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Citation for the published paper:
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"Early Cellular Pathways of Mouse Natural Killer Cell Development."

Journal of Innate Immunity
2011 Mar 26

<http://dx.doi.org/10.1159/000323925>

Access to the published version may require journal subscription.

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EARLY CELLULAR PATHWAYS OF MOUSE NK CELL DEVELOPMENT

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ABSTRACT

Natural killer (NK) cells are large granular lymphocytes that are components of the innate immune system. These cells are key players in the defence against viral and other microbial infections and cancer, and have important function during pregnancy, autoimmunity and allergy. Furthermore, NK cells play important roles in hematopoietic stem cell (HSC) transplantation by providing the graft versus leukemia effect and preventing the development of graft versus host disease. Thus, understanding the developmental pathway/s from multipotent hematopoietic stem cells (HSCs) to the NK cell lineage-restricted progenitors is of significant clinical value. However, despite extensive progress in the delineation of mature blood cell development, including the B and T cell lineages, the early stages of NK cell lineage commitment and development have been less well established and characterized. Here, I review the progress made thus far in dissecting the developmental stages, from HSCs in the bone marrow to the lineage committed NK cells in mouse.

KEY WORDS

Lineage commitment, hematopoietic progenitors, NK cell progenitors

I. INTRODUCTION

Due to the short half life of the majority of blood cell lineages, during steady state, millions of different mature blood cells are replenished every second in man ¹.

The integrity of this process is strictly dependent on the existence of a rare population of hematopoietic stem cells (HSCs) with the extensive self-renewing properties as well as the ability to commit and generate all blood cell lineages to persistently support hematopoiesis throughout the life of an individual ².

NK cells are lymphocytes primarily participating in the innate immunity but also having important roles in regulating the adaptive immune responses. Diverse NK cell functions include an early defence that can directly eliminate infected and transformed cells by immediate cytotoxic activity, but also via cytokine (tumour necrosis factor (TNF) and interferon gamma (IFN- γ)) and chemokine production, with the latter serving to generate and sustain an inflammatory environment and influence adaptive immunity ³. Thus, NK cells prime B and T cell responses and cross-talk between NK cells and dendritic cells (DC) is required to generate efficient immune response ⁴.

After bone marrow transplantation, donor NK cells play a protective role against alloreactive T cells initiating the graft versus host disease ⁵, and mismatched NK cells provide graft versus leukemia effect in acute myeloid leukemia (AML) patients. Also, by producing hematopoietic growth factors such as granulocyte/macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) and interleukin 3 (IL-3), NK cells are involved in modulating hematopoiesis ³. Excitingly, NK cells were recently demonstrated to also have the capacity for memory-like responses, a property previously thought to be limited to adaptive immunity ⁶.

A current prevailing model suggests that the bone marrow in the adult, is the primary site of NK cell development in steady state ⁷. However, NK cells have also been shown to

arise at the sites that were traditionally thought to be occupied only by the adaptive immune cells⁸ such as the thymus⁹ and lymph nodes¹⁰. NK cells are also found in the gut-associated lymphoid tissues and are specialized in IL-22 production^{11,12}. Further, the subset of NK cells in the uterine mucosa plays an important function during fetal development by regulating the trophoblast invasion and development of the placenta¹³. Characterization and understanding the functional diversity of NK cell subsets and how these distinct effector cells develop is important to fully realize the potential of NK cell based therapies¹⁴.

Lineage commitment is the process by which a multipotent stem or progenitor cell becomes increasingly restricted in its lineage fate choices, to ultimately develop into a fully committed progenitor of a single cell lineage. Decades of extensive research provided the evidence that the lineage commitment of HSCs occurs as a stepwise process, however the cellular pathways and restriction sites, to generate uni-potent progenitors of the lymphoid lineage, remain the subject of debate and investigation^{15,16}.

