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Establishment and Functional Characterization of Novel Natural Killer Cell Lines Derived from a Temperature-Sensitive SV40 Large T Antigen Transgenic Mouse

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Natural killer (NK) cells belong to an important lymphocyte population that eliminates transformed cells and invading pathogens without any prior sensitization. NK cells possess not only natural killing activity against non-self and altered-self cells but also exhibit cytokine production and antibody-dependent cell-mediated cytotoxicity (ADCC). Despite their important roles in the innate immune system, little is known about the details of NK cell biology. In spite of that several murine NK cell clones have been established, studies have mainly focused on their natural killing activity but not their cytokine production or ADCC. In this study, we established and characterized eight novel, immortalized murine NK cell clones derived from a temperature-sensitive SV40 large-T antigen transgenic mouse. These NK cell lines continuously proliferated for more than 30 months in a culture medium supplemented with interleukin 2. All cell lines contained azurophilic granules in the cytoplasm, and a few clones retained the NK cell functions, such as natural killing activity, cytokine production, and ADCC. In addition, one clone could serve as a host for transient as well as stable gene transfection. Taken together, these findings indicate that the cell lines could constitute useful tools for detailed analysis of murine NK cell biology.

Key words: cell-culture, cell-differentiation, cell-sorting, immuology-cellular response, receptor-lyeukocyte/lymphocyte.

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; FcγRIII, type III Fc receptor for IgG; LAK, lymphokine-activated killer; NK, natural killer; rh, recombinant human; SVNK, temperature-sensitive SV40 large T antigen–transduced natural killer cell line; TNP, trinitrophenol; tsSV40LT, temperature-sensitive mutant of SV40 large T transgenic.

Natural Killer (NK) cells represent a relatively minor, but substantial population of lymphocytes in the immune system, and are important for eliminating transformed cells and virally infected cells (*1*). Unlike T and B cells, which rearrange their receptors to acquire antigen specificity, NK cells express non-rearranged, germline-encoded antigen receptors on their surface (*2*). Recognition of the ligands on the target cell surface by NK cell receptors leads to cytotoxicity without prior sensitization. Accordingly, NK cells are thought to participate in early host defense, including the precocious recognition of tumors and stressed self-cells infected by viruses. This process is called natural killing and is generally accepted as the most prominent aspect of NK cells.

Cytokine/chemokine production and antibody-dependent cell-mediated cytotoxicity (ADCC) are other important functions of NK cells (*3*, *4*). Upon activation, NK cells release various cytokines and chemokines such as IFN-γ, TNF-α, GM-CSF, and CC-chemokines, which activate other immune cells for innate responses as well as for acquired antigen-specific responses. Moreover, IgG antibody-coated cells are recognized *via* type III Fc receptor for IgG (FcγRIII) on NK cells, and are efficiently lysed in a perforin/granzyme-dependent manner. Therefore, NK cells have critical influence on both innate and acquired immunity.

Despite their important roles in host defense against tumor cells and infectious pathogens, the details of NK cell biology remain unclear. Lymphokine-activated killer (LAK) cells prepared from mouse splenocytes have been utilized to investigate NK cell biology *in vitro*. However, this is time-consuming and the prepared LAK cells do not proliferate for more than 3 weeks, hence immortalized NK cell lines with the primary functions are desired. Recently, several murine NK cell clones were established from splenocytes of BALB/c nude mice or wild-type mice (*5*), C57BL/6 fetal liver cells (*6*), and p53-deficient C57BL/6 splenocytes (*7*, *8*). Although NK cells can exhibit not only natural killing activity but also cytokine/chemokine production and ADCC, these preceding studies mainly focused on

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the natural killing activity of and the expression of surface antigens on the NK cell lines generated, and hence few NK cell lines have been analyzed regarding all of these functions of NK cells.

SV40 large T (SV40LT) antigen is often utilized to immortalize rodent and human cells (*9*). Various cell lines have been established by introducing the SV40LT gene into primary rodent and human cells, and this method provides basic strategies for inducing cell immortalization (*10*–*15*). In 1991, a transgenic mouse line harboring the temperature sensitive-SV40LT (tsSV40LT) gene was generated to circumvent the need for gene insertion *in vitro* in order to establish various immortalized cell lines (*16*). The transgenic mice show stable and identifiable expression of the tsSV40LT gene in all tissues, and the cultured cells can be readily immortalized by activating the tsSV40LT gene at 33°C, a permissive temperature (*16*). The immortalized cells proliferate at 33°C because large T antigen binds to the tumor suppressor gene product, p53, and/or the retinoblastoma gene product, Rb, which regulate cell proliferation (*16*). At temperatures over 37°C, which are non-permissive temperatures, large T antigen becomes unstable and is degraded, which stops the growth of the cells. Many studies have verified the utility of the transgenic mice for the generation of cell lines retaining their primary functions, such as hepatocytes (*17*), kidney tubule cells (*18*), gastric surface mucous cells (*19*), brain capillary endothelial cells (*20*), and bone marrow stromal cells (*21*). We recently established immortalized dendritic cells with sufficient antigen presentation ability, and several immortalized mast cell lines with enhanced proliferative and degranulative abilities by culturing bone marrow cells from tsSV40LT mice in the presence of GM-CSF or IL-3, respectively (*22*, *23*). These were the first studies on immortalization of myeloid-lineage cells with specialized functions from tsSV40LT mice.

