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Differential Roles for IL-15R α-Chain in NK Cell Development and Ly-49 Induction[1]

Toshihiko Kawamura,* Rima Koka,† Averil Ma,† and Vinay Kumar[2]*

IL-15Rα-deficient (IL-15R$\alpha^{-/-}$) mice lack NK cells. However, when bone marrow (BM) progenitors from IL-15R$\alpha^{-/-}$ mice were cultured with IL-7, stem cell factor and flt3 ligand, followed by IL-15, they were able to differentiate into functional NK cells, indicating that IL-15Rα is not critical for NK cell development. Whereas NK cells generated in vitro from IL-15R$\alpha^{-/-}$ BM progenitors expressed CD94/NKG2, they failed to express Ly-49 receptors. In keeping with this, when IL-15R$\alpha^{-/-}$ BM cells were transferred into wild type recipients, they gave rise to NK cells in vivo, but with greatly reduced expression of Ly-49 receptors. Furthermore, the small numbers of NK cells found in IL-15$^{-/-}$ as well as IL-15R$\alpha^{-/-}$ but not flt3 ligand$^{-/-}$ mice expressed much lower levels of Ly-49 receptors than those from wild type mice. These results indicate a novel role for IL-15Rα-chain in Ly-49 induction on developing NK cells. *The Journal of Immunology,* **2003, 171: 5085–5090.**

Natural killer cells play important roles in innate immunity against viral infections and cancer by exerting cytotoxicity and cytokine production (1). NK cell development occurs in the bone marrow (BM)[3] , and the marrow microenvironment provides necessary signals for developing NK cells (2). IL-15 is one of the soluble factors produced by BM stromal cells (3), and numerous studies have demonstrated that IL-15 plays a pivotal role in the development, survival, and function of NK cells (2, 4–6). Recently, it has been shown that the transcription factor IFN-regulatory factor-1 (IRF-1) is required for the induction of IL-15 in the BM microenvironment, and NK cells are absent in IRF-1-deficient mice in an IL-15-dependent manner (7). Lack of NK cells in IL-15-deficient mice (8) and the expansion of NK cells in mice overexpressing IL-15 (9) further confirm the importance of this cytokine in regulating the development and/or expansion of NK cells. IL-15 shares IL-2/15Rβ and the common γ (γ_c) subunits with IL-2, but also utilizes a private IL-15Rα subunit (6). It is thought that IL-15Rα confers high-affinity binding of this cytokine, whereas IL-2/15R$\beta\gamma$ complex signals through Janus kinase/STAT pathways (6). Mice lacking IL-15R subunits, IL-15Rα (10), IL-2/15Rβ (11), and γ_c (12) have greatly reduced numbers of NK cells. Taken together, these studies show that the IL-15/IL-15R system is critical for the development of NK cells (13). However, the relative contributions of IL-15R subunits, α, β, and γ to NK cell development remain to be investigated.

NK cell function is regulated by activating and inhibitory receptors (14). In the mouse, two families of C-type lectin-like receptors, Ly-49 and CD94/NKG2, have been identified, which recognizes classical and non-classical MHC class I molecules, respectively (15, 16). We have recently established an in vitro system in which early-acting cytokines, IL-7, stem cell factor (SCF), and flt3 ligand (flt3L) act on lineage marker (Lin)$^-$c-*kit*$^+$*Sca*2$^+$ BM progenitor cells to induce IL-2/15Rβ^+NK1.1$^-$ precursor cells, and subsequent culture of these precursor cells with IL-15 leads to the generation of NK.1$^+$ cells (17). The NK cells generated in this stroma-free culture express CD94/NKG2 but not Ly-49 receptors. The latter are expressed when precursor cells are exposed to marrow stromal cells as well as IL-15, suggesting that Ly-49 induction requires factors produced by and/or direct interactions with the BM microenvironment (18). Two factors, TCF-1 and PU.1, have been implicated in modulating Ly-49 expression. TCF-1$^{-/-}$ mice show a selective reduction in Ly-49A expression on NK cells (19), and PU.1$^{-/-}$ NK cells that develop in Rag-2/$\gamma_c^{-/-}$ mice reconstituted with PU.1$^{-/-}$ fetal liver cells fail to express Ly-49A and Ly-49D (20). However, the external signals that regulate the transcriptional activity of these factors remain unclear, and the molecular mechanisms that regulate Ly-49 induction remain to be further investigated.

