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IL-15–High-Responder Developing NK Cells Bearing Ly49 Receptors in IL-15$^{-/-}$ Mice

Katsumi Yoshizawa,*,†,1 Shinsuke Nakajima,*,1 Tsuyoshi Notake,*,† Shin-Ichi Miyagawa,† Shigeaki Hida,* and Shinsuke Taki*

In mice lacking IL-15, NK cell development is arrested at immature stages, providing an opportunity to investigate the earliest developing NK cells that would respond to IL-15. We show in this study that immature NK cells were present in the spleen as well as bone marrow (BM) and contained IL-15–high-responder cells. Thus, mature NK cells were generated more efficiently from IL-15$^{-/-}$ than from control donor cells in radiation BM chimeras, and the rate of IL-15–induced cell division in vitro was higher in NK cells in the spleen and BM from IL-15$^{-/-}$ mice than in those from wild-type mice. Phenotypically, NK cells developed in IL-15$^{-/-}$ mice up to the minor but discrete CD11b$^-$CD27$^+$DX5^{hi}CD51^{dull}CD127^{dull}CD122^{hi} stage, which contained the majority of Ly49G2$^+$ and D$^+$ NK cells both in the spleen and BM. Even among wild-type splenic NK cells, IL-15–induced proliferation was most prominent in CD11b$^-$DX5^{hi} cells. Notably, IL-15–mediated preferential expansion (but not conversion from Ly49$^-$ cells) of Ly49$^+$ NK cells was observed in vitro only for NK cells in the spleen. These observations indicated the uneven distribution of NK cells of different developing stages with variable IL-15 responsiveness in these lymphoid organs. Immature NK cells in the spleen may contribute, as auxiliaries to those in BM, to the mature NK cell compartment through IL-15–driven extramarrow expansion under steady-state or inflammatory conditions. *The Journal of Immunology*, 2011, 187: 5162–5169.

Natural killer cells play crucial roles in host defense against viral infection, surveillance of neoplasms, as well as rejection of allogenic bone marrow (BM) transplants. Development of NK cells occurs mainly, yet not exclusively, in BM where both stromal and cytokine signals are believed to play indispensable, perhaps distinct, roles (1, 2). A cytokine known to be essential for NK cell development is IL-15. Thus, mature NK cells were virtually absent in mice deficient for IL-15 itself (IL-15$^{-/-}$ mice) or the α-chain of IL-15R (3, 4) that was required for the *trans*-presentation of IL-15 to responding cells (5, 6). All NK lineage cells, from the earliest committed NK cell progenitors to mature NK cells, expressed IL-2/IL-15R β-chain (CD122) (7), suggesting that IL-15 could potentially act throughout the course of NK cell development. In fact, IL-15 promoted not only early NK cell development but also the differentiation of CD11b$^+$CD27$^+$ NK cells into CD11b$^+$CD27$^-$ fully matured NK cells and survival of mature NK cells in the periphery (8–12).

Several NK cell developmental stages were defined with the signature cell surface molecules, including NK cell receptors, integrins, and cytokine receptors, expressed uniquely on NK lineage cells of particular stages (2, 13). As was formulated by Kim and colleagues (2, 14), most immature NK cells expressed integrin

α$_v$ (CD51) as well as NKG2D, NK1.1, CD94/NKG2A, NKp46, and CD127. As they progressed through the developmental stages, CD51 expression was lost and CD49b (DX5) and CD11b expression upregulated, with concomitant acquisition of Ly49 receptors (2, 13). Mature NK cells could further be separated into subpopulations based on the expression of CD43 or CD27 (14, 15). NK cell effector functions, including cytotoxicity and the ability to produce IFN-γ, were also acquired during the progression. It was reported that Ly49 receptors were not fully expressed on the residual NK cells in IL-15$^{-/-}$ mice, although they expressed other NK receptors such as NKG2D and NKp46 and exhibited a weak, yet demonstrable, cytotoxicity (16–18). NK cell development was halted at the earliest stage(s), and even very immature NK cells with the NK1.1$^+$DX5$^-$CD43$^-$CD11b$^-$ phenotype were reduced in these mice (16). These earlier observations suggested that IL-15 expanded very early NK cell progenitors and accelerated Ly49 expression (18, 19).

To obtain insights into what IL-15 did on developing NK cells and where IL-15–mediated progression of NK cell development occurred, we characterized NK lineage cells remaining in the spleen and BM in IL-15$^{-/-}$ mice. These cells were found to proliferate faster in vitro and to replenish mature NK cell compartment in vivo more efficiently than wild-type NK cells. NK cell development in IL-15$^{-/-}$ mice seemed to be arrested at the discrete CD11b$^-$DX5^{hi}CD51^{dull}CD127^{dull} immature NK cell stage, and the great majority of Ly49G2$^+$ or Ly49D$^+$ NK cells in these mice, which were present at reduced frequencies, exhibited the same phenotype. IL-15 failed to induce Ly49 expression but expanded these preexisting Ly49$^+$ NK cells preferentially over Ly49$^-$ NK cells in IL-15$^{-/-}$ spleen cells in vitro. Notably, such a preferential expansion of Ly49$^+$ cells was not observed in IL-15$^{-/-}$ BM cell cultures. Our current study thus indicated that IL-15 responsiveness was dynamically regulated as NK cells developed, and the majority of IL-15–high-responder NK cells in the spleen bore Ly49 receptors, whereas those in BM were both Ly49$^+$ and

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Abbreviation used in this article: BM, bone marrow.

