originally identified to mark immature NK cells during *in vitro* development, as well as IL-7R α (CD127)^{33, 53}. Interestingly, CD56 is variably expressed within the stage 3 population, and it is also detectable on some cells at stage 2⁴⁴. The significance of this is unknown as is the role of CD56 during human NK cell development.

The acquisition of CD94 coexpression marks the transition to stage 4 of human NK cell development within SLT. CD94 is uniformly and strongly expressed on all peripheral blood CD56^{bright} NK cells, and due to many other immunophenotypic similarities between stage 4 cells and CD56^{bright} NK cells in the blood, the latter is thought to represent the stage 4 NKDI. Likewise, the acquisition of CD16 on some CD94⁺ cells in SLT is empirically held as the marker of the stage 5 cells, whose blood counterpart is the CD56^{dim} NK cell, particularly the CD94^{high}CD56^{dim}CD16⁺ population described by Yu *et al*²⁸. Thus, peripheral blood CD56^{bright} and CD56^{dim} NK cells are considered to represent the two terminal stages of human NK cell maturation (Figure 2). *Ex vivo* analyses of human stage 3 and stage 4 cells demonstrated that the latter can produce IFN- γ and can lyse the NK-sensitive targets, K562 and Jurkat, *in vitro*, indicating that functional maturity is acquired at stage 4^{44, 48}. Nonetheless, a comprehensive analysis comparing CD94⁺CD16⁻ stage 4 and CD94⁺CD16⁺ stage 5 cells from SLT has not yet been performed; therefore we do not yet know with certainty how these two SLT-derived populations may compare in terms of their functional capabilities.

Cell intrinsic and extrinsic factors regulating human NK cell development in SLT

Despite a relatively clear view of the NK cell maturation continuum within SLT, the internal and external regulatory mechanisms controlling this process in SLT are largely unknown. IL-15, which is produced by antigen-presenting cells in the T cell interfollicular regions of SLT, is a key external factor that supports NK cell development in SLT. However, whether developing NK cells have access to this membrane-bound ligand throughout their development or whether they receive IL-15 at certain checkpoints is not yet known. IL-2,

which is produced by activated T cells, can also support *in vitro* NK cell development from CD34⁺ progenitor cells by signaling through the shared IL-2/IL-15 intermediate-affinity receptor composed of CD122 and CD132; however, there is no evidence to date to suggest that IL-2 is necessary for NK cell development *in vivo* under steady state conditions^{54, 55}. Nonetheless, it is very interesting that NKDI at stages 2, 3, and 4 in SLT constitutively express IL-2R α (CD25), which in combination with CD122 and CD132, forms the high-affinity IL-2 receptor and enables these populations to compete for picomolar concentrations of IL-2 released from activated T cells^{11, 33}. Moreover, *in vitro* coculture of autologous activated T cells with stage 2 or stage 3 cells is sufficient to support maturation to stage 4. These data suggest that T cell-derived IL-2 could promote NK cell maturation in SLT during T cell activation although this has not been tested using an *in vivo* experimental model system. Other growth factors, such as flt3 ligand (FL), c-kit ligand (KL), IL-7, IL-21, and many others may influence NK cell development in SLT, but under what circumstances these factors act has not been investigated. Gaining a thorough understanding of the microenvironment(s) of developing NK cells in SLT should provide key insights into these as yet unaddressed questions.

Stage 3 cells are relatively abundant in comparison to the other NKDI within SLT, especially in comparison to stage 1 and stage 2 cells, and it has been shown that this population can proliferate in response to IL-1 α that acts in synergy with IL-15 and that is produced by local CD11c⁺ dendritic cells⁴⁸. Interestingly, at least *in vitro*, IL-1 α stimulation of stage 3 cells maintains a stage 3/ILC3-like phenotype at the expense of differentiation/maturation to the stage 4 NK cell⁴⁸. Therefore, IL-1 α potentially acts *in vivo* as a major regulator of NK cell maturation. IL-1 α stimulation, in conjunction with IL-23 activation, of this population also promotes the release of the epithelial growth factor, IL-22, which has been shown to be vital for adequate mucosal immunity in experimental mouse models⁴⁸. Therefore, humans may have evolved such that NKDI can participate in immunity as well as serve as a reservoir of NK cell precursors. Studies are clearly warranted to investigate the interplays between various immune responses and the stage 3 to stage 4 transition. A recent study shows that death receptor 3 (DR3) is expressed by IL-22-producing ILCs (most likely ILC3)⁵⁶. The ligand of DR3, tumor like antigen-1 (TL1A), activates IL-22 production in ILCs in the presence of IL-1 α and IL-23. The combination of TL1A, IL-1 α and IL-23 led to significantly higher amounts of CD25 on the surface of ILCs, resulting in higher responsiveness of ILCs to IL-2⁵⁶.

The transcriptional regulation of human NK cell development has largely been studied in the context of *in vitro* culture systems^{33, 57–60}. Various transcription factors and microRNAs have been shown to qualitatively and/or quantitatively impact NK development, and it will be important to correlate these data with ex vivo analyses of SLT-derived NKDI.

Challenges in studying human NK cell development in SLT and future directions

One of the major caveats in studying human NK cell development, especially within SLT, is the fact that these aforementioned populations may have the capacity to traffic in and out of the tissues. Therefore, when NKDI from a given tissue are evaluated by flow cytometry, it is

impossible to know which, if any, NK cells actually matured in that tissue. Moreover, when stage 4 and stage 5 cells are evaluated *ex vivo*, one cannot be sure that these cells do not represent activated mature NK cells that have trafficked from the circulation. In support of the hypothesis that at least a subset of NK cells in SLT indeed matured *in situ* is the observation that by flow cytometry one can visualize continuums of antigen expression to suggest maturation (Figure 3). Indeed in essentially every sample of SLT we personally have evaluated (more than 700 pediatric tonsils to date), there are always some cells with immunophenotypes that are intermediate between the major described stages. This finding is similar to what is observed when evaluating B cell development in the BM or T cell development in the thymus^{2, 61, 62}.

