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Distinct and Overlapping Patterns of Cytokine Regulation of Thymic and Bone Marrow-Derived NK Cell Development

Min Cheng, ‡* Hojjatollah Nozad Charoudeh, ‡* Petter Brodin, † Yanjuan Tang, * Tadepally Lakshmikanth, † Petter Höglund, † Sten Eirik W. Jacobsen, *‡ and Ewa Sitnicka 3*

Although bone marrow (BM) represents the main site for postnatal NK cell development, recently a distinct thymic-dependent NK cell pathway was identified. These studies were designed to investigate the role of cytokines in regulation of thymic NK cells and to compare with established regulatory pathways of BM-dependent NK cell compartment. The common cytokine receptor γ-chain (IL2rg) essential for IL-15-induced signaling, and FMS-like tyrosine kinase 3 (FLT3) receptor ligand (Flt3l) were previously identified as important regulatory pathways of the BM NK cell compartment based on lack of function studies in mice, however their complementary action remains unknown. By investigating mice double-deficient in IL2rg and Flt3l (Flt3l−/−Il2rg−/−), we demonstrate that FLT3L is important for IL2Rg-independent maintenance of both immature BM as well as peripheral NK cells. In contrast to IL-7, which is dispensable for BM but important for thymic NK cells, IL-15 has a direct and important role in both thymic and BM NK cell compartments. Although thymic NK cells were not affected in Flt3l+/− mice, Flt3l−/−Il2rg−/− mice lacked detectable thymic NK cells, suggesting that FLT3L is also important for IL-2Rg-independent maintenance of thymic NK cells. Thus, IL-2Rg cytokines and FLT3L play complementary roles and are indispensable for homeostasis of both BM and thymic dependent NK cell development, suggesting that the cytokine pathways crucial for these two distinct NK cell pathways are largely overlapping. The Journal of Immunology, 2009, 182: 1460–1468.

Natural killer cells, members of the innate immune system, represent a third lymphoid lineage distinct from T and B lymphocytes. NK cells recognize and eliminate tumor cells and cells infected by viruses or parasites, and promote adaptive immune responses by producing cytokines (1). Although significant progress has been made in understanding the regulation and function of mature NK cells, the cellular and molecular pathways of the earliest stages of NK lineage commitment and development remain largely unknown. In adult mice, the bone marrow (BM) is the main site of NK cell production and it harbors both NK cell progenitors and mature thymus-independent NK cells (2, 3). However, recent studies demonstrated the existence of another, seemingly distinct, thymus-dependent pathway of NK cell development (4). Importantly, it remains to be established whether the same or different molecular pathways are involved in regulation of these two distinct NK developmental pathways.

The first step during NK cell development in BM involves generation of the common lymphoid progenitor (CLP) from hematopoietic stem cells, which can give rise to NK, T, and B cells, but not to myeloid cells (5, 6). The acquisition of the IL-2/15 receptor β-chain (CD122) marks the transition into the earliest committed NK cell progenitor (NKP) that can be identified in the adult BM based on the Lin−CD122+DX5−NK1.1− phenotype (7). The differentiation of mouse NKPs into mature NK cells starts with up-regulation of the NK1.1 molecule and the subsequent expression of CD94/NKG2 and Ly49 receptors (2). In later stages, the markers DX5, Mac-1, and CD43 are up-regulated (8).

To establish which cytokine pathways are important for NK development, several cytokine-deficient mouse lines have been studied. For example, mice deficient in Il15 or Il15 receptor expression are severely deficient in NK cell-mediated cytotoxicity (9, 10) demonstrating that IL-15 plays a key role in the generation and maintenance of mature NK cells (11–14). Of important note, a small number of mature NK cells can however be generated in the absence of IL-15 signaling (11, 15), but the regulators of NK cell development that appears to act in a IL-15-independent manner remain to be established. Notably, Il15-deficient mice as well as mice lacking expression of the common cytokine receptor γ-chain (IL2rg), essential for the function of several cytokines including...
IL-15 and IL-7 (16), have been suggested to not have any changes in the NK committed progenitor pool, raising the possibility that other cytokine pathways might be involved in NKp regulation (17). The FMS-like tyrosine kinase-3 (FLT3 also called FLK2) is a cytokine tyrosine kinase receptor important for early lymphopoiesis (18, 19), but its expression pattern and role during NK cell development is not well understood. Mice lacking Flt3 ligand (Flt3l−/−) have deficiencies in mature NK cells as well as NK cytotoxic activity (20) and FLT3L has been shown to be important for generation of mature NK cells in vitro (21). However, it has not been investigated whether FLT3L is important for the earliest stages of NK cell development.