Turning on lineage specific gene programs leads to lineage specification, while repression of lineage inappropriate gene programs, and concomitant restriction of alternative lineage differentiation potential drives the lineage commitment^{17,18}. Such transcriptional events are coordinated by networks of transcription factors and their associated chromatin remodelling factors, presumably under the guidance of extrinsic signals from the local environment.

On the road map of NK lineage commitment and development several questions remain. Are all diverse mature NK cell subsets generated from the same NK lineage restricted progenitor (NKP)? What is the relationship between NKP and other lymphoid progenitors? What is the direct predecessor cell upstream NKP? Where and when do NK cells develop and differentiate? What is the relationship between the thymic and bone marrow NK developmental pathways? From the perspective of these outstanding questions, I have here

attempted to review the current knowledge and controversies in the field, with a focus on mouse NK cell development.

II. NK CELL DEVELOPMENT IN ADULT MICE

From hematopoietic stem cells to immature NK cells – the bone marrow dependent pathway

All the blood cell types are derived from hematopoietic stem cells (HSCs) that reside in bone marrow in the adult individual¹⁵. HSCs have unique properties to self-renew required to maintain the blood cell production over the lifetime of the host.

A considerable amount of data supports the hierarchical model of hematopoiesis in which mature blood cells are generated from HSCs in an ordered fashion through a stepwise loss of self-renewal and lineage potentials. This process of HSCs differentiation starts with the loss of self-renewing ability and generation of multipotent progenitors (MPPs), while the developmental stages downstream of MPPs are less defined. The prevailing model of HSCs commitment established by the Weissman group predicts that HSCs and MPPs gradually lose self-renewing ability and sustain pluripotentiality, whereas downstream progenitors exhibit lineage restriction which occurs in a stepwise manner¹⁵.

A number of recent improvements in methodology, used for the prospective purification of candidate progenitors, as well as assays evaluating their lineage potential, allow an alternative road map for blood development to be drawn. The classical model of hematopoiesis proposed a first branching point – a strict lineage commitment/restriction into lymphoid and myelo-erythroid lineages - which was supported by identification of the common lymphoid (CLPs)¹⁹ and the common myeloid (CMPs) progenitors²⁰. The CLPs can generate only lymphoid cells (T, B and NK cells)¹⁹, while CMPs can produce myeloid and erythroid / megakaryocyte cells²⁰. However, identification of lymphoid primed multipotent progenitors (LMPPs) that lack erythroid / megakaryocyte potential but retain both lymphoid

and myeloid potential²¹ suggested an alternative hematopoietic hierarchy. Based on this finding, a revised model of hematopoiesis proposes the existence of lymphoid-myeloid progenitors as an intermediate stage in T, B, NK and myeloid development²¹.

The most immature hematopoietic progenitors in adult mouse bone marrow have a Lineage negative (Lin⁻), stem-cell antigen 1 positive (SCA-1⁺), tyrosine kinase receptor C-KIT high (KIT^{high}) phenotype (so called LSK cells)²². The LSK compartment is very heterogeneous and can be further sub-fractionated into different stem and progenitor populations based on multiple additional markers. Self-renewing long term reconstituting HSCs in adult mice are contained within a small fraction of LSK cells that lack the expression of fms-tyrosine kinase receptor FLT3^{21, 23}, do not express CD34 molecule²⁴, but express Slam family receptor CD150 (LSK FLT3⁻CD34⁻CD150⁺)²⁵. Single LSK FLT3⁻CD34⁻CD150⁺ HSCs can permanently sustain efficient production of all blood lineages after transplantation into lethally irradiated recipient mice^{24, 25}.