The questions that we will address here are 1) Is IL-15Rα on developing NK cells required for NK cell development? 2) Is IL-15Rα involved in Ly-49 induction on NK cells?

Materials and Methods

Mice

IL-15R$\alpha^{-/-}$ mice were previously described (10). IL-15$^{-/-}$ (8) and flt3L$^{-/-}$ (21) mice were purchased from Taconic Farms (Germantown, NY). C57BL/6 (B6) (CD45.2$^+$) and B6-Ly-5.2 (CD45.1$^+$) mice were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Six- to 12-wk-old mice were used.

Cell preparation

Spleen and bone marrow cells were obtained as previously described (18). Lung resident mononuclear cells were isolated as described previously (22). Briefly, after the lung tissues were digested by collagenase and trypsin inhibitor, mononuclear cells were isolated by a Percoll gradient centrifugation.

Flow cytometric analysis

The following FITC-, PE-, or biotin-conjugated mAbs were used for flow cytometry: anti-CD3 (145-2C11), anti-NK1.1 (PK136), anti-CD49b (DX5), anti-CD122 (TM-β1), anti-CD244 (2B4), anti-CD2 (RM2-5), anti-CD94 (18d3), anti-NKG2A/C/E (20d5), anti-Ly-49A (A1), anti-Ly-49C/I (5E6), anti-Ly-49D (4E5), anti-Ly-49G2 (4D11), anti-CD45.1 (A20), and

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[3] Abbreviations used in this paper: BM, bone marrow; IRF-1, IFN-regulatory factor-1; γ_c, common γ; SCF, stem cell factor; flt3L, flt3 ligand; Lin, lineage marker; RMA, MHC class I positive target cell; RMA-S, MHC class I negative target cell;WT, wild type; mIL, murine IL.

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anti-CD45.2 (104). All mAbs and their isotype controls were purchased from BD PharMingen (San Diego, CA). Standard flow cytometric analysis was performed as previously described (18).

Culture of spleen and BM cells with IL-15

A total of 1×10^6 whole spleen or BM cells from wild type (WT) or IL-15Rα$^{-/-}$ mice were cultured in RPMI 1640 medium containing 10% FBS and 20 ng/ml murine (m) IL-15 (BioSource International, Camarillo, CA) for 10 days in 24-well plates. The cells were fed with fresh IL-15 every 3 days.

In vitro generation of NK cells from bone marrow progenitor cells

BM Lin$^-$c-*kit*$^+$ progenitor cells were isolated by magnetic cell sorting as described (17). Briefly, after Lin$^+$ cells were depleted by negative selection, c-*kit*$^+$ cells were positively selected. mAbs specific for Mac-1 (M1/70), Gr-1 (RB6-8C5), B220 (RA3-6B2), CD2 (RM2-5), NK1.1 (PK136), and erythroid cells (TER119) were used to detect Lin$^+$ cells. The purity of Lin$^-$c-*kit*$^+$ cells was routinely 85- 90%. For the generation of NK cells in vitro, magnetically enriched Lin$^-$c-*kit*$^+$ cells were cultured as previously described (17). Briefly, sorted BM Lin$^-$ c-*kit*$^+$ cells were cultured with the combinations of mIL-7, mSCF, and mflt3L for 5 days (primary culture). On day 5, cells were harvested, washed, and replated in 20 ng/ml mIL-15 for 6 days (secondary culture). For the induction of Ly-49 receptors on NK cells generated in vitro, OP9 (Riken Cell Bank, Tsukuba, Japan) cells were used as stromal cells in the secondary culture (18). In some experiments, a high dose (5000 U/ml) of human IL-2 (Chiron, Emeryville, CA) was used instead of mIL-15 in the secondary culture (17, 18).

Bone marrow chimera

Reciprocal BM chimeras were generated between WT and IL-15Rα$^{-/-}$ mice. A total of 1×10^7 donor BM cells isolated from WT or IL-15Rα$^{-/-}$ mice were i.v. injected via the tail vein into lethally irradiated (10 Gy) recipient mice. To distinguish donor-derived cells, B6-Ly-5.2 (CD45.1$^+$) mice were used. The chimeric mice were analyzed 8 wk after transplantation at which time >95% of the leukocytes in the spleen and lung were donor-derived.

Cytotoxicity assays

Cytotoxicity assays were performed by a standard 4 h ^{51}Cr-release assay using MHC class I positive (RMA) and negative (RMA-S) as target cells as previously described (17).