IL-7 has also been identified as a key regulator for both B and T lymphopoiesis (22–24) however, mice deficient in IL7 showed only mild reductions in NK cells, suggesting that IL-7 is dispensable for BM-dependent NK cell development (25). In contrast, IL-7 has been implicated as an important regulator of the thymic-dependent NK pathway (4), although the regulatory role of other cytokines, such as IL-15, in thymic NK cell development has yet to be explored.

These studies were designed to investigate the role, individually and complementarily, of cytokines in regulation of thymic NK cells and to compare with established regulatory pathways of BM-dependent NK cell compartment.

Previous studies have shown crucial roles and complementary interactions between hematopoietin and tyrosine kinase family cytokines such as FLT3L and IL-7 during B and T lymphopoiesis (26, 27). FLT3L (20) and IL-2Rg cytokines (17) have been shown to be important for NK cell development, however they were only investigated as single cytokines and their interdependence was not investigated.

Through studies of Flt3l−/− and Flt3l−/−Il2rg−/− mice, we demonstrate a critical role of FLT3L in regulation of the BM NK cell compartment, and specifically in IL-2Rg-independent homeostasis of different BM-derived peripheral NK cell subsets. In contrast to IL-7, which has been implicated as an important regulator of the thymic but not BM NK cell pathway, we further demonstrate that loss of IL-15 function results in severe reductions in both thymic and BM NK cell pools. We also provide evidence in support of IL-2Rg cytokines and FLT3L playing complementarily and essential roles in regulation of thymic and BM NK cell compartments.

**Materials and Methods**

**Mouse strains**

Mice deficient in Flt3l (20) (on C57BL/6 background), mice lacking Il15 (9), mice deficient in Il7 (24), and mice lacking Il2rg expression (28–30) (backcrossed into C57BL/6 background for 11 generations) were reported previously. Mice double deficient in Flt3l and Il2rg expression were obtained by cross-breeding of single Flt3l−/− and Il2rg−/− and subsequent interbreeding of heterozygous Flt3l+/-Il2rg+/- mice. Mice used for experiments were obtained through Flt3l−/−Il2rg−/− homozygous breeding. Mice double deficient in Flt3l and Il7 (Flt3l−/−Il7−/−) were reported previously (31). Genotyping was performed by PCR on genomic DNA derived from the tails (20, 30) and C57BL/6 mice were used as wild type (WT) controls in all experiments. All mice were maintained under specific pathogen-free conditions at Lund University Animal Facility and were used at 8–15 wk of age. The Ethical Committee at Lund University approved all performed experiments.

**Tissues**

BM cells were collected as previously described (26). Cell suspensions were prepared from spleens and thymuses by gently breaking up the tissues in PBS containing 5% of FCS (Invitrogen). Total tissue cellularities were counted by automated hematology analyser (Sysmex KX21, Sysmex Corporation).

**mAbs and flow cytometry**

Before staining single cell suspensions with Abs against specific surface markers, Fc receptors were blocked by incubation with 2.4G2 (anti-FcγRIII). All Abs were from BD Biosciences unless otherwise indicated. mAbs (conjugated with different fluorochromes) were used to stain cell surface. Abs were: CD3 (17A2), CD122 (TM-β1), CD49D (DX5), NK1.1 (PK136), Ly49G2 (4D11), CD11b/Mac-1 (M1/70), CD43 (S7), CD127 (A7R34), Ly49D (4E5), CD25 (3C7), Ter119 (LY-76), CD19 (ID3), Gr-1 (RB6-8C5), CD4 (H129.19), and CD8 (53-6.7). Isotype-matched controls labeled with appropriate fluorochromes were used. Biotinylated Abs were visualized by streptavidin-PE or streptavidin-PECy7 and purified Abs by polyclonal goat anti-rat-Tricolor (both Caltag). PKp46 (NCR1)-biotin was purchased from R&D Systems. 7-AAD (Sigma-Aldrich) was used to exclude dead cells from the analysis.