Ly49⁻. IL-15–high-responder immature NK cells present in the spleen may participate in the establishment of the peripheral mature NK cell compartment as driven by constitutively produced and/or inflammation-induced IL-15.

Materials and Methods

Mice

IL-15$^{-/-}$ mice on the C57BL/6 background (3) were purchased from Taconic Farms. These mice were crossed to mice lacking RAG-1 (RAG$^{-/-}$ mice), back-crossed at least 10 times with C57BL/6 mice (17), to generate IL-15$^{-/-}$RAG$^{-/-}$ double mutant mice. C57BL/6 mice on the Ly5.1 background (B6.Ly5.1 mice) were from Sankyo Labo (Tsukuba, Japan). All mice were maintained in the animal facility at the Research Center for Human and Environmental Science, Shinshu University, under specific pathogen-free conditions and used at 8–12 wk of age. All animal experiments were preapproved by the Division of Laboratory Animal Research of Shinshu University and performed in accordance with the Regulation for Animal Experimentation of Shinshu University.

Abs and flow cytometry

The following fluorochrome- or biotin-conjugated Abs were used for flow cytometry: anti-Ly49G2 (4D11), anti-Ly49D (4E5), anti-Ly49A (A1), anti-CD11b (M1/70), anti-CD122 (TM-β1), anti-CD43 (S7), anti–c-Kit (2B8) and anti-Ly5.1 (A20), purchased from BD Pharmingen; anti-Ly49I (YLI90), anti-TCRβ (H57-597), anti-NK1.1 (PK136), anti-CD127 (A7R34), anti-CD49b (DX5), anti-CD51 (RMV-7), anti–Sca-1 (D7), anti-CXCR3 (CXCR3-173), anti-NKG2A/C/E (20d5), and anti-Ly5.2 (104), from eBioscience; and anti-CD27 (LG-3A10) from BioLegend. FITC–streptavidin and PE–Cy7–streptavidin (eBioscience) and PE–Texas red–streptavidin (BD Pharmingen) were used to develop biotin-conjugated Abs. Cells were stained on ice in the presence of anti-CD16/32 Ab (culture supernatant from 2.4G2 hybridoma) to block Fc-mediated nonspecific staining and analyzed on the Cytomics FC500 flow cytometer (Beckman-Coulter). Data analysis was carried out with the RXP software (Beckman-Coulter).

Cell preparation and culture

Spleen or BM cells (4×10^5) were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and 2 mM L-Gln in the presence of 50 ng/ml of murine recombinant IL-15 (Peprotech) in 48-well plates for 8 d. To deplete Ly49$^+$ NK cells, spleen cells were stained with FITC–anti-Ly49 Abs, followed by staining with anti-FITC microbeads (Miltenyi Biotec), and depleted on the AutoMACS as instructed by the manufacturer (Miltenyi Biotech). To obtain NK cell subpopulations, spleen cells were depleted with anti-TCRβ, anti-CD19, and anti-TER119 Abs, followed by staining with appropriate Abs and microbeads, and purified on the AutoMACS. For the analysis of cell division, cells were labeled for 8 min with 1 μM CFSE (Molecular Probe), washed with FBS-containing medium, and cultured as above for 3 d in vitro in 24-well plates (1×10^6 cells/well). When CFSE-labeled NK cell subpopulations were cultured, wild-type spleen cells depleted of NK1.1$^+$ cells were added as "feeder" cells (1×10^6 cells/well).

Radiation BM chimeras

Radiation BM chimeras were established by reconstituting irradiated (9.5 Gy) C57BL/6 or IL-15$^{-/-}$ mice with red cell-depleted BM cells (5×10^7) derived from B6.Ly5.1 mice through the tail vein and analyzed 10 d later. For mixed BM chimeras, we reconstituted irradiated (9.5 Gy) IL-15$^{-/-}$ mice with a 1:1 mixture of red cell-depleted BM cells prepared from IL-15$^{-/-}$ and B6.Ly5.1 mice (2.5×10^7 cells from each donor) and analyzed 2 or 8 wk later.

Results

IL-15–deficient BM cells were highly efficient in rapid repopulation of the mature NK cell compartment in radiation BM chimeras

To examine the potential of the residual immature NK cells in IL-15$^{-/-}$ mice to mature in vivo, we generated radiation BM chimeras through cotransplantation of BM cells from IL-15$^{-/-}$ (Ly5.2) and those from wild-type (Ly5.1) mice into irradiated IL-15$^{-/-}$ recipients. As we used irradiated IL-15$^{-/-}$ mice as recipients, only the accompanying wild-type donor BM cells, likely