To obtain useful tools for investigating NK cell biology, we established novel, immortalized murine NK cell lines derived from a tsSV40LT transgenic C57BL/6 mouse, and characterized their natural killing activity, cytokine production, surface marker profiles, IL-2 dependency of proliferation, and ADCC. In addition, we examined their competency as hosts for the transfer of an exogenous gene into NK cell lines. This paper is the first description of the immortalization of lymphoid cells with specialized functions from tsSV40LT mice.

MATERIALS AND METHODS

Mice—Female C57BL/6 mice were obtained from Charles River Japan Inc. (Yokohama, Japan) and were used at 8–12 weeks of age. The founder mice for tsSV40LT mice were donated by Dr. K. Ishibashi, Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan) and used at 8 weeks of age. The transgenic mice were maintained in the animal facility of the Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan), an environmentally controlled and specific pathogen-free facility, according to the guidelines for experimental animals defined by the facility, by artificial insemination because of their spontaneous development of colloid plexus tumors within 5 months of age. All the experiments were performed according to protocols approved by the institutional animal care committee.

Reagents, Medium and Antibodies—Recombinant human (rh)IL-2 was obtained from Strathmann Biotech (Hamburg, Germany) or Peprotech (Rocky Hill, NJ). RPMI-1640 was obtained from Sigma-Aldrich (St. Louis, MO). Antibiotics were obtained from ICN (Costa Mesa, CA). Fetal bovine serum (FBS) was obtained from Nichirei Co., Ltd. (Tokyo, Japan). A 100× non-essential amino acid solution and geneticin were obtained from Invitrogen (Carlsbad, CA). NK cells cultures were performed in RPMI-1640 supplemented with 50 μM penicillin, 50 μg/ml streptomycin, 50 μM 2-ME, 10% heat-inactivated FBS, 1 mM sodium pyruvate, 1× non-essential amino acid solution (complete RPMI-1640), and 1,000 IU/ml rhIL-2. YAC-1, RL♂1, EL-4 and 293T cells were maintained in the same medium as that used for NK cells except for rhIL-2. The following antibodies for flow cytometric analysis were obtained from PharMingen (SanDiego, CA): anti-NK1.1 (PK136); anti-CD49b (DX5); anti-FcγRIIB/III (2.4G2); anti-CD3ε (145-2C11), and anti-CD5 (53-7.3). Anti-Ly49H (3D10) and anti-CD94 (Yuri-3) were donated by Dr. Wayne M. Yokoyama (Washington University School of Medicine, St. Louis, MO) and Dr. Noriko Toyama-Sorimachi (Research Institute, International Medical Center of Japan, Tokyo, Japan), respectively. Anti-SV40LT antibodies were obtained from Oncogene (Cambridge, MA). Anti-G3PDH antibodies were obtained from Chemicon (Temecula, CA). Anti-trinitrophenol (TNP) IgG1 was purified from ascites fluid.

Cell Lines—The following murine cell lines were used as targets or stimulators. YAC-1: a highly NK-susceptible T cell line established from moloney virus-transformed newborn A/Sn mice. RL♂1: a moderately NK-susceptible T cell line established from X-ray irradiated BALB/c mice. EL-4: an NK-resistant T cell line established from C57BL/6 mice. 293T is a human embryonic kidney cell line and an efficient host for transfection. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in our laboratory.

Preparation of LAK Cells—A single cell suspension was prepared from spleen samples from C57BL/6 mice. NK cells were labeled with DX5 microbeads (Miltenyi Biotech, Auburn, CA) and purified using a MACS magnetic cell-sorting instrument. Freshly isolated NK cells were expanded for 7–14 days in complete RPMI-1640 medium supplemented with 1,000 IU/ml rhIL-2 (*24*, *25*). These cells, hereafter termed B6-LAK, were used as the experimental control.

Establishment of NK Cell Lines—An 8-week-old female tsSV40LT mouse was killed and a spleen cell suspension was prepared under sterile conditions. Red blood cells were lysed in 0.14 M NH_4Cl, 0.17 M Tris-HCl (pH 7.4), and the remaining cells were washed with complete RPMI-1640 medium. Cells were labeled with 100 μl of 5 μg/ml biotin-conjugated anti-NK1.1 antibodies (PharMingen) for 15 min on ice, and then washed. The cells were labeled with 100 μl of diluted streptoavidin–conjugated magnetic microbeads (Miltenyi Biotech) according to the manufacturer's instructions, and then extensively washed. Labeled cells were collected using a MACS magnetic cell sorting instrument (Miltenyi Biotech). These cells were expanded in complete RPMI-1640 medium supplemented with 1,000 IU/ml

rhIL-2 for 1 month, and then cloned by limiting dilution at a density of 0.2 cell/well in flat-bottom 96-well culture plates. Proliferated cells were harvested with PBS containing 2 mM EDTA and culture scales were extended in a stepwise fashion. We utilized a relatively high concentration (1,000 IU/ml) of IL-2, which is considered to be sufficient for the induction of human and rodent LAK cells (*24–26*), as described above.

Wright-Giemsa Staining—1×10^6 cells were cytocentrifuged on a slide glass and then completely air-dried. Cells were fixed with methanol, and then stained with Wright's solution (Merck Japan, Tokyo, Japan) for 5 min, followed by Giemsa's solution (Merck Japan) for 20 min. Stained cells were observed under a light microscope and photographed.