For the evaluation of NK cell and NKP compartments in bone marrow the mixture of following Abs was used to define the lineage negative (Lin−) population: anti-CD19, Gr-1, Mac-1, CD3, CD4, CD8, and Ter119.

Samples were analyzed on a FACSCalibur (BD Biosciences), LSR II (BD Biosciences), or FACSCan (BD Biosciences). Between 50,000 and 500,000 events were collected and analysis was performed using FlowJo (Tree Star) software.

**NK cell killing activity**

Cytotoxicity was measured using a standard Na251Cr-release assay using RMA-Rae1γ cells as targets. In this assay, RMA-Rae1γ cells are specifically killed by NK cells but not by NKT or conventional T cells (32). To enhance resolution in the killing assay and obtain positive readout in killing two cell strains in vitro, NK cell activity was augmented in vivo using tilorone analog T8014 before testing (33). Tilorone enhances NK cell activity in the spleen via IFN-induction (34), acting in a nonspecific global fashion to provide NK cell activation in vivo irrespective of the genetic control of NK cell activity or the initial number of NK cells (35). Ten to 15-wk-old mice were given 0.2 mg tilorone analog (Sigma-Aldrich) orally; 18 h later, mice were sacrificed and spleens removed. Single cell suspensions from two to three pooled spleens per strain were used at different ratios as effector cells and incubated for 4 h at 37°C in triplicate in 96-well V-bottom microtiter plates with RMA-Rae1γ target labeled cells for 1 h at 37°C with 100 μCi Na251CrO4 (Nycomed) per 106 cells. The 10 μl of supernatant was harvested from each well and its radioactive content was measured in a gamma-irradiation counter. The mean percentage of specific target cell death of triplicate wells was calculated using the following formula: % specific killing = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.

**Statistics**

All results were expressed as means ± SD. The statistical significances between groups were determined using the Student’s t test.

**Results**

**Key and complementary roles of FLT3L and IL-15 in maintaining BM-derived NK cell compartment**

FLT3L (20) and IL-2Rg cytokines (17) have been previously shown to be important for NK cell development, however they were only investigated as single cytokines and their interdependent interactions were not addressed. Of relevance, previous studies demonstrated the complementary roles of hematopoietin and tyrosine kinase family cytokines such as FLT3L and IL-7 to be crucial during B and T lymphopoiesis (26, 27). Thus, in this study, we investigated for the first time, how the concomitant loss of FLT3L and IL-2Rg signaling affects different NK cell compartments.

In agreement with the previous studies, the loss of FLT3L, IL-2Rg, or IL-15 resulted in significant reductions in the BM NK cell pool, while IL-7 deficiency had no effect (9, 17, 20, 24). Specifically, the total number of Lin−CD122 subset NK1.1+ Dx5+ NK cell population that contains both immature and mature NK cells was reduced in Flt3l−/−, Il2rg−/−, and I15−/− mice compared with WT mice (Fig. 1, A and C). Interestingly, Flt3l−/−Il2rg−/− mice had a further extensive reduction (182- and 12-fold, as compared with Flt3l−/− and Il2rg−/− mice, respectively) in the Lin−CD122 subset NK1.1+ Dx5+ NK cell compartment (Fig. 1, A and C). Of important note, Flt3l−/− and Flt3l−/−Il2rg−/− mice had a significant decrease in the total
BM cellularity compared with all other different mouse lines (Fig. 1B). Because IL-7 had no direct effect on NK cell compartments in BM, the impairment of NK development in \( \text{Il2rg}^{-/-}/\text{Il15}^{-/-} \) appears to be mainly due to the loss of IL-15. Taken together, these data demonstrate crucial and complementary roles of FLT3 ligand and IL-15 in the regulation of the BM NK cell pool.