The compartment of multipotent progenitors (MPPs) downstream of HSCs is composed of cells that have little or no self-renewing ability and maintain multilineage potential. The MPPs are heterogeneous: both the LSK FLT3⁻CD34⁺CD150⁺ cells²¹ as well as LSK FLT3^{low/+}CCR9⁻VCAM-1⁺ cells²⁶ are progenitors that retain both lymphoid and myelo-erhthroid / megakaryocyte potential. The fraction of LSK cells that expresses high levels of FLT3 (LSK FLT3^{high}) represents lymphoid primed multipotent progenitor (LMPP), that has both lymphoid (B, T, NK) and myeloid potentials but has little or no ability to generate the erythroid or megakaryocyte lineages, and expresses high levels of transcripts for multiple lymphoid genes^{16, 21, 27}. Thus, the LMPP population is the earliest lineage restricted lympho-myeloid progenitor identified in mouse hematopoiesis¹⁶.

Downstream of the LSK compartment (HSCs, MPPs and LMPPs), there are several progenitors that possess NK cell lineage potential including the common lymphoid progenitors

(CLPs) defined based on the expression of low levels of SCA-1 and KIT and high levels of interleukin-7 receptor α chain and FLT3 ($\text{Lin}^- \text{SCA-1}^{\text{low}} \text{KIT}^{\text{low}} \text{IL-7R}\alpha^+ \text{FLT3}^+$)^{19, 28}.

Transplantation studies have demonstrated that CLPs are generated in the bone marrow from LMPPs^{28, 29}. Although CLPs have been shown to generate B, T and NK cells, recent studies have shown that a fraction of CLPs still maintains myeloid potential^{30, 31}. Further purification of CLPs based on LY6D expression suggests the branching point between the $\text{Lin}^- \text{SCA-1}^{\text{low}} \text{KIT}^{\text{low}} \text{IL-7R}\alpha^+ \text{FLT3}^+ \text{LY6D}^+$ population that has primarily B cells potential with little or no ability to generate NK and T cells, whereas the $\text{Lin}^- \text{SCA-1}^{\text{low}} \text{KIT}^{\text{low}} \text{IL-7R}\alpha^+ \text{FLT3}^+ \text{LY6D}^-$ CLPs produced B, T and NK cells³².

The acquisition of the IL-2 receptor β chain (CD122) that together with IL-15R α and the common γ chain is a part of the IL-15 receptor complex, marks the commitment into the NK lineage and CD122 continues to be expressed through all the NK cell developmental stages^{7, 33}. A candidate NK cell restricted progenitor (NKP) has been identified in adult mouse bone marrow as lacking the expression of lineage specific as well as mature NK cell markers and expressing the IL-2 receptor β chain (CD122) ($\text{Lin}^- \text{CD122}^+ \text{NK1.1}^- \text{DX5}^-$)³³, however the lineage potentials of this NKP progenitor has not been extensively studied, and its *in vivo* reconstituting ability was not evaluated. We have recently demonstrated that the $\text{Lin}^- \text{CD122}^+ \text{NK1.1}^- \text{DX5}^-$ cells in addition to having a robust NK cell potential, sustain the ability to generate T cells *in vivo*, and *in vitro* at the single cell level, thus representing a bipotent NK/T cell progenitor³⁴. Therefore, the identity of the progenitor restricted to NK cell lineage is yet to be established. The CLPs are negative for CD122 and the hierarchical relation between early lymphoid progenitors as well as the progenitor directly upstream NKPs remain unclear. The fact that the fraction of NKPs expresses IL-7R α , KIT and FLT3³³⁻³⁶, suggests that they are downstream CLPs.

The bone marrow is the main site of NK cell development as the ablation of the bone marrow environment is associated with dramatic defects in NK cell compartment and function³⁷. Essentially all the stages of NK cell development take place in the bone marrow including the generation of NKPs, differentiation and maturation of developing NK cells, NK cell education and the emergence of fully competent functional NK cells⁷.

NKPs were also found in the other mouse tissues including the thymus, spleen and lymph nodes^{7, 33, 38}, suggesting that NKPs and immature NK cells are not unique to the bone marrow and that the other sites can support NK cell differentiation. Alternatively, NKPs and immature NK cells generated in the bone marrow might seed the other tissues through the circulation.