Flow Cytometry—A single cell suspension was prepared and stained with appropriate FITC- or PE-labeled monoclonal antibodies directly, or indirectly stained with FITC- or PE-labeled secondary antibodies, and then analyzed with BD-LSR (Becton Dickinson Japan, Tokyo, Japan). Debris was excluded by a gating based on light scatter parameters. To avoid non-specific staining via Fc receptors, cells were first incubated with a saturating concentration of 2.4G2 except for in the case of 2.4G2 staining. Fc receptor–blocked cells not stained with specific antibodies were used as negative controls.

RT-PCR—Total mRNA was isolated with Trizol reagent (Invitrogen). 1st strand cDNA was reverse transcribed from 2 μg total RNA with a random 6 mer-primer in a volume of 40 μl. 2 μl of cDNA was used as a template for PCR. The primers used for RT-PCR and the annealing temperatures were as follows: NKG2A, sense 5′-CGAAGCAAAGGCACAGA-3′, anti-sense 5′-ATGGCACAGTTACATTCATCA-3′, 53°C. NKG2C, sense 5′-GCTGAACTGAAGAAGCAGATCC-3′, anti-sense 5′-TGGGGAATTTACACTTACAAAG-3′, 58°C. NKG2D, sense 5′-ACACATTGATGTGGCTTG-3′, anti-sense 5′-TGGTATTTCCACCAGAGT-3′, 46°C. NKG2E, sense 5′-TATATAAACCACAGTCTA-3′, anti-sense 5′-GTTACATTCATCATGGAG-3′, 44°C. G3PDH, sense 5′-CAGCAATGCATCCTGCACCA-3′, anti-sense 5′-GATCCACGACGGACACATTG-3′, 50°C. The gene specificity of these primers was confirmed with GENETYX software (Genetyx Co., Tokyo, Japan).

Genomic PCR—Tail tip DNA or intracellular DNA was extracted according to the standard procedure as a template. PCR was performed at an annealing temperature of 58°C for 35 cycles. The amplified DNA was run on a 2% agarose gel and stained with ethidium bromide. The following primers were used for PCR: SV40LT, sense 5′-GGAGGAGTAGAATGTTGAG-3′, anti-sense 5′-GTGTTGATGCAATGTACTGC-3′; and FcεRIγ, sense 5′-GCCCTGGGAGAGCCGCAGCTCTGCTATATCCTGGA-3′, anti-sense 5′-CTCACGGCTGGCTATAGCTGCCTTTCGGACCTGGA-3′.

Western Blotting—2.5×10^6 cells were lysed in 40 μl of TNE (10 mM Tris-HCl (pH 7.8), 1% NP-40, 0.15 M NaCl, 1 mM EDTA (pH 8.0), 1 mM PMSF) for 1 h on ice, and then centrifuged. 30 μl of the supernatant was mixed with an equal volume of 2× SDS-PAGE sample buffer [20% glycerol, 4% SDS, 0.125 M Tris-HCl (pH 6.8), 0.01% BPB, 4% 2-ME], and boiled, and then 20 μl was subjected to 7.5% SDS-PAGE. The fractionated proteins were transferred to a Fluoro Trans W Membrane (Pall, East Hills, NY) and then probed with anti-SV40LT antibodies or anti-G3PDH antibodies, followed by HRP-conjugated anti-mouse IgG secondary reagent (Amersham Bioscience, Piscataway, NJ). Detection of chemiluminescence was performed with an ECL Western blotting analysis system (Amersham Bioscience).

MTT Cell Growth Assay—Cells were plated at 1×10^5/100 μl in 96-well culture plates. 10 μl of a 10 mg/ml MTT (Sigma-Aldrich) solution (dissolved in PBS) was added, followed by culturing for 4 h, and then the cells were lysed by adding 100 μl of 0.04 N HCl (diluted with 2-propanol). The degree of color development was measured at 584 nm, the background OD_{630} being subtracted. Assays were performed in triplicate.

Natural Killing—The YAC-1 or RL♂1 cell line was used as the target. ^{51}Chromium (^{51}Cr) incorporated 1×10^4 target cells were plated with various numbers of effector cells in a volume of 200 μl. Subsequently, the plates were spun and cytotoxicity was measured as the release of ^{51}Cr into the supernatant. After 4-h incubation at 37°C, 100 μl of the supernatant was collected, and radioactivity was measured with a scintillation counter. The maximal and spontaneous release was determined by the addition of 1 N HCl or medium. Percentage specific lysis was determined using the following equation: (experimental mean cpm – spontaneous release mean cpm)/(maximal release mean cpm – spontaneous release mean cpm) × 100. Assays were performed in triplicate.

ADCC—The opsonized EL-4 cell line was used as the target. ^{51}Cr incorporated EL-4 was surface-labeled with a TNP labeling solution (50 mg $NaHCO_3$ and 50 mg 2,4,6-trinitrobenzenesulfonic acid sodium salt dihydrate, dissolved in 10 ml PBS) for 15 min at room temperature. Cells were extensively washed with complete RPMI-1640 medium, and then incubated with various concentrations of anti-TNP IgG1 on ice for 15 min. Cells were extensively washed, and then used as the target. Conventional ^{51}Cr release assays were performed as described in the section on natural killing activity.