**Critical role of FLT3 and IL-2Rg signaling for maintaining homeostasis in the peripheral NK cell pool**

Because the absence of FLT3L and IL-2Rg signaling resulted in severe reductions in the BM immature NK cell compartment, we next analyzed different subsets of peripheral mature NK cells in
FIGURE 2. Critical role of FLT3 ligand and IL-15 signaling in regulation of peripheral NK cells. A, Mean (SD) total spleen cell numbers of 8–12-wk-old WT (n = 8), Flt3l−/− (n = 11), Il2rg−/− (n = 7), and Flt3l−/−Il2rg−/− (n = 7) mice (each from two to three litters). B and C, FACS profiles (B) and mean (SD) total numbers (C) of CD3−NK1.1+DX5+ NK cells in spleens of 8–12-wk-old WT (n = 8), Flt3l−/− (n = 11), Il2rg−/− (n = 7), and Flt3l−/−Il2rg−/− (n = 7) mice (each from two to three litters). D, FACS profiles of CD3−DX5+NKp46+ NK cells in spleens of 10–15 wk old WT (n = 5), Flt3l−/− (n = 5), Il2rg−/− (n = 5), and Flt3l−/−Il2rg−/− mice (n = 5) mice (each from two litters). E, FACS profiles and mean (SD) total numbers of CD3−NK1.1+Ly49G2− NK cells in spleen of WT (n = 6), Flt3l−/− (n = 6), Il2rg−/− (n = 7), and Flt3l−/−Il2rg−/− (n = 5) mice (each from two litters). F, FACS profiles and mean (SD) total numbers of CD3−NK1.1+CD43+ Mac-1+ NK cells in spleen of 8–12-wk-old WT (n = 8), Flt3l−/− (n = 11), Il2rg−/− (n = 7) and Flt3l−/−Il2rg−/− (n = 7) mice (each from two to three litters). Numbers in FACS profiles show mean frequencies of populations within the indicated gates or quadrants of total spleen cells. The text above the flow profiles describes the gate. **, p < 0.01; ***, p < 0.001.
Flt3l−/−, Il2rg−/−, and Flt3l−/−Il2rg−/− mice. Although NK cell populations defined based on the expression of specific surface markers such as NK1.1, DX5, NKp46, CD43, and Mac-1 partly overlap, each of these subsets have been shown to also have distinct functions (36–38). As previously shown, the total spleen cellularity were reduced in Flt3l−/− and Il2rg−/− mice, however, Flt3l−/−Il2rg−/− mice had a further 2.8- and 3.5-fold reduction, compared with Flt3l−/− and Il2rg−/− mice, respectively (Fig. 2A).

In agreement with previous studies (17, 20), the CD3− NK1.1+ DX5+ NK cells in the spleen were reduced 5- and 82-fold, respectively, in Flt3l−/− and Il2rg−/− mice compared with WT controls (Fig. 2, B and C). Strikingly, Flt3l−/−Il2rg−/− mice had a further 127- and 8-fold reduction in the CD3− NK1.1+ DX5+ NK cells compared with Flt3l−/− and Il2rg−/− mice, respectively (Fig. 2, B and C), further supporting the crucial role of FLT3L in regulation of the IL-15-independent NK cell pool. Similar results were obtained when NK cells were identified using an Ab against NKp46, a highly NK cell-specific marker (37, 38) (Fig. 2D).

