Orderly and Nonstochastic Acquisition of CD94/NKG2 Receptors by Developing NK Cells Derived from Embryonic Stem Cells In Vitro[1]

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In mice there are two families of MHC class I-specific receptors, namely the Ly49 and CD94/NKG2 receptors. The latter receptors recognize the nonclassical MHC class I Qa-1^b and are thought to be responsible for the recognition of missing-self and the maintenance of self-tolerance of fetal and neonatal NK cells that do not express Ly49. Currently, how NK cells acquire individual CD94/NKG2 receptors during their development is not known. In this study, we have established a multistep culture method to induce differentiation of embryonic stem (ES) cells into the NK cell lineage and examined the acquisition of CD94/NKG2 by NK cells as they differentiate from ES cells in vitro. ES-derived NK (ES-NK) cells express NK cell-associated proteins and they kill certain tumor cell lines as well as MHC class I-deficient lymphoblasts. They express CD94/NKG2 heterodimers, but not Ly49 molecules, and their cytotoxicity is inhibited by Qa-1^b on target cells. Using RT-PCR analysis, we also report that the acquisition of these individual receptor gene expressions during different stages of differentiation from ES cells to NK cells follows a predetermined order, with their order of acquisition being first CD94; subsequently NKG2D, NKG2A, and NKG2E; and finally, NKG2C. Single-cell RT-PCR showed coexpression of CD94 and NKG2 genes in most ES-NK cells, and flow cytometric analysis also detected CD94/NKG2 on most ES-NK cells, suggesting that the acquisition of these receptors by ES-NK cells in vitro is nonstochastic, orderly, and cumulative. ***The Journal of Immunology,*** **2002, 168: 4980–4987.**

The Ly49 receptors and the CD94/NKG2 heterodimers recognize MHC class I on target cells and regulate NK cell functions. The former recognizes specific classical MHC class I, whereas CD94/NKG2 receptors interact with the nonclassical class I molecule, Qa-1^b (1). Both receptor families comprise inhibitory and activating members. The inhibitory receptors harbor the immunoreceptor tyrosine-based inhibitory motif (ITIM)[4] in their cytoplasmic domains (2–6), while the activating receptors associate with a signaling molecule termed DAP12 (7–11). Within the CD94/NKG2 family, CD94/NKG2A functions as an inhibitory receptor, while CD94/NKG2C and CD94/NKG2E are thought to be activating receptors (1, 7, 12). NKG2D is divergent from the other NKG2 molecules and does not form a heterodimer with CD94. It associates with DAP10 and functions as an activating receptor that recognizes the retinoic acid early inducible protein and the H60 minor histocompatibility Ag. Both proteins show homology to MHC class I (1, 7, 12–14).

Adult mouse NK cells express both the Ly49 family and the CD94/NKG2 heterodimers. As many as six of these receptors can be coexpressed on individual adult NK cells in various combinations (15–19). The receptor expression is generally stochastic, although the activating Ly49D and Ly49H receptors appear to show nonstochastic expression patterns (20). The diverse receptor repertoires of adult mouse NK cells are thought to enable the NK cell population as a whole to recognize subtle changes in MHC class I expression on target cells while maintaining self-tolerance. Unlike adult NK cells, most fetal and neonatal NK cells express CD94/NKG2 but not Ly49 receptors, with the only exception being Ly49E, which appears to be expressed on fetal NK cells (21, 22). As the mouse matures, NK cells begin to acquire Ly49 receptors while CD94/NKG2 positive NK cells decline to ~50% of the population (5, 15, 16, 23). Recent studies have examined how NK cells acquire Ly49 receptors following transfer of NK cell subsets or NK progenitors into irradiated hosts (5). The acquisition of Ly49 receptors by developing NK cells during their differentiation from bone marrow progenitors in vitro has also been examined (19, 24, 25). These studies have demonstrated that the acquisition of Ly49 receptors is a stochastic but nonrandom event, although the precise order of individual Ly49 being acquired appears to vary according to the culture system and detection method used in these studies.

In contrast to the acquisition of Ly49 receptors, relatively little is known about how CD94/NKG2 receptors are acquired by developing NK cells. During differentiation of adult bone marrow progenitors into functional NK cells in vitro, CD94 and NKG2 gene expression precedes the expression of the Ly49 family (16, 18, 19, 24). Among different NKG2 genes, adult NK cells seem to predominantly express the NKG2A gene (1, 12). Single-cell

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[4] Abbreviations used in this paper: ITIM, immunoreceptor tyrosine-based inhibitory motif; ES, embryonic stem; SCF, stem cell factor; ES-NK, ES-derived NK.

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RT-PCR analysis also indicates that the frequency of CD94 and NKG2A coexpression in individual adult NK cells is much higher than those of CD94 and NKG2C or NKG2E (26). Fetal NK cells also express CD94/NKG2 receptors and are inhibited by MHC class I on target cells, suggesting that CD94/NKG2A may be responsible for the maintenance of self-tolerance in fetal NK cells (18, 19, 22, 27). Similarly, NK cell clones generated from fetal thymus also express various CD94/NKG2 receptors in a stochastic manner and differentially recognize MHC class I-deficient targets (21).

This study was undertaken to determine the acquisition pattern of CD94/NKG2 receptors in the NK cell developmental pathway through the use of an embryonic stem (ES) cell culture system. In this system, ES cells are induced to differentiate into the hemopoietic lineage, then they further differentiate into cells expressing markers of the lymphoid lineage that finally become cells displaying NK cell phenotypes and functions. Analysis of developing NK cells in this culture system shows that they acquire CD94/NKG2 receptor gene expression in an orderly manner.