ELISA—The YAC-1 or EL-4 cell line was used as a stimulator. 1×10^5 of the stimulator and 1×10^5 of the NK clone were plated on a 96-well round-bottom culture plate in a volume of 200 μl and then spun down. After 24-h incubation at 33°C, the culture supernatant was subjected to standard sandwich ELISA analysis using an OptEIA anti-mouse IFN-γ ELISA kit (PharMingen). Assays were performed in duplicate.

Transfection—An enhanced green fluorescent protein (EGFP) expression vector, pIRES2-EGFP (BD Biosciences, San Jose, CA), was used for gene transfer to SVNK 6. The 293T cell line was used as the experimental control. Various amounts of circular plasmid were transfected with Lipofectamine 2000 (Invitrogen) or Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions or electroporated at 250 kV, 975 μF in a Gene Pulser (Bio-Rad, Hercules, CA). Transfected cells were seeded onto a 60 mm plastic culture dish, and then incubated at 33°C. After 48 h, the cells were harvested and subjected to flow cytometric analysis of EGFP expression. Alternatively, geneticin (Invitrogen) was added to a final concentration of 1,000 μg/ml to the culture medium after 24 h of electroporation, and the medium was replaced with fresh medium containing geneticin once a week.

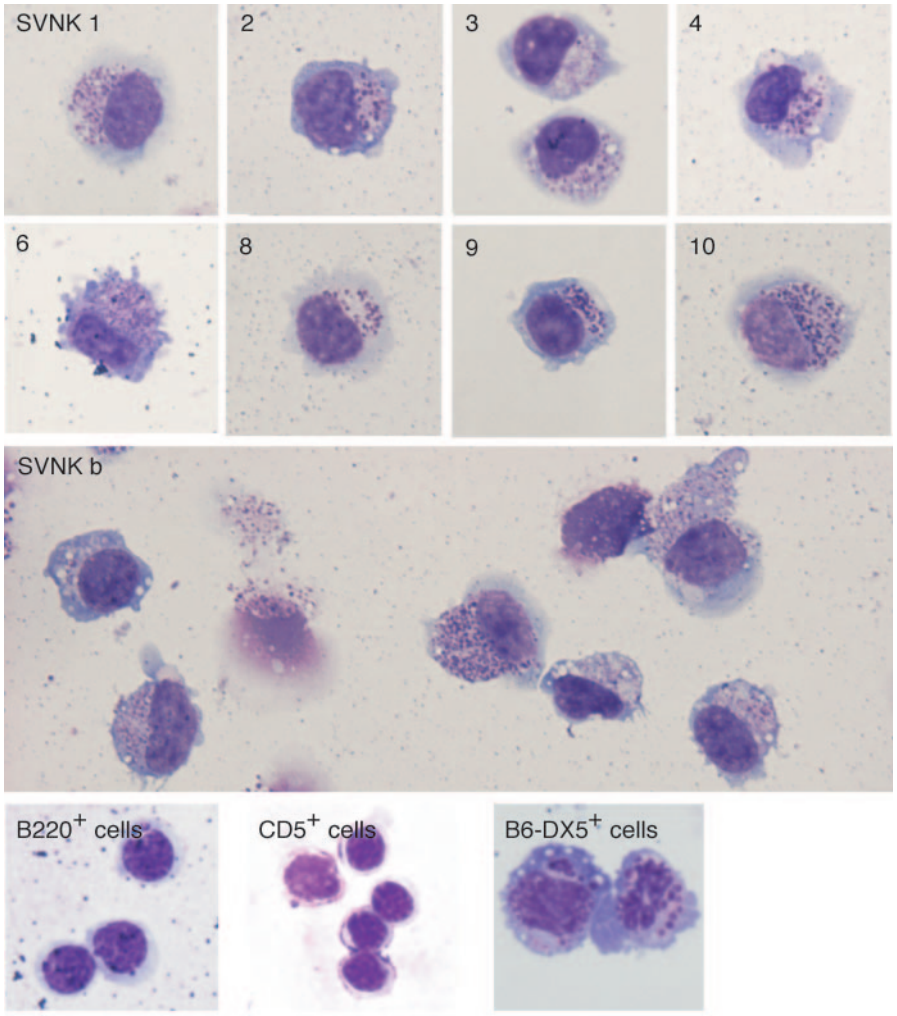

Fig. 1. **Morphology of SVNK lines.** Single cell suspensions were cytocentrifuged on slide glasses, and then stained by the Wright-Giemsa method. The typical morphology of each clone is shown. Freshly isolated $B220^+$ (B cells), $CD5^+$ (T cells), and C57BL/6 (B6) DX5 LAK cells were stained as experimental controls. Samples were photographed under a phase contrast microscope (original magnification, ×400).