The CD3− NK1.1+ DX5+ NK cell pool in the spleen contains different NK cell populations that can be further characterized based on the expression of integrins such as Mac-1 and CD43 (36) as well as inhibitory and activating MHC class I-binding receptors of the Ly49 family (1). The frequency of NK cells expressing a given Ly49 receptor is influenced by interactions with host MHC class I and thus reflects NK cell “education” (1). The division of NK cells into Mac-1 and CD43 subpopulations have been suggested to represent different NK cell subsets with different functions (36). It was therefore of interest to investigate whether lack of function in either FLT3L, IL-2Rg, or both affected the compositions of these subsets. Consistent with a general reduction in the proportion of NK cells in the spleen, Flt3l−/− mice showed a 6-fold reduction in the CD3− NK1.1+ Ly49G2+ NK cells, whereas these were reduced as much as 398-fold in Il2rg−/− mice, although clearly detectable (Fig. 2E). Strikingly, the CD3− NK1.1+ Ly49G2+ NK cells were undetectable in the Flt3l−/−Il2rg−/− spleens (Fig. 2E). Interestingly, whereas in WT mice ~36% of NK cells expressed Ly49G2 receptor, the Ly49G2+ population was reduced to 25% in Flt3l−/− mice and only 3% in Il2rg−/− mice (Fig. 2E). Whereas the total number of CD3− NK1.1+ CD43+ Mac-1+ NK cells was reduced 5- and 191-fold, respectively in Flt3l−/− and Il2rg−/− mice compared with WT (Fig. 2F), in the Flt3l−/−Il2rg−/− mice they were further reduced 171- and 5-fold compared with Flt3l−/− and Il2rg−/− mice, respectively (Fig. 2F).

Because NK cell populations expressing NKp46 and Mac-1 receptors, which are both involved in NK target cell killing (1), were severely reduced in the absence of FLT3 ligand and IL-15, we next examined NK cell-mediated cytotoxicity in Flt3l−/−Il2rg−/− mice. The purpose of performing the killing assay experiments was primarily to verify that the decreased numbers of phenotypically defined NK cells seen in these mice would, as expected, translate into a decrease in NK cell activity. These functional studies were essential to verify that NK activity was really lost at the system level, and thereby to exclude that the decreased frequency of NK cells seen was not just due to loss of any NK cell phenotypic marker in the genetically modified mice. With this in mind, sorting phenotypically defined NK cells from the different mouse strains before performing the functional NK cell assay would carry the risk of biasing the assay to include only cells with the specific phenotype, rather than as performed on whole spleen cell population, being done in unbiased manner. Importantly, the target cell population used to measure NK cell-mediated killing provided a NK cell-specific assay, because RMA-Rae1y cells have been shown to be specifically killed by NK cells but not by NKT or conventional T cells (32). In agreement with the severe reduction in the number of phenotypically defined NK cells (Fig. 2, B–F), Flt3l−/−Il2rg−/− spleen cells showed little or no ability to kill NK-specific target cells (Fig. 3). Specifically, at the E:T cell ratio of 300:1, only 3.3% specific killing of RMA-Rae1y target cells was seen using Flt3l−/−Il2rg−/− spleen NK cells as effector cells, while specific target death was 22% using WT effector cells. At a 11:1 ratio, the cytotoxic activity of Flt3l−/−Il2rg−/− NK cells was undetectable compared with 2.4% in WT control (Fig. 3). Thus, the cytotoxic assay as performed provided a reliable functional correlate to the reduction in phenotypically defined NK cell numbers in Flt3l−/−Il2rg−/− mice, providing additional evidence for the observed NK cell deficiencies in the different mutant mice investigated.

Taken together, these data support a critical role of FLT3L for generation/maintenance of a functional NK cell pool, in the presence as well as the absence of IL-15.

**Role of FLT3 ligand in regulation of thymic NK developmental pathway**

Recent studies have demonstrated the existence of a thymus-dependent pathway of NK cell development (4). Importantly, thymus-derived NK cells can be distinguished from BM-dependent NK cells based on the expression of IL-7Rα, the absence of Ly49D (4), and also through their suggested IL-7-dependent development (4). The latter implicates an important functional distinction to BM-derived NK cells, which primarily depend on intact IL-15 function for maturation (1, 9, 10, 17). However, the regulatory role of other cytokine pathways, including IL-15 and FLT3L, remains to be investigated for thymic NK cells. To address this, we examined the thymic NK cell compartment in mice single and double deficient in FLT3L, IL-2Rg, IL-7, and IL-15 signaling (Flt3l−/−, Il2rg−/−, Il7−/−, Il15−/−, and Flt3l−/−Il2rg−/−, respectively). Interestingly, the number of thymic NK cells was reduced >2000-fold in Il2rg−/− mice compared with WT mice, and as much as 201-fold compared with Il7−/− mice, suggesting that IL-15 may act as an important regulator also of thymic NK cells (Fig. 4, A and C). Analysis of the thymic NK cell compartment in Il15−/− mice revealed 9-fold reduction compared with WT mice but no significant difference compared with Il7−/− mice (Fig. 4, A and C), suggesting that IL-7 and IL-15 both signaling through IL-2Rg play complementary roles in maintaining the thymic NK cell pool. Interestingly, although total number of thymic NK cells showed comparable reductions in both Il7−/− and Il15−/− mice, as a consequence of loss of IL-15, the frequency of NK cells was clearly reduced without affecting thymus cellularity. In contrast, the loss