In contrast to the early stages of NK cell development, the more terminal steps of NK cell maturation are better understood. Based on the studies from the DiSanto and Yokoyama laboratories a model of NK cell development downstream NKP has been established in adult mouse bone marrow^{7, 39}. In this model, the differentiation of NKP to immature NK cells involves progressive acquisition of NK cell receptors starting with CD161c (NKR1C, a family of C-type lectins receptors) recognized by the anti-NK1.1 antibody in C57BL/6 and C57BL/10 mouse strains, a marker that can also be detected on NK T cells and subsets of $\gamma\delta$ T cells^{40, 41}. The natural cytotoxicity receptor NKp46 (NCR1) is restricted to the NK lineage and starts to be expressed on immature NK cells downstream of NKPs⁴². The NKp46 is considered to be the most specific NK cell lineage marker. The differentiation of immature NK cells into functionally mature NK cells involves sequential acquisition of multiple cell surface receptors such as CD94/NKG2A/C/E and Ly49 (recognizing MHC class I) and DX5/CD49b, with the final maturation being associated with increased expression of CD11b/Mac-1 and CD43 molecules^{7, 43}. Mature NK cells leave the bone marrow and migrate to the periphery and are primarily found in the spleen, blood, lungs, lymph nodes as well as some of them re-circulating through the bone marrow⁴⁴.

The thymic NK cell developmental pathway

A distinct population of NK cells that has reduced cytotoxic activity and is highly efficient in secreting cytokines has been recently identified in the thymus of adult mice⁹. In contrast to the conventional bone marrow dependent NK cells, thymic NK cells (tNK) lack the expression of Ly49, Mac-1 and CD43 receptors but express IL-7R α ⁹. The cells with the tNK cell phenotype are also present in the lymph nodes, but not in the spleen or bone marrow and do not develop in athymic mice (Foxn1^{-/-})⁹. The origin of thymic NK cells remains controversial and the developmental relationship between bone marrow and thymic NK pathways is not known. Although tNK cell depend on the thymic environment, it is not clear whether they are generated from the progenitors that seed the thymus or whether they are derived from the bone marrow NK cells and undergo final maturation in the thymus. In support for the first possibility, previous studies have shown that the early thymic progenitors (ETPs) can generate both T and NK cells *in vitro*⁴⁵. We have recently shown that thymic NK cells and bone marrow dependent NK cells can be generated from both NKPs as well as ETPs at the single cell level³⁴ and their generation is not restricted by Notch signalling. Recent studies using Notch-1 deficient and RBP(CSL)-deficient bone marrow chimeras demonstrated that loss of Notch signalling did not altered generation of thymic NK cells, suggesting that thymic NK cells can developed independently of T-cell progenitors⁴⁶. However, further studies are needed to determine to what degree NKPs and ETPs contribute to generate NK cells in the thymus *in vivo*.

A distinct population of NK cell progenitors have been also identified in the mouse lymph nodes (LN)¹⁰. These progenitors resemble double negative (DN) thymocytes with Lin⁻CD44⁺CD25⁻ (DN1) and Lin⁻CD44⁺CD25^{lo} (pre-DN2) phenotypes, and express very low levels of IL-7R α ¹⁰. However, both IL-7R⁺ and IL-7R⁻ DN progenitors in the lymph nodes have been shown to generate NK cells *in vitro*¹⁰. It remains to be determined how much these

LN NK progenitors contribute to the different NK cell compartments as well as their origin. The LN NK progenitors are undetectable in the athymic mice, suggesting that they might develop from thymic progenitors migrating and seeding the LN^{9, 10}.

