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Roles for Common Cytokine Receptor γ-Chain-Dependent Cytokines in the Generation, Differentiation, and Maturation of NK Cell Precursors and Peripheral NK Cells in Vivo[1]

Christian A. J. Vosshenrich,[2,3] Thomas Ranson,[3] Sandrine I. Samson, Erwan Corcuff, Francesco Colucci, Eleftheria E. Rosmaraki, and James P. Di Santo

NK cells differentiate in adult mice from bone marrow hemopoietic progenitors. Cytokines, including those that signal via receptors using the common cytokine receptor γ-chain (γ_c), have been implicated at various stages of NK cell development. We have previously described committed NK cell precursors (NKPs), which have the capacity to generate NK cells, but not B, T, erythroid, or myeloid cells, after in vitro culture or transfer to a fetal thymic microenvironment. NKPs express the CD122 Ag (β chain of the receptors for IL-2/IL-15), but lack other mature NK markers, including NK1.1, CD49b (DX5), or members of the Ly49 gene family. In this report, we have analyzed the roles for γ_c-dependent cytokines in the generation of bone marrow NKP and in their subsequent differentiation to mature NK cells in vivo. Normal numbers of NKPs are found in γ_c-deficient mice, suggesting that NK cell commitment is not dependent on IL-2, IL-4, IL-7, IL-9, IL-15, or IL-21. Although IL-2, IL-4, and IL-7 have been reported to influence NK cell differentiation, we find that mice deficient in any or all of these cytokines have normal NK cell numbers, phenotype, and effector functions. In contrast, IL-15 plays a dominant role in early NK cell differentiation by maintaining normal numbers of immature and mature NK cells in the bone marrow and spleen. Surprisingly, the few residual NK cells generated in absence of IL-15 appear relatively mature, expressing a variety of Ly49 receptors and demonstrating lytic and cytokine production capacity. *The Journal of Immunology,* **2005, 174: 1213–1221.**

The importance of common cytokine receptor γ-chain (γ_c)[4] cytokines for the development of NK cells has been demonstrated by natural mutations in man and by induced genetic deletions in mice. Defects in the gene coding for γ_c (*il2rg*) cause an X-linked severe combined immune deficiency characterized by several immune defects, including an absence of mature NK cells (1, 2). The common cytokine receptor γ chain is essential for the function of at least six cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (3). Each of these cytokines, except for IL-9, has been implicated in NK cell biology, although it is unclear whether their effects on the NK cell compartment are direct or indirect. For example, IL-2 plays an essential role in regulating the size of the peripheral T cell pool (4) and is reported to influence NK cell differentiation and function (5). However, in the absence of IL-2, activated T cells accumulate and provoke a lethal multiorgan system inflammatory syndrome (6). Under these conditions, "in trans" effects of T cell activation leading to the loss of B cells have been described (7). As such, indirect vs direct effects of IL-2 on the functional differentiation of hemopoietic cells must be carefully considered when examining tissues from IL-2-deficient (IL-2°) mice. Although IL-4° mice were not shown to have a major defect in the number of peripheral NK cells (8), IL-4 has been suggested to be involved in the terminal differentiation of human NK cells in vitro (9–11). IL-7 is the major γ_c cytokine involved in B and T cell development (12–14). IL-7° mice have a 10-fold reduction in B cells and αβ T cells (14, 15), whereas γδ T cells are absent (16). Splenic NK cells are reduced approximately threefold (16), although it is not known at what stage of NK cell differentiation this occurs. It is possible that IL-7 is involved in NK cell generation or in the peripheral maintenance of NK cells. In this respect, IL-7 was shown to play a minor role in NK homeostasis in an adoptive transfer model (17). Whether similar, though so far undiscovered, functions of IL-4 or IL-7 operate during murine NK cell differentiation has not yet been analyzed.

The essential role of IL-15 in the generation of NK cells has been demonstrated by the marked reduction in NK cell numbers and NK-mediated cytotoxic activity found in mice lacking IL-15 or IL-15Rα (18, 19). As such, the defect in NK cell production in IL-15° mice mirrors that of γ_c° mice. Still, it is not known at what stage the IL-15 deficiency impacts on NK cell development and by what mechanism IL-15 exerts its effects on the NK cell developmental process.

IL-21, the most recently described γ_c-dependent cytokine (20), has activity on all lymphoid lineages (20, 21). IL-21 acts on activated (but not resting) NK cells, which is likely due to the activation-dependent expression of the IL-21R by NK cells (22). IL-21 was shown to increase the NK cell natural cytotoxicity and to stimulate IFN-γ production by activated NK cells (23, 24). Its

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[3] C.A.J.V. and T.R. contributed equally to this work.

[4] Abbreviations used in this paper: γ_c, common cytokine receptor γ-chain; °, deficient; BM, bone marrow; NKP, NK cell precursor; iNK, immature NK cell; $DiOC_6(3)$, 3,3-dihexyloxacarbocyanine iodide; DC, dendritic cell; CLP, common lymphoid progenitor.

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involvement in early NK cell development has not yet been studied. As mature NK cell numbers in IL-21R-deficient mice are undisturbed (21), the role of IL-21 in this process may be limited.

In this study, we analyzed the relative contributions of several different γ_c cytokines to the generation of mature, peripheral NK cells, by analyzing the NK compartment in γ_c°, IL-2°, IL-4°, IL-7°, and IL-15° mice. To exclude any potential "trans" effects of other lymphoid cells caused by the absence of a specific cytokine, all mice were analyzed on the Rag2-deficient (Rag2°) background (the absence of Rag2 does not impinge on NK cell development (25)). Because possible redundant functions in NK cell biology could be maintained through cytokines using a common receptor subunit, the lack of a single γ_c cytokine might result in an attenuated phenotype. To address the potential redundancy among γ_c cytokines in NK differentiation and functional activities, we also generated a series of Rag2° mice simultaneously lacking two or three γ_c-dependent cytokines.

Materials and Methods

Mouse strains

Mice doubly deficient in Rag-2 and in γ_c (Rag°γ_c° mice), as well as those deficient in Rag2 alone (Rag° mice), on the C57BL/6 background have been described. Rag° mice deficient in IL-2 (Rag°2°), IL-4 (Rag°4°), IL-7 (Rag°7°), or IL-15 (Rag°15°) were obtained by intercrossing Rag° mice with the corresponding cytokine mutant mice (8, 14, 18, 26). These cytokine-deficient Rag° mice were intercrossed to obtain Rag° mice deficient for multiple cytokines (Rag°2°7°, Rag°4°7°, Rag°2°4°7°, Rag°4°15°, Rag°7°15°, and Rag°4°7°15°). Genotyping was performed by PCR on genomic DNA derived from ear punches, and primer sequences and conditions are available from the authors upon request. C57BL/6 mice were obtained from Iffa-Credo. All mice described in this study were maintained in specific pathogen-free conditions at The Pasteur Institute (Paris, France) and were used at 4–10 wk of age.