Results and Discussion

Expansion of NK cells in vitro by IL-15 in the absence of IL-15Rα expression

We first examined the responsiveness of spleen and BM cells from IL-15Rα$^{-/-}$ mice to IL-15 in vitro. Whole spleen and BM cells isolated from WT or IL-15Rα$^{-/-}$ mice were cultured with IL-15 in vitro for 10 days (Fig. 1). As previously shown (10), the percentages of NK cells in the spleen and BM of IL-15Rα$^{-/-}$ were extremely low. However, NK cells were readily induced from the spleen and BM cells of IL-15Rα$^{-/-}$ after the exposure to IL-15, to an extent similar to those from WT mice. Since whole spleen and especially BM contain mature NK cells as well as their precursors and progenitors (23), the NK cells observed following culture in IL-15 could have resulted not only from the expansion of the few mature NK cells but also from differentiation of NK cell precursors responsive to IL-15. It is unlikely that the induction of NK cells by IL-15 resulted from differentiation from multipotent progenitors because they do not respond to IL-15 alone (17). In any case, this result suggests that IL-15Rα is not required for the expansion of NK cells in response to IL-15. IL-15Rα has high affinity for IL-15, but IL-15 can bind and signal through IL-2/15Rβγ with intermediate affinity (6, 13). Although both IL-15Rα mRNA and IL-15Rβγ proteins are expressed by mature NK cells (17, 23), IL-15 signaling through IL-15Rβγ complex may be sufficient for NK cell expansion.

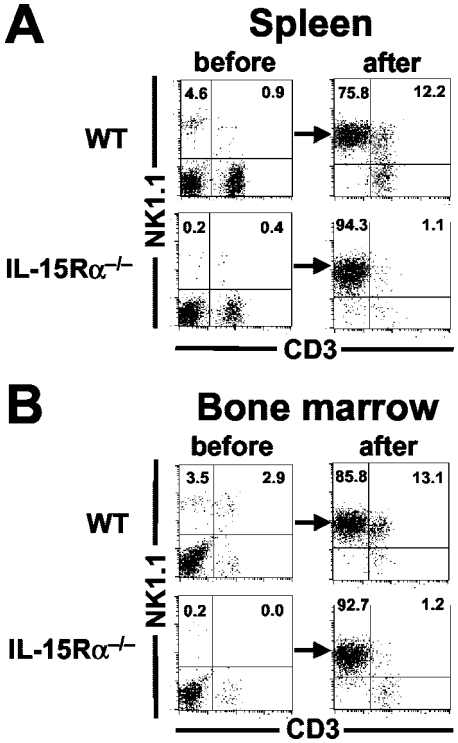

FIGURE 1. Induction of NK cells in vitro by IL-15 in the absence of IL-15Rα. Whole spleen or BM cells isolated from WT or IL-15Rα$^{-/-}$ mice were cultured with IL-15 for 10 days. Before and after the culture, cells were stained with FITC-anti-CD3 and PE-anti-NK1.1 and were analyzed by flow cytometry.

Generation of NK cells in vitro from BM progenitor cells of IL-15Rα$^{-/-}$ mice

We next examined the requirement of IL-15Rα for NK cell development from BM progenitor cells using the in vitro system we have recently developed (17). In this system, culture of BM Lin$^-$c-*kit*$^+$ progenitor cells with IL-7, SCF, and flt3L (primary culture), followed by IL-15 in a stroma-free condition (secondary culture), gives rise to lytic NK1.1$^+$CD94/NKG2$^+$Ly-49$^-$ cells. According to this protocol, Lin$^-$c-*kit*$^+$ progenitor cells were purified from the BM of WT or IL-15Rα$^{-/-}$ mice as described in *Materials and Methods*, and were cultured for 5 days in the primary culture cytokines, followed by the secondary culture cytokines for 6 days as shown in Table I. The cells were then harvested, counted, and examined for NK1.1 expression. As we previously demonstrated (17), Lin$^-$ c-*kit*$^+$ progenitor cells from WT mice did not respond to IL-15 alone, but the combination of IL-7/SCF/flt3L in the primary culture and IL-15 in the secondary culture effectively induced the differentiation of NK cells. Under these conditions, we found that progenitor cells from IL-15Rα$^{-/-}$ mice also give rise to NK cells to an extent similar to those from WT mice (Table I). Further phenotypic analysis showed that the NK1.1$^+$ cells generated in vitro from IL-15Rα$^{-/-}$ mice expressed most NK cell markers, including DX5, CD122, 2B4, CD2, and CD94/NKG2 at levels comparable to those from WT mice (Fig. 2*A*). NK cells generated from IL-15Rα$^{-/-}$ BM progenitors were found to be cytotoxic against RMA-S (class I^{low}) cells but not against RMA (class I^{high}) cells, presumably due to the recognition of Qa-1^b molecules by CD94/NKG2 complex on class I^{high} cells (Fig. 2*B*). There was no significant difference in the lytic activity of in vitro-derived NK cells between WT and IL-15Rα$^{-/-}$ mice. These results suggest that IL-15Rα is not essential for the differentiation of lytic NK cells from their progenitors.

