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Location and cellular stages of NK cell development

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Abstract

The identification of distinct tissue-specific natural killer (NK) cell populations that apparently mature from local precursor populations has brought new insight into the diversity and developmental regulation of this important lymphoid subset. NK cells provide a necessary link between the early (innate) and late (adaptive) immune responses to infection. Gaining a better understanding of the processes that govern NK cell development should allow us to better harness NK cell functions in multiple clinical settings as well as to gain further insight into how these cells undergo malignant transformation. In this review, we summarize recent advances in understanding sites and cellular stages of NK cell development in humans and mice.

What are NK cells?

Natural killer (NK) cells are founding members of a specialized cohort of leukocytes that have been recently collectively referred to as innate lymphoid cells (ILC) [1]. ILC are distinguished from B and T lymphocytes by their lack of somatic rearrangement of immunoglobulin and T cell receptor genes. All ILC are hypothesized to derive from a common ILC precursor. Moreover, three subsets of mature ILC have been described to date (ILC1, ILC2, and ILC3) and are currently characterized based on divergent expression of key transcription factors and distinct functional profiles. NK cells are one of two ILC1 subsets described to date [1], and their prototypic phenotypic characteristics include the production of cytokines, particularly interferon gamma (IFNγ), and a variety of chemokines as well as the mediation of major histocompatibility (MHC)-independent and antibody-dependent cellular cytotoxicity. These functional attributes enable NK cells to survey the body in search of pathogen-infected or malignant cells and to help protect the host by directly killing such cells as well as by augmenting and recruiting other immune cells. NK cells are known to be important in a variety of clinical settings including viral infection, solid organ and stem cell transplantation (SCT), and pregnancy, among others [2–5]. Given the above as well as recent compelling evidence that NK cells are also capable of

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immunological memory, there is great interest and rationale in deciphering the process of NK cell development in the hopes of manipulating NK cell numbers and/or functions for therapeutic benefit.

Human NK cells normally constitute 5–15% of peripheral blood (PB) lymphocytes, and they are also present in relative abundance in the bone marrow (BM), liver, uterus, spleen, and lung, as well as to a lesser extent in secondary lymphoid tissues (SLT), mucosal associated lymphoid tissues (MALT), and the thymus. Like all leukocyte populations, NK cells ultimately derive from self-renewing pluripotent hematopoietic stem cells (HSC) that reside in the BM. Moreover, NK cell development is similar to that of other leukocytes, occurring through a series of coordinated differentiation and maturation steps that result in the progressive restriction towards the NK cell lineage and acquisition of functional competency, respectively. Here, we review the major cellular intermediates that have been described to date as well as discuss recent advances in the regulation of the developmental pathway in mice and humans. We will also discuss the various anatomical sites in which NK cells appear to develop *in vivo*.

NK cell developmental intermediates

Knowledge of the maturational stages of NK cell development provides a fundamental framework to enable in-depth prospective studies of the regulation of this process. Indeed a large area of investigation in this field has been dedicated to the elucidation of the immunophenotypes of human and mouse NK cell developmental intermediates (NKDI) that represent distinct maturational stages. As the field is rapidly evolving, there is as yet no formal consensus as to how NKDI should be defined. Herein we use terminology to refer to human and mouse NKDI based on contemporary publications and reviews published by leaders in the field [4, 6–14]. Figures 1 and 2 depict schematic representations of human and mouse NK cell developmental pathways, respectively.

Early stages of NK cell development

Most studies in humans and in mice favor a model whereby NK cells develop from BM-derived HSC through a common lymphoid progenitor (CLP) capable of giving rise to all lymphocyte subsets [15, 16]. Human HSC and CLP populations are contained within the lineage negative (Lin$^-$) CD34$^+$CD38$^{dim/-}$CD45RA-CD10$^-$ and CD34$^+$CD38$^+$CD45RA$^+$CD10$^+$ fractions of the total BM-derived CD34$^+$ hematopoietic progenitor cell (HPC) pool, respectively [17]. Murine HSCs are minimally defined as Lin$^-$ stem cell antigen 1 (Sca-1)$^+$c-Kit (CD117)$^+$ “LSK” cells[18], whereas mouse CLPs have been defined as Lin$^-$Sca-1^{lo}CD117^{lo}CD127 (IL-7Rα)$^+$CD135 (flt3)$^+$[19]. Interestingly, similar CLP-like populations are also found in extramedullar tissues and may represent the earliest precursors in pathways of T or NK cell development outside of BM [20, 21]. For example, Freud *et al* identified a population of CD34$^+$CD45RA$^+$CD10$^+$CD117$^-$ “stage 1” cells in human SLT that retains potential for NK cell, T cell, and dendritic cell (DC) differentiation under supportive conditions *ex vivo* [8]. Bipotent T/NK cell precursors have also been described in the thymus [6, 22, 23].

The acquisition of the IL-15 receptor (IL-15R) beta chain (CD122) marks an important step in NK cell differentiation downstream of the CLP [24]. In both species, IL-15 selectively promotes NK cell differentiation, functional maturation, and survival [25]. Mice deficient in IL-15 or its signaling components, such as Jak3 and STAT5a/b, exhibit markedly reduced NK cell development, while IL-15 transgenic mice possess increased NK cell numbers, and a subset develops NK or NKT cell leukemia [14, 26–28]. The IL-15R is composed of three subunits: the common gamma chain (CD132), the shared IL-2/IL-15R beta chain (CD122), and the high affinity IL-15R alpha chain (CD215) [29]. Interestingly, although soluble IL-15

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drives NK cell development *in vitro* from human and mouse BM-derived HPCs [30], *in vivo* IL-15 is presented *in trans* in the form of a membrane-bound ligand coupled to CD215 to recipient cells expressing CD122 and CD132 [31]. IL-15-responding cells therefore need only express the intermediate-affinity IL-2/IL-15 receptor composed of CD132 and CD122, although CD215 expression has been detected on cells that respond to IL-15 [32].