macrophages and/or dendritic cells (5, 9), would supply IL-15. With such a restricted supply of IL-15, IL-15$^{-/-}$ BM donor cells could generate CD11b$^+$DX5^{hi} mature NK cells in these mixed chimeric mice as efficiently as did wild-type donor cells in long-term (8 wk) settings (Fig. 1A), whereas in the chimeras established with IL-15$^{-/-}$ BM donor cells alone, the BM donor cells could not generate CD11b$^+$DX5^{hi} NK cells, obviously due to the absence of the source of IL-15. The presence of CD11b$^+$ CD51$^-$ as well as Ly49I$^+$ and Ly49G2$^+$ cells confirmed the mature phenotype of these NK cells (data not shown). Rather surprisingly, similarly efficient maturation of NK cells from IL-15$^{-/-}$ BM donors was observed when these chimeras were examined 2 wk after transfer (Fig. 1B), a duration perhaps not sufficient for early lymphoid progenitors to differentiate into mature NK cells but sufficient for CD11b$^-$ NK cells to mature to CD11b$^+$ NK cells (14). NK1.1$^+$ cells as well as B cells in these chimeras were almost equally derived from both donors; NK cells derived from IL-15$^{-/-}$ donors occupied 51.6 ± 3.6% and 52.7 ± 6.9% in spleen ($n = 7$) and BM ($n = 6$), respectively, on average. IL-15$^{-/-}$ donor-derived NK cells in the spleen were as mature as those derived from wild-type donors as judged from the abundance of CD11b$^+$DX5^{hi} or Ly49$^+$ cells (Fig. 1B). Given the paucity of NK1.1$^+$ cells in IL-15$^{-/-}$ donor BM cells ($5.6 ± 2.3 \times 10^5$ and $0.47 ± 0.22 \times 10^5$ cells in two femurs in wild-type and IL-15$^{-/-}$ mice, respectively; $n = 4$ for each genotype), these results suggested a possibility that residual NK cells in IL-15$^{-/-}$ mice possessed higher potential in IL-15–driven expansion and/or maturation than that of wild-type NK cells. In a separate series of BM chimeras in which wild-type BM cells were transferred into irradiated IL-15$^{-/-}$ mice, we confirmed that virtually all (97.0 ± 0.75%, $n = 3$) of splenic NK cells were of donor origin on day 10 after transfer. Thus, NK cells derived from IL-15$^{-/-}$ recipients contributed minimally, if at all, to mature NK cells in these mixed chimeras.

Residual NK cells in IL-15$^{-/-}$ mice proliferated faster in response to IL-15 than wild-type NK cells

To examine such a possibility more directly, we compared IL-15–mediated proliferation of NK cells from IL-15$^{-/-}$ and wild-type mice in vitro. To preclude the influences of non-NK cells on IL-15–induced NK cell proliferation, wild-type (Ly5.1) and IL-15$^{-/-}$ (Ly5.2) cells were cultured together in the presence of IL-15 and enumerated separately 8 d later. In these mixed cultures, NK cells derived from wild-type BM and spleen cells expanded 92.6- and 29.6-fold, respectively, whereas IL-15$^{-/-}$ BM and splenic NK cells exhibited much higher degrees of expansion of 463- and 154-fold, respectively (Fig. 2A). Accordingly, the frequencies of IL-15$^{-/-}$ NK cells were elevated from 5.8% before culture to 33.8% at the end of culture for BM cells and those of IL-15$^{-/-}$ splenic NK cells from 10.1 to 31.2% during the culture (Fig. 2B). Furthermore, when CFSE-labeled spleen and BM cells were cultured for 3 d with IL-15, CFSE dilution was more prominent in NK1.1$^+$ cells derived from IL-15$^{-/-}$RAG$^{-/-}$ mice than that in those from RAG$^{-/-}$ mice (Fig. 2C). These results indicated that immature NK cells in IL-15$^{-/-}$ spleen and BM were more efficient than those in wild-type spleen in IL-15–driven proliferation.

NK cell development was arrested at a unique immature stage in IL-15$^{-/-}$ mice

In agreement with previous reports (3, 16), NK1.1$^+$ cell numbers in BM and spleen in IL-15$^{-/-}$RAG$^{-/-}$ double deficient mice were reduced up to 10-fold in comparison with those in RAG$^{-/-}$ mice. The reduction became progressively more severe as NK cells matured. As depicted in Fig. 3A, virtually all NK1.1$^+$ cells in IL-15$^{-/-}$RAG$^{-/-}$ mice lacked CD11b expression, in contrast to
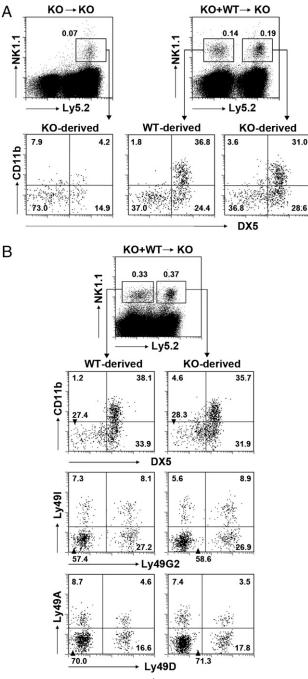
FIGURE 1. Maturation of IL-15$^{-/-}$ NK cells in mixed BM chimeras. *A*, Irradiated IL-15$^{-/-}$ mice were reconstituted with IL-15$^{-/-}$ BM cells (KO→KO) or a 1:1 mixture of BM cells derived from B6.Ly5.1 and IL-15$^{-/-}$ mice (KO+WT→KO). TCRβ$^-$ cells derived from B6.Ly5.1 (Ly5.2$^-$, "WT-derived") or IL-15$^{-/-}$ (Ly5.2$^+$, "KO-derived") donors were analyzed 8 wk after transfer for the expression of NK1.1, CD11b, and DX5. Representative results of three independent chimeras for each combination. *B*, Splenic NK cells in mixed BM chimeras established as in *A* were analyzed 2 wk after transfer for the expression of the markers indicated. Representative of three mixed chimeras. In two of these mixed chimeras, B cells of wild-type origin occupied 44.7 and 54.2% of all B cells 2 wk after transfer (data not shown). In *A* and *B*, numbers indicate the percentages of cells within each quadrant or region.