Table I. *Generation of NK cells in vitro from bone marrow progenitors in the absence of IL-15R α chain on hematopoietic cells*[a]

Mice[b]	Primary Culture		Secondary Culture			
	Cytokines	Yield[c]	Cytokines	Yield[c]	Total Yield[d]	% NK1.1$^+$
WT	IL-15	0.3	IL-15	0.9	0.3	1.2
	IL-7/SCF/flt3L	9.5	IL-7/SCF/flt3L	5.9	56.1	1.5
	IL-7/SCF/flt3L	9.5	IL-15	4.6	43.7	96.8
IL-15R$\alpha^{-/-}$	IL-15	0.1	IL-15	0.5	0.1	0.9
	IL-7/SCF/flt3L	11.3	IL-7/SCF/flt3L	7.2	81.4	2.3
	IL-7/SCF/flt3L	11.3	IL-15	5.2	58.8	95.5

[a] Sorted Lin$^-$ c-kit$^+$ cells were cultured for 5 days in the indicated primary culture cytokines. On day 5, the cells were harvested, washed, and replated in the indicated secondary culture cytokines for 6 days. On day 6, the cells were harvested, counted, and stained for NK1.1 expression.
[b] BM Lin$^-$ c-kit$^+$ cells were purified from WT or IL-15R$\alpha^{-/-}$ mice.
[c] Yield represents the fold increase in total cell number.
[d] Total yield is the product of the yield in the primary and secondary cultures.

Requirement of IL-15Rα on hematopoietic cells for Ly-49 induction

Although culture of BM progenitor cells with cytokines in a stroma-free condition gives rise to CD94/NKG2$^+$ NK cells, these NK cells fail to express Ly-49 (17). Interaction of developing NK cells with BM-derived stroma cells is critical for the induction of Ly-49 receptors (18, 24). However, the signals that induce Ly-49 expression in developing NK cells are not fully characterized. To assess the requirement of IL-15Rα on developing NK cells for Ly-49 induction, we examined whether or not Ly-49 receptors are induced on NK cells derived from IL-15R$\alpha^{-/-}$ mice in vitro in the presence of BM-derived stromal cell line, OP9 (18). Lin$^-$c-*kit*$^+$ cells from WT or IL-15R$\alpha^{-/-}$ mice were cultured with IL-7/SCF/flt3L, followed by IL-15 on monolayers of OP9 cells (Fig. 3). NK cells derived from WT mice expressed Ly-49C/I and Ly-49G2 (Fig. 3, *left*). However, we found that no Ly-49 receptors were induced on NK cells derived from IL-15R$\alpha^{-/-}$ mice (Fig. 3, *center*). These results suggest that IL-15Rα expression on developing NK cells is essential for Ly-49 induction. Although IL-15Rα has been characterized as a high affinity binding subunit for IL-15, there is some evidence that IL-15Rα can transduce an intracellular signal in certain cases (25–27). This raises the possibility that IL-15 signaling through IL-15Rα may be involved in Ly-49 induction on NK cells. Alternatively, signaling through IL-2/15R$\beta\gamma$ complex may be qualitatively different when it is in a complex with IL-15Rα. Interestingly, when IL-2 instead of IL-15 was used in the culture, Ly-49 C/I and Ly-49 G2 were induced on NK cells derived from IL-15R$\alpha^{-/-}$ mice (Fig. 3, *right*). Since both IL-2 and IL-15 bind IL-2/15R$\beta\gamma$ with intermediate affinity (compared with their respective α subunits), this result was surprising. There are several possible interpretations of this data. One possibility is that the signaling through IL-2/15R$\beta\gamma$ is modified when the α subunit of IL-2R or IL-15R is a part of the receptor complex. Alternatively these α subunits may themselves contribute to the signaling to the Ly-49 induction pathway. We observe that NK1.1$^-$ CD122$^+$ NK cell precursor cells which are induced in vitro by IL-7, SCF, and flt3L (17, 18) express cell surface IL-2Rα (our unpublished observation).