Given this receptor biology, NK cell precursors (NKP) are defined as cells that 1) demonstrate NK cell developmental potential in response to IL-15 (i.e. they express CD122 and CD132), 2) lack functional and immunophenotypic features of mature NK cells (mNK), and 3) lack other lineage (Lin)-specific surface antigens (e.g. CD3, CD14, and CD19). Indeed murine NKPs were first discovered in mouse BM as Lin^- CD122+NK1.1$^-$DX5$^-$ cells [33], and for many years this population was considered to be the earliest committed NKP [12]. Recently, a novel $Lin^-CD27^+CD244^+CD117^{lo}CD127^+CD122^-CD135^-$ population was identified in BM that represents an NK lineage-committed precursor immediately downstream of the CLP and directly upstream of $Lin^-CD122^+NK1.1^-DX5^-$ NKP in the NK cell developmental continuum. This population has therefore been referred to as “pre-NKP” [34]. Using a reporter mouse model system in which the gene encoding green fluorescent protein was introduced into the *Id2* locus, Carotta and colleagues also provided evidence for the existence of a similar pre-NKP population which expresses high levels of ID2, CD127, CD244, and NKG2D, yet lacks detectable CD122 expression [35].

CD122 expression is rarely if ever detected by flow cytometry on the surface of human NKDI prior to the expression of CD94 [7], thus requiring surrogate immunophenotype(s) for the identification of IL-15-responsive early NKDI. Two populations of IL-15-responsive Lin-CD94$^-$ NKDI have been indentified that are naturally enriched in the interfollicular T cell-rich areas of SLT where their putative progeny, $CD56^{bright}$CD94+ NK cells, also reside: $Lin^-CD34^{dim}CD45RA^+\alpha_4\beta_7^{bright}CD117^+CD161^{+/-}$ $CD94^-$ “stage 2” and $Lin^-CD34^-\alpha_4\beta_7^-CD117^+CD161^+CD94^-$ “stage 3” NKDI [7, 8, 36]. Similar stage 3 cells were described in cord blood as well as in NK cell development cultures *in vitro* [37]. Stage 2 and stage 3 populations both respond to IL-15 and can give rise to mNK *in vitro.* Moreover, stage 2 NKDI can give rise to T cells and DC as well as stage 3 cells *in vitro*, whereas stage 3 cells do not retain T or DC developmental potential nor do they revert to a stage 2 immunophenotype *in vitro*, supporting the conclusion that stage 3 cells are the direct progeny of stage 2 cells [8].

Although human stage 3 cells were originally hypothesized to represent committed NKDI given their inability to become T cells or DC *in vitro* [7, 8], it is notable that the stage 3 population in human SLT (originally minimally defined as Lin^-CD34^- $CD117^+CD94^-$) is heterogeneous with regard to expression of a number of other surface antigens including CD7, CD56, CD121A (IL-1R1), CD127, CD336 (NKp44, NCR2), and leukocyte function antigen-1 (LFA-1) [7, 38–40]. In fact, recent data support the existence of immunophenotypically overlapping, yet functionally distinct ILC subsets (including *bona fide* stage 3 human NKDI) present within the total Lin-CD34-$CD117^+CD94^-$ SLT population [41–44]. These subsets appear to include 1) an ILC3 population that is characterized by the expression of retinoid-related orphan receptor gamma (RORγt), CD336, CD121A and IL-17 and/or IL-22 [1, 39, 44–47]; 2) a minor proportion of ILC2 cells (most are $CD117^-$) that are characterized by the expression of CD161, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2, CD294), RORα, GATA3, and type 2 cytokines (e.g. IL-5 and IL-13) [42, 48]; and 3) *bona fide* stage 3 NKDI which may be distinguishable by their unique expression of LFA-1 and capacity for robust NK (i.e. ILC1) cell differentiation in response to IL-15 [40]. Similar populations have also been described in mice [1]. Each of these ILC subsets appears to be commonly reliant on the transcription factor, Id2, for their development *in vivo*, suggesting origination from a common ILC

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precursor population [1, 49–51]. However, a number of recent mouse experiments provide strong evidence that these are distinct lineages that rely on unique extracellular factors for their terminal differentiation and maturation [1]. For example, NK cells in mice ($CD3^{-}$CD122+NK1.1+NKp46+) require IL-15 and lymphotoxin-β (LTβ) but not IL-7 for their development, whereas the opposite is true for ILC3 cells [43, 51, 52]. Furthermore, RORγt is indispensable for mouse ILC3 development, whereas NK cells do not require RORγt for their development and apparently never express this gene during their lifespan *in vivo* as demonstrated by fate-mapping studies [51, 53]. Although human *in vitro* studies indicate some -amount of developmental plasticity between NK cell and ILC3 development [39, 45, 54], the physiologic and clinical relevance of this is not yet known.

Late stages of NK cell development

Maturing NKDI acquire functional receptors in an orderly fashion that is similarly observed during *in vitro* NK cell development, following adoptive transfer of purified NKDI *in vivo*, and in the setting of human SCT (Figures 1 and 2) [8, 55, 56]. In mice, committed NKDI first acquire NK1.1, CD94/NKG2A, and NKp46 followed by Ly49 receptors, DX5, and finally CD43 and Mac-1 (CD11b) [57]. A progressive and orderly acquisition of functional receptors likewise occurs during human NK cell development in the following order: 1) CD161; 2) CD56, CD94/NKG2A, NKp46, and NKG2D; and 3) killer immunoglobulin-like receptors (KIR) and CD16 [8, 37, 56]. Some surface antigens and receptors, such as CD56 in humans and CD117, NKp46, CD94/NKG2A, and the TNF family receptor, CD27, in both species, are partially downregulated or lost at the final stages of NK cell development. Similarly ordered patterns of surface antigen expression are also observed when comparing NK cells derived from mice and humans at different ages, such that fetal and neonatal NK cells are largely $CD94^{+}Ly49/KIR^{-}$ whereas NK cells in older mice and humans are more often $CD94^{-}Ly49/KIR^{+}$[58–62].