**FIGURE 3.** Loss of cytotoxic activity in mice double deficient in FLT3 and IL-15 signaling. Ten- to 15-week-old mice were given 0.2 mg tilorone analog (Sigma-Aldrich) orally, were sacrificed 18 h later, spleens were removed, and single cell suspensions were used as effector cells in standard 51Cr-release assays. 300:1 to 11:1 ratios of effector cells were incubated for 4 h at 37°C with the NK sensitive RMA-Rae1y target cells labeled with 100 μCi 51Cr per 106 cells for 1 h at 37°C. Data represent mean (SD) percentage of specific killing from three independent experiments, each in which two to three spleens per genotype were pooled.
FIGURE 4. Role of FLT3 ligand and IL-2Rg cytokines in regulation of thymic dependent NK cell development. A, Representative FACS profiles of Lin<sup>−</sup>CD25<sup>−</sup>CD122<sup>−</sup>NK1.1<sup>−</sup>IL-7R<sub>A</sub><sup>−</sup>Ly49D<sup>−</sup> NK cells in the thymus (4) of different mice. B, Mean (SD) numbers of total thymus cellularities of 8- to 12-wk-old WT (n = 15), Flt3<sup>−/−</sup> (n = 14), Il2rg<sup>−/−</sup> (n = 9), Flt3<sup>−/−</sup>Il2rg<sup>−/−</sup> (n = 10), Il7<sup>−/−</sup> (n = 16), Il15<sup>−/−</sup> (n = 6) and Flt3<sup>−/−</sup>Il7<sup>−/−</sup> (n = 11) mice (each from two to four litters). C, Mean (SD) numbers of total Lin<sup>−</sup>CD25<sup>−</sup>CD122<sup>−</sup>NK1.1<sup>−</sup>IL-7R<sub>A</sub><sup>−</sup>Ly49D<sup>−</sup> thymic NK cells of different mice. Il2rg<sup>−/−</sup> and Flt3<sup>−/−</sup>Il2rg<sup>−/−</sup> thymuses from each litter were pooled for the analyses. Lineage negative (Lin<sup>−</sup>) = negative for CD3, CD4, CD8, CD19, Ter119, Mac-1, and Gr-1. Numbers in FACS profiles show mean frequencies of populations within the indicated gates of total thymocytes. **, p < 0.01; ***, p < 0.001; ns, not significant. The arrows indicate the gating, and the text above the flow profiles describes the gate.
FIGURE 5. FLT3 ligand and IL-15 are dispensable for homeostasis of committed NK progenitors. A, Representative FACS profiles of BM Lin^−CD122^+NK1.1^+DX5^+ committed NK progenitors (NKP) in BM (7) in different mice. Numbers in FACS profiles show mean frequencies of populations within the indicated gates of total BM cells. The arrows indicate the gating, and the text above the flow profiles describes the gate. B, Mean (SD) numbers of total BM cellularities of 8- to 12-wk-old WT (n = 6), Flt3^−/−^ (n = 6), Il2rg^−/−^ (n = 6), Il7^−/−^ (n = 6), Il15^−/−^ (n = 6), and Flt3^−/−^Il2rg^−/−^ (n = 6) mice (each from two to three litters). C, Mean (SD) of total numbers of Lin^−CD122^^−^NK1.1^−^DX5^−^NKPs in BM. Lineage negative (Lin^−^) cells were defined as negative for: CD19, Gr-1, Mac-1, CD3, CD4, CD8, and Ter119. Total BM cellularities (B) and absolute numbers of NKPs (C) are expressed per two tibiae and two femora. *, p < 0.05; ns, not significant. FACS profiles are the same as those shown in Fig. 1A.
of IL-7 resulted only in reduced thymic cellularity, but had no effect on NK cell frequency, suggesting that whereas IL-15 might act directly on NK cell development, IL-7 might primarily affect NK cell development by reducing the numbers of early thymic progenitors known to also have NK lineage potential (39–43) (Fig. 4, A–C). Although the total number of thymic NK cells was not affected in Flt3l−/− mice, NK cells were undetectable in the thymus of Flt3l−/−Il2rg−/− mice (Fig. 4, A and C), demonstrating a critical role of FLT3L in IL-2Rg-independent thymic NK cell generation. Taken together these results suggest that both IL-15 and IL-7 are critically involved in regulation of thymic NK cells, but most likely at distinct stages of development, and that FLT3L plays an important role in IL2Rg-independent thymic NK cell development.