those in RAG$^{-/-}$ mice of which >80% and around one half in the spleen and in BM, respectively, were CD11b$^+$. The majority of these CD11b$^-$ NK cells in IL-15$^{-/-}$RAG$^{-/-}$ mice expressed CD27 (Fig. 3*A*) and accordingly CXCR3 (20) at frequencies comparable with those in RAG$^{-/-}$ mice (data not shown). Nota-
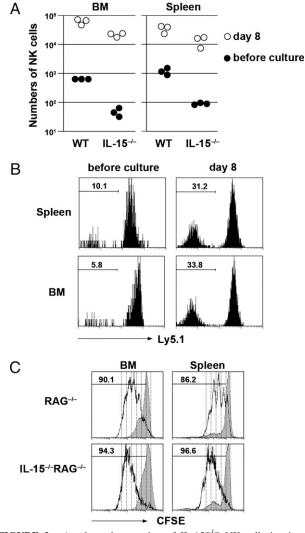

FIGURE 2. Accelerated expansion of IL-15$^{-/-}$ NK cells in vitro in response to IL-15. *A*, BM or spleen cells derived from B6.Ly5.1 (control) or IL-15$^{-/-}$ mice were mixed 1:1 and cultured in the presence of IL-15 for 8 d. The numbers of Ly5.1$^+$ and Ly5.1$^-$ NK1.1$^+$TCRβ$^-$ cells in recovered cells were calculated separately. Each symbol represents the mean NK cell numbers before (●) and after (○) culture for an independent culture with duplicated wells. Cumulative data from three independent cultures. *B*, Spleen or BM cells from B6.Ly5.1 and IL-15$^{-/-}$ (Ly5.2) mice were cultured as in *A*. Recovered NK1.1$^+$TCRβ$^-$ cells were analyzed for Ly5.1 expression. The percentages of NK1.1$^+$TCRβ$^-$ cells derived from IL-15$^{-/-}$ mice are shown. Representative of three independent trials with similar results. *C*, CFSE-labeled BM and spleen cells prepared from RAG$^{-/-}$ or RAG$^{-/-}$IL-15$^{-/-}$ mice were cultured as in *A* but for 3 d. CFSE fluorescence is shown for NK1.1$^+$ cells, together with the CFSE profiles of NK1.1$^-$ cells (shaded histograms). The numbers indicate the percentages of divided cells. Representative of five independent experiments.

bly, whereas most of DX5^{hi} cells in RAG$^{-/-}$ mice lacked CD51 expression, a substantial fraction of DX5^{hi} NK cells in IL-15$^{-/-}$RAG$^{-/-}$ mice expressed CD51 at levels slightly lower than those of DX5 $^{lo/-}$ cells (Fig. 3*A*). In addition, when CD11b$^-$ NK cells from these two strains of mice were compared (Fig. 3*B*), the majority of DX5^{hi} cells in IL-15$^{-/-}$RAG$^{-/-}$ mice were found to be largely CD27$^+$ and to express higher levels of CD127 and c-Kit than those in RAG$^{-/-}$ mice. NK cell development seemed thus to advance up to the CD11b$^-$CD27$^+$DX5^{hi} stage in IL-15$^{-/-}$RAG$^{-/-}$ mice, but these cells had not yet downregulated CD51, CD127, and c-Kit fully to the levels seen on mature CD11b$^+$ NK cells.
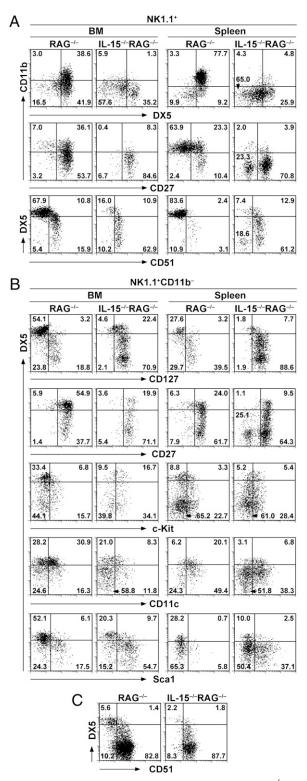
FIGURE 3. Immature phenotype of residual NK cells in IL-15$^{-/-}$ mice. BM and spleen (*A*, *B*) and liver (*C*) cells prepared from RAG$^{-/-}$ or IL-15$^{-/-}$RAG$^{-/-}$ mice were stained for the markers indicated. Profiles for NK1.1$^+$ (*A*) and NK1.1$^+$CD11b$^-$ (*B*, *C*) cells are shown. Because anti-DX5 Abs with different fluorochromes were used in combination with other Abs, the intensities for DX5 staining are not necessarily equal among different stainings. Numbers indicate the percentages of cells within each quadrant. Profiles for the CD27 in *A* and for CD27 and c-Kit in *B* are representative of two animals, whereas the other profiles are representative of more than three animals for each genotype.

Higher frequencies of splenic CD11b$^-$DX5^{hi} NK cells were observed to express CD11c than did those in the BM. Only a small fraction of CD11b$^-$DX5^{hi} NK cells in IL-15$^{-/-}$RAG$^{-/-}$ mice expressed Sca-1 (Fig. 3*B*), which was expressed almost exclusively on CD11b$^-$CD51$^+$ NK cells (data not shown). These DX5$^{dull/-}$Sca-1$^+$CD51$^+$ cells, scarcely present in the spleen in RAG$^{-/-}$ mice, seemed to represent most immature cells within CD11b$^-$ NK cells. CD11b$^-$DX5^{hi}CD51^{dull} NK cells were also present, yet at extremely low frequencies, in the liver (Fig. 3*C*), whereas these cells were practically difficult to identify clearly in lymph nodes where NK cells were normally much less frequent than in the other organs examined (data not shown).