We have previously shown that culture of BM progenitors with IL-7, SCF, and flt3L, followed by IL-15 in a stroma-free condition gives rise to lytic CD94/NKG2$^+$ NK cells without Ly-49 expression (17, 18). Also, fetal and neonatal NK cells express CD94/

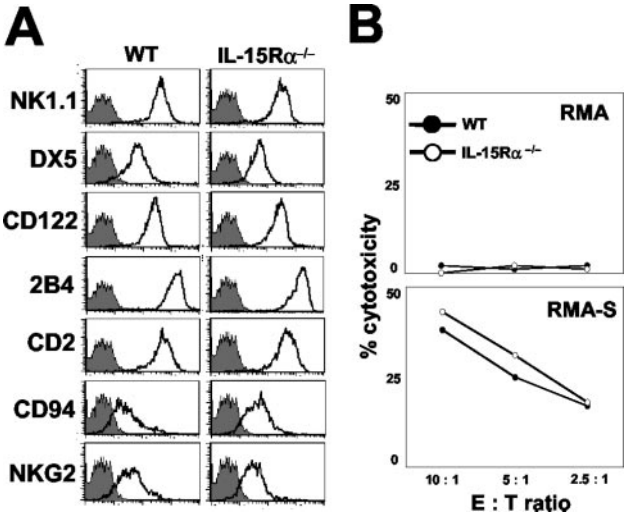

FIGURE 2. NK cells derived from WT and IL-15R$\alpha^{-/-}$ BM progenitor cells in vitro in the absence of stroma are phenotypically and functionally identical. Surface phenotype (*A*) and cytotoxic activity (*B*) of NK cells generated in vitro from BM Lin$^-$c-*kit*$^+$ cells of WT or IL-15R$\alpha^{-/-}$ mice in a stroma-free condition. Sorted Lin$^-$c-*kit*$^+$ cells from WT or IL-15R$\alpha^{-/-}$ mice were cultured with IL-7, SCF, and flt3L for 5 days, followed by IL-15 for 6 days. The cells were then harvested and examined for the expression of the indicated cell surface markers by flow cytometric analysis (*A*) and the cytotoxicity against RMA and RMA-S target cells by a 4 h ^{51}Cr-release assay (*B*).

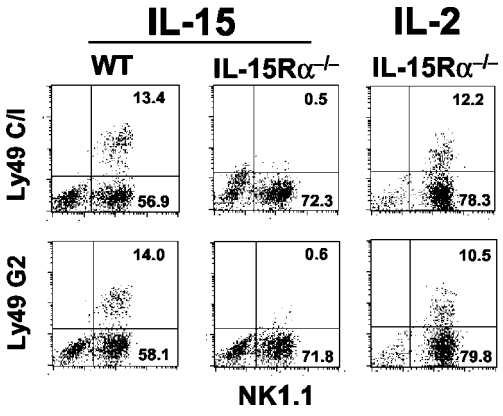

FIGURE 3. Ly-49 receptors are not expressed by NK cells generated in vitro from IL-15R$\alpha^{-/-}$ mice when cultured with IL-15. Sorted BM Lin$^-$c-*kit*$^+$ cells from WT or IL-15R$\alpha^{-/-}$ mice were cultured with IL-7, SCF, and flt3L for 5 days, followed by IL-15 on confluent monolayers of OP9 stroma for 6 days. On day 6 (day 11 of total culture time), the cells were harvested and stained with anti-NK1.1 and anti-Ly-49 C/I or anti-Ly-49 G2 and were analyzed by flow cytometry. In some experiments, IL-2 was used instead of IL-15. The data are representative of five independent experiments with similar results.

NKG2 but not Ly-49 (28-30), and expression of Ly-49 is a relatively late event in NK ontogeny (30, 31). These studies suggest that acquisition of CD94/NKG2 and lytic activity precedes Ly-49 expression by NK cells. We found in this study that CD94/NKG2$^+$Ly-49$^-$ NK cells with lytic activity were successfully induced in vitro from IL-15R$\alpha^{-/-}$ BM progenitors, suggesting that this process is IL-15Rα-independent, but that IL-15Rα is required for further maturation of NK cells, including acquisition of Ly-49.