The stages of murine NK cell maturation have been elucidated using many of the aforementioned markers, and the capacity for IFNγ production and perforin-mediated cellular cytotoxicity has been found to occur late in development, primarily at the $CD11b^{+}$ stage and after the acquisition of CD94 [57]. As such, immature NK cells (iNK) in mice have been designated as $Lin^{-}CD122^{+}NK1.1^{+}CD94^{+}Ly49^{-}DX5^{-}CD11b^{-}$ cells [63]. These functional capacities are likewise only detected subsequent to the acquisition of the human stage 4 immunophenotype ($Lin^{-}CD34^{-}CD117^{+/-}CD94^{+}CD56^{bright}CD16^{-}$), indicating that CD94 expression similarly precedes functional maturity during human NK cell development [8, 37, 64]. Human stage 3 NKDI have been previously referred to as iNK; however, unlike murine iNK, human stage 3 cells are $CD94^{-}$[8]. The possibility that an orthologous human $CD94^{+}$ iNK population exists downstream of stage 3 and within the total $LinCD34^{-}CD117^{+/-}CD94^{+}CD56^{bright}CD16^{-}$ stage 4 population (that likely also contains activated mature NK cells that immigrated from the blood [65–67]) requires further investigation. Nonetheless, the notion of an iNK stage of mouse (and human) maturation is in keeping with the prevailing view that the acquisition of certain functional receptors is a prerequisite to achieving functional competency *in vivo* as a means of ensuring NK cell self-tolerance [68, 69].

In contrast to iNK, mNK in mice and humans can produce IFNγ and mediate perforin-dependent cellular cytotoxicity. At least two functionally distinct PB mNK subsets exist in humans and in mice [70], and other unique NK cell populations are also present in the thymus, liver, and uterus [9, 13, 71]. mNK subsets differ in their surface receptor expression, proliferative and functional capacities, and *in vivo* trafficking. Human mNK subsets were first described as $CD56^{bright}$ and $CD56^{dim}$, and they have been extensively characterized [72, 73]. $CD56^{bright}$ NK cells ($Lin^{-}CD34^{-}CD117^{+/-}CD94^{+}CD16^{-}$) are

relatively enriched in SLT and have high capacity for cytokine production but relatively lower cytotoxicity in comparison to CD56^{dim} NK cells (Lin^{-}CD34^{-}CD117^{-}CD94$^{+/-}$CD16^{+}) that predominate in PB and display lower levels of monokine-induced cytokine production but more potent target cell-induced cytokine production and cytotoxicity [70]. Accumulating evidence indicates that CD56^{bright} NK cells are precursors to CD56^{dim} NK cells [70, 74, 75] consistent with their designation as stage 4 and stage 5 human NKDI, respectively [7]. CD56^{bright} NK cells are more prevalent in neonatal tissues and umbilical cord blood, and they also are the predominant PB mNK population to be detected at engraftment following SCT [76]. In contrast, CD56^{dim} NK cells accumulate later in life as well as later in time following SCT, and CD56^{dim} NK cells also display shorter telomeres compared to CD56^{bright} NK cells [77, 78]. In further support of this precursor-progeny relationship, a functionally and immunophenotypically intermediate population of human PB CD94^{hi}CD56^{dim} NK cells was identified that likely represents a transitional stage between CD56^{bright} and CD56^{dim} subsets [67]. Subsequent studies from three different groups found that CD62L and CD57 can also identify a unique functional CD56^{dim} subset and perhaps mark a progressive progression of NK-cell maturation from CD56^{bright} to CD56^{dim} as well [79–81].

Murine CD11b^{+} mNK subsets have also been dissected CD11b^{+} mNK cells into CD27^{hi} and CD27^{lo} populations [82]. CD27^{hi} NK cells produce relatively more cytokines following monokine stimulation, and they also show more robust cellular cytotoxicity in comparison to CD27^{lo} NK cells. Furthermore, CD27^{hi} cells give rise to CD27^{lo} NK cells but not vice versa *in vivo* following adoptive transfer [82]. These data are consistent with a subsequent study characterizing a terminal four-stage NK cell maturational program as follows: CD11b^{lo}CD27^{lo} → CD11b^{lo}CD27^{hi} → CD11b^{hi}CD27^{hi} → CD11b^{hi}CD27^{lo}[83]. Thus mNK subsets in both mice and humans likely fulfill distinct roles in immunity yet also represent sequential stages of NK cell maturation [67, 74, 75]. In light of these data, we note that activated mouse and human mNK cells can reportedly change their surface antigen expression profiles upon stimulation [56, 84, 85]. For example, culture of human PB CD56^{dim} cells in IL-18 induces a CD56^{bright}-like phenotype *in vitro* with upregulation of CCR7, CD83, and CD25 and downregulation of CD16 [85]. Therefore, many apparent CD56^{bright} cells *in vivo* may actually be activated CD56^{dim} cells [86].