Ly49 expression and upregulation of CD122 on developing NK cells during the transition from the DX5$^{dull/-}$CD51$^+$ to DX5^{hi} CD51$^-$ stages

One of the differences of residual NK cells in IL-15$^{-/-}$ mice from those in wild-type mice was the low frequencies of Ly49$^+$ cells among them (16–19), whereas NKG2A/C/E$^+$ cells were as frequent in IL-15$^{-/-}$ mice as in wild-type mice; 47.5 ± 5.1% (*n* = 4) and 48.8 ± 8.4% (*n* = 5) of splenic NK cells in wild-type and IL-15$^{-/-}$ mice, respectively. These observations were consistent with the expression of NKG2A/C/E already on CD51$^+$ immature NK cells as previously reported (14). We chose Ly49G2 and Ly49D for examinations thereafter, as these two were more abundant both in the spleen and BM in IL-15$^{-/-}$ mice than the others (data not shown). The paucity of Ly49$^+$ cells in IL-15$^{-/-}$ mice appeared to be due largely to the lack of CD11b$^+$DX5^{hi}CD51$^-$ mature NK cells, the majority (>70%) of which expressed either Ly49G2 or D (Fig. 4*A*). Ly49G2/D$^+$ cells were found in RAG$^{-/-}$ mice in CD11b$^-$DX5^{hi}CD51$^-$ immature NK cells less frequently than in CD11b$^+$ NK cells, but almost not at all in DX5$^{lo/-}$ and CD51$^+$ NK cells (Fig. 4*A*). In IL-15$^{-/-}$RAG$^{-/-}$ mice, small numbers of Ly49G2/D$^+$CD11b$^-$DX5^{hi} NK cells were also present, but unlike those in RAG$^{-/-}$ mice, they were CD51^{dull} (Fig. 4*A*) and CD127^{dull} as can be expected from the data shown in Fig. 3*B*. A slight difference was noted between NK cells in the BM and spleen in IL-15$^{-/-}$RAG$^{-/-}$ mice in that Ly49G2/D$^+$ cells in spleen contained more DX5^{dull} cells than those in BM. We also noted that the expression of CD122 molecules, the β-chain of functional IL-15R, was higher on DX5^{hi} or CD51$^{dull/-}$ cells among CD11b$^-$ NK cells both in RAG$^{-/-}$ and IL-15$^{-/-}$RAG$^{-/-}$ mice (Fig. 4*B*). CD122 expression was kept at relatively high levels thereafter during normal NK cell development, as the levels of CD122 displayed on mature CD11b$^+$ NK cells were slightly lower, yet significantly higher, compared with those on CD11b$^-$DX5^{hi} and CD11b$^-$DX5$^{dull/-}$ NK cells, respectively, in RAG$^{-/-}$ mice (Fig. 4*C*). The majority, if not all, of Ly49G2/D$^+$ NK cells in IL-15$^{-/-}$ RAG$^{-/-}$ mice appeared to be included in these CD122^{hi} cells.

IL-15 expanded preexisting Ly49$^+$ NK cells in the spleen preferentially but failed to induce Ly49 expression

IL-15 was suggested to elevate Ly49 expression (18, 19). When cultured in vitro with IL-15, the frequencies of Ly49$^+$ NK cells were elevated prominently in splenic NK cells derived from IL-15$^{-/-}$ mice, whereas those in wild-type spleen cells were only slightly elevated (Fig. 5). In contrast, we did not observe such an elevation of Ly49$^+$ cell frequencies in both wild-type and IL-15$^{-/-}$ BM-derived cultured NK cells, except for a moderate difference in Ly49G2$^+$ cell frequencies in IL-15$^{-/-}$ BM-derived NK cells (Fig. 5). We then tested if IL-15 induced Ly49 expression on splenic NK cells from IL-15$^{-/-}$ mice in the in vitro cultures. As seen in Fig. 6*A*, IL-15 failed to induce Ly49G2 or D expression on NK cells depleted of Ly49$^+$ cells prepared from IL-15$^{-/-}$ spleen. Even
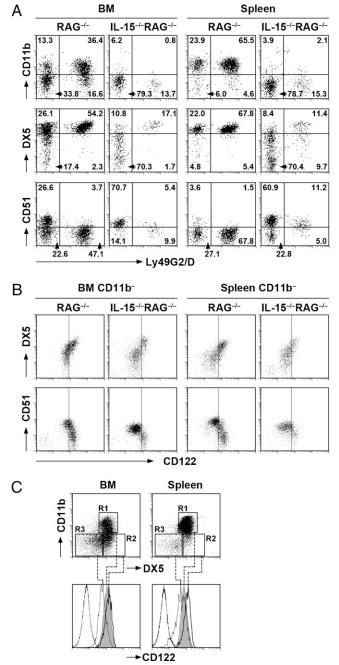
FIGURE 5. IL-15 elevated Ly49$^+$ cell frequencies in splenic, but not BM, NK cells from IL-15$^{-/-}$ mice. Spleen and BM cells from control and IL-15$^{-/-}$ mice were cultured in vitro with IL-15 for 8 d. The ratio of the frequencies of NK1.1$^+$TCRβ$^-$ cells bearing the indicated Ly49s before culture to those after culture was calculated for four independent cultures except for IL-15$^{-/-}$ BM cells (three cultures). The means ± SD are shown. Values significantly higher for IL-15$^{-/-}$ NK cells than control as determined with Student t test are marked. $*p < 0.05$, $**p < 0.01$.