Development of NK cells in vivo from IL-15Rα $^{-/-}$ BM progenitors

To determine the in vivo relevance of these results, reciprocal BM chimeras between WT and IL-15R$\alpha^{-/-}$ mice were generated as described in *Materials and Methods*. Eight weeks after BM transplantation, spleen and lung lymphocytes were obtained from these chimeric mice and were analyzed by flow cytometry (Fig. 4, *A* and *B*). More than 95% of the leukocytes in these organs were donor-derived (data not shown). We first asked whether BM progenitors from IL-15R$\alpha^{-/-}$ could develop into NK cells when transplanted into irradiated WT mice. When IL-15R$\alpha^{-/-}$ BM was transferred into WT mice (KO→WT chimera), the percentage of NK cells in the spleen was low compared with that in WT→WT chimera (Fig. 4*A*, *lower right*). However, a substantial proportion of NK cells (67% of that in WT→WT chimera) was found in the lung of the WT mice reconstituted with IL-15R$\alpha^{-/-}$ BM cells (KO→WT chimera) (Fig. 4*B*, *lower right*). Thus, as in vitro, IL-15R$\alpha^{-/-}$ BM progenitors are capable of differentiating into NK cells especially in certain non-lymphoid tissues of WT mice. These BM chimera experiments reinforce the in vitro data and suggest that IL-15Rα on hematopoietic cells is not critical for NK cell development in vivo. At present, we do not know the reasons why NK cells develop in vivo from IL-15R$\alpha^{-/-}$ BM progenitors preferentially in the lung. Even in the lung of normal mice, ~20% of all lymphocytes are NK cells compared with ~3–5% in the spleen (22). Whether this is due to a higher local concentration of IL-15 in the lung than in the spleen is unknown. Recent study has shown that IL-15Rα on activated monocytes presents IL-15 to neighboring cells that express IL-2/15R$\beta\gamma$ upon cell-cell interaction (32). Certain radiation-resistant cells in the lung that express IL-15Rα may present IL-15 to IL-15Rα-deficient NK cells in a similar manner. In contrast, when BM cells from WT or IL-15R$\alpha^{-/-}$ mice were transferred into IL-15R$\alpha^{-/-}$ mice (WT→KO or KO→KO chimera), few NK cells were found even in the lung (Fig. 4*B*, *lower left*), suggesting that IL-15Rα expression on radiation-resistant stromal cells is important for the maintenance of NK cells in these BM chimeras. Lack of NK cells in IL-15R$\alpha^{-/-}$ mice is largely due to the absence of IL-15Rα expression on stromal cells rather than NK cells themselves. We have recently shown that IL-15Rα expressed by stromal cells promotes the survival of NK cells (33), possibly by presenting IL-15 to IL-15R$\beta\gamma^+$ NK cells (32).

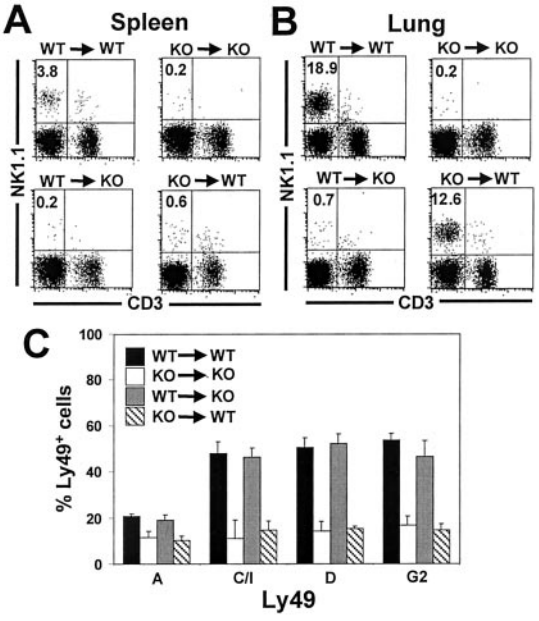

FIGURE 4. In vivo development of NK cells with greatly reduced Ly-49 expression from IL-15R$\alpha^{-/-}$ BM progenitor cells. FACS profiles of the spleen (*A*) and lung (*B*) lymphocytes isolated from the reciprocal BM chimeras between WT and IL-15R$\alpha^{-/-}$ mice. Eight weeks after the transplantation, spleen and lung lymphocytes isolated from the BM chimeras of the indicated combinations were examined for the presence of NK (CD3$^-$ NK1.1$^+$) cells by flow cytometry. The data are representative of four independent experiments with similar results. NK cells recovery in spleens of KO → KO, WT → KO, and KO → WT is not statistically different. *C*, Ly-49 expression by splenic NK cells in the BM chimeras. Eight weeks after the transplantation, spleen cells isolated from the BM chimeras of the indicated combinations were stained with anti-CD3, anti-NK1.1, and anti-Ly-49A, C/I, D or G2 mAbs. NK cells (CD3$^-$NK1.1$^+$) were gated and examined for the expression of Ly-49 receptors by flow cytometry. The data are shown as mean ± SD from four mice in each group.