Although an in-depth discussion of NK cell memory is beyond the scope of this review, memory NK cells could be considered to represent the final stage of NK cell maturation/differentiation in both species (Figures 1 and 2). Notably, however, it is not yet clear whether memory formation occurs as a single terminal event along a linear pathway similar to the aforementioned stages of NK cell development, or whether memory NK cells may arise from various NKDI depending upon the stimulatory milieu. For example, human memory-like NK cells have been derived from both CD56^{bright} and CD56^{dim} NK cells [87]. In addition, memory formation appears to be a diverse process in that memory NK cells may be induced through at least three distinct pathways: 1) cytokine stimulation; 2) viral exposure due to multiple viruses but most thoroughly characterized to date in the setting of murine cytomegalovirus (MCMV) infection; and 3) hapten-mediated with specificity for liver-restricted NK cell memory [88]. To date, the definitive and specific immunophenotype(s) of memory NK cells remain elusive. In a recent study, no immunophenotypic differences were observed between cytokine-induced memory-like versus naïve NK cells in mice [89]. In contrast, MCMV-specific memory NK cells showed relatively increased expression of Ly49H, Ly6C, CD43, and KLRG1 and decreased expression of CD27 [11], suggesting overlap of mouse MCMV-specific memory NK cells with the CD11b^{+}CD27^{lo} mNK subset. Liver-restricted NK memory cells that mediate contact hypersensitivity have been similarly shown to be enriched within the CD11b^{+}CD27^{lo} subset, and expression of Thy1, CXCR6, and/or Ly49C/I as well as CD49a

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may also be useful to identify these cells [88, 90–92]. However, the specificity of these antigens is not yet known. In humans, NKG2C and/or CD57 expression are associated with acute CMV infection and CMV reactivation following HSCT [93–95], and CD57 expression on $CD56^{dim}$ NK cells denotes the most terminally differentiated human NK cell population [81]. Therefore, it is possible that these antigens on human NK cells may identify memory NK cells, at least in the setting of viral-induced NK cell memory [93–95]. However, an alternative hypothesis is that increased NKG2C and/or CD57 expression may be due to continued priming of naïve NK cells. These hypotheses await further investigation.

Factors involved in NK cell differentiation

Elucidation of the immunophenotypes of NKDI in mice and humans has greatly facilitated investigation into the extrinsic and intrinsic factors that regulate NK cell development. Indeed the regulation of NK cell development is an ever-expanding field, and a comprehensive discussion of the genetic regulation of this process goes beyond the scope of this review. Here we focus our discussion toward that of external factors (e.g. cytokines, other surface receptors) that influence NK cell development and refer the reader to recent excellent reviews on the topic of transcriptional regulation of NK cell development [96–99].

The traditional two-step model of cytokine-mediated early NK cell development holds that IL-15-non-responsive HPC become IL-15-reponsive NKDI and then subsequently mature in response to IL-15 [100, 101] (Figures 1 and 2). In agreement with this model, IL-3, IL-7, c-kit ligand (KL) and flt3 ligand (FL) increase the frequency of IL-15-responsive NKDI following *in vitro* culture of unfractionated human and mouse BM-derived HPC or purified SLT-derived human stage 1 cells [8, 101, 102]. Moreover, HSC and CLP as well as human stage 1 cells lack CD122 expression and hence do not respond to IL-15, whereas NKP (in mice) and stage 2 and stage 3 cells (in humans) express CD122 (albeit not necessarily detectable by flow cytometry) and can develop into mNK in response to IL-15 without additional cytokines [6, 8, 14]. *In vivo*, stromal cells residing in BM and other sites are important sources of IL-3, IL-7, FL, and KL [103–106]. It is likely that these cytokines also act indirectly by promoting the homeostasis of accessory hematopoietic cells that can facilitate increased NK cell production. For example, FL administration to mice and humans expands DC, an important source of IL-15 for NK cell survival [107].

Chemokines also influence NKDI *in vivo* by directing their localization to distinct microenvironments [108]. For example, CXCR4 and sphingosine 1 phosphate receptor 5 (S1P5) are reciprocally regulated on developing NK cells in mouse BM, such that early NKDI express CXCR4 and are retained in this tissue where the concentration of the CXCR4 ligand, stromal-derived factor 1, is high. In contrast, mNK lose CXCR4 expression and upregulate S1P5, which binds S1P and promotes egress from the BM [109].

Other regulators of NK cell development include the tyrosine kinase receptor Axl/Gas6 that enhances IL-15-, KL-, and FL-induced human NK cell development *in vitro* [110, 111]. In contrast, TGF-β represses human NK cell development from $CD34^+$ HPC [112]. IL-1β also decreases NK cell maturation in the presence of IL-15, and the mechanism may involve the promotion of the ILC3 phenotype at the expense of conventional NK cell development [39].

Notch signaling may also play a role in human NK cell development [113]. The Notch ligands Jagged2, Delta1, and Delta4, but not Jagged 1 and Delta3, promote the development of cord blood $CD34^+$ HPC into human NK cells *in vitro* [113]. In addition, microRNA (miR)-181 positively regulates human NK cell development, potentially via regulation of Notch signaling [114]. Despite these human data, studies in mice indicate that NK cell

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development occurs via a Notch-independent mechanism, suggesting that Notch signaling may be permissible but not required for mouse NK cell development *in vivo* [115].

It is well established that NK cell inhibitory receptors, such as Ly49 and KIR, function in "educating" NK cells during their development [116]. In contrast, the potential roles for many NK activating receptors during the NK cell developmental process are less clear [117]. For example, NK cell development reportedly occurs normally in CD27-deficient and CD94-deficient mice [118, 119]. Signaling through NKG2D, which is detected on mouse pre-NKP and NKP populations but is not detected by flow cytometry on human SLT-derived NKDI prior to stage 4 [8, 9], appears to qualitatively influence murine NK cell development in that NK cells developing in the absence of NKG2D (*Klrk1*$^{-/-}$ mice) undergo higher rounds of cellular division and are more susceptible to apoptotic stimuli [120]. Moreover, *Klrk1*$^{-/-}$ mice showed a relative loss of NKDI at the iNK to mNK transition with reduced NKDI expressing CD117, integrin α_V (CD51), and Ly49 as well as reduced CD11b$^+$CD43$^+$Klrg1$^+$ mNK cells in the BM. Surprisingly, *Klrk1*$^{-/-}$ NK cells were nonetheless better able to secrete IFNγ and to protect against infection by murine cytomegalovirus (MCMV) compared to controls [120].