Recent studies identified a population of NK-like lymphoid cells in the intestine that do not express NK1.1 receptor, but are positive for NKp46 and produce IL-22^{11, 12}. These cells were named natural cytotoxicity receptor 22 (NCR22) or NK-22 based on the expression of NK cell lineage markers and the ability to produce IL-22^{11, 47}. However, further characterization of intestinal NKp46⁺ cell subsets suggests that they are different from conventional NK cells, and represent distinct innate cell lineage. It has been recently postulated that the innate lymphoid cells (ILCs) include: 1) NK cells, 2) lymphoid tissue – inducer (LTi) cells and 3) cells that produce interleukins: IL-5, IL-13, IL-17 and/or IL-22⁴⁸. The different subsets of ILCs are developmentally related and depend on the expression of transcription factor Id2 and cytokine signalling through the common gamma chain (γ c) and IL-2 receptor^{47, 48}. Further studies are needed to establish direct progenitors, and signals governing their development, to resolve the controversy in the field.

III. NK CELL DEVELOPMENT IN MOUSE EMBRYO

The relationship between fetal and adult NK cell development is poorly understood. Multiple fetal tissues (liver, thymus, spleen) contain hematopoietic progenitors that have potential to generate NK cells, however the biological importance of fetal NK cell development is not clear.

The liver is the main site of hematopoiesis during fetal development⁴⁹. The HSCs in the embryo originate within the intra embryonic sites: in the aorta-gonado-mesonephros (AGM) region derived from the paraaortic splanchnopleura (P-Sp) and in the placenta^{49, 50}, as well as in the extra embryonic site – in the Yolk sac (YS)⁵¹. The fetal liver starts to be

colonized by hematopoietic progenitors between days E10 and E12 and from day E12 to E15 the number of HSCs increases exponentially⁵². The fetal spleen is colonized by HSC and hematopoietic progenitors from day E13.5 and is considered to be a transient hematopoietic organ as it becomes an adult immune organ⁵².

Using RAG1 expression as a marker for the most primitive lymphoid progenitors, Yokota et al provided a detailed chart of the emergence and expansion of cells giving rise to the cells of the immune system⁵³. The first RAG1-EGFP-expressing cells were found in the embryo proper at day E10.5, at day E11 in fetal liver and at day E12.5 within the thymus⁵³. These RAG-1⁺EGFP⁺CD45⁺ progenitors have ability to generate B and T cells *in vitro*, however their NK cell lineage potential was not investigated. The fetal equivalent of CLP that has been identified in the liver in 14.5-day-old fetus retain, in addition to T, B and NK lymphoid potential, significant myeloid potential⁵⁴.

The NK lineage restricted progenitors have not been yet identified in the fetal liver, however hematopoietic progenitors with combined NK/T/DC and NK/T potentials have been found in different embryonic sites. The Lin⁻KIT⁺IL-7R⁺ progenitor cells able to generate NK/T/DC cells have been identified in the fetal blood at day E10.5⁵⁵ and found to persist in circulation until day E15. Bi-potent NK/T cell progenitors have been located to multiple sites during embryonic development, including the fetal thymus at day E13 as NK1.1⁺KIT⁺CD44⁺CD25⁻ cells^{56,57}, at day E 14.5 as CD44⁺CD25⁻FcγR⁻⁵⁸ and CD4⁻CD8⁻TCR⁻Fcγ⁺ thymocytes⁵⁹ as well as in the fetal liver at day E15 as B220^{low}KIT⁺CD19⁻ cells⁶⁰. It remains unclear whether these bi-potent NK/T progenitors are directly upstream NK restricted progenitors NKPs. In most of studies the lineage potential of these progenitor cells was only evaluated *in vitro*, thus their physiological contribution to NK and other lymphoid lineages in the developing embryo is not known.