Since Ly-49 receptors were not induced in NK cells generated in vitro from IL-15R$\alpha^{-/-}$ mice by IL-15 even in the presence of BM stroma (Fig. 3), we asked whether or not splenic NK cells generated in vivo from these mice in WT microenvironment express Ly-49. Splenic NK cells from the BM chimeras were examined for Ly-49 expression (Fig. 4*C*). When WT BM cells were transferred into WT recipients (WT→WT chimera), Ly-49 receptors were expressed at expected frequencies. However, when IL-15R$\alpha^{-/-}$ BM cells were transferred into WT mice (KO→WT chimera), the generated NK cells expressed much lower levels of Ly-49. In contrast, when WT BM cells were transferred into KO recipients (WT→KO chimera), the numbers of NK cells were small (Fig. 4*A*, *lower left*), but they expressed Ly-49 receptors at normal levels (Fig. 4*C*). Similar results were obtained when lung NK cells in these chimeric mice were examined (data not shown). These results are consistent with the in vitro observation that NK cells derived from IL-15R$\alpha^{-/-}$ BM progenitors fail to fully express Ly-49 and suggest that IL-15Rα expression on developing NK cells plays an essential role in Ly-49 induction. Therefore, our studies suggest that while presentation of IL-15 in *trans* by host IL-15Rα expressing cells to donor IL-15R$\alpha^-\beta\gamma^+$ cells is adequate for expansion of NK cells (32), it seems insufficient for Ly-49 induction both in vivo and by IL-15Rα expressing stromal cells in vitro.

Reduced expression of Ly-49 receptors by NK cells that develop in vivo from IL-15Rα$^{-/-}$ progenitors

Absence of IL-15/IL-15Rα results in reduced Ly-49 expression

Although severely reduced, a few NK cells are found in the spleen of IL-15$^{-/-}$ (8) and IL-15R$\alpha^{-/-}$ (10) mice. In these mice IL-15 actions mediated by IL-15Rα would be expected to be absent. If such effects of IL-15Rα are important for Ly-49 induction, Ly-49 expression by NK cells in IL-15$^{-/-}$ and IL-15R$\alpha^{-/-}$ mice should be also reduced. By contrast if reduction in NK cells is due to an IL-15-independent step of NK development, there should be no reduction of Ly-49 expression. To address this, we examined the Ly-49 expression on splenic NK cells freshly isolated from IL-15$^{-/-}$ and IL-15R$\alpha^{-/-}$, as well as flt3L$^{-/-}$ mice. In the last mentioned, NK development is also impaired (21). Spleen cells isolated from B6, IL-15R$\alpha^{-/-}$, IL-15$^{-/-}$, or flt3L$^{-/-}$ mice were

gated on CD3$^-$ NK1.1$^+$ cells as shown in Fig. 5*A*, and were examined for Ly-49 expression (Fig. 5*B*). There was a severe reduction of Ly-49 expression in NK cells isolated from IL-15$^{-/-}$ as well as IL-15R$\alpha^{-/-}$ mice. In contrast, flt3L$^{-/-}$ mice, which have reduced numbers of NK cells in an IL-15-independent mechanism (21), expressed Ly-49 receptors at levels comparable to B6 mice (Fig. 5*B*), indicating the specificity of IL-15/IL-15Rα in Ly-49 expression. This result further supports that IL-15 binding or signaling to IL-15Rα expressed on developing NK cells is important for Ly-49 induction.