Abs and reagents

Monoclonal Abs directly conjugated to FITC, PE, biotin, or allophycocyanin and specific for the following Ags (clone name in parenthesis) were used in this study: CD49b (DX5), CD69 (H1.2F3), CD94 (18d3), CD122 (TM-β1), CD127 (A7R34), Ly49A (JR9), Ly49G2 (4D11), Ly49C/I (5E6), NK1.1 (PK136), IFN-γ (XMG1.2), IL-21R (4A9), NKG2D (CX5), CD19(1D3), Ter119, CD117 (2B8), and CD135 (A2F10.1). Abs were purchased from BD Biosciences and eBioscience. The anti-IL-21R mAb 4A9 (22) was generously provided by T. Malek and H. Jin (University of Miami, FL). FCS and PBS were purchased from Invitrogen Life Technologies. Percoll was from Pharmacia.

Isolation of lymphoid cells and flow cytometric analysis

Single cell suspensions were generated from bone marrow (BM), spleen, and liver as described (27) and were treated with Fc-Block (2.4G2; BD Pharmingen) before staining with fluorochrome-conjugated Abs. For surface Ab staining, cells were incubated with saturating amounts of mAb for 20 min on ice. Biotinylated Abs were revealed with PerCP-Cy5.5 or allophycocyanin-conjugated streptavidin (BD Pharmingen). Bcl-2 expression was detected as described (28) using directly conjugated FITC-labeled Abs specific for mouse (3F11) Bcl-2 and their isotype control Abs from BD Pharmingen. Samples were analyzed using a FACSCalibur flow cytometer with CellQuest 3.3 software (BD Biosciences). Dead cells were excluded by staining with propidium iodide, and an electronic gate was set to acquire 10^4 lymphoid cells.

To evaluate rare NK progenitors, 20×10^6 BM cells were incubated with purified rat mAbs for CD19 and TER-119 on ice for 20 min. After washing, cells were incubated with goat anti-rat IgG coupled with magnetic beads (Miltenyi Biotec), and Lin^+ cells were removed on a magnet before staining for surface markers. To enrich for BM or splenic NK cells, cell suspensions were incubated with anti-DX5 coupled microbeads (MACS) and $DX5^+$ cells isolated using MidiMACS (Miltenyi Biotec) purification columns according to manufacturer's instructions.

NK cell lytic activity

A standard ^{51}Cr release assay was used to measure NK lytic activity in vitro as described. YAC-1 cells (mouse thymoma; H-2^a) were used as target cells and were maintained in complete medium (RPMI 1640 with 10% FCS, 10^{-5} M 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin). Target cells were labeled with 100 μCi of ^{51}Cr (Valeant Pharmaceuticals), and $2.5–5 \times 10^3$ cells were incubated with graded numbers of effector cells in 200 μl of medium for 4 h. Effector cells were NK cells that were freshly isolated from splenocytes and expressed NK1.1 and CD49b. The radioactivity released into the cell-free supernatant was measured, and the percent specific lysis was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). For the functional analysis of NK cells from Rag°15° mice, spleens from 10 mutant mice were pooled and NK cells were enriched using DX5 Microbeads before the assay.

In vivo tumor rejection assay

RMA-S cells (a selected H2-deficient variant of the RBL-5 (ethyl methane sulfonate) mutagenization series A-derived subline RMA) (29) were grown in complete medium. For tumor rejection studies, 10^7 RMA-S cells were washed once with PBS and then incubated for 10 min at 37°C in 1 ml of a 5 μM solution of CFSE. The cells were then washed twice with PBS and 3×10^5 cells (in a total volume of 100 μl) were injected i.p. into recipient mice of the indicated genotype. Two days later, cells of the peritoneal cavity were harvested, stained with PE-conjugated NK1.1, and analyzed using a FACSCalibur flow cytomter (BD Biosciences).

In vitro cytokine production

Splenocytes or DX5-enriched NK cells (2×10^4 cells/200 μl) were cultured in flat-bottom microtiter plates in human IL-2 (1000 U/ml) and were stimulated with mouse IL-12 (2 ng/ml; Peprotech). After 8 h, cells were incubated in presence of brefeldin A (10 μg/ml) for 4 h at 37°C to inhibit cytokine secretion and raise intracellular stores. Cells were then washed, fixed in 2% paraformaldehyde for 30 min on ice, and thereafter stained with PE-conjugated anti-IFN-γ mAb (BD Pharmingen) in 0.5% saponin. As controls, cells were incubated in medium alone or with PMA (50 ng/ml) and ionomycin (1 μg/ml).

Statistics

Only p values below 0.05 using the Mann-Whitney t test were considered significant.

Results

BM NK cell precursors develop in the absence of γ_c

We have previously described NK cell precursors (NKPs) in the BM of adult mice (30). NKPs represent a committed NK cell progenitor having lost the capacity to generate B, T, erythroid, and myeloid cells. NKPs can be identified by expression of the CD122 marker and by the absence of mature NK cell surface Ags including NK1.1, CD49b, and members of the Ly49 family of inhibitory and activating receptors for MHC class I (30–32). CD122 is an essential subunit of the γ_c-dependent cytokines IL-2 and IL-15 (33). We found that NKPs, in addition to CD122, also expressed γ_c, and that a subset of these cells also expressed CD127 (IL-7Rα), IL-21R, CD117 (c-Kit), and CD135 (Flk2), but were CD25 (IL-2Rα) negative (Fig. 1). To assess the role of γ_c-dependent cytokines in the generation of the steady-state NKP pool, we compared NKP numbers and phenotype in the BM of adult Rag° vs Rag°γ_c° mice.