Previous studies have shown that the NK1.1⁺ mature NK cells are detected first in the fetal thymus at day E14, and then at day E16 in the fetal liver as well as in the spleen⁶¹. Studies in adult mice suggested that commitment to the NK lineage coincides with the up-regulation of the surface expression of the β chain of IL-2 receptor (IL-2R β) CD122³³. To determine the time and the sites when different NK cell populations arise during development we performed detailed flow cytometric analyses of different mouse fetal tissues. Cells negative for mature lineage markers and expressing CD122 (Lin⁻CD122⁺) arise first in the fetal liver at day E13.5, while the CD3⁻NK1.1⁺DX5⁺ NK cells can be first detected in the fetal liver at day E14.5 and spleen at day E15.5. Mature NK cells with lytic activity are first found in the fetal liver at day E16.5 and spleen at day E17.5 (C. Stolz, Y. Tang and E. Sitnicka, unpublished observation). The fetal equivalent of bone marrow Lin⁻CD122⁺NK1.1⁻DX5⁻ NK cell progenitor (NKP) arises first in the fetal liver at day E13.5 and the numbers of NKPs expand between day E14.5 and E17.5 (Y. Tang, C. Stolz and E. Sitnicka, unpublished observation). These Lin⁻CD122⁺NK1.1⁻DX5⁻ cells can be also found in the fetal thymus, spleen, blood and bone marrow (C. Stolz, Y. Tang and E. Sitnicka, unpublished observation). It is currently unclear whether the fetal NKPs first expand in the fetal liver and then migrate to the other tissues through the circulation or whether they arise independently in the different embryonic sites. Likewise, it remains to be determined what the contribution to the generation of the mature NK cell compartments these differentially located NKPs have.

SUMMARY

Recent progress in understanding NK cell biology have identified multiple NK cell populations, each with distinct properties that appear to be generated as the result of distinct developmental programs that are regulated by alternative intrinsic and extrinsic mechanisms. Further studies are needed to understand the relationship between these diverse NK

progenitors, their origin as well as their contributions to the mature NK cell compartments. Uncovering how different microenvironments/ tissues support the development of NK cell subsets will help to identify critical extrinsic factors supporting NK cell lymphopoiesis and to understand how they affect developing NK cells. Identifying the cellular NK developmental pathway/s and its regulatory networks will further drive the therapeutic application of NK cells, and establish the molecular basis for diseases that result from altered NK cell development including syndromes manifesting as NK cell deficiencies and NK cell malignancies.

ACKNOWLEDGEMENTS

I apologize to all of my colleagues whose work could not be cited directly here owing to space constraints.

I thank Dr David Bryder for critical comments and the members of my laboratory for helpful and stimulating discussions.

Work in my laboratory is supported by grants from the Swedish Cancer Foundation, Swedish Paediatric Cancer Foundation, Swedish Research Council and Hemato-Linne grant.