Ly-49 induction has been shown to be regulated by certain transcription factors. TCF-1 selectively regulates Ly-49A expression (19), and PU.1 regulates Ly-49A and Ly-49D (20). Also, the src family kinase, Fyn, has been shown to be involved in the regulation of Ly-49A (34). In contrast to selective contributions to certain Ly-49 receptors by these factors, IL-15Rα expression on developing NK cells seems to contribute to the broader regulation of Ly-49 receptors because the absence of IL-15/IL-15Rα resulted in the reduction of all Ly-49 families examined here including Ly-49A, C/I, D, and G2 (Fig. 3, Fig. 4*C*, and Fig. 5*B*). Thus, IL-15 plays important roles not only in NK cell development and homeostasis but also in Ly-49 induction. Although a severe reduction of Ly-49 expression was observed in NK cells from IL-15R$\alpha^{-/-}\rightarrow$WT chimera (Fig. 4*C*), IL-15R$\alpha^{-/-}$ and IL-15$^{-/-}$ mice (Fig. 5*B*), Ly-49 expression was not completely absent, suggesting that other unknown factors may partially regulate Ly-49 induction. When BM progenitors are cultured with IL-15 in vitro, other cooperating factors are not available. IL-2 signaling is one of the candidates, since Ly-49 receptors were induced on NK cells derived from IL-15R$\alpha^{-/-}$ BM progenitors when IL-2 was used in the culture (Fig. 3). IL-21 would be another likely candidate (35).

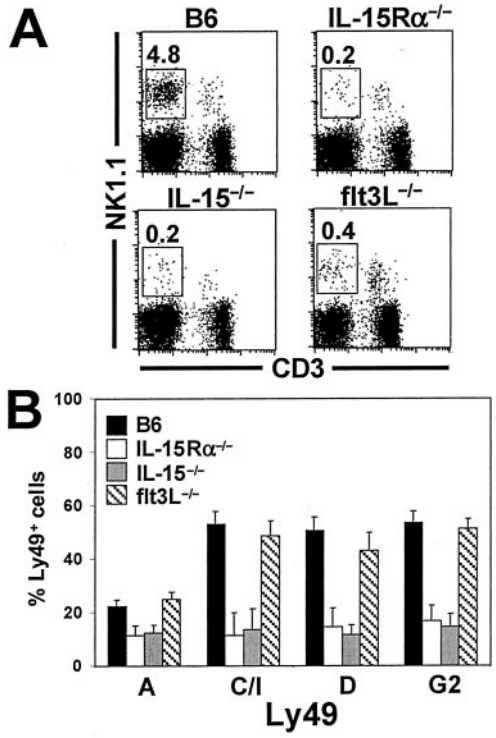

FIGURE 5. Reduced expression of Ly-49 by splenic NK cells from IL-15R$\alpha^{-/-}$ and IL-15$^{-/-}$ mice. Freshly isolated spleen cells from B6, IL-15R$\alpha^{-/-}$, IL-15$^{-/-}$ or flt3L$^{-/-}$ mice were gated on CD3$^-$ NK1.1$^+$ cells (*A*) and were examined for Ly-49 expression on NK cells (*B*). The data are shown as mean ± SD from four mice in each group.

In addition to its non-redundant role in the development of NK cells, IL-15 has been shown to be essential for peripheral NK cell homeostasis. When normal NK cells were adoptively transferred into IL-15-deficient mice, these cells could not survive (4, 5). In addition, we and others have recently reported that IL-15Rα on radiation-resistant stromal cells is important for NK cell homeostasis (33, 36). These studies, together with the finding that IL-15Rα presents IL-15 to neighboring cells (32), suggest that IL-15 presented by IL-15Rα on stromal cells plays an important role in NK cell homeostasis. In contrast to the role of IL-15Rα on stromal cells in NK cell homeostasis (33, 36), expression of IL-15Rα on developing NK cells but not on stromal cells is required for Ly-49 induction. In support of this, when IL-15R$\alpha^{-/-}$ BM was transferred into irradiated normal mice that express IL-15Rα on stromal cells, the resulting NK cells fail to fully express Ly-49. Conversely, when WT BM was transferred into IL-15R$\alpha^{-/-}$ mice, the few NK cells that developed expressed normal levels of Ly-49, suggesting that presentation of IL-15 bound to IL-15Rα on stromal cells is not essential for Ly-49 expression. At present we cannot distinguish whether IL-15Rα on NK precursors sends unique signals for Ly-49 induction or modifies the signals mediated by IL-15R$\beta\gamma$ complex in the *cis*-position.

In summary, we have demonstrated that BM progenitors of IL-15R$\alpha^{-/-}$ mice could differentiate into NK cells both in vitro and in vivo, but with greatly reduced expression of Ly-49 receptors. IL-15Rα expression on developing NK cells is not critically important for the development of CD94/NKG2$^+$ lytic NK cells, but is required for further acquisition of Ly-49 receptors by NK cells.

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