As we have previously reported (30), distinct subpopulations of $CD122^+Lin^-$ BM cells can be identified in wild-type mice based on NK1.1 and CD49b expression (Fig. 1*b*). $CD122^+NK1.1^-CD49b^-$ cells include NKPs, whereas $CD122^+NK1.1^+CD49b^-$ cells have features of immature NK cells (iNK) and may represent an intermediate stage preceding the mature $CD122^+NK1.1^+CD49b^+$ NK cell (34). In contrast with Rag° mice, the profile of NK1.1/CD49b staining on $CD122^+$ cells from Rag°γ_c° mice was different (Fig. 1*b*). Percentages of both iNK and mature NK cells were reduced, whereas cells bearing the phenotypic characteristics of NKPs appeared proportionally increased in Rag°γ_c° mice. Calculations of absolute cell numbers revealed that NKPs were normally generated in the absence of γ_c, whereas iNKs and mature NK cells were severely reduced. These results suggest that stimulation via γ_c-dependent cytokines is not essential for commitment of progenitors to the NK cell lineage, but it

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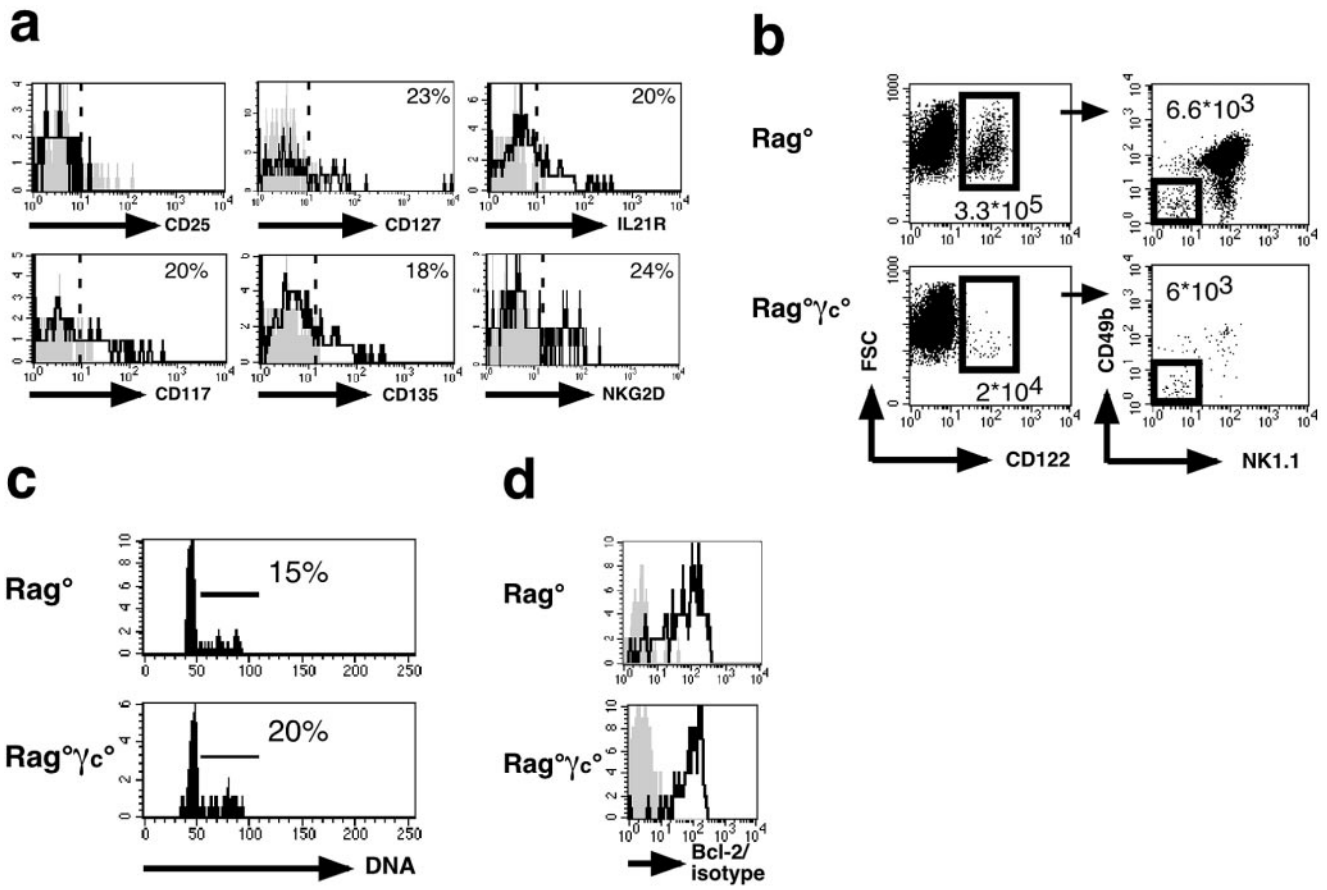

FIGURE 1. Characterization of BM NK cell progenitors. *a*, Surface markers (thick lines) expressed by NKPs from adult Rag° mice. Isotype controls are shown as shaded histograms. Dotted lines indicate threshold of expression based on isotype controls. NKPs were gated as BM $CD122^+DX5^-NK1.1^-$ cells. *b*, Expression profiles of NK1.1 and CD49b (right panels) gated on $CD122^+$ cells from total BM of Rag° and Rag°γ_c° mice (left panels). In both panels the average numbers of the boxed cells found in the indicated mice are given (see also Fig. 2*a*). *c*, NKPs from Rag° and Rag°γ_c° mice contain similar numbers of dividing cells. Shown are histograms of Hoechst 33342 staining on electronically gated $CD122^+NK1.1^-CD49b^-$ cells from the indicated mice. Given are the percentages of cells in G_1/S-phase among gated NKPs. *d*, Analysis of intracellular Bcl-2 expression in NKPs from the indicated mice. Shown are histograms of electronically gated $CD122^+NK1.1^-CD49b^-$ cells after fixation and intracellular staining for Bcl-2 protein (thick lines) and the isotype control (shaded histogram). A representative experiment of at least three is shown.

plays an important role once this is achieved. We did not find phenotypic differences between NKPs generated in presence (Rag°) or absence of γ_c cytokine signaling (Rag°γ_c°) with regard to expression of CD25, CD117, CD127, CD135, and IL-21R (Fig. 1*a* and data not shown). NKPs from both types of mouse contained similar frequencies of cycling cells as revealed by Hoechst staining (Fig. 1*c*) and expressed the anti-apoptosis protein Bcl-2 intracellularly at comparable levels (Fig. 1*d*). In line with the latter, NKPs from Rag° and Rag°γ_c° mice demonstrated equal labeling with 3,3-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (data not shown), indicating a healthy (nonapoptotic) cell status.

NKPs do not express CD94, NKG2A/C/E, or Ly49 family members (30). We found that NKPs express NKG2D on their cell surface and that this occurs independently of γ_c (Fig. 1a and data not shown). Therefore, NKG2D expression coincides with the commitment of lymphocyte precursors to the NK lineage and appears as one of the first NK cell-target recognition molecules expressed by NKPs.