NK cell functional maturation

As discussed above, early NKDI are in part defined by their inability to produce IFNγ and mediate perforin-dependent cytotoxicity [6, 8]. Interestingly, it has been reported that following culture of CD34$^+$ HPC in IL-2 and IL-4, *in vitro*-derived Lin$^-$CD34$^-$CD161$^+$CD56$^-$CD94$^-$ (stage 3-like) NKDI were capable of producing type-2 cytokines, IL-5 and IL-13, as well as TNFα and GM-CSF. These cells could not produce IFNγ unless subsequently cultured in IL-12 in the presence of feeder cells, and this notably also correlated with the acquisition of CD94 expression [121, 122]. The same *in vitro*-derived NKDI could also mediate TRAIL-dependent cytotoxicity but not Fas/FasL-or perforin-dependent cytotoxicity [123]. Freud *et al* observed that SLT-derived stage 3 cells *ex vivo* could produce GM-CSF upon stimulation with IL-2, phorbol 12-Myristate 13-Acetate (PMA) and ionomycin, although they could not detect IL-13 production or TRAIL-mediated killing of Jurkat cells [8]. These disparate findings may relate to enrichment of an ILC2-like population *in vitro* in the studies by Perussia and colleagues, low sensitivity of type 2 cytokine detection by the heterogeneous bulk "stage 3" population in the study by Freud *et al*, and/or fundamental differences in deriving NK cells *in vitro* versus *in vivo*. Regardless, these findings raise the possibility that immature NKDI may function in immunity and warrant further investigation to determine if *bona fide* stage 3 NKDI are capable of the aforementioned functions. In newborn mice and adult mouse liver, TRAIL expression has also been described to denote "immature" NK lineage cells [124]. However, these TRAIL$^+$ NK cells were capable of robust cytotoxicity indicating that they were likely a unique subset of mNK [124].

It is challenging to determine whether a given cytokine influences functional maturation *per se* or has an effect on the subsequent functionality of mNK that develop in experimental systems, especially in light of the fact that cytokine stimulation often induces immunophenotypic changes to mNK [85, 121, 122, 125]. Nonetheless, there is great interest in learning what external signals promote NK cell functional maturation, as these may have high clinical utility in the settings of SCT and immunodeficiency [126]. IL-15 undoubtedly promotes NK maturation, but even in the absence of IL-15 functionally competent NK cells are still detected [127]. Interestingly, enforced expression of the anti-apoptotic molecule, Bcl-2, in CD122-deficient mice restored NK cell numbers but did not restore NK cell cytotoxicity indicative of at least two qualitatively distinct roles for IL-15 that may relate to separate signaling pathways [128]. For example, IL-15 regulates perforin expression through

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Stat5a and Stat5b [129]. In addition, Horng *et al* demonstrated that IL-15R signaling also influences the signaling of NKG2D via JAK3-mediated phosphorylation of the NKG2D-adaptor molecule, DAP10 [130]. Therefore cross-talk among different signaling pathways is likely to result in qualitatively distinct effects on NK cell development.

Many other cytokines, including IL-2, IL-4, IL-7, IL-10, IL-12, IL-18, IL-21, type I and type II IFN, and TGFβ, appear to also influence NK cell maturation [56, 121, 122, 125]. For example, as mentioned above, IL-4 can promote a Th2-like phenotype among human $CD34^{-}CD161^{+}CD56^{-}CD94^{-}$ NKDI derived *in vitro*, whereas IL-12 promotes their full maturation [121, 122]. IL-21 induces umbilical cord blood $CD34^{+}Lin^{-}$ cells to mature into pseudo-mature lytic NK cells [131]. Despite these mostly *in vitro* data, it remains unclear how, when, and at what concentrations during NK cell development *in vivo* these various cytokines act, but this is an important area of ongoing investigation.

Sites of NK cell development

During intrauterine development, hematopoiesis occurs in the yolk sac, aortogonad mesonephric region, and fetal liver [132]. However, during post-natal life, NK cell differentiation and maturation have been traditionally thought to occur primarily within the BM, because selective BM ablation abrogates NK cell development in mice; BM-derived stromal cells produce the necessary cytokines and can support the development of NK cells from human BM-HSCs and other BM-derived progenitor cells *in vitro*; and a full complement of mouse NKDI has been thoroughly described in this tissue [9, 34, 35, 57, 133, 134]. Nevertheless, there is no definitive evidence precluding the possibility of extramedullar NK cell maturation for at least a subset of mNK. Indeed cytokines that can support NK cell development *in vitro*, including FL, KL, IL-7, and IL-15, have been shown to be produced by stromal cells derived from human liver, spleen, and SLT [103, 105, 135, 136]. In addition, monocytes, DCs, and T cells may also produce IL-15 and/or IL-2 [31, 137–140]. Thus, each of these cell populations that are normally present in extramedullary sites might facilitate NK cell development *in vivo*. Interestingly, recent data suggest that neutrophils may also be required for NK cell development [141].

Previous analysis of fresh human tissues demonstrated that IL-15-responsive stage 2 cells represent less than one percent of total $CD34^{+}$ HPC in normal adult BM, whereas these cells comprise 5–10% of circulating $CD34^{+}$ HPC and display high surface expression of CD62L (L-selectin), LFA-1, and $\alpha_4\beta_7$ integrin, which are molecules that have been shown to facilitate entry into SLT [36]. Indeed human stage 2 cells are substantially enriched in SLT [8, 36], and early NKDI have also been described in mouse LNs [142, 143]. A full complement of NKDI (stage 1 – stage 5 cells) was identified within human SLT with evidence that ongoing NK cell maturation *in situ* likely occurs based on flow cytometry data showing continuums of antigen acquisition and loss, similar to what has been observed for early B and T cell development in the BM and thymus, respectively [7]. These human data provide strong support for a model whereby BM-derived early NKDI traffic through the blood and develop into mNK within SLT (Figure 3).