FLT3L and IL-15 are dispensable for maintaining NK cell progenitors in bone marrow

The earliest committed NKPs have been identified in the BM of adult mice based on the Lin–CD122+ NK1.1+ DX5− phenotype (7). However, little is known about cytokines involved in NKP generation and maintenance. Mice deficient in IL-15 (Il15−/− or Il2rg−/−) have been suggested to have normal numbers of NKPs, but severely reduced immature and mature NK cell pools (17), indicating that other cytokine pathways may be involved in NK cell commitment and NKP generation. Because FLT3L is important in regulation of early lymphoid development as well as mature NK cells (18–20), we hypothesized that it could be also involved in regulation of the early stages of NKPs. Surprisingly, the lack of FLT3L, IL-2Rg, or IL-15 alone or in combination, did not have any significant effect on the total pool of committed NKP progenitors (Fig. 5, A and C). Specifically, the total number of NKPs in Flt3l−/−, Il2rg−/−, Il15−/−, and Flt3l−/−Il2rg−/− showed only 34, 50, 40, and 20% reductions, respectively, compared with WT control mice (Fig. 5, A and C). Taken together, these data suggest that FLT3L and IL-2Rg cytokines play important roles in generation and maintenance of immature and mature NK cell compartments but are dispensable for generation of committed NK progenitors.

Discussion

The present studies investigated and established a number of important and novel aspects of cytokine regulation of the NK cell compartment. IL-15 has been identified as the key regulator of mature NK cells (11–14), however, a small fraction of NK cells can develop in the absence of IL-15 (11, 15) and the potential role of other cytokines in regulation of IL-15-independent generation of NK cells remained to be investigated. Recent studies had implicated that BM and thymic pathways for NK cell development might be regulated by distinct rather than the same cytokines (4, 44). Synergistic interactions between cytokines that belong to hematopoietin and tyrosine kinase families play key roles in development of different blood lineages: for example FLT3L and IL-7 are indispensable for B and T cell development (26, 27) however, such interactions had not been studied in NK cell development. Herein, we provide new and important knowledge in each of these areas.

FLT3L has been identified as a critical regulator of early lymphopoiesis (18, 19), but its role in NK cell development has not been explored in detail. It was previously shown that Flt3l−/− mice have deficiencies in the functional NK cell pool however, only the NK1.1+CD3− peripheral NK cells were investigated in the spleen (20). In this study, we explored both the BM and spleen NK cell compartments and demonstrate that FLT3L has a distinct role in maintaining the BM immature NK cell compartment, as well as in the transition to mature NK cells. It has been shown previously that a significant proportion of NKP expressed IL-7Rα and FLT3, and all NKP are IL2Rβ+ (CD122+) (7, 17). In addition, the CLP upstream of the NKP also expresses IL-7Rα and FLT3, and both IL-7 and FLT3L are critical for generation and maintenance of the CLP population (5, 6, 19). However, the loss of FLT3L, IL-15, or IL-2Rg cytokines alone, or in combination, resulted only in mild reductions in the NKP pool, suggesting that these cytokines are dispensable for NKP generation and thus the key NKP cytokine regulatory pathway remains to be identified.