Role of IL-2, IL-4, IL-7, and IL-15 in NK cell differentiation

Given that NKPs can be generated in a γ_c-independent fashion, we next analyzed individual roles of several different γ_c-dependent cytokines in the generation and differentiation of mature peripheral NK cells. For this purpose, we derived a series of Rag° mice having additional deficiencies in one or more γ_c-dependent cytokines (including IL-2, IL-4, IL-7, and IL-15). These cytokine-deficient Rag° mice included Rag°2°, Rag°4°, Rag°7°, Rag°15°, Rag°2°7°, Rag°4°7°, Rag°2°4°7°, Rag°4°15°, Rag°7°15°, and Rag°4°7°15°. These mutant mouse strains were viable and healthy when maintained under specific pathogen-free conditions (data not shown). Furthermore, the "autoimmune" or inflammatory syndromes reported in some of the corresponding cytokine-deficient mice on a RAG-competent background (as is the case for IL-2° mice) (35), were not observed in this series of cytokine-deficient mice on the Rag° background, clearly implicating a role for B and/or T cells in the corresponding disease process (36).

We analyzed BM $CD122^+$ subsets expressing NK1.1 alone or in combination with CD49b as well as mature NK cells in the spleen in these cytokine-deficient Rag° mice (Fig. 2*a* and data not shown). The following observations were made: 1) single or multiple deficiencies in IL-2, IL-4, and IL-7 had no impact on the development of BM $CD122^+$ NK subpopulations or on the generation of mature splenic NK cells, 2) IL-15 was required for the generation of iNK and mature NK cells in the BM and mature NK cells in the spleen, 3) in the absence of IL-15, the cellular expansion that takes place when BM NK cells acquire maturation markers (and which results in a 50-fold increase from NKPs to the $NK1.1^+/CD49b^+$ stage) was not observed, and 4) γ_c-dependent cytokines other than IL-15 did not appear to have a major role in

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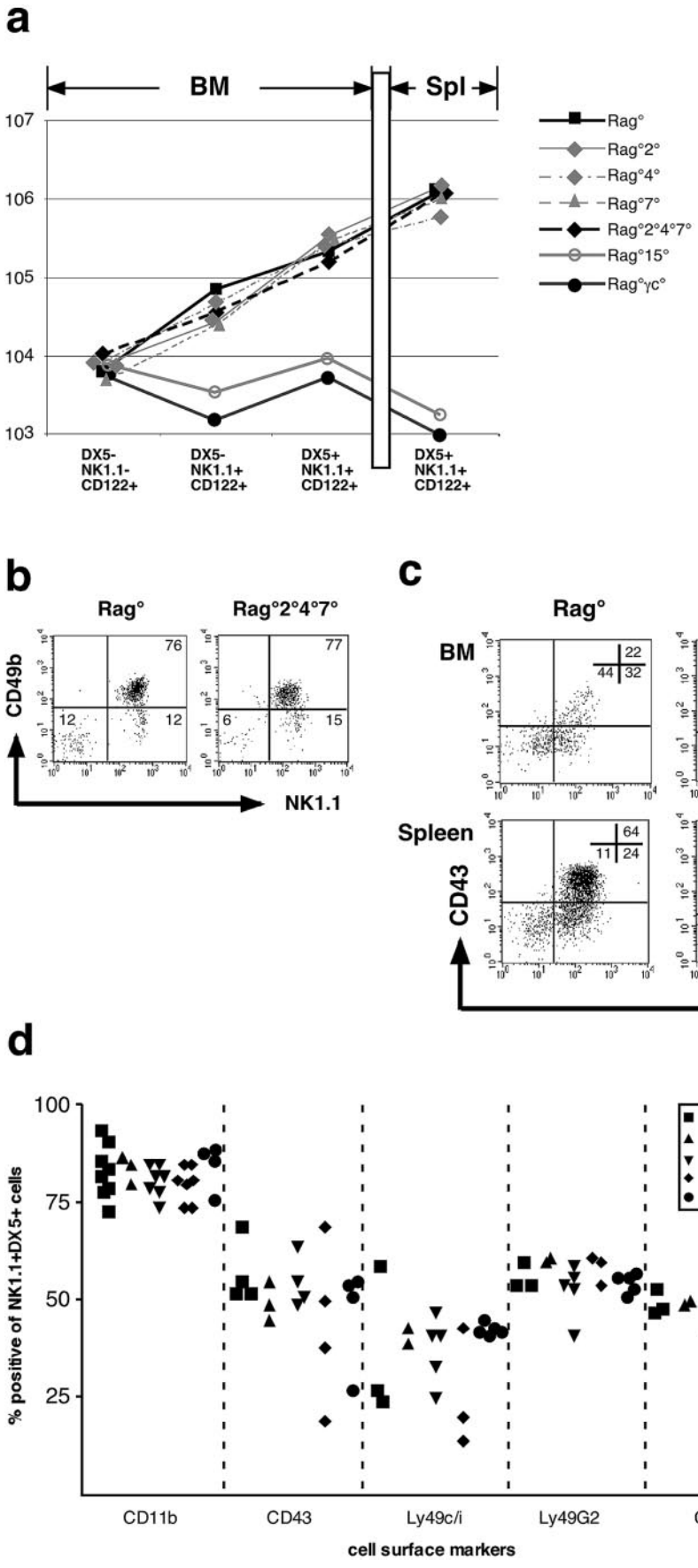

FIGURE 2. The NK cell compartment in mice lacking one or more γ_c cytokines compared with controls. *a*, Absolute numbers of indicated cells in the BM and spleen of Rag° ($n = 8$), Rag°2° ($n = 5$), Rag°4° ($n = 10$), Rag°7° ($n = 7$), Rag°2°4°7° ($n = 4$), Rag°15° ($n = 7$), and Rag°γ_c° ($n = 6$). The data obtained from Rag°4°15° ($n = 5$), Rag°7°15 ($n = 4$), and Rag°4°7°15° ($n = 6$) mice were indistinguishable from those of Rag°15° and Rag°γ_c° mice (data not shown). The differences between the cell numbers from Rag°15° and Rag°γ_c° mice were not statistically significant. *b*, Expression profiles of NK1.1 and CD49b on BM CD122$^+$ cells from the indicated mice. *c*, Expression profile of CD11b vs CD43 on NK1.1$^+$CD49b$^+$ cells isolated from BM and spleen of the indicated mice. The numbers are the percentages of the respective subsets among the gated cells. *d*, Phenotypic analysis of splenic NK1.1$^+$CD49b$^+$ cells isolated from the indicated mice. Results show the percentage of gated cells expressing the respective cell surface markers. Shown are results for CD11b, CD43, Ly49C/I, Ly49G2, and CD94. Each symbol represents one mouse.