FIGURE 4. Ly49G2/D expression and CD122 upregulation on NK cells during the transition from DX5$^{dull/-}$CD51$^+$ to DX5^{hi}CD51$^-$. *A* and *B*, Expression of Ly49G2/D (*A*) and CD122 (*B*) is shown for total NK1.1$^+$ (*A*) and NK1.1$^+$CD11b$^-$ (*B*) cells from the BM and spleen in RAG$^{-/-}$ or IL-15$^{-/-}$RAG$^{-/-}$ mice. *C*, CD122 expression on NK1.1$^+$ cell subpopulations in RAG$^{-/-}$ mice is shown, with controls representing CD122 expression on NK1.1$^-$ cells (the *leftmost* histogram in each panel). Numbers in *A* represent the percentages of cells within each quadrant among NK1.1$^+$ cells. Representative staining of more than three (*A*, *C*, and DX5 in *B*) and two (CD51 in *B*) independent experiments.

in whole spleen cell cultures, the levels of cell surface Ly49s were not altered (Fig. 6*A*). These results argued against the role for IL-15 in Ly49 induction or upregulation on NK cells and rather favored the hypothesis that preexisting Ly49$^+$ NK cells proliferated more efficiently in response to IL-15 than the other NK cells in the spleen in IL-15$^{-/-}$ mice, thereby resulting in the elevation of overall Ly49$^+$ cell frequencies. Consistent with this hypothesis,
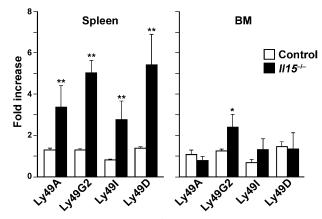
splenic NK cells depleted of Ly49G2$^+$ and D$^+$ cells were slightly less efficient in IL-15–induced proliferation in vitro (Fig. 6*B*). Moreover, when unseparated NK cells were cultured, NK cells that underwent only a few rounds of cell division were more abundant in Ly49G2$^-$ cells than in Ly49G2$^+$ cells, whereas highly divided NK cells were equally present in both subpopulations, perhaps representing NK cells expressing other Ly49s than Ly49G2 (Fig. 6*C*). Removal of Ly49G2$^+$ and D$^+$ cells from wild-type spleen cells prior to the cultures did not alter the rate of cell division (data not shown). These observations suggested that CD11b$^-$DX5^{hi} NK cells were most efficient in IL-15 responses among splenic NK cells in IL-15$^{-/-}$ mice.

CD11b$^-$DX5^{hi} NK cells in wild-type mice proliferated faster than the other NK cell subpopulations in the spleen

To test if the differential responsiveness to IL-15 was observed also among splenic NK cells in wild-type mice, we next examined the proliferation of splenic NK cells of various developmental stages in response to IL-15. Upon stimulation with IL-15 in vitro, c-Kit, Sca-1, and CD51 were rapidly upregulated on almost all NK1.1$^+$ cells within 1 or 2 d (data not shown). It was thus not likely that the phenotypes of in vitro-expanded NK cells mirrored those of initial IL-15–responding NK cells. We hence took another approach in which NK cells were separated into subpopulations and labeled with CFSE before culture. Fully matured CD43$^+$ NK cells were found to proliferate to a lesser extent than did CD43$^-$ NK cells (Fig. 7*A*), and immature CD11b$^-$ cells underwent more cell divisions than did whole or CD11b$^+$ NK cells (Fig. 7*B*). Furthermore, CD11b$^-$DX5^{hi} NK cells proliferated faster than whole CD11b$^-$ NK cells, whereas CD11b$^-$DX5$^{dull/-}$ NK cells proliferated very poorly. These observations indicated that IL-15 did not uniformly expand all NK cells present in the spleen in wild-type mice, and the CD11b$^-$DX5^{hi} subpopulation, among them, contained NK cells that had the highest potential to expand in response to IL-15.

Discussion

IL-15 is considered to play important roles in immune responses mediated by NK cells and CD8$^+$ T cells, as this cytokine promotes not only NK cell development but also the survival in the steady state and activation during infection of mature NK and CD8$^+$
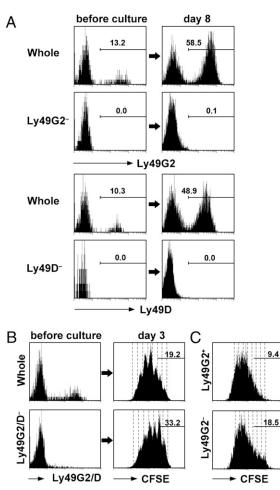
FIGURE 6. IL-15 failed to induce Ly49 expression but expanded pre-existing Ly49+ NK cells. *A,* Untreated spleen cells (Whole) and those depleted of Ly49G2+ (Ly49G2−) or Ly49D+ (Ly49D−) cells prepared from IL-15−/− mice were cultured with IL-15 for 8 d. Histograms denote Ly49G2 or Ly49D expression on NK1.1+TCRβ− cells before (*left panels*) and after (*right panels*) culture. *B,* Spleen cells from IL-15−/− mice were depleted of cells bearing either Ly49G2 or Ly49D, labeled with CFSE, and cultured with IL-15. Ly49G2/D expression before culture and CFSE fluorescence on day 3 are shown for NK1.1+TCRβ− cells. *C,* CFSE-labeled unseparated spleen cells from RAG−/−IL-15−/− mice were cultured for 3 d, and Ly49G2+ and Ly49G2− NK1.1+ cells were gated and examined separately for CFSE fluorescence. The numbers in *B* and *C* represent the percentages of cells that underwent two or less cell divisions. Representative results of more than three (*A, C*) and two (*B*) independent experiments.