RESULTS

Establishment of NK Cell Lines—We used a tsSV40LT transgenic C57BL/6 mouse, which is an effective tool for establishing immortalized murine cell lines (*16*). The original tsSV40LT mouse was generated by injecting DNA fragments derived from tsA58 (a temperature-sensitive mutant strain of SV40) into fertilized C57BL/6 eggs. When cells are isolated from tsSV40LT mice and cultured at 33°C, the SV40LT protein binds to and inactivates the p53 and Rb proteins (*27*), which results easily in cell immortalization. We purified NK cells from the splenocytes of an eight-week-old female tsSV40LT mouse by MACS magnetic cell sorting, and seeded them onto a 100 mm plastic culture dish and subcultured them once a week. After 1 month of *in vitro* culture, limiting dilution was performed to obtain single cell clones at a density of 0.2 cell/well. In a parallel culture, we expanded the bulk NK cells without any limiting dilution. Among 480 wells initially prepared for the limiting dilution (96-well plate ×5), approximately 20 growing clones were subcultured further on a 24-well culture plate. Some of the clones stopped proliferating or showed slower growth than others. We chose vigorously growing clones for further subculture on 6-well culture plates, and finally obtained eight NK cell clones (termed SVNK 1–4, 6, 8–10) as well as a bulk culture of NK cells (termed SVNK b) (Fig. 1). These cell clones proliferated for more than 30 months with the passage of a 1/5 to 1/10 volume of cells to new medium once a week. These cell clones were cryopreserved with 10% DMSO-containing culture medium by gradually decreasing the temperature to –130°C. To confirm that these cell clones maintained NK cell features, morphological analysis was conducted on the isolated NK cell clones. As shown in Fig. 1, all the SVNK cell clones, and SVNK b and DX5-positive LAK cells of a primary culture exhibited azurophilic granules in their cytoplasm on Wright-Giemsa staining, whereas CD5-positive T cells and B220-positive B cells isolated from murine splenocytes did not contain any azurophilic granules. This observation indicates that the SVNK clones and SVNK b retain the morphological aspect of freshly isolated LAK cells. Based on these and the following observations, we refer to these NK clones and SVNK b as NK cell lines hereafter.

Expression of NK-Specific Surface Antigens—To exclude the possibility that these cell lines were NK-like T cells, we further analyzed several NK-specific surface antigens and T cell antigens (Fig. 2). Flow cytometric analysis showed that NK1.1, an NK-specific marker, was negative for SVNK 1, 2 and 8 but very weakly positive for SVNK 3,

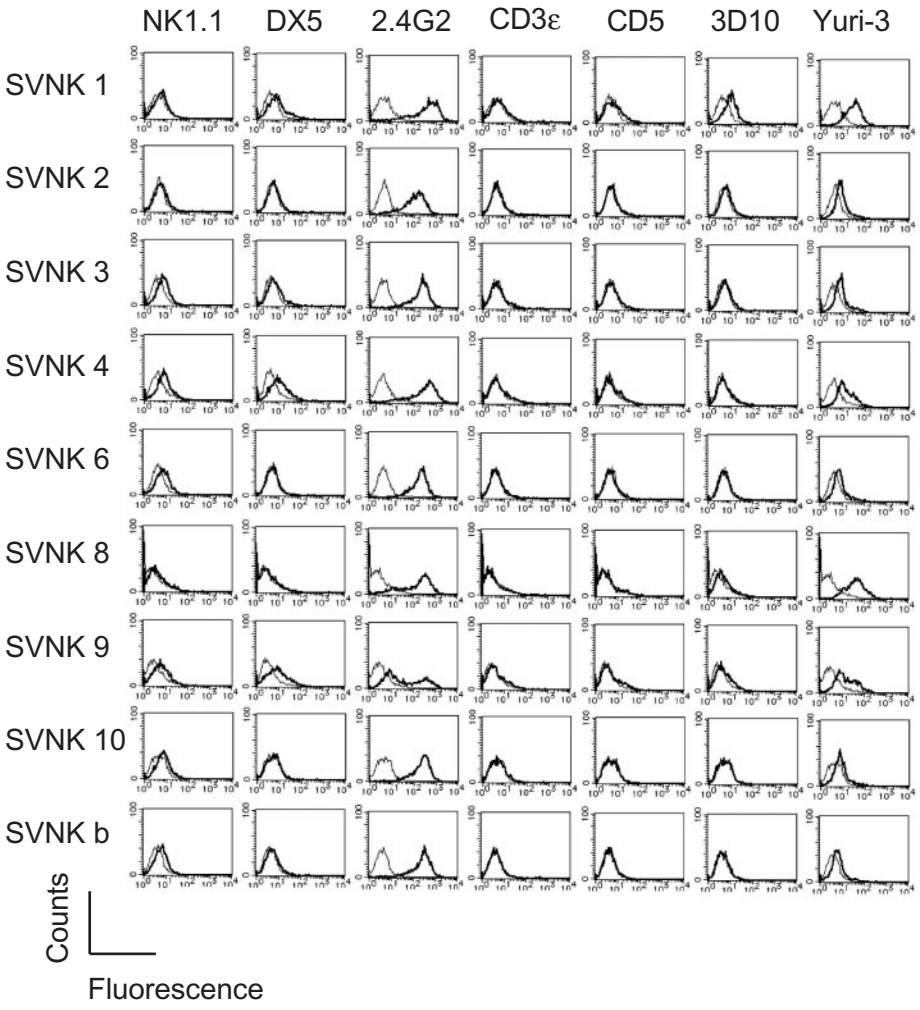

Fig. 2. **Surface antigen analysis of SVNK lines.** To avoid non-specific staining, cells were first blocked with 2.4G2 for 10 min, and subsequently labeled directly or indirectly with specific monoclonal antibodies for 15 min and analyzed (thick line). Unstained cells were used as negative controls (thin line). The data for 1×10^4 cells were corrected and analyzed with BD-LSR using CellQuest software.