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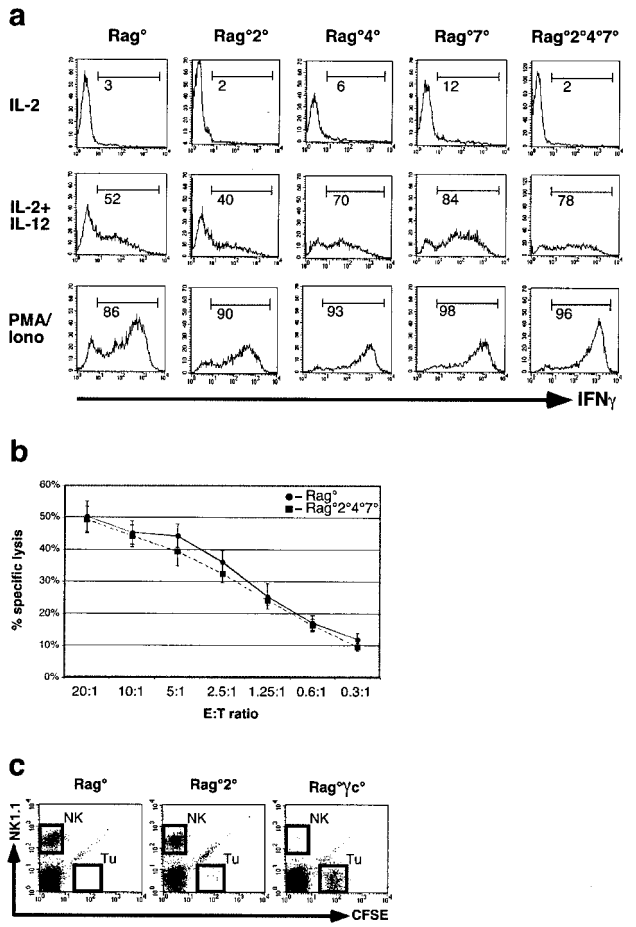

FIGURE 3. Functional capacities of NK cells derived from cytokine-deficient mice. *a*, In vitro IFN-γ production by NK cells was assessed by intracellular cytokine staining. Splenocytes were stimulated with IL-2 alone (top row), IL-2 and IL-12 (middle row), or phorbol ester and ionomycin (bottom row). *b*, In vitro natural cytotoxicity of NK cells derived from the indicated mice. The numbers of mature NK cells in the spleen of the indicated mice were determined, and a 4-h killing assay was set up with the indicated ratios of NK cells to ^{51}Cr-labeled YAC-1 target cells. Shown are results of one representative of two experiments. All E:T ratios were set up in triplicate and the variations are shown as the error bars. Results of the cytotoxicity assays from NK cells derived from Rag°2°, Rag°4°, and Rag°7° were similar to that obtained from the Rag°2°4°7° mice (data not shown). *c*, In vivo tumor rejection assay. RMA-S tumor cells were labeled with CFSE, and 3×10^5 cells were injected i.p. into the mice of the indicated genotype. Two days later, the peritoneal cavity was lavaged and cells were stained for NK1.1. The profiles of NK1.1$^+$ cells (NK) vs CFSE$^+$ tumor cells (Tu) are shown. Dead cells were excluded by electronically gating on propidium iodide-negative cells.

the generation of BM CD122$^+$ NK1.1$^+$ subsets, because Rag°15° and Rag°γ_c° mice had similar profiles of NK cell development; in fact, we did not observe differences of statistical significance between Rag°15° and Rag°γ_c° mice.

Concerning the accumulation of mature NK1.1$^+$CD49b$^+$ cells in the spleen, IL-15 was the dominant cytokine required for this process (Fig. 2*a*). In the absence of IL-15, peripheral NK cell numbers in Rag° mice were reduced by a factor of ~750. A small but reproducible effect of IL-4 deficiency was also observed: Rag°4° and Rag°4°7° mice had approximately threefold fewer splenic NK cells compared with controls (Fig. 2*a* and data not shown). Although widely used to stimulate the growth of NK cells from BM progenitors or to generate lymphokine-activated killer cells from splenocytes in vitro, we found no role for IL-2 in the development of NK cells in vivo in that normal numbers of peripheral NK cells were generated in Rag°2° mice (Fig. 2*a*). The fact that Rag°2°4°7° had normal numbers of splenic NK cells, whereas Rag4° and Rag4°7° mice consistently showed a threefold reduction in these cells, may result from the increase (~25%) in BM NK cells in Rag°2° mice compared with Rag°4° mice (Fig. 2*a*).

Normal NK cell differentiation in the combined absence of IL-2, IL-4, and IL–7

To determine potential redundancy among γ_c cytokines, we analyzed the NK cell compartment from mice lacking cytokines IL-2, IL-4, and IL-7. We found that those mice contained normal numbers of NKPs, iNKs, and mature NK cells (Fig. 2, *a* and *b*). Several reports have demonstrated that NK cells from the BM, spleen, and liver differ with regard to their phenotype and function (37–39). Kim et al. (38) reported that expression of integrin α_M (CD11b) and sialophorin (CD43) was markedly reduced on NK1.1$^+$ cells in the BM (CD11b^{low}, CD43^{low}), whereas the majority of splenic NK1.1$^+$ cells were CD11b^{high} and CD43^{high}. We confirm these observations and further demonstrate that IL-2, IL-4, and IL-7 do not play an appreciable role in the acquisition of CD11b or CD43 expression by BM or peripheral NK cells (Fig. 2*c*). The phenotype of splenic NK cells that differentiate in the absence of IL-2, IL-4, and IL-7 appeared normal, expressing 2B4, CD69, CD94, NKG2A/C/E, and Ly49A, C/I, and G2 at similar intensity and frequency (Fig. 2*d* and data not shown).

NK cells developed in absence of IL-2, IL-4, and IL-7 exhibit normal effector functions

We assessed whether NK cells developing in the absence of IL-2, IL-4, and IL-7 could produce cytokines (IFN-γ) after stimulation with IL-12 or nonspecific activators such as phorbol ester plus calcium ionophore. We failed to observe any defect in the capacity of NK cells derived from these mice to produce IFN-γ after ex vivo stimulation (Fig. 3*a*). Moreover, lytic activity of freshly isolated splenocytes from Rag° mice compared with Rag°2°, Rag°4°, or Rag°7° double or Rag°2°4°7° quadruple mutant mice against YAC-1 cells were similar (Fig. 3*b* and data not shown).