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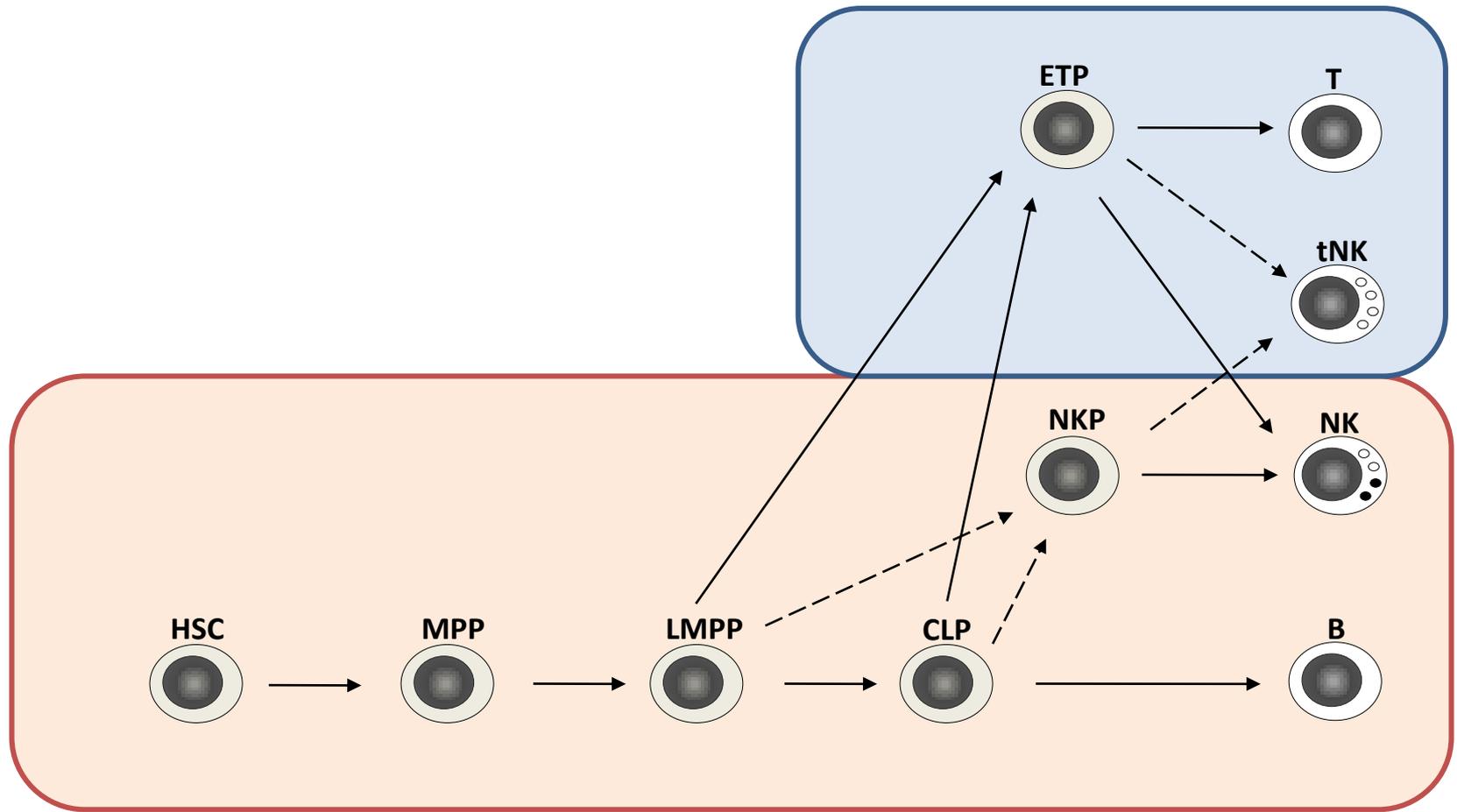


Figure 1. Schematic drawing of the early stages of lymphopoiesis in adult mouse. Solid arrows indicate established pathways, while the fragmented arrows indicate suggested pathways. HSC: hematopoietic stem cell, MPP: multipotent progenitor, LMPP: lymphoid primed multipotent progneitor, CLP: common lymphoid progenitor, ETP: early thymic progenitor, NKP: NK cell progenitor, NK: natural killer cells, tNK: thymic NK cells. Pink area indicates bone marrow pathway, blue area thymic dependent pathway.