Various other groups have reported the identification of human leukocyte populations that are functionally and immunophenotypically similar to SLT-derived NKDI in uterine, intestinal, and hepatic tissues, suggesting that other anatomical locations also support human NK cell maturation *in vivo* (Figure 3) [144–146]. For example, a stage 3-like Lin-CD34-$CD117^{+}CD94^{-}$ population has been recently identified in human liver that expresses surface CD127 and *ID2* transcript but does not express *RORC* or *IL22* mRNA [145]. This distinct liver-resident population gives rise to functionally mature NK cells *ex vivo*, suggesting that the hepatic $Lin^{-}CD34^{-}CD117^{+}CD94^{-}$ population may be more enriched for conventional

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stage 3 human NKDI in comparison to the SLT $Lin^{-}CD34^{-}CD117^{+}CD94^{-}$ population that may be more enriched for human ILC3 cells. Further in-depth comparison of NKDI populations in various tissues is clearly warranted and may lend new insight into the regulation in ILC development. Moreover, these data suggest that the hepatic microenvironment might preferentially direct cells towards conventional NK cell development, whereas SLT and MALT likely allow for robust production of ILC3 cells as well as conventional NK cells [39, 43, 45, 54, 145, 147].

Insofar as human NKDI have been identified in various extramedullary tissues, it is interesting that many of the mature NK populations that reside in these tissues are somewhat distinct from most circulating NK cells in PB. For example, human uterine NK cells show distinct attributes in that they are $CD56^{bright}$ but show high expression of KIRs and CD9, features not observed on most PB $CD56^{bright}$ NK cells [148–150]. Moreover, as mentioned above, distinct $TRAIL^{+}$ NK cells have been characterized in murine livers, and thymic-dependent $CD127^{+}$ NK cells have been described in the murine thymus [13, 124]. These antigenic differences among NK cell subsets may relate to local activation signals, which, as mentioned above, can induce immunophenotypic changes on mNK [56, 84–86]. Alternatively, unique signals derived from local parenchymal and hematopoietic accessory cells may qualitatively influence NK cell development *in situ* to result in distinct tissue-specific mNK subsets from common NKDI. It is also possible that each of these subsets may derive from completely separate developmental pathways apart from that of PB NK cells [6, 151]. These hypotheses await further investigation.

Concluding remarks

Tremendous progress has been made in the field of NK cell development. Studies in mice and humans over the last decade have provided much clearer models of the cellular stages of NK cell development in these species. Moreover, the recent discovery that NKDI circulate through PB and are normal cellular constituents of multiple extramedullary tissues strongly supports the notion that NK cells can and likely do develop outside of the BM. Nonetheless, many fundamental questions remain. What are the mechanism(s) of action of how non-NK lineage accessory cells and other extrinsic factors regulate NK cell development *in vivo*? Is programmed trafficking to discrete anatomical compartments required for some NKDI to complete the developmental process? Might NK cell maturation from local precursors result in the functional diversity of NK lineage cells that is observed in various tissues, or do all subsets of NK cells develop at the same site(s) and from the same precursor populations? How relevant to *in vivo* NK cell development is *in vitro* lineage plasticity? Do early NKDI participate in immunity (e.g. by production of type-2 cytokines)? Continued research is clearly warranted to answer these and many other interesting and important questions in this field. Such answers will not only further our basic understanding of the immune system, they will no doubt bring new insight into the pathophysiology of NK cell malignancies as well as guide us toward the most appropriate and beneficial use of NK cell-based immunotherapies.

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Highlights

- Hematopoietic progenitor cells follow discrete stages to differentiate into mature NK cells.
- NK cells develop in bone marrow as well as in some extramedullar sites, such as lymph nodes, thymus, liver, and uterus.
- NK cell development is controlled by both extracellular and intracellular factors.

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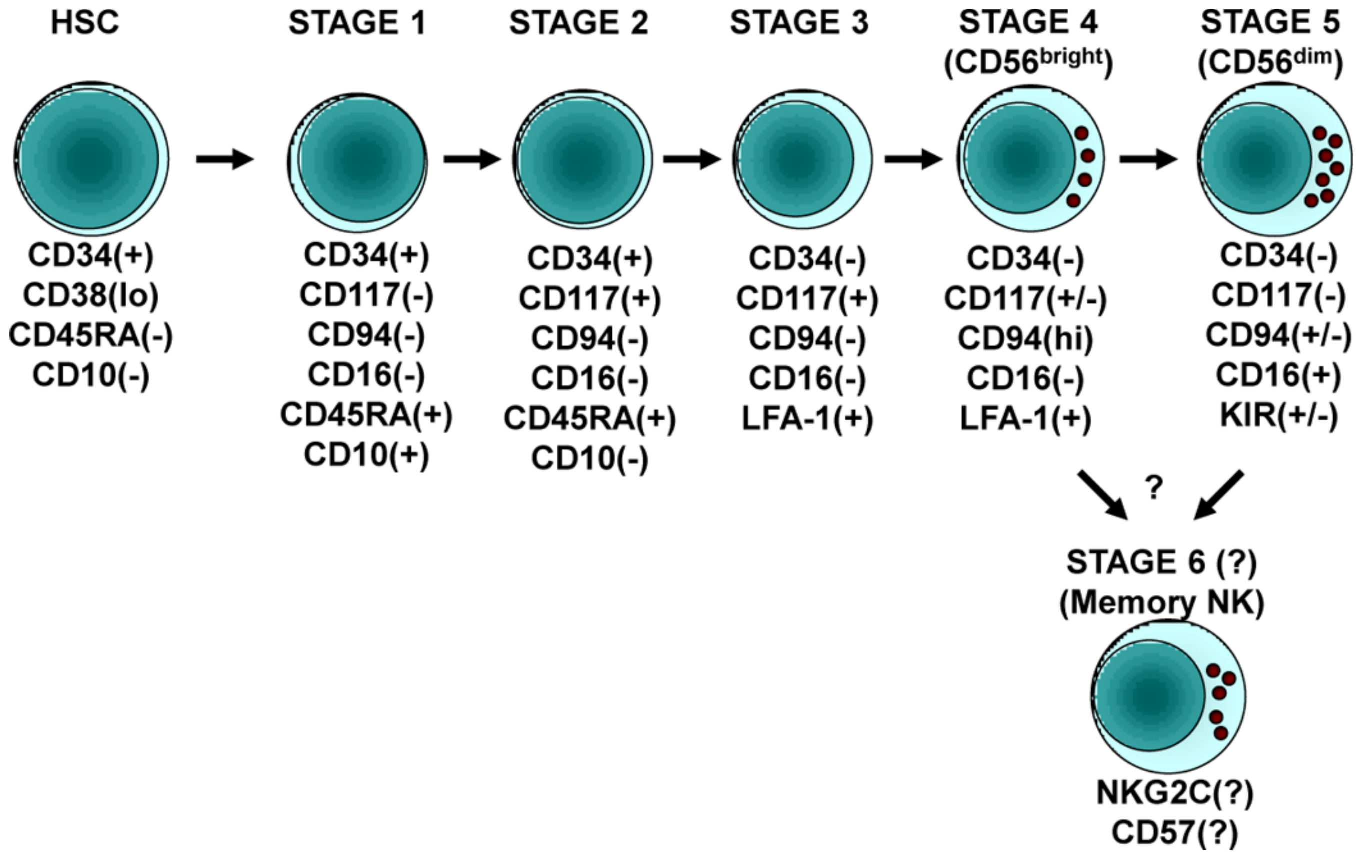