A recent report suggested an important role for bacterially activated dendritic cell (DC)-derived IL-2 in mediating NK cell anti-tumor responses in vivo (40). Because this study made use of DCs derived from IL-2° mice, it remained possible that DC differentiation in this setting was indirectly compromised secondary to "in trans" effects from activated T cells (7). Our Rag°2° mice offered the possibility to assess a role for IL-2 in NK cell anti-tumor responses. We performed an in vivo tumor rejection assay using the NK-sensitive target RMA-S (41). Relative NK cell activity in this assay was controlled using NK-deficient Rag°γ_c° mice. Both Rag° and Rag°2° mice could efficiently clear the injected RMA-S cells after transfer (Fig. 3*c*), whereas Rag°γ_c° mice were unable to eliminate the tumor cells. Therefore, IL-2 appears dispensable for NK cell-mediated tumor cell clearance in this model. Mice deficient in IL-2, IL-4, and IL-7 also cleared RMA-S tumor cells in vivo (data not shown). Collectively, these results rule out any essential or redundant roles for IL-2, IL-4, and IL-7 in NK differentiation.

NK cell differentiation in the absence of IL-15

Despite the overall reduction in absolute numbers of NK cells in Rag°15° mice, we analyzed residual NK cells in these mice to determine to what extent NK cell differentiation could proceed in the absence of IL-15. Phenotypic analysis revealed a surprising degree of NK cell surface marker acquisition in Rag°15° BM and

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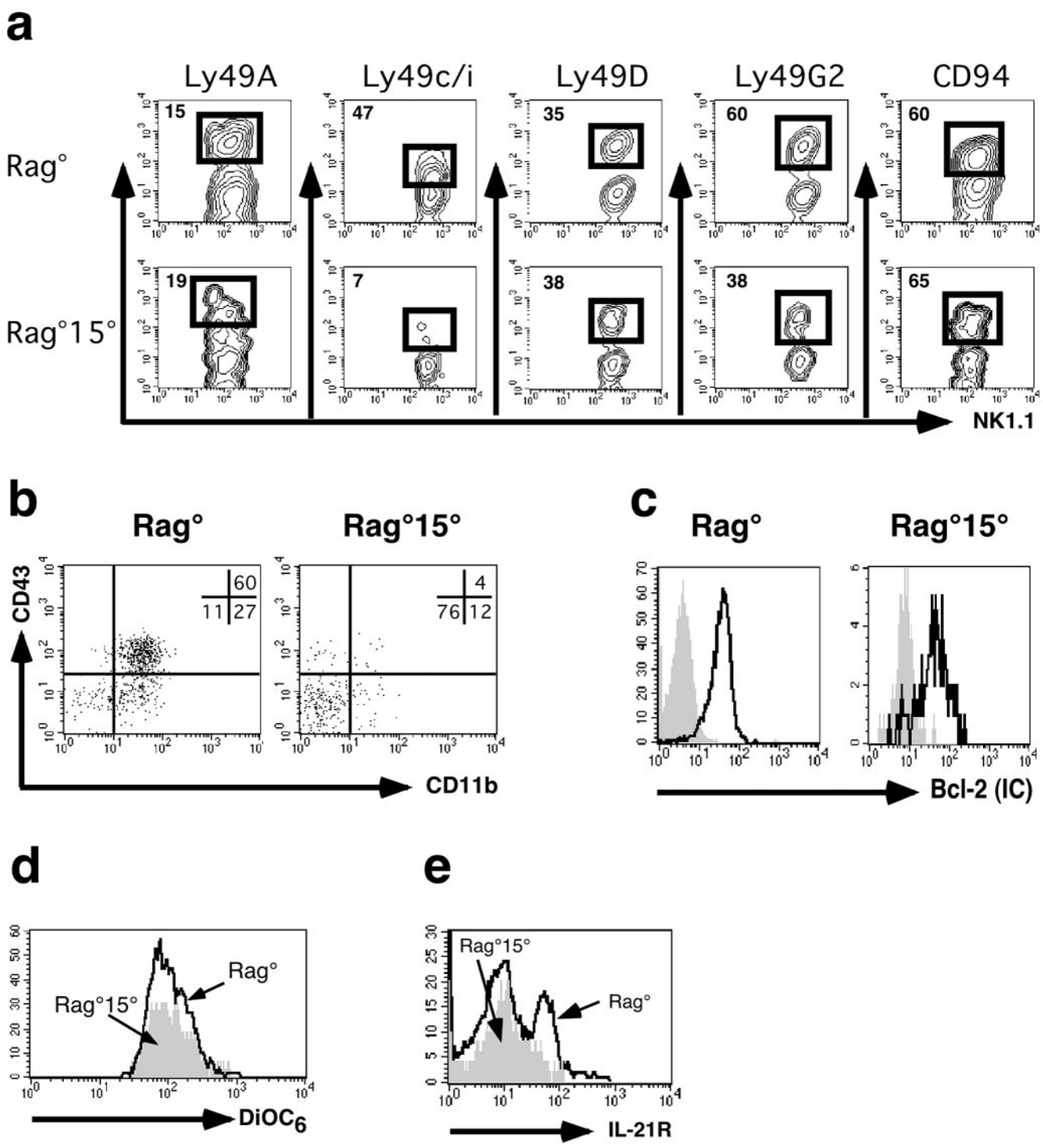

FIGURE 4. Analysis of NK cells isolated from Rag°15° mice compared with Rag° controls. *a*, NK receptor expression profiles on splenic NK cells from Rag° (upper panels) and Rag°15° mice (lower panels). Percentages of cells expressing a given marker among NK1.1$^+$CD49b$^+$ cells are indicated on the plots. *b*, Expression profiles of CD11b vs CD43 on splenic NK cells from Rag° (left panel) and Rag°15° mice (right panel). *c*, Intracellular staining for expression of endogenous Bcl-2. Histograms show Bcl-2 expression level (thick line) in NK cells from Rag° (left panel) or Rag°15° (right panel) compared with isotype control (shaded histogram). *d*, Assessment of mitochondrial membrane potential using DiOC6(3) on NK cells from Rag° mice (thick line) or Rag°15° mice (shaded histogram). *e*, IL-21 receptor expression on CD122$^+$NK1.1$^+$ NK cells from the BM of Rag° (thick line) or Rag°15° mice (shaded histogram). Note the bimodal histogram for IL-21R expression on NK cells from Rag° mice compared with the unimodal histogram obtained from Rag°15° NK cells.

splenic NK cells (Fig. 4*a*). This indicates that, despite the reduction in more mature NK cells in absence of IL-15, the phenotypic differentiation of precursor cells was not abrogated. NK1.1$^+$ cells from Rag°15° mice normally expressed CD2, 2B4, CD94, and NKG2A/C/E molecules. Concerning the Ly49 family of activating and inhibitory receptors, Rag°15° NK cells expressed normal levels and proportions of Ly49D, but lower percentages of Ly49C/I and G2 (Fig. 4*a* and data not shown). Interestingly, NK cells from Rag°15° spleen were CD11b^{low} and CD43^{low} (Fig. 4*b*). Because CD11b^{low}CD43^{low}NK1.1$^+$ cells in the spleen of Rag° mice are normally considered immature (because they express lower levels of the Ly49C/I, G2, and D compared with their

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$CD11b^+$ counterparts; data not shown), we conclude that NK cells from IL-15-deficient Rag° mice have substantial, but not complete, phenotypic differentiation.