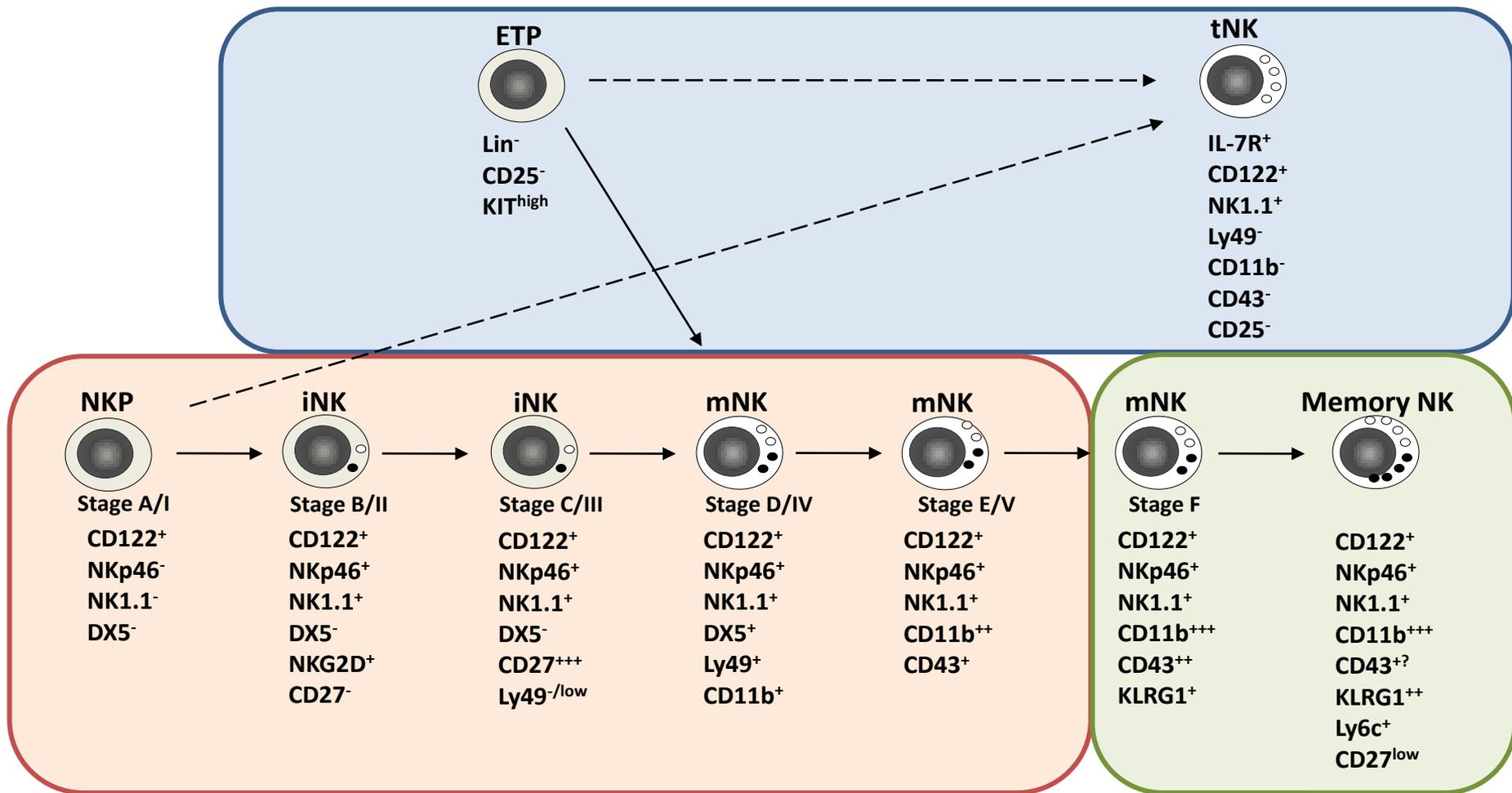


Figure 2. Schematic model for NK cell development in adult mouse. Stages of developing NK cells in bone marrow are defined based on expression of cell surface markers. For the each sequential maturation stage differentially expressed markers are listed. Solid arrows indicate established pathways, while the fragmented arrows indicate suggested pathways. ETP: early thymic progenitor, NKP: NK cell progenitor, iNK: immature NK cell, mNK mature NK cell, tNK: thymic NK cell. White granules illustrate cytokine production, black granules illustrate lytic activity and increased number of granules represents increased functional activity of developing NK cells. Stages A-F and I – V proposed by DiSanto and Yokoyama.

Pink area indicates bone marrow, blue area represents thymus, green indicates periphery and purple represents the gut. The intestinal NKp46⁺ cells represent heterogeneous population of innate lymphoid cells, distinct from conventional NK cells.