Materials and Methods

mAbs and flow cytometry

mAbs to CD34, CD2, CD3, CD4, CD8, DX-5, CD127, CD132, CD44, CD122, CD117, and H-2K^b were purchased from BD PharMingen (San Diego, CA). The hybridomas 2.4G2 (anti-FcRγ), M1/69.16.11.HL (anti-heat stable Ag), 7D4 (anti-IL-2Ra), HO-13-4 (anti-Thy1.2), anti-LFA-1(TIB213), and anti-Mac-1 (TIB218) were obtained from the American Type Culture Collection (Manassas, VA) and were purified from the hybridoma culture supernatants. The anti-murine ICAM-1 (CD54) mAb YN1/1.7.4 has been described (28). The mAb 4D11 has been previously described (29), and the 4E5 mAb was a kind gift from Dr. V. Kumar (University of Chicago, Chicago, IL). Biotinylated anti-murine CD94 (18d3) and FITC-labeled anti-murine NKG2 (20d5) have been described (1). Secondary Abs were purchased from Jackson Laboratories (Mississauga, Canada). For all cell stainings and sortings, cells were first preincubated with 2.4G2 supernatant followed by primary mAbs. All incubations were performed on ice for 30 min and stained cells were subsequently analyzed on a FACSCaliber (BD Biosciences, Mississauga, Canada) equipped with the CellQuest software (BD Biosciences). For cell sorting, a FACStar^{Plus} was used. For the analysis of ES-derived NK (ES-NK) cells, dead cells, and the stroma OP9 cells were excluded by the forward and wide angle scatter gates, and the efficiency of the gating was confirmed by the expression of K^b on all ES-NK cells thus analyzed.

RT-PCR analysis of NK cell-associated genes

The amplification of total cDNA from a limiting number of cells or single cells was based on a method previously described (15). Gene expression of NK markers and receptors were analyzed by subjecting the amplified total cDNA derived from each cell to a second round of PCR using elongase or platinum *Taq* DNA polymerase (Life Technologies, Burlington, Canada) as described. The following primers were used: NKR-P1A (sense) CCCT GCTCACCAGTTCAG, (antisense) CCATAACCCACATAGTTGCTC; granzyme A (sense) TTGACTGCTGCCCACTGTAAC, (antisense) ATCG GCGATCTCCACACTTC; granzyme B (sense) GATATGTGGGGGCTTC CTTA, (antisense) CTCACACTCCCGATCCTTCTG; perforin (sense) AG GTTCCTGAGGCCTGACCGC, (antisense) ACCGATGCTGACCTGGGC CTC; Ly49B (sense) ATCGTACTTTACATACTTCCAAGATG, (antisense) GCTTCTGCTCTGTTAAGTCTGTTG; Ly49I (sense) ATGAGTGAGCCA GAGGTTACTTAC, (antisense) CTTTAACTCTGGTTGGAAAGTTAATC; CD94 (sense) TTTCTTGATGGTTACTTTGGGAGTT, (antisense) AAAC GCTTTTGCTTGGACTGTA; NKG2A (sense) CGAAGCAAAGGCA CAGA, (antisense) ATGGCACAGTTACATTCATCAT; NKG2C (sense) GCTGAACTGAAGAAGCAGATCC, (antisense) TGGGGAATTTACACT TACAAAG; NKG2E (sense) ATAAACCACAGTCTATCTTCAGCA, (antisense) same as for NKG2C; NKG2D (sense) GAGATGAGCAAATGC CATAA, (antisense) TAGCCCTCTCTTGAGGAAA. The PCR were performed with an initial denaturation at 94°C followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, with a final 7 min extension at 72°C. The PCR products were transferred onto ZetaProbe membranes (Bio-Rad, Mississauga, Canada) and analyzed by standard Southern blot hybridization. The following probes were used for Southern hybridization: NKR-P1A, CAGCT GTGCTGGGCTCATC; granzyme A, TCAACATCACTGTCATAGACAG; granzyme B, AGAGGACTAGAGCTGTGAGG; perforin, CACAGTAG AGTGTCCGATGTA; CD94, AACAATTGCACTGATGCCCAA; NKG2A/C, TCCTTCGGAAGGGCAGAGGTCA; NKG2E, CAAACTTATGCTCTT TGTCCA; NKG2D, GAGTCCTTGCTATAGCCTTG; β-actin, CAAGT GCTTCTAGGCGGACTGT. Probes for Ly49B and Ly49I were previously described (15, 30, 31). The oligonucleotides were ^{32}P-labeled using terminal transferase and hybridized at 58°C to Southern blots. After hybridization, the blots were washed in 3 × SSC, 1% SDS. The filters were exposed to x-ray films at −70°C overnight. Although the PCR for NKG2E also amplified NKG2A cDNA, the oligoprobe detected only NKG2E in Southern blot analysis, enabling specific detection of individual NKG2 gene expression. All other PCR primers specifically amplified the appropriate cDNA. RT-PCR analyses were done in blind.