T cells (3, 8, 10–12, 21, 22). All the NK lineage cells express CD122 and would potentially respond to IL-15 (2, 13). We showed in this article that NK lineage cells responded to IL-15 not uniformly but to a variety of extents, and residual immature NK cells in IL-15−/− mice contained NK cells of the IL-15–high-responder stage.

BM cells from IL-15−/− mice, despite the paucity of NK lineage cells, replenished mature NK cells within 2 wk as efficiently as wild-type BM cells in mixed chimeras. This rapid replenishment might be attributable to the very immature committed progenitors for NK cells or even multipotent hematopoietic stem cells that would likely be present normally in IL-15−/− mice. However, CD11b−NK1.1+ cells were shown to give rise to mature CD11b+ NK cells within 2 wk in vivo (14, 23), and CD11b− NK cells were reported to undergo vigorous expansion through ho-

meostatic proliferation within 2 wk after transfer into an NK cell-deficient environment in a manner dependent on IL-15 (10). Thus, it was possible that at least a fraction of those mature NK cells were direct descendants of immature NK cells present in IL-15−/− donor BM cells. If this was the case, the equal contribution of IL-15−/− and wild-type donor BM cells to mature NK cells in the short-term mixed chimeras would suggest that residual NK cells in IL-15−/− mice were more potent in IL-15–driven expansion.

Consistent with this prediction, splenic and BM NK cells derived from IL-15−/− mice expanded more efficiently than wild-type NK cells in response to IL-15 in vitro. Furthermore, CD11b−DX5hi NK cells in wild-type mice proliferated most efficiently among all NK cells in the spleen. Reinforcing the report that proliferating NK cells in vivo exhibited CD43−CD11b−DX5hi phenotype as measured by BrdU incorporation assay (14), these observations suggested that such a steady-state proliferation in vivo was mediated by IL-15. A somewhat different picture reported recently, however, is that the highest proliferation rate was observed for CD11b−CD27−DX5dull/− NK cells (23). The basis for this difference is unclear but may possibly be due to the different markers used to define NK lineage cells; whereas Chiossone et al. (23) defined NK lineage cells with NKp46 expression, we and Kim et al. (14) used the NK1.1 marker. Another recent report showed that injection of IL-15/IL-15Rα complexes acutely and transiently expanded CD11b−CD27+NK1.1+CD3− cells in the spleen and BM (24). Because CD11b−DX5hi cells were a subset of CD11b−CD27+ NK cells (Fig. 3B), this observation was not contradictory to ours.

More than a half of NK cells in the BM were CD11b− in RAG−/− mice, whereas the great majority of NK cells in the spleen were CD11b+ (Fig. 3A). Even within CD11b− NK cells, the IL-15–high-responder CD11b−DX5hi cells were twice more abundant in the BM than in the spleen (Fig. 3B). This unequal distribution of developing NK cell subpopulations in these two organs seemed to account, at least in part, for the superior IL-15–induced proliferation of wild-type NK cells in BM to that of splenic NK cells (Fig. 2C). Likewise, in addition to the absence of less-proliferative CD11b+ NK cells, the relative abundance of CD11b−DX5hi NK cells in the spleen in IL-15−/−RAG−/− mice (25.9 versus 9.2% in RAG−/− mice, see Fig. 3A) might also be involved in the accelerated proliferation of splenic NK cells in IL-15−/− mice compared with wild-type NK cells. In this regard, CD11c+ cells were less abundant in CD11b−DX5hi NK cells in the BM than in those in the spleen in IL-15−/−RAG−/− mice (Fig. 3B), suggesting that CD11b−DX5hi NK cells in these two organs differed not only quantitatively but also qualitatively. Further examinations are certainly required to determine the relative importance of these characteristics of NK cells in IL-15−/− mice in the accelerated IL-15 responses. Nevertheless, it is clear that IL-15 responsiveness was regulated dynamically during NK cell development, although essentially all NK lineage cells including fully mature NK cells responded to IL-15 at least to certain extents.

Detailed flow cytometric examination showed that NK cells in IL-15−/− mice developed up to the NK1.1+CD11b−CD27+DX5hi Sca-1− stage but were still on the way to downregulate the expression of CD51, CD127, and c-Kit, and hence distinct apparently from the majority of CD11b−DX5hi NK cells in wild-type mice that were CD51−, CD127−, and c-Kit−. DX5 upregulation and CD127 downregulation were reported to occur during the transition from the CD27+CD11blo to CD27+CD11bhi stages in the recently proposed four-step development model (23). In this regard, CD11b−DX5hiCD51dullCD127dullc-Kitdull NK cells in IL-15−/− mice were unique as they constituted a subset within the CD11b− CD27+ stage and seemed to represent an intermediate between the stages III (DX5+CD51+c-Kit+) and IV (DX5+CD51−c-Kit+)
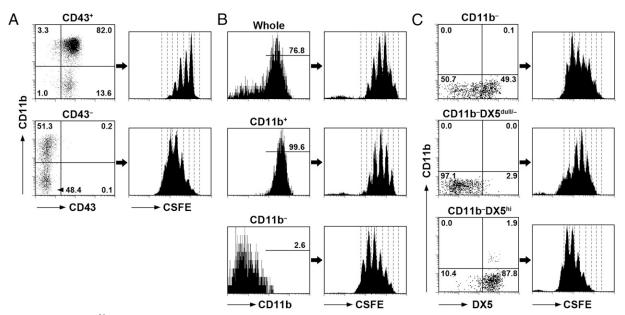
FIGURE 7. CD11b⁻DX5ʰⁱ NK cells underwent cell division most efficiently among NK cell subpopulations in the spleen in wild-type mice. *A*, Spleen cells isolated from wild-type mice were depleted of T and B cells, followed by separation into CD43⁺ and CD43⁻ subsets. These cells were labeled with CFSE and cultured with IL-15 in the presence of NK cell-depleted wild-type spleen cells. CD11b and CD43 expression before culture (*left panels*) and CFSE fluorescence on day 3 (*right panels*) on NK1.1⁺TCRβ⁻ cells are shown. *B*, T and B cell-depleted wild-type spleen cells were separated into CD11b⁺ and CD11b⁻ subpopulations, labeled with CFSE, and cultured as in *A*. "Whole" denotes T and B cell-depleted spleen cells. CD11b expression before culture (*left panels*) and CFSE fluorescence on day 3 (*right panels*) on NK1.1⁺TCRβ⁻ cells are shown. *C*, T and B cell-depleted wild-type spleen cells were separated into CD11b⁻, CD11b⁻DX5ᵈᵘˡˡ/⁻, and CD11b⁻DX5ʰⁱ subpopulations and cultured as in *A*. *Left* and *right panels* represent NK1.1⁺TCRβ⁻ cells before and after culture, respectively. Repeated three (*A, C*) and five (*B*) times with similar results.