How does IL-15 function in NK cell development? IL-15 might act as survival factor for NK cells at different developmental stages or it might act to support proliferation of developing NK cells. We found that $NK1.1^+CD49b^+$ cells generated in absence of IL-15 expressed Bcl-2 protein (Fig. 4*c*) and displayed a comparably strong $DiOC_6(3)$ staining than did control NK cells (Fig. 4*d*). However, NK cells from these mutants also contained a similar frequency of cycling cells as compared with controls (data not shown), which suggests that this cytokine is involved in neither $NK1.1^+CD49b^+$ NK cell expansion nor their survival. However, these findings do not explain why NK cells generated in the absence of IL-15 acquire only a partially mature phenotype. It was previously suggested that IL-21 might be involved in the maturation of NK cells (23, 24). As shown in Fig. 1, a subset of NKPs express the IL-21R and, in addition, we have found that expression of high levels of IL-21R on more mature NK cells in the BM was dependent on IL-15 (Fig. 4*e*). A previous report has shown that expression of IL-21R by mature splenic NK cells can be detected after IL-15 activation (22). These results suggest that IL-15 may have indirect effects on developing NK cells by regulating their susceptibility to other maturation factors, like IL-21.

NK cells generated in the absence of IL-15 develop functional activity

We analyzed the effector capacity (cytotoxicity and cytokine production) of NK cells from Rag°15° mice. In agreement with their less mature phenotype, freshly isolated NK cells from Rag°15° mice demonstrated reduced cytotoxicity toward YAC-1 targets compared with control NK cells, although they were clearly capable of cytolysis (Fig. 5*a*). After stimulation with IL-12, Rag°15° NK cells produced reduced but measurable levels of IFN-γ compared with controls (Fig. 5*b*). Collectively, our observations support a role for IL-15 in sustaining developing NK cells, but rule out an absolute requirement for IL-15 in NK cell differentiation.

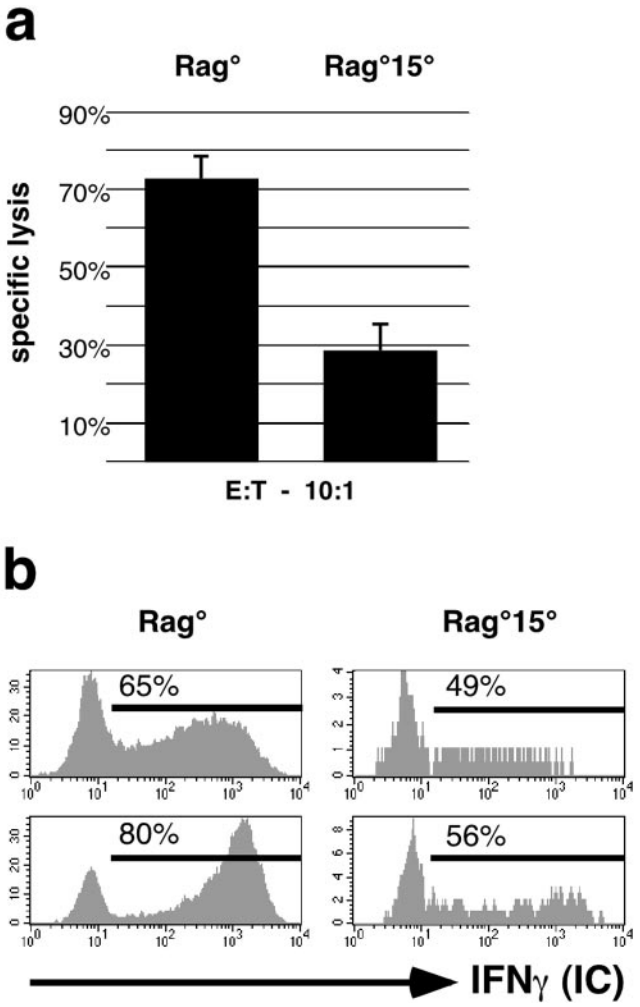

FIGURE 5. Effector functions of NK cells from Rag°15° mice. *a*, Cytotoxicity assay of splenic NK cells ($NK1.1^+CD49b^+$) derived from Rag° (left bar) or Rag°15° mice (right bar). To have sufficient NK cell number for the assay, NK cells from Rag°15° mice were obtained from a pool of 10 animals. Shown are the percentages of specific lysis of NK cells against YAC-1 target cells at an E:T ratio of 10:1. *b*, In vitro IFN-γ production by NK cells from Rag° (left panels) and Rag°15° (right panels). Cells were stimulated with IL-2 and IL-12 (upper panels) or with phorbol ester and ionomycin (lower panels).

Discussion

The γ_c family of cytokines plays essential roles in the development, differentiation, and homeostasis of all lymphocyte subsets (17, 27, 42, 43) and thus act as central mediators and modulators of both the innate and adaptive arms of the immune system. We demonstrate that γ_c cytokines, although essential for the generation of mature NK cells, are dispensable for the commitment of hemopoietic precursors to the NK cell lineage because normal numbers of NKPs are found in the absence of γ_c. In contrast, further differentiation of NKP along the NK cell lineage is γ_c dependent.