In vitro ES culture system

The ES cell line R1 was maintained on gelatin-coated tissue culture flasks in the presence of DMEM containing 15% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 ng/ml of leukemia inhibitory factor and 100 μM monothioglycerol (Sigma-Aldrich, Oakville, Canada). The protocol for the differentiation of ES cells into NK cells was divided into three stages. In the first stage, ES cells were trypsinized, resuspended in IMDM, and added to methylcellulose media containing 15% FBS, 2 mM L-glutamine, 150 μM monothioglycerol, 40 ng/ml stem cell factor (SCF), and 20 ng/ml vascular endothelial growth factor and dispensed at a concentration of 350 cells/ml into 35-mm petri dishes (StemCell Technologies, Vancouver, Canada). The plated cells were incubated at 37°C and 5% CO_2 for 8 days. To harvest, cells (now in clusters) were washed to remove methylcellulose agar and then trypsinized. Although in trypsin solution, the cells were made into a single-cell suspension by passing them through a 21-gauge 1½-inch needle three times. Subsequently, the cells were stained with anti-CD34-FITC mAb and sorted on the FACStarplus for isolation of CD34$^+$ cells. In the second stage, the CD34$^+$ cells were seeded onto the OP9 stroma in six-well plates at a concentration of 10^4 cells/well and cultured for 7 days with 30 ng/ml IL-6, IL-7 (10% of hybridoma supernatant), 40 ng/ml SCF, and 100 ng/ml Flt3-ligand. After the first 3 days of incubation, half the media was removed and fresh medium containing the same growth factors was added. After another 2 days, the cells were trypsinized, vigorously pipetted, washed, and transferred to a new culture of OP9 stroma. In the third stage of the culture protocol, the growth factor medium was replaced with fresh medium containing 1000 U/ml IL-2, 20 ng/ml IL-15, 20 ng/ml IL-18, and 1 ng/ml IL-12. The developing ES cells were incubated in this cytokine mixture for 7 days, with a transfer onto new OP9 layers after the first 3 days of incubation. On day 8, differentiated ES cells were harvested by vigorous pipetting for analysis. For some experiments, ES-NK cells were further expanded by culturing for additional 2 days with IL-2, IL-12, and IL-15 without OP9 layers. The growth factors and cytokines SCF, Flt3-ligand, IL-6, and IL-2 were purified by an in-house facility (Terry Fox Laboratory, Vancouver, Canada). Vascular endothelial growth factor, IL-15, IL-18, and IL-12 were purchased from R&D Systems (Minneapolis, MN).

LAK cells

IL-2-activated splenic NK cells from 129S1/SvImJ (129/SvJ) mice were generated as described (32). Flow cytometric analysis of the cells showed that >95% of the cells were DX5$^+$CD3$^-$.

NK cytotoxicity assay

For specific lysis of target cells, the standard ^{51}Cr-release assay was performed as previously described (32). Target cells used were YAC-1, A20, K562, 721.221, C1498, and Con A blasts. For Con A blast generation, splenocytes were obtained from C57BL/6, β_2m$^{-/-}$, and Tap-1$^{-/-}$ mice 2 days before the cytotoxicity assay. The cells were treated with 2 μg/ml Con A (Sigma-Aldrich) and allowed to incubate for 2 days at 37°C and 5% CO_2 before being labeled with ^{51}Cr.

Results

Phenotypic and functional characterization of ES-derived NK cells

The differentiation protocol to generate NK cells from ES cells (R1 from 129/SvJ mouse) was divided into three stages, each one associated with the addition of specific cytokines (details described in *Materials and Methods*). In the first stage, ES cells differentiated to form embryoid bodies that contained hemopoietic progenitor

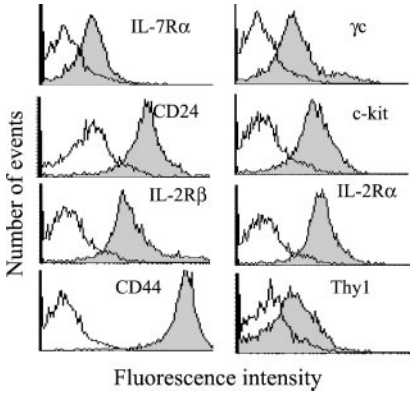

FIGURE 1. Flow cytometric analysis of day 6 ES-derived population. ES-derived cells were harvested 6 days after the initiation of cultures of sorted CD34$^+$ cells with the OP9 stromal cells in the presence of appropriate cytokines. Cells were first gated on the H-2K^b positive and propidium iodide negative fraction and then analyzed for expression of the indicated cell surface molecules. The filled histograms represent staining with the appropriate mAbs and open histograms represent control stainings. The *x*-axis and *y*-axis show fluorescence intensity and relative cell number, respectively. The results are consistent in two independent experiments.

cells expressing CD34. In the second stage, sorted CD34$^+$ cells differentiated into cells resembling common lymphoid progenitors that expressed c-*kit*, IL-7Rα (CD127), the common γ chain (CD132), IL-2Rα (CD25), low Thy1.2 (CD90), and high CD44 and CD24 (Fig. 1). In the third stage, these cells were cultured in the presence of a combination of IL-2, IL-12, IL-15, and IL-18. The resulting product of this differentiation system was a population of cells that resembled NK cells. The M-CSF-deficient OP9 stromal cell line previously shown to support in vitro lymphopoiesis of bone marrow and ES cells (19, 24, 33–36) seemed essential for the generation of NK cells from ES cells. In the absence of OP9 in the second and the third stages, no NK cells were generated. Flow cytometric analyses showed that the ES-NK cells expressed CD16, CD2, LFA-1 (CD11a/CD18), ICAM-1 (CD54), and Mac-1 (CD11b/CD18), but not CD3, CD4, or CD8 (Fig. 2*A*). The majority of the cells did not express DX5, a pan NK cell marker. This surface phenotype of ES-NK cells was very similar to that of IL-2-stimulated adult 129 mouse splenic NK (LAK) cells (Fig. 2*B*), with the exception of CD2 and DX5, which were detected on most LAK cells but not on most ES-NK cells. Transcripts for the NK-associated proteins NKR-P1A, granzymes A and B, and perforin were detected by RT-PCR (Fig. 2*C*), indicating that the cells derived from the ES differentiation system were of the NK lineage.