Another major hurdle to gaining a comprehensive understanding of human NK cell development in SLT is deciphering the true nature of the stage 3 human NKDI in SLT. As mentioned above, stage 3 cells bear an immunophenotype (Lin⁻CD34⁻CD117⁺CD161⁺CD127⁺CD94⁻) that has also been assigned to other ILC populations including group 2 and group 3 ILCs that appear to represent distinct lineages, at least in mice^{51, 52, 63, 64}. In particular, human ILC3 cells, which are enriched in SLT, appear to have an immunophenotype that overlaps considerably with that of stage 3 NKDI, such that to date it is not entirely clear whether these cells are not one in the same population (Figure 3). On the one hand, fate-mapping studies in mice evaluating the expression patterns of the retinoid-related orphan receptor (ROR) γ t indicate that group 3 ILCs are distinct from the NK cell lineage, yet *in vitro* data with human SLT-derived cells shows some level of lineage plasticity *ex vivo*^{65–67}. The physiologic (and clinical) significance of the latter observations is not yet known. Further *ex vivo* characterization of SLT-derived Lin⁻CD34⁻CD117⁺CD161⁺CD127⁺CD94⁻ cells is therefore warranted.

There are other very interesting and clinically relevant aspects of NK cell biology that have not yet been explored in the context of NK cell development in SLT. For instance, NK cell education, which is the process that maturing and mature NK cells continually undergo as a means of balancing autoreactivity with functional competence, has not yet been studied with SLT-derived populations. Likewise, NK cell memory, which is another large area of ongoing investigation and which may represent the final stage of an NK cell's life^{68, 69}, should be evaluated in SLT-derived NK populations. That SLT normally contains a pool of memory NK cells, which overlap with stage 4 and/or stage 5 cells, is likely and should be investigated.

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Highlights

1. In vivo cellular stages of human natural killer (NK) cell development are reviewed.
2. Human NK cell development in secondary lymphoid tissues is presented.
3. Future directions in the field of human NK development are discussed.
4. Distinctions between NK precursors and other innate lymphocytes are discussed.
5. Potential interactions between NK precursors and other immune cells are discussed.

Relative frequencies of CD34⁺CD45RA⁺CD117⁺integrin β_7^+ HPC among total CD34⁺ HPC

Figure 1. Human IL-15 responsive CD34⁺ NK cell precursors (stage 2 cells) are naturally relatively enriched in secondary lymphoid tissues

Schematic representation of the relative frequencies of human stage 2 NK cell precursors (CD34⁺CD45RA⁺CD117⁺integrin β_7^+) among total CD34⁺ HPC in bone marrow, blood, and lymph node. Stage 2 NK cell precursors are extremely rare in the body (<0.1% of total mononuclear cells in all hematolymphoid tissues). However, it is notable that among total CD34⁺ HPC,

NK cell precursors represent <1% and <10% in the bone marrow and blood, respectively, whereas in secondary lymphoid tissues, such as lymph nodes (as depicted in the figure), stage 2 CD34⁺ NK cell precursors represent close to half of all CD34⁺ HPC, of which >95% are CD34⁺CD45RA⁺ ¹¹. Bone marrow, blood, and lymph node illustrations with licensure for publication were purchased from iStockphoto and Fotosearch.

Figure 2. Morphology and immunophenotypes of the stages of human NK cell maturation in secondary lymphoid tissues
NK cell developmental intermediates were viably purified from a pediatric tonsil specimen as previously described⁷⁰ followed by centrifugation of the cells onto glass slides and subsequent staining with Wright-Giemsa stain. Original magnification = 1000X.

Figure 3.

Ex vivo immunophenotypic analysis of enriched NK cell developmental intermediates by flow cytometry and schematic representation of the stage 3 to stage 4 transition in human secondary lymphoid tissues. (A) Dot plots were generated from *ex vivo* flow cytometry immunophenotyping of enriched NK cell developmental intermediates from a pediatric tonsil specimen. Enriched NK cells were obtained via negative selection of total T cells, B cells, monocytic cells, and granulocytes. The images were obtained by gating on CD3/CD14/CD20-negative live cells (dye exclusion) and then gating on lymphoid cells based on low forward and side scatter characteristics on a Becton Dickinson LSR II flow cytometry. Analyses were performed using FlowJo software. Stage 3 cells, as originally described, express CD117 and CD161 and lack expression of CD94. Here they are shown to coexpress IL-1R1 and show dim partial expression of NKp46, CD122, and NKG2D. Stage 4 cells (CD117⁺/CD94⁺) show strong surface expression of NKp46, CD122, and NKG2D and partial coexpression of CD161. Note the intermediate events between the two major stage 3 and stage 4 populations suggestive of ongoing NK cell maturation within the tonsil. (B) Schematic representation of two possible scenarios of human NK cell development as it relates to Group 3 ILCs: In scenario I. (left) ILC3 cells represent a distinct lineage apart from NK cells. This scenario is the prevailing contemporary view held by many leaders in the field of ILC biology, and it is not yet known to what extent ILC3 cells may derive, at least in part, from the NK cell maturation pathway and/or may give rise to mature NK cells. In scenario II. (right), an alternative hypothesis, stage 3 cells represent the same population as ILC3 cells. As represented by the overlapping cells in between I. and II. in the figure, significant immunophenotypic overlap between cells described as stage 3 NK cell precursors and cells described as ILC3 have thus far made it challenging to reliably distinguish between the two cell types.