Figure 1. Schematic representation of the cellular intermediates of human NK cell development

Human NK cell development from hematopoietic stem cell (HSC) to mature stage 5 CD56^{dim} NK cell is represented as a linear pathway from left to right. Memory NK cells are depicted as a possible terminal stage of maturation (stage 6), with experimental evidence that memory NK cells may be derived from CD56^{bright} as well as CD56^{dim} NK cells [87]. Of note, memory NK cells are depicted here as one population although evidence to date indicates that there are at least three distinct pathways of NK cell memory formation [88]. Shown for each developmental stage is the expression of each surface antigen useful in distinguishing populations of NK developmental intermediates. Expression is designated as "+" (expression), "–" (no expression), "hi" (high expression), or "lo" (low expression).

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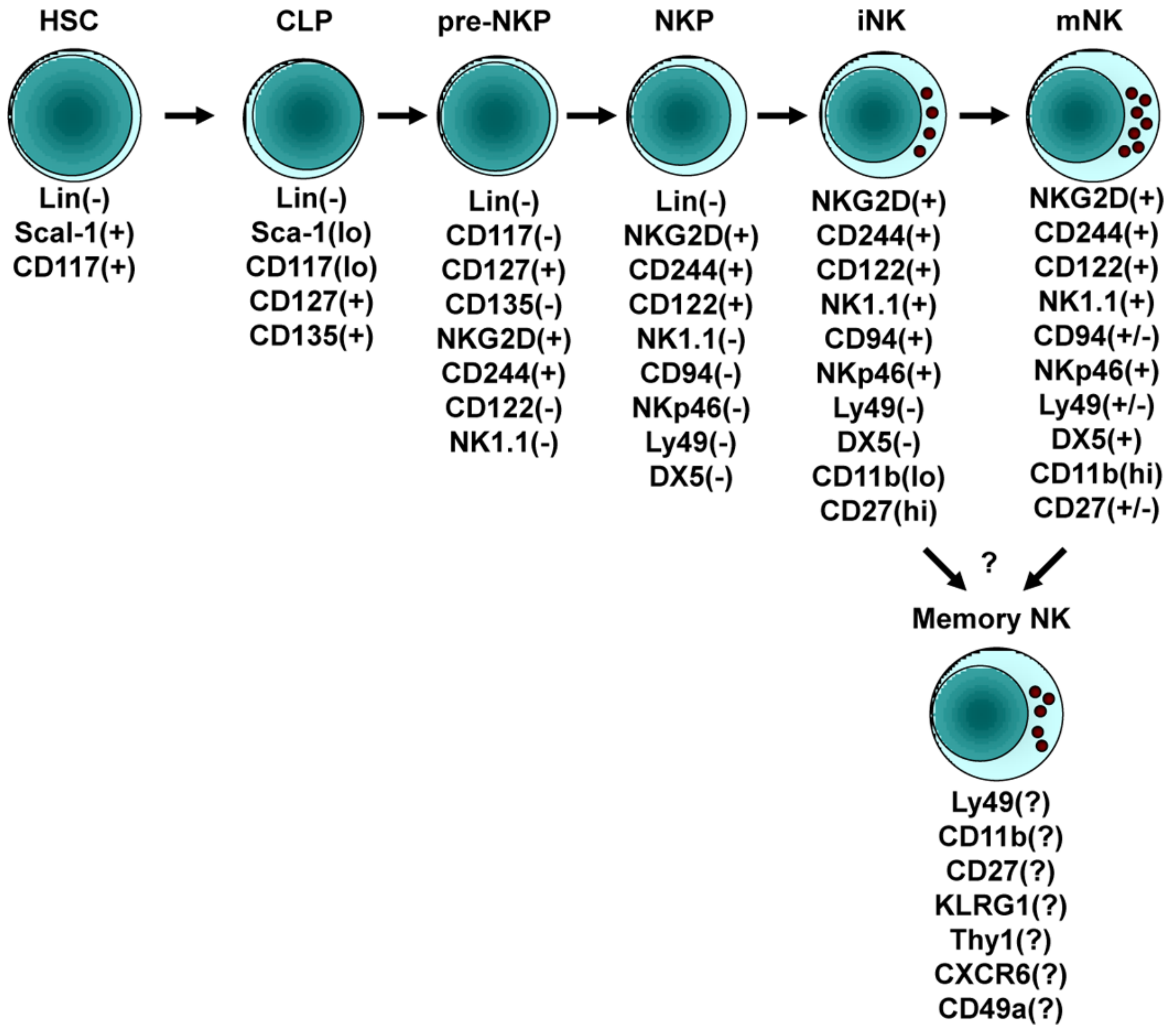