The ES-NK cells were also tested for their ability to kill a panel of tumor cells. As shown in Fig. 2*D*, ES-NK cells killed the prototypic mouse NK target YAC-1 as well as the lymphoma line A20 and, to a lesser extent, the C1498 lymphoma cell line. They did not kill the human cell lines 721.221 and K562. These cells may lack the proper activation ligands for ES-NK cells. The specificity of

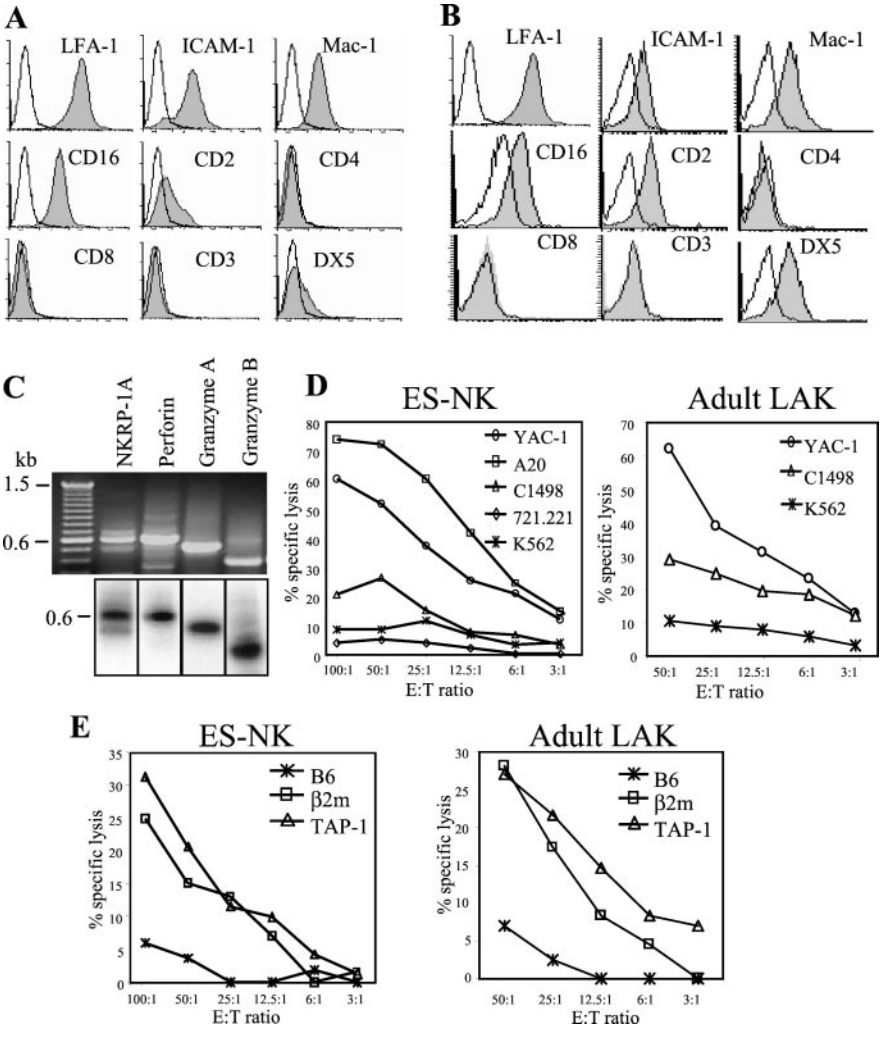

FIGURE 2. ES-NK cells phenotypically and functionally resemble normal NK cells. *A*, Flow cytometric analysis of typical NK cell-associated proteins on ES-NK cells harvested at day 16. *B*, Expression of NK cell-associated proteins on LAK cells generated from adult 129 mouse splenocytes. *C*, RT-PCR results are shown in an agarose gel (*top panel*) and confirmed by Southern blotting (*bottom panel*) to indicate that transcripts for typical NK cell-associated genes are found. *D*, Cytotoxicity of ES-NK cells (*left panel*) and adult LAK cells (*right panel*) against tumor cell lines. *E*, Cytotoxicity of ES-NK cells (*left panel*) and adult LAK cells (*right panel*) against MHC class I$^+$ vs class I$^-$ Con A blasts.

ES-NK cytotoxicity (Fig. 2*D*, *left panel*) was similar to that of adult 129 mouse LAK cells (Fig. 2*D*, *right panel*). More importantly, ES-NK cells, like adult 129 LAK cells, were able to distinguish between MHC class I-positive (C57BL/6) vs class I-negative ($\beta_2m^{-/-}$ or Tap-$1^{-/-}$) lymphoblasts and lysed only the latter (Fig. 2*E*). These results indicate that the NK cells generated from our in vitro ES culture system are capable of killing some tumor cell lines and MHC class I-deficient, but not normal, lymphoblasts.

Cytokine production is another important function of NK cells. To examine whether the ES-NK cells were capable of producing cytokines, they were stained for intracellular IFN-γ and analyzed by flow cytometry according to a method previously described (37). Results (data not shown) indicated that the ES-NK cells do produce IFN-γ.

Expression and functions of MHC class I-specific NK cell receptors on ES-derived NK cells

Anti-Ly49 mAbs 4E5 and 4D11 that have been shown to recognize Ly49R, O, V, and Ly49G2 and T of 129/SvJ origin, respectively, (38) as well as anti-CD94 (18d3) and anti-NKG2 (20d5) mAbs, were used to detect the expression of MHC class I-specific receptors on ES-NK cells. Flow cytometric analysis showed that most ES-NK cells expressed CD94/NKG2, but not Ly49 receptors (Fig. 3*A*). By contrast, large proportions of LAK cells generated from adult 129 mouse spleen expressed Ly49 at high levels (Fig. 3*B*). The expression of these receptors was also examined by RT-PCR. ES-NK cells contained transcripts for CD94, NKG2A, NKG2C, NKG2E, NKG2D, and DAP12 (Fig. 3*C*). Using Ly49 consensus primers, RT-PCR products that hybridized to a mixture of Ly49 cDNA probes were detected by Southern blot analysis. However, the PCR products were not detectable by ethidium bromide staining of agarose gel. Therefore, the amount of Ly49 cDNA amplified by the consensus primers appeared to be very low. It should be noted that the consensus primers readily amplified all known Ly49 (except Ly49B) from the splenocytes of the C57BL/6 mouse and multiple Ly49 from 129/SvJ spleen cells (data not shown). When specific primers for Ly49I, O, and P were used, transcripts for all three genes were detected by Southern blot, but not by ethidium bromide staining of the gel. The sizes of the PCR products for Ly49O and P were smaller than expected and they seemed truncated (data not shown). Only the PCR product of the expected size for Ly49I was detected by Southern blot analysis. The PCR-amplified Ly49I cDNA was subsequently cloned an sequenced to confirm that it was indeed Ly49I (data not shown). Ly49B transcripts were also detected when Ly49B-specific primers were used. Full-length Ly49B cDNA clones from the ES-derived cells were subsequently isolated and sequenced (GenBank accession no. AF395446). These results suggest that the ES-NK cells resemble fetal NK cells that have been reported to express high levels of CD94 and NKG2, but not a significant level of Ly49 receptors.