4, 6, 9, 10 and b, and DX5, another NK-specific marker, was negative for SVNK 2, 3, 6, 8, 10 and b but weakly positive for 1, 4 and 9. Staining with 2.4G2, an antibody against FcγRIIB/III, was positive for SVNK 1–4, 6, 8, 10 and b, but SVNK 9 showed a heterogeneous expression profile, presumably due to the expansion of mutated cells. CD3ε, a component of the TCR complex, and CD5, a T cell marker, were negative for all SVNK cell lines. Ly49 molecules consist of the multigene family and are expressed heterogeneously on NK cells (*28*). One of these antigens, Ly49H, which is a critical recognition receptor for murine cytomegalovirus-infected cells (*29*), was analyzed with a specific monoclonal antibody, 3D10. Ly49H expression was negative for SVNK 2–4, 6 and 10 but weakly positive for SVNK 1, 8 and 9. CD94 is expressed on all NK cells (*30*) as well as a minor population of conventional T cells (*31*, *32*). We also inspected CD94 expression with Yuri-3 monoclonal antibodies, and found that CD94 was weakly positive for SVNK 2–4, 6, 9, 10 and b but positive for SVNK 1 and 8. In summary, the expression of most NK cell markers on the SVNK cell lines decreased on the cell surface except for that of FcγRIIB/III and CD94.

NKG2 family receptors, which pair with CD94 in general, are also expressed on the NK cell surface and function as recognition receptors for Qa-1^b, a non-classical MHC

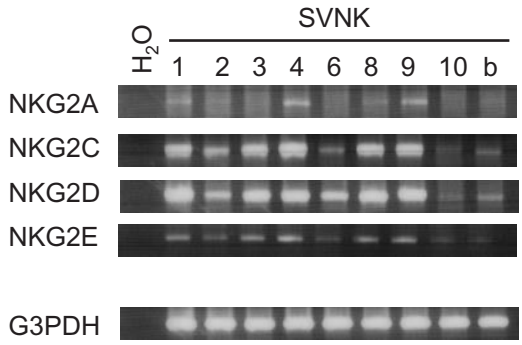

Fig. 3. **Expression analysis of NKG2 mRNAs in SVNK lines.** First strand cDNA of NK cell lines was generated from total RNA with random 6-mers, and PCR was performed with specific primers. Amplified DNA was electrophoresed through a 2% agarose gel. H_2O represents PCR amplification without a template.

class I molecule (*33*), and transmit activatory or inhibitory signaling to regulate various functions of NK cells. To determine which subtypes of NKG2 family molecules were expressed on SVNK lines, RT-PCR was performed in order to detect the expression of mRNAs for NKG2A, C, D, and E (Fig. 3). NKG2A mRNA was detected in SVNK