NKPs are the earliest precursors committed to the NK cell lineage in the adult BM (30) and are characterized by the CD122 (IL-2Rβ) expression in the absence of more mature NK cell markers (including DX5, NK1.1, and Ly 49 molecules). NKPs were shown to give rise to NK cells with a frequency of 1/12 under the tested conditions (30). Here we have further characterized the phenotype of these cells. NKPs express cell surface markers of early hemopoietic precursors like CD127 (IL-7Rα), CD135 (Flk-2), and CD117 (c-Kit). Whereas these cytokine and growth factor receptors are expressed by common lymphoid progenitors (CLPs), CD122 is not (44). A developmental sequence, therefore, is suggested whereby CLPs committed to or directed toward the NK cell lineage would up-regulate CD122, while continuing to express CD117, CD127, and CD135. Still, it remains to be shown whether NKPs are direct progeny of CLPs. Regarding the expression of NK cell lineage-related genes by NKPs, we found that NKG2D was expressed by this population at a frequency similar to the in vitro-precursor frequency determined before (30). Therefore, NKG2D coexpression by CD122-positive BM cells may clonally identify NK-committed precursor cells. This hypothesis is currently under investigation. The expression of NKG2D by NK cells is normally associated with effector functions. Because NKPs were shown to be nonlytic, the function of NKG2D in NKP biology remains elusive.

Cytokine redundancy within the γ_c-dependent network has been suggested from in vitro studies of lymphocyte growth and function (45). Our analysis of cytokine-deficient Rag° mice provided an opportunity to address the unique and redundant roles of IL-2, IL-4, and IL-7 in NK cell differentiation. Previous reports have suggested that IL-2 may play a role in the development and function of B cells, NK cells, and DCs (5–7, 26, 35, 46) in addition to its essential role in regulating T cell homeostasis (4). Nevertheless, evidence to support a direct role for IL-2 in murine NK cell differentiation is lacking: we found that Rag°2° mice harbor mature

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NK cells that are phenotypically and functionally indistinguishable from control NK cells in Rag° mice. Our results contrast with recent data indicating an important role for DC-derived IL-2 in the development of NK cell-mediated anti-tumor activity (40). What differs in these experiments are the source of IL-2° cells: we studied NK cells from Rag° mice in which T cell-mediated autoimmune disorders (which characterize IL-2° mice (35)) cannot develop. In contrast, Granucci et al. (40) used DCs derived from IL-2° mice that harbored T cells. It remains possible that the inability of IL-2° DCs to promote NK cell activation would result from an "in trans" effect secondary to dysregulated T cells in the IL-2° mice. A similar phenomenon has been described to account for B cell loss in IL-2° mice (7). T cell-deficient IL-2° mice (nude × IL-2°) could provide a system to confirm or refute this explanation.

Our results clearly rule out any major role for the cytokines IL-2, IL-4, and IL-7 in the generation of fully mature NK cells. We observed a slight reduction in the absolute numbers of mature splenic NK cells in Rag°4° mice. A role for IL-4 as an amplifier of immature human NK cells has been suggested from in vitro studies (47). However, Rag°4° mice do not manifest alterations in the percentage or absolute number of immature NK cells, and mature NK cells in these mice are phenotypically and functionally indistinguishable from control NK cells. Furthermore, cytokine redundancy among IL-2, IL-4, and IL-7 does not appear to operate in vivo for murine NK cell development, in that mice lacking all three of these cytokines (Rag°2°4°7°) developed normal numbers of phenotypically mature NK cells with normal capacity to exert natural cytotoxicity in vitro, produce IFN-γ, and kill tumor cells in vivo.

Our results confirm the essential role of IL-15 in the generation of NK1.1$^+$ NK cells in the BM and spleen (18). The fact that IL-15 deficiency is not further compounded by additional deficiencies in IL-4 and IL-7 demonstrates the singular ability of IL-15 to promote NK cell development and clearly identifies it as the major γ_c cytokine in this process. Nevertheless, a demonstrable population of NK1.1$^+$ NK cells is generated in absence of IL-15. The existence of these cells provides an opportunity to distinguish which facets of NK cell development are driven by IL-15 from those that are IL-15 independent. We found that NK cells from Rag°15° mice show clear evidence of maturation as judged by their cell surface phenotype: NK cells from Rag°15° mice express CD94, NKG2A/C/E, and Ly49D molecules at a normal frequency and intensity, whereas the percentage of cells expressing Ly49G2 and Ly49C/I was reduced, although clearly present. These data confirm and extend a recent report using IL-15° mice (31).

Recently, Kim et al. (38) demonstrated that expression of CD11b and CD43 differed on BM and splenic NK cells (34). These authors further suggested that NK cells bearing higher or lower expression of CD11b or CD43 were functionally distinct and that increased CD11b/CD43 expression correlated with NK cell maturity (34). We found that BM and splenic NK cells generated in absence of IL-15 expressed low levels of CD11b and CD43, which would suggest that they were not mature. We found that IL-15° NK cells were capable of killing YAC-1 target cells and producing IFN-γ upon stimulation in vitro, although at a reduced level compared with wild-type NK cells. These results support the notion that the acquisition of CD11b and CD43 by NK cells may parallel their functional maturation.

IL-15 may promote NK cell development indirectly by controlling the responsiveness of maturing NK cells to other growth and survival factors. We found that IL-15° NK cells expressed lower levels of the IL-21R. This observation suggests that interactions between IL-15 and IL-21 occur in NK cells. One possibility is that NK cells exhibit sequential sensitivity to these cytokines: NK cells first respond to IL-15 and subsequently to IL-21. This would provide a means to distinguish immature and mature NK cells and to control the homeostasis of these two NK cell populations. Whether signaling through IL-21 would modify IL-15 responsiveness (via a feedback loop) is unknown, although it has been shown that IL-21 can limit IL-15 effects in vitro (21). Alternatively, IL-15 might be required to allow NK cells to mature to a stage where they receive other signals that induce IL-21R expression. Finally, a distinct subset of IL-15-dependent NK cells may exist that constitutively coexpress the IL-21R. Additional experiments are required to distinguish between these different hypotheses.

Concerning IL-21, initial reports have suggested a role of this cytokine in the stimulation of preactivated NK cells (21), counteracting at the same time the proliferative effect of IL-15 activation (21, 24). The role of IL-21 in the development of NK cells appears to be limited because IL-21R° mice contain normal numbers of NK cells. However, the phenotype of these cells has not been fully explored. Because IL-15 may control IL-21 responsiveness of early NK cell precursors, it would be interesting to analyze whether NK cells from IL-21R° mice display a similar impairment in expression of CD11b, CD43, and Ly49 family members, as observed in IL-15° NK cells.

Taken together, our data reveal IL-15-independent and -dependent steps in the development of NK cells. Although the acquisition of several NK cell surface markers and the effector functions appear IL-15 independent, full NK cell maturation (both phenotypically and functionally) depends on IL-15. These results are compatible with the notion that IL-15 acts as a general amplifier of NK cell development and may bridge early and late events in NK cell differentiation by allowing developing NK cells to respond to additional critical factors such as IL-21.

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