proposed by Kim et al. (14). CD11b⁻DX5ʰⁱ cells expressing CD11c in the spleen in IL-15⁻/⁻RAG⁻/⁻ mice (Fig. 3*B*) might be identical to NK1.1⁺DX5⁺CD11c⁺B220⁺ cells observed at extremely low frequencies in the spleen of RAG-2⁻/⁻γc⁻/⁻ mice (25). With no surface markers available to distinguish these unique NK cells clearly from others, it was practically impossible at this moment to enumerate unequivocally CD11b⁻DX5ʰⁱ NK cells expressing low levels of CD51, CD127, and c-Kit in the spleen and BM in wild-type mice and to examine if these CD51ᵈᵘˡˡ CD127ᵈᵘˡˡc-Kitᵈᵘˡˡ NK cells proliferated better than CD127⁻ CD51⁻ cells among CD11b⁻DX5ʰⁱ NK cells.

The molecular basis for the dynamic regulation of IL-15 responsiveness on developing NK cells is not clear at this moment, and little has been studied even as to the mechanisms for the regulation of IL-15 responses in general. As a possible mechanism, we found in this article that CD122 expression was upregulated during the transition from DX5ᵈᵘˡˡ/⁻CD51⁺CD127⁺ to DX5ʰⁱCD51⁻ CD127⁻ stages. The modulation of CD122 expression might also contribute to the rather poor responses of mature CD11b⁺ NK cells to IL-15, as CD122 expression was slightly, but significantly, lower on CD11b⁺ NK cells than on CD11b⁻DX5ʰⁱ NK cells particularly in the spleen (Fig. 4*C*). It is, however, to be explored how much this mechanism contributed to the variable magnitudes in IL-15 responses of developing NK cell subpopulations. Because an array of genes, such as those for cell cycle regulation, transcription, and cell activation, were reported to be up- and down-modulated during the CD11b⁻ to CD11b⁺ transition (23), intracellular or cell surface events other than CD122 modulation would likely be involved, too.

As NK cells matured, Ly49 receptor expression was induced (13, 14), and IL-15 elevated the frequencies as well as absolute numbers of Ly49⁺ NK cells in vivo (5, 18). In our in vitro cultures with IL-15, Ly49A⁺, I⁺, G2⁺, or D⁺ NK cell frequencies were elevated

prominently in spleen cells from IL-15⁻/⁻ mice, not through Ly49 induction but apparently through preferential expansion of pre-existing Ly49⁺ NK cells. We noted that the majority of NK cells bearing Ly49G2 or D were CD11b⁻DX5ʰⁱ in IL-15⁻/⁻ mice, which appeared to be included in the IL-15–high-responder cell population in wild-type mice. Curiously, however, such an elevation of Ly49⁺ cells was much less prominent for BM NK cells from IL-15⁻/⁻ mice, indicating that Ly49⁺ and Ly49⁻ NK cells in BM in IL-15⁻/⁻ mice proliferated at comparable efficiencies, unlike those in the spleen, in response to IL-15. We envisage that a certain NK cell subpopulation(s) responding to IL-15 in BM was not present in the spleen, perhaps because only particular subpopulations of developing NK cells generated in the BM migrated to the spleen. In this regard, the difference in developing NK cell composition between these two organs was pointed out by our previous finding that IFN regulatory factor-2 deficiency affected Ly49⁺ NK cells in BM less severely than it affected those in the spleen (17). Such a difference might be reflected in the lower frequencies of CD11c⁺ cells within CD11b⁻DX5ʰⁱ NK cells in the BM than in the spleen (Fig. 3*B*), an interesting possibility that requires future studies on these extremely rare cells.

Although a large body of evidence indicated that BM was a principal site of NK cell development, other organs such as the thymus, spleen, and liver were also shown to accommodate immature NK cells (14, 26). IL-15, which is constitutively produced and *trans*-presented by macrophages and dendritic cells in those organs (5, 6, 27), would promote these Ly49⁺ NK cells to proliferate and possibly mature in situ, thereby contributing to the homeostasis of peripheral NK cells in the steady state. Because IL-15 was also shown to be induced upon viral infection (28, 29), these IL-15–high-responder NK cells in the spleen and liver may undergo acute expansion and possibly participate in the host defense against viral infection.

Disclosures

The authors have no financial conflicts of interest.

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