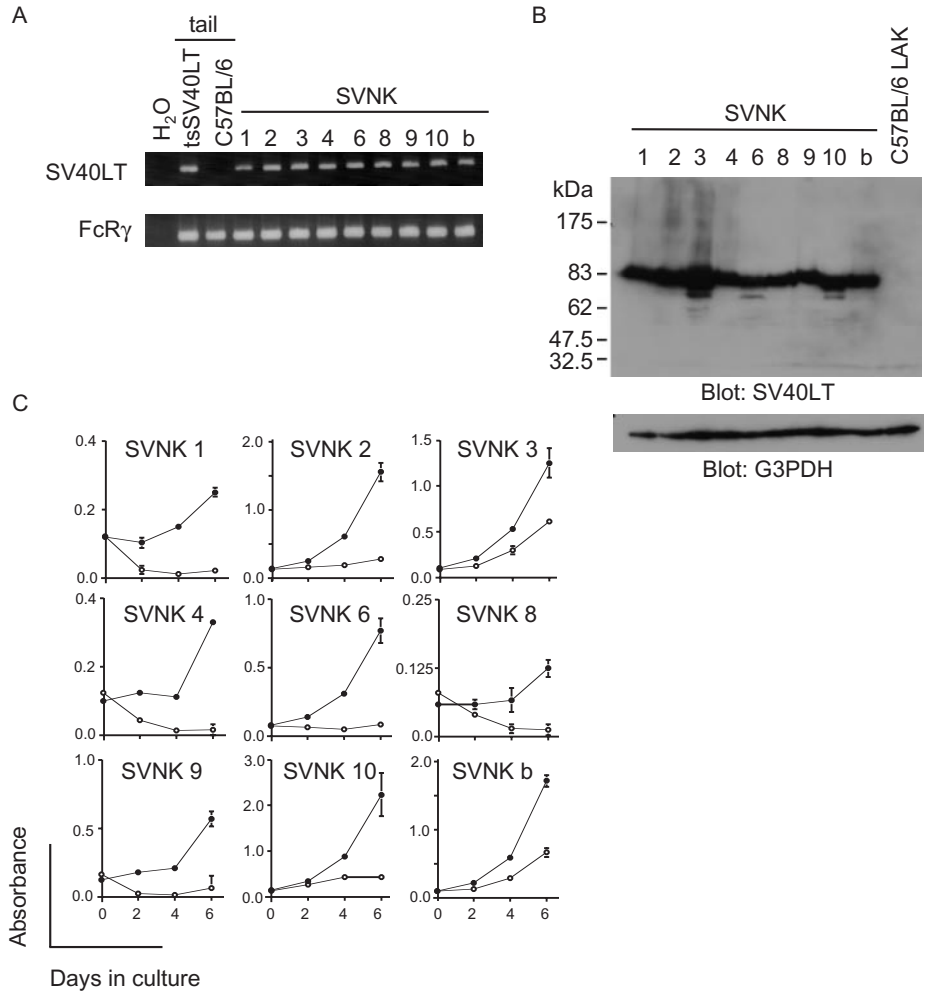

Fig. 4. **Validation of tsSV40LT mouse-derived cell lines.** A: Presence of the tsSV40LT transgene was confirmed by genomic PCR. Genomic DNA was extracted as a template, and PCR was performed with tsSV40LT transgene–specific primers. The PCR results for tail DNA of a tsSV40LT transgenic mouse and a C57BL/6 mouse are shown as experimental controls. B: Expression of the SV40LT protein was verified by Western blot analysis. Cellular protein of NK cell lines and C57BL/6 LAK cells was fractionated by SDS-PAGE, and probed with specific antibodies. The positions of protein markers are shown on the left. C: Temperature-dependent cell proliferation was examined by MTT analysis. Duplicate 96-well culture plates were prepared, and incubated at 33°C (closed circle) or 39°C (open circle). On days 0, 2, 4, and 6, cell proliferation was determined by MTT analysis. The means ± SD for triplicate wells are shown.

1, 4, 8 and 9 but not in SVNK 2, 3, 6, 10 and b. The NKG2C mRNA was abundant in SVNK 1, 3, 4, 8 and 9, but was less abundant in SVNK 2, and scarcely detectable in SVNK 6, 10 and b. NKG2D mRNA was abundant in SVNK 1–4, 6, 8 and 9, but was less significant in SVNK 10 and b. The NKG2E mRNA signal was weakly detected in all SVNK lines. Thus, the established NK cell lines showed the mRNA expression of nearly all NKG2 family receptors other than NKG2A.

Validation of a tsSV40LT Mouse–Derived Cell Line—In order to verify that these cell lines were derived from the tsSV40LT transgenic mouse, the presence of the SV40LT transgene and protein, and temperature-dependent cell proliferation were examined. Cellular DNA was extracted from each NK cell line as a template, and genomic PCR was performed with SV40LT-specific primers. Figure 4A shows that all SVNK cell lines contained the SV40LT transgene, while control DNA from a C57BL/6 mouse did not show any signal for it. The presence of the SV40LT protein was determined by Western blot analysis (Fig. 4B). While a cell lysate of C57BL/6 LAK cells as a control showed no apparent signal, all SVNK cell lines gave a clear band corresponding to 83 kDa. It has been shown that the tsSV40LT gene is expressed in cells at 33°C (a permissive temperature). However, once the cells are cultured at 39°C (a non-permissive temperature), expression of the tsSV40LT gene disappears within 2 days, leading to growth arrest and apoptotic cell death. The temperature dependency of the proliferation of all SVNK cell lines was examined by MTT analysis (Fig. 4C). The cells showed active proliferation at 33°C, but not at 39°C. Collectively, these results show that all the SVNK cell lines were derived from the tsSV40LT mouse and exhibit temperature-dependent growth characteristics of the tsSV40LT transgene.

Rh IL-2 Dependency—A number of cytokines, such as IL-2, IL-7, IL-12, IL-15 and SCF, have intrinsic effects on NK cell development and/or proliferation. IL-2 is considered the most effective cytokine for the *in vitro* expansion of mature NK cells. We established cell lines by culturing splenic NK cells in complete RPMI-1640 medium containing 1,000 IU/ml rhIL-2, the concentration used for efficient isolation of human and rodent LAK cells (*24–26*). To clarify the rhIL-2 dependency, SVNK lines were plated on a 96-well culture plate at various concentrations (100–1,000 IU/ml) of rhIL-2 and then incubated at 33°C, and cell growth was determined on day 6 by MTT analysis. All SVNK lines showed most vigorous growth with the concentration of 1,000 IU/ml rhIL-2 (Fig. 5). Lowering of the concentration below 1,000 IU/ml significantly attenuated their proliferation. These data indicate that rhIL-2 at 1,000 IU/ml is suitable for effective SVNK cell growth, and

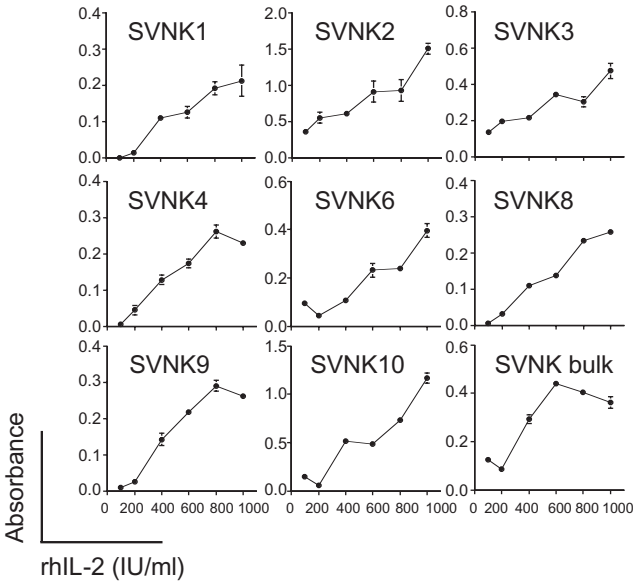

Fig. 5. **IL-2–dependent growth of SVNK lines.** SVNK lines were plated on a 96-well culture plate with various concentrations (100–1,000 IU/ml) of rhIL-2 and incubated at 33°C, and cell growth was determined on day 6 by MTT analysis. All SVNK lines showed vigorous growth with 800–1,000 IU/ml rhIL-2.

suggest that reduction of the rhIL-2 concentration is not recommended for effective proliferation in *in vitro* culture.