The functional role of CD94 and NKG2 on ES-NK cells was tested by their effects on cytotoxicity against target cells transfected with Qa-1^b, the ligand for the CD94/NKG2 receptors. The RMA tumor cell line, which expresses a very low level of Qa-1^b (Fig. 3*D*), was sensitive to ES-NK killing, whereas the RMA/Qa-1^b transfectants were resistant (Fig. 3*E*). Therefore, CD94/NKG2 on ES-NK cells are functional and capable of recognizing Qa-1^b on target cells and inhibiting cytotoxicity. Although ES-NK cells expressed transcripts for both the inhibitory CD94/NKG2A and potentially stimulatory CD94/NKG2C and CD94/NKG2E, the inhibitory receptor seemed dominant.

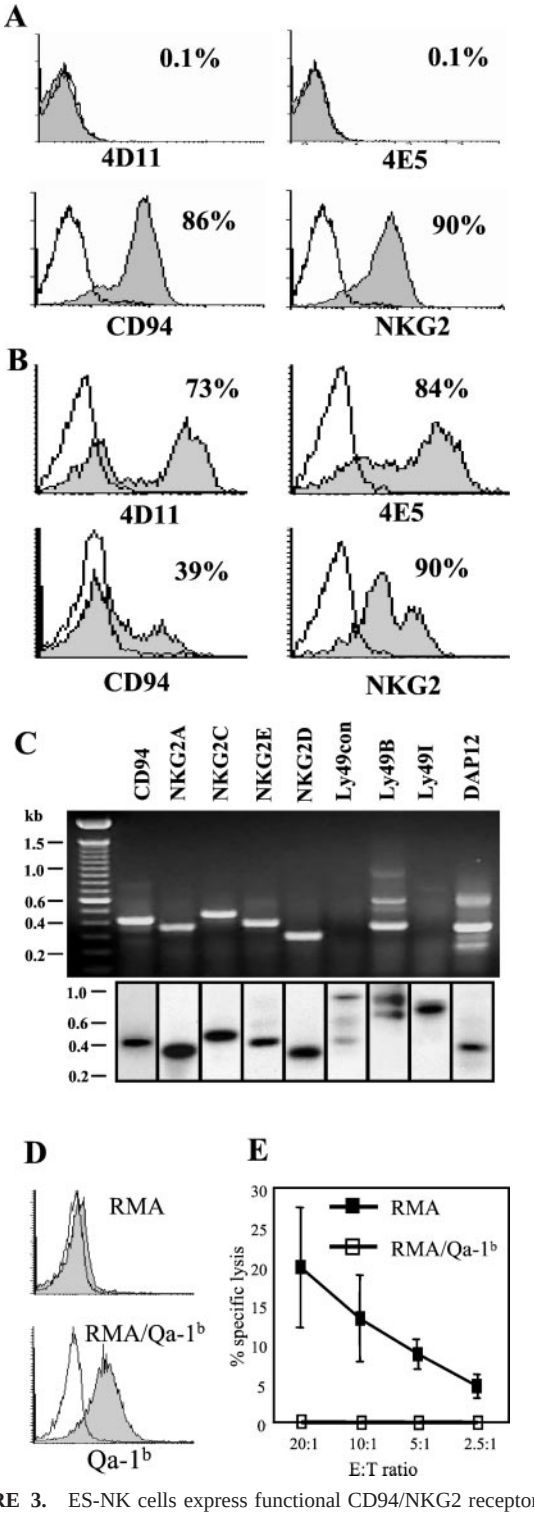

FIGURE 3. ES-NK cells express functional CD94/NKG2 receptors. *A*, FACS analysis of ES-NK cells harvested at day 16, using mAbs to CD94 and NKG2, and with anti-Ly49 mAbs, 4D11, and 4E5. Stainings with mAbs 12A8 and YE1/48 were negative and are not shown. *B*, FACS analysis of LAK cells generated from adult 129 mouse splenocytes stained with the same Abs as in *A*. *C*, RT-PCR (*top panel*) and Southern blot (*bottom panel*) analysis of day 16 ES-NK cells. Ly49con refers to amplification using Ly49 consensus primers. *D*, FACS analysis of parental and Qa-1^b-transfected RMA cells stained with anti-Qa-1^b mAb. *E*, Cytotoxicity assay using the RMA cells and Qa-1^b-transfected RMA as target cells.

CD94/NKG2 expression in individual ES-NK cells

The above results showed that almost all ES-NK cells expressed CD94/NKG2 receptors and contained transcripts for CD94 and all