In an analysis of a limited set of additional NK cell markers, we found some unexpected alterations, suggesting that specific processes related to maturation and MHC class I-mediated education may also be affected. Although more extensive data will be needed to draw more firm conclusions, it is not unexpected that signaling from growth-promoting cytokines such as FLT3L, and in particular IL-15, would not only be critical for filling up the NK cell niche, but also to promote specific education events in which the NK cell repertoire is adapted to the MHC class I phenotype of the host. In fact, previous studies indicated that IL-15 is important for Ly49 induction (15, 17) as well as CD43 and Mac-1 expression (17), however the direct involvement of FLT3L was identified in this study for the first time. Additionally, the reduced production of mature NK cells may be secondary to the reductions in the immature NK cell compartment.

The role of FLT3L in NK lymphopoiesis was particularly evident in IL-2Rg-independent NK cell development, as reflected in Flt3l−/−Il2rg−/− mice having severe and almost undetectable cytotoxic activity.

Although the BM is the main site of postnatal NK cell development, recently a seemingly distinct thymic NK pathway was identified (4). In the same studies, IL-7 was proposed to be a key regulator of thymic NK cells (4), although the possible regulatory role of other cytokines was not explored. In addition, recent studies have shown that the transcription factor Id2 is required for BM-dependent but not thymic-dependent NK cells (45), suggesting distinct extracellular as well as intrinsic regulatory mechanisms of these two NK cell pathways. However, through the present studies, of Flt3l−/− and/or Il2rg−/− mice, we provide evidence for distinct but largely overlapping patterns of cytokine pathways mediated by FLT3L and IL-2Rg, being critical for regulation BM- and thymic-dependent NK cell development.

We have previously demonstrated a crucial role of FLT3L in IL-7-independent B and T lymphopoiesis, as Flt3l−/−Il7r−/− double deficient mice completely lack all stages of fetal and adult B cell development (26), and have extensive reductions in fetal and postnatal thymic progenitors resulting in loss of active thymopoiesis in early adult life (27). Herein, we show for the first time, that in a similar fashion FLT3L is critical for IL2Rg-independent NK lineage development.

Among several cytokines that signal through the common cytokine receptor chain IL2Rg (16), IL-15 has been shown to be the most important for BM-dependent NK cell development (11–14), as supported by the NK phenotype of Il2rg−/− mice being identical with that of Il15−/− mice (17). IL-7, another member of IL2Rg cytokines, has been shown to affect thymic NK cell development (4), but not BM NK cells (4, 17). Comparison of thymic NK cells in Il2rg−/− and Il7−/− mice revealed a much more severe NK cell reduction in Il2rg−/− than Il7−/− mice suggesting the complementary role of IL-7 and IL-15 in thymic NK cell development. Interestingly, although Il7−/− and Il15−/− mice had similar reductions in total number of thymic NK cells, the frequency of NK cells was reduced only in Il15−/− mice suggesting a direct and specific effect of IL-15 on committed NK cells, whereas reductions observed in Il7−/− mice were reflecting reduced thymic...
cellularity. IL-7 has unique role in maintenance thymic progenitors (22–24), known to possess NK lineage potential, thus, reduction in thymic NK cells could largely be a consequence of an effect of IL-7 at the thymic pre-NK cell level.

The concomitant loss of FLT3L and IL-2Rg cytokines resulted in complete depletion of thymic NK cells. Collectively, these findings implicate FLT3L as a critical regulator of IL-2Rg independent but not IL-7 independent thymic NK cell development.

In summary, we have identified distinct and partly overlapping patterns of FLT3L and IL-2Rg cytokine regulation of thymic- and BM-dependent NK cell generation, and establish a complementary role between hematopoietin and tyrosine kinase cytokines: IL-2Rg cytokines and FLT3L, as indispensable NK cell development regulators.

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Disclosures

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