Figure 2. Schematic representation of the cellular intermediates of mouse NK cell development

Mouse NK cell development from hematopoietic stem cell (HSC) to mature CD11b^{high}DX5^{+} NK cell is represented as a linear pathway from left to right. Memory NK cells are depicted as a possible terminal stage of maturation, and memory NK cells are depicted here as one population although evidence to date indicates that there are at least three distinct pathways of NK cell memory formation [88]. Shown for each developmental stage is the expression of each surface antigen useful in distinguishing populations of NK developmental intermediates. Expression of each antigen in a particular stage is designated as “+” (expression), “–” (no expression), “hi” (high expression), or “lo” (low expression). CLP – common lymphoid progenitor, NKP –NK cell precursor, iNK – immature NK cell, mNK – mature NK cell.

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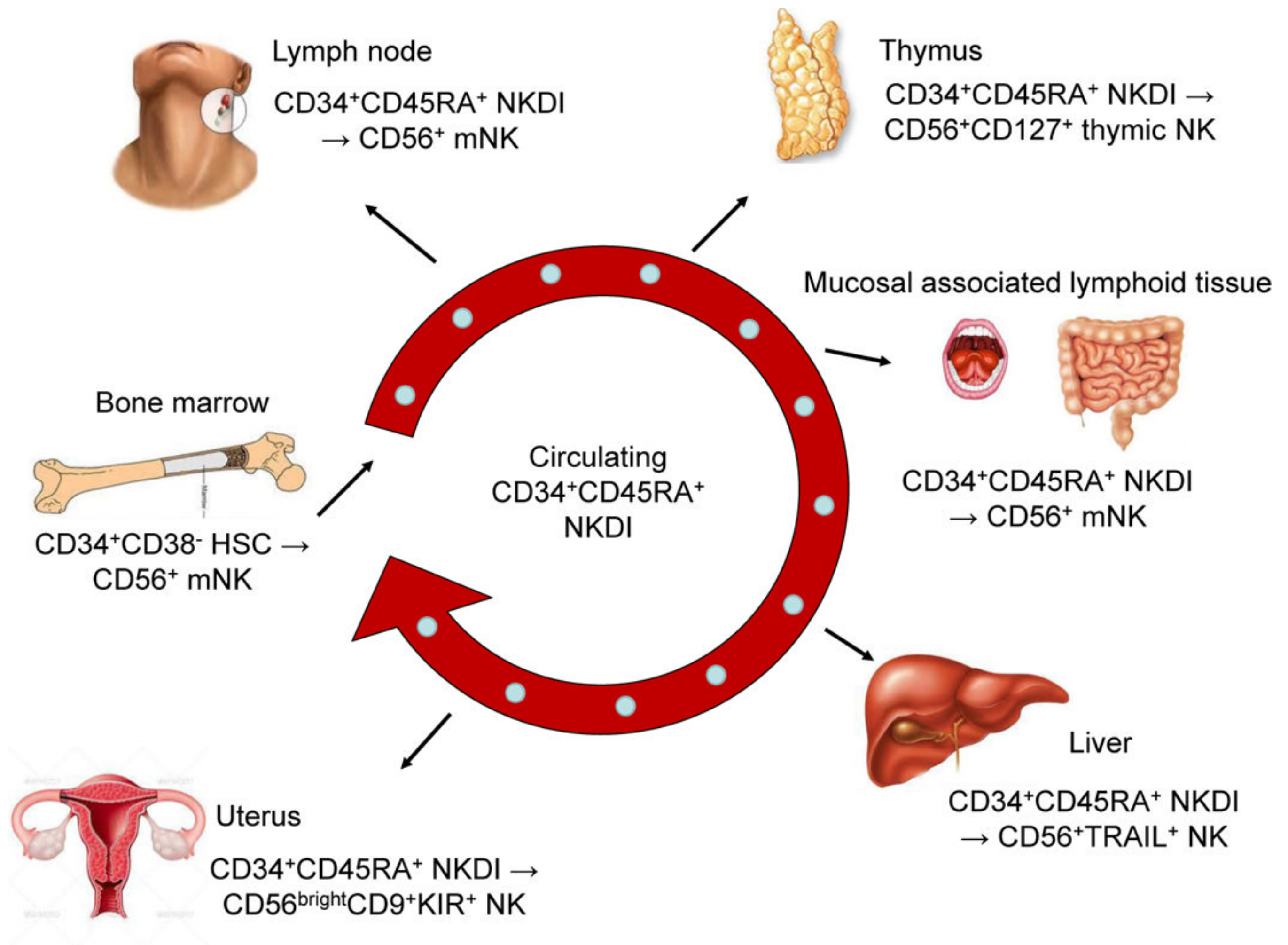

Figure 3. Extramedullary human NK cell development as a means of generating functionally distinct and tissue-specific NK cells

$CD34^+CD45RA^+$ NK cell developmental intermediates (NKDI) with *ex vivo* potential for NK cell differentiation are present in and likely originate within human bone marrow, but they are also present as normal constituents of circulating peripheral blood and extramedullary tissue leukocytes. In humans, IL-15-responsive $CD34^+CD45RA^+$ NKDI comprise a relatively higher proportion of total CD34+ progenitor cells (5–10%) in the blood compared to bone marrow (<1%), and in SLT they comprise the major subset of CD34+ HPC (>90%) [36]. Similar $CD34^+$ HPC have been detected in MALT, liver, and the gravid uterus where downstream NK cell developmental intermediates have also been identified [144–146]. Given the evidence for functionally distinct NK cell subsets in these various tissues as well as in the thymus [2, 6, 13, 124, 152], the findings support a model whereby $CD34^+CD45RA^+$ NKDI originate in the bone marrow and traffic to extramedullary tissues where they give rise to tissue-specific and functionally distinct mature NK cell subsets.