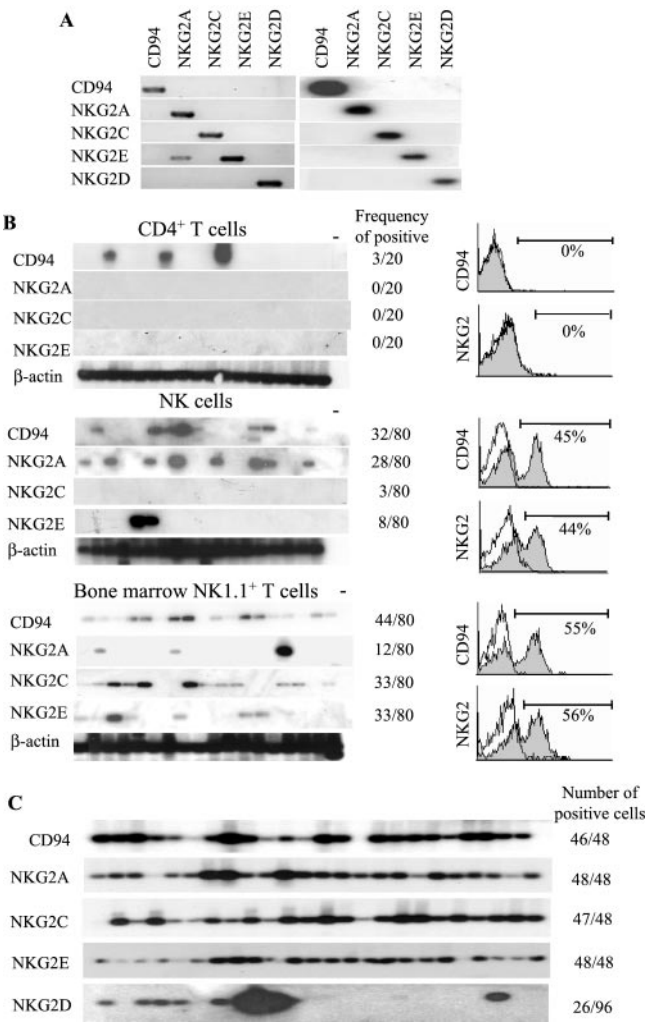

FIGURE 4. Single-cell RT-PCR analysis of CD94 and NKG2 expression in ES-derived NK cells. *A, left panel,* ~1 pg each of cloned cDNA (*top*) was subjected to PCR using primers (*left*), separated by agarose gel electrophoresis and stained with ethidium bromide. *Right panel,* The PCR products from the *left panel* were blotted and hybridized to oligonucleotide probes specific for each gene (*top*). *B, left panel,* splenic $CD4^+$ T cells, NK cells ($CD3^-NK1.1^+$), and bone marrow NKT cells ($CD3^+NK1.1^+$) were individually sorted and subjected to single-cell RT-PCR analysis. Each lane represents a single cell. For negative control (−) no cells were used for RT-PCR. *Middle panel,* Frequencies of cells positive for the expression of each gene, based on larger panels of cells analyzed. *Right panel,* Flow cytometric analysis of the same cell populations as those for the RT-PCR analysis. *C,* Individual ES-NK cells, harvested after 16 days of culture following the initiation of cultures of $CD34^+$ cells, were sorted and analyzed for the presence of transcripts of CD94 and the NKG2 gene family. The PCR products amplified were confirmed by oligonucleotide probes specific for each gene. Southern blot results from the first 24 cells are depicted. Also shown are the numbers of positive clones from the total number of cells analyzed.

known NKG2 genes. To determine whether different receptors were coexpressed on ES-NK cells, individual ES-NK cells were directly sorted into microtiter wells by flow cytometer, and the expression of individual receptor genes in each cell was determined by single-cell RT-PCR. The validity of this method was first confirmed with cloned cDNA. All the PCR specifically amplified appropriate cDNA with the exception of PCR for NKG2E that partially amplified NKG2A cDNA as well (Fig. 4*A, left panel*). However, Southern blot analysis with specific oligonucleotide

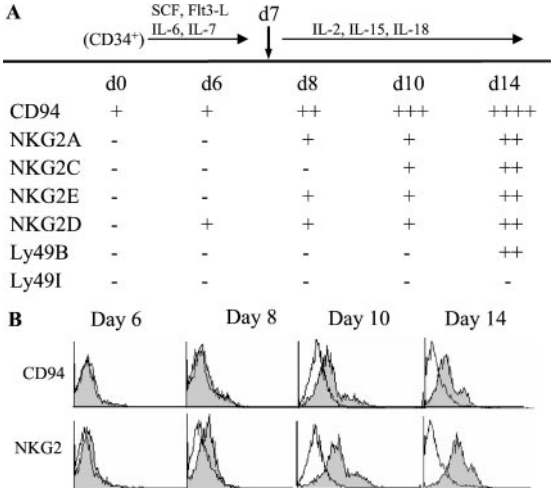

FIGURE 5. Acquisition of CD94 and NKG2 receptors by developing ES-NK cells. *A,* Increasing numbers (1, 10, 100, and 1000) of ES-derived cells were sorted at various stages of development (days 0, 6, 8, 10, and 14) and subjected to RT-PCR analyses. Day 0 was the day when $CD34^+$ cells were sorted and placed on OP9 cultures. The cytokine mixture was changed at day 7 of the ES culture system. The PCR products were detected by Southern hybridization. When no PCR products were detected with 1000 cells, they were considered negative. Detection of PCR products with 1, 10, 100, or 1000 cells was shown by ++++, +++, ++, or +, respectively. *B,* Surface expression of CD94 and NKG2 was determined by flow cytometry.

probes specifically detected the individual genes (Fig. 4*A, right panel*). The single-cell RT-PCR method was applied to analyze three different cell populations. Flow cytometric analysis showed that $CD4^+$ splenic T cells do not express a detectable level of CD94/NKG2 (Fig. 4*B, right panel*), and no cells among 20 tested by single-cell RT-PCR were positive for NKG2 gene expression (Fig. 4*B, left panel*). Three cells were positive for CD94 mRNA. Approximately 45% of freshly isolated splenic NK cells were found to be $CD94/NKG2^+$ by flow cytometry, whereas similar percentages of positive cells were detected by single-cell RT-PCR. As previously reported, most $CD94/NKG2^+$ splenic NK cells express NKG2A mRNA, but not NKG2C or NKG2E mRNA. $CD3^+NK1.1^+$ cells in bone marrow also expressed CD94/NKG2. However, unlike splenic NK cells, mRNA for NKG2C and NKG2E is more frequently expressed in these cells than that for NKG2A. The frequency of bone marrow NKT cells coexpressing transcripts for CD94 and NKG2 was comparable to that expressing the protein products, as determined by flow cytometry. Overall, these results show close correlation between the frequency of CD94/NKG2 expressing cells determined by flow cytometry and that determined by single-cell RT-PCR. The same method was applied to ES-NK cells. ES-NK cells cultured with OP9 were further incubated for 2 additional days with IL-2, 1L-12, 1L-15, and IL-18 in the absence of OP9 and individually sorted for the RT-PCR. The results revealed that most ES-NK cells coexpressed CD94, NKG2A, NKG2C, and NKG2E mRNA, whereas 25% expressed NKG2D mRNA (Fig. 4*C*). Only 2% of the cells expressed Ly49B or Ly49I mRNA (data not shown).

Orderly acquisition of CD94/NKG2 expression by ES-NK cells

Having verified that the ES-NK cells expressed functional CD94/NKG2, we examined how these receptors were acquired. Cells at different stages of development were obtained from the ES-NK differentiation protocol and assessed for expression of CD94 and

NKG2. This was achieved by collecting the cells on days 0, 6, 8, 10, and 14 of the culture system, day 0 being the day $CD34^+$ cells were isolated from embryoid body. Cells on day 6 likely represent common lymphoid progenitors as discussed above. On day 7, cytokines were switched from a mixture of IL-6, IL-7, SCF, and Flt3-ligand to a mixture of IL-2, -12, -15, and -18 to induce differentiation of lymphoid progenitors into the NK cell lineage. The expression of NK cell receptors was determined by flow cytometry and RT-PCR. For semiquantitative detection of the individual receptor transcripts, increasing numbers (1, 10, 100, and 1000) of cells were sorted and used for RT-PCR. CD94 transcripts were detected as early as day 0 of the ES-NK differentiation pathway (Fig. 5*A*). The first NKG2 transcript observed was NKG2D, an activating receptor that is significantly divergent from the rest of the NKG2 family. It was followed by NKG2A and E on day 8, and finally by NKG2C on day 10 (Fig. 5*A*). Although NKG2A and NKG2E mRNA could be detected on the same day, the level of NKG2E expression was considerably lower than that of NKG2A (data not shown). The amount of transcripts detected for all genes gradually increased with time. By FACS analyses, both CD94 and NKG2gradually increased over time after the addition of IL-15 to the culture system (Fig. 5*B*). These results were reproducible in four separate experiments.

Discussion

NK cells are thought to be potentially self-reactive but rendered self-tolerant due to the expression of inhibitory receptors specific for MHC class I. In contrast to adult mouse NK cells that express both Ly49 and CD94/NKG2, most, if not all, fetal and neonatal mouse NK cells express CD94/NKG2 receptors, but not the Ly49 family (16, 39–41). Therefore, CD94/NKG2 receptors are likely responsible for the recognition of missing-self and self-tolerance of NK cells in fetal and neonatal life. In this study, we examined how NK cells acquire these receptors as they differentiate in vitro from ES cells. The advantage of the ES cell system is that the differentiation process can be dissected into distinct steps. ES cells first differentiate into $CD34^+$ hemopoietic progenitor cells capable of differentiating into the myeloid and lymphoid lineages (42–44). They then become putative common lymphoid progenitors, which finally differentiate into functional NK cells. This multistep differentiation process allowed us to follow the acquisition of individual receptors by developing NK cells. Because the currently available Abs and Qa-1^b tetramers react with all CD94/NKG2 heterodimers, we examined the expression of CD94/NKG2 by a combination of RT-PCR and flow cytometry. The most significant finding from this study is that the initiation of the receptor gene expression is orderly and nonstochastic. The expression of the CD94 gene was detected as early as in $CD34^+$ cells. The level/frequency of CD94 expression remained low until the last step to induce NK cell differentiation. Similarly, NKG2D gene expression was detected, albeit at low level/frequency, at the step of the putative common lymphoid precursor cells, whereas no other NKG2 expression was detected until the final step of NK cell differentiation. It should be noted that the detection of NKG2D expression by single-cell RT-PCR is not efficient. Flow cytometric analysis showed that all adult mouse NK cells express NKG2D (13, 14), whereas only 25–30% were positive by single-cell RT-PCR. In the final step, expression of NKG2A and NKG2E was detected first, and NKG2C expression was detected last. The results of flow cytometric analyses were consistent with the RT-PCR results and confirmed that the expression of the receptor genes resulted in the expression of the protein products on the cell surface. Almost all ES-NK cells coexpressed transcripts for CD94, NKG2A, C, and E. The expression of some Ly49 genes, including Ly49B and Ly49I, was also detected in this culture system. However, the frequencies of the Ly49 gene expression were very low, and the protein products were undetectable by flow cytometry.

The detection of CD94 and NKG2D transcripts in $CD34^+$ cells in embryoid bodies and the putative common lymphoid precursor cells, respectively, suggests that the expression of these genes may be regulated differently from that of other NKG2 genes. It also suggests that the expression of these genes may not be restricted to the NK cell lineage, but may be more widely distributed in the hemopoietic lineages. The expression of NKG2A, C, and E genes was rapidly initiated by IL-15 and their expression, both at the mRNA and the protein levels, increased in the subsequent culture period of 7–9 days. IL-15 and the OP9 stroma cells appear to be critical for this culture system. In the absence of IL-15 or OP9, no NK cells were generated. In contrast, IL-12 and IL-18 were not essential for the expression of CD94/NKG2, but seemed to enhance the cytotoxicity of ES-NK cells. Although most ES-NK cells coexpressed all the CD94 and NKG2 mRNA, the inhibitory CD94/NKG2A receptor seemed dominant over the possible stimulatory CD94/NKG2C and CD94/NKG2E receptors as demonstrated by the inhibition of cytotoxicity mediated by Qa-1^b on target cells. ES-NK cells can kill MHC class I-deficient, but not normal, Con A blasts suggesting that CD94/NKG2A is responsible for the self-tolerance of ES-NK cells. Thus, induction of NKG2A at an early point during NK cell differentiation ensures that developing NK cells remain self-tolerant.

ES-NK cells resemble fetal NK cells in many ways. Both express CD94/NKG2 but not Ly49, and they also differentially kill MHC class I-deficient cells. Toomey et al. (21) generated NK cell clones from fetal thymus in the presence of IL-2 and IL-4. Although they all contained CD94 and NKG2 transcripts, the Qa-1^b receptor protein was detected on some, but not all, fetal NK clones. Single-cell analysis of those clones suggested that the acquisition of the Qa-1^b receptor on fetal NK cells is stochastic. In contrast to the NK clones generated from fetal thymus, almost all ES-NK cells expressed the CD94/NKG2 proteins, consistent with the CD94/NKG2 expression pattern of fetal NK cells (22). Currently, no mAbs are available to differentially detect individual CD94/NKG2 heterodimers, and it remains to be determined whether the CD94 and NKG2 mRNA detected in most ES-NK cells encode functional proteins. However, the frequencies of cells expressing these mRNA determined by single-cell RT-PCR in other cell populations thus far tested are very similar to the percentages of cells positively stained with anti-CD94/NKG2 mAb. Therefore, most ES-NK cells may coexpress all CD94/NKG2 receptors, and thus, the acquisition of CD94/NKG2 receptors by ES-NK cells appears to be nonstochastic. The expression of NKG2 genes in NK progenitors seems to be initiated by IL-15 and follow a predetermined order of expression. Whether the chromosomal locations of the genes influence the order of individual NKG2 expression is currently unknown. In both humans and mice, the *Cd94* gene is most centromeric followed by *Nkg2d*, *Nkg2e*, *Nkg2c*, and *Nkg2a* (1, 45). Although Ly49E has been reported to be expressed on fetal NK cells from B6 mice (22, 46), transcripts for Ly49E were not detected in ES-NK cells by RT-PCR. The sequence of 129 Ly49E cDNA is almost identical with that of B6 origin, and it should be amplified by PCR using the consensus primers in this study. It is still unknown why Ly49E transcripts are not detected in our studies. Ly49B transcripts were previously detected in immature NK cells generated from adult mouse bone marrow progenitors in vitro (19). The sequence of Ly49B is divergent from those of other Ly49, and the gene is distantly located from the Ly49 gene cluster. The sequence of Ly49B cDNA from 129 mice indicates that it is

highly conserved in the two strains. It remains to be determined whether Ly49B plays a role in NK cell differentiation.

In bone marrow cell culture systems described by Roth et al. (24) and Williams et al. (19), stromal cells were required for the induction of Ly49. In the absence of stromal cells, bone marrow progenitor cells isolated from adult C57BL/6 mice differentiated into NK1.1$^+$ cells that were cytotoxic but lacking Ly49 expression (47). However, coculturing with the OP9 stromal cell line yielded ~50% Ly49$^+$ NK cells, the majority of them acquiring Ly49C/I and Ly49G receptors. In the ES cell system, Ly49 receptors were undetectable on developing NK cells despite being cocultured almost the entire period on OP9 stromal cells. It has recently been reported that fetal liver hematopoiesis has a different developmental capacity than that of adult bone marrow hematopoiesis despite both having the ability to generate lymphoid cells (19, 48). In this case, the common lymphoid progenitor in adult bone marrow was found to be more restricted in its differentiation potential, suggesting a switch in mechanisms regulating development from fetal to adult life. It was proposed that the variable expression of Pax-5, a myeloid-suppressing transcription factor, was responsible for that switch. It seems likely that the ES cell culture system in this study mimics the fetal, but not adult, NK cell differentiation pathway.

Nakayama et al. (43) reported an in vitro method to generate LAK cells from ES cells in which CD34$^+$ embryoid body cells were also enriched as differentiation intermediates. From their method, two types of LAK cells were obtained, both of which were cytotoxic in response to IL-2. However, there were differences between them in terms of their cytotoxic capabilities and specificities. Also, there did not appear to be a controlled preference for generating one type of LAK over the other. One of the main differences between the culture system described by Nakayama et al. (43) and that described in this study was the cytokines used. Based on studies showing the importance of IL-15 and Flt3-ligand in the bone marrow microenvironment (24, 49), these cytokines were added to our developing ES cultures. Because Nakayama et al. (43) did not analyze their ES-derived LAK cells for the expression of MHC class I-specific receptors, a detailed comparison cannot be made between those cells and the ES-NK cells described in this study.

For CD94/NKG2, their order of expression reflects one of the models proposed by D. H. Raulet (23) describing how individual NK cells obtain a proper balance of activating and inhibitory signals. According to this model, individual NK cells first express inhibitory receptors, and once sufficient expression is obtained to prevent autoaggression, expression of activating receptors is initiated. There is also evidence suggesting that activating receptors for non-MHC self-ligands are expressed at the earliest stages of receptor acquisition. Their expression subsequently drives the expression of the MHC-specific receptors, beginning with the inhibitory and followed by the activating receptors. The NKG2 acquisition pattern observed in developing ES-NK cells appear to fit this description. Expression of NKG2D, a non-MHC-specific activating receptor, occurred very early in ES-NK development and preceded the expression of all the other NKG2 genes. The next receptor expressed was NKG2A, which is inhibiting and MHC-specific, followed by NKG2E and finally NKG2C, both of which are MHC-specific activating molecules. Even though the ES-NK results seem to conform to this model, further investigation is required to fully understand how these genes are developmentally regulated.

Future experiments using the culture method to generate NK cells from ES cells described in this study will be very useful for examining and identifying molecular events and genes that regulate NK cell differentiation and receptor acquisition. In addition, these future studies will also be important in evaluating the precise role of CD94/NKG2 in the NK developmental process.

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