Natural Killing, ADCC and Cytokine Production—NK cells possess two distinct target recognition mechanisms to induce cytotoxicity. One is dependent on the NK cell receptors encoded in the germline, which recognize their ligands on target cells, and the other is dependent on the FcγRIII expressed on the NK cell surface, which binds to the Fc portions of IgG antibodies coating target cells. These phenomena are known as natural killing activity and ADCC, respectively. To estimate natural killing activity, ^{51}Cr release analysis was performed, for which YAC-1 or RL♂1 cells were used as the target of SVNK cells (Fig. 6A). SVNK 8 and b appeared to have higher NK activity than others. However, the rest of the SVNK lines showed remarkably lower NK activity than that of C57BL/6-derived LAK cells when RL♂1 was used as the target. On the other hand, for ADCC assessment, non-susceptible cell line EL-4 was chemically labeled with the TNP group, coated with various amounts of anti-TNP IgG1, and then used as the target cells (Fig. 6B). SVNK 6, 10 and b showed higher ADCC comparable to that of C57BL/6-derived LAK cells in an anti-TNP IgG1 dose-dependent manner. However, SVNK 1, 3, 4, 8 and 9 did not show any cytotoxicity against anti–TNP antibody–coated EL-4 target cells at any antibody concentration tested.

To explore the ability of cytokine production in response to target cells, the SVNK lines were co-cultured with either EL-4 or YAC-1 cells in a 96-well round bottom culture plate for 24 h, and the supernatants were subjected to ELISA for IFN-γ (Fig. 6C). SVNK 4, 6, 8, 9, and b showed significant but lower IFN-γ release than that by C57BL/6 LAK cells, but other clones produced a minute amount of IFN-γ in response to YAC-1 cells. On the contrary, no SVNK clone produced a significant amount of IFN-γ on co-culture with EL-4. Judging from these findings together, SVNK 8 exhibited outstanding natural killing activity and cytokine production, whereas SVNK 6, 10, and b showed distinct ADCC activity. On the other hand, SVNK 1 and 3 exhibited little natural killing activity, ADCC and cytokine production, and thus seemed to possess negligible NK cell functions. SVNK 2 exhibited a moderate level of ADCC, but no significant natural killing activity or cytokine production. SVNK 4 and 9 exhibited moderate cytokine production, but marginal natural killing activity and ADCC.

SVNK as a Host for Exogenous Gene Expression—The exogenous gene expression in a cell line permits us to efficiently investigate the cellular function of a transfected gene-encoded protein. Since NK cell lines able to accept an extraneous gene were not available, we determined whether the established SVNK lines had sufficient transfection competency to express an extrinsic gene-encoded protein. Various amounts of pIRES2-EGFP were introduced into SVNK 6 with Lipofectamine 2000 or Effectene or an electroporation method. After 48 h, the cells were harvested and subjected to flow cytometry to monitor the transgene expression. As summarized in Table 1, the most efficient gene transfer was achieved with the electroporation method. Although Lipofectamine 2000 and Effectene effectively introduced the EGFP gene into the 293T cell line, they induced less EGFP expression in SVNK 6 cells. Figure 7A shows typical results of EGFP transfection, indicating that 7.1% of SVNK 6 cells expressed the introduced EGFP in the amount of 40 μg EGFP DNA. However, this EGFP expression efficiency probably does not support any transient expression assays. In order to obtain a stable EGFP transfectant, the transfected cells were subjected to pharmacological cell selection in geneticin-containing medium for 1 month, which yielded a single EGFP-positive cell population, indicating that stable transfectants of SVNK lines could be isolated with an adequate selection method (Fig. 7B).

DISCUSSION

We established novel murine NK cell lines from a tsSV40LT transgenic mouse. Important aspects of these cell lines are described below. First, continuous maintenance of the cell lines for over 30 months was accomplished by supplementing rhIL-2 alone, and no other cytokines or stimulating reagents such as antibodies or special chemicals were required. Second, some clones, which maintain natural killing, ADCC and cytokine production activities, are useful tools for investigating NK cell functions *in vitro*. The present report is the first of immortalized murine NK cell lines that could express an exogenous gene.

Several reports have described the establishment of immortalized murine NK cell lines, which were derived from splenocytes of BALB/c nude or wild-type mice in Con A–stimulated conditioning medium (*5*), and from C57BL/6 fetal liver cells in a medium supplemented with IL-2, IL-4 and PMA (*6*), from a p53 C57BL/6 mouse in a medium containing rhIL-2, feeder cells, and anti-NK1.1 and anti-K^b antibodies (*7*), or rhIL-2 and poly(IC) (*8*), respectively. The present study indicates that NK cell lines can be established by a simpler method, namely cell isolation from tsSV40LT mice and culture with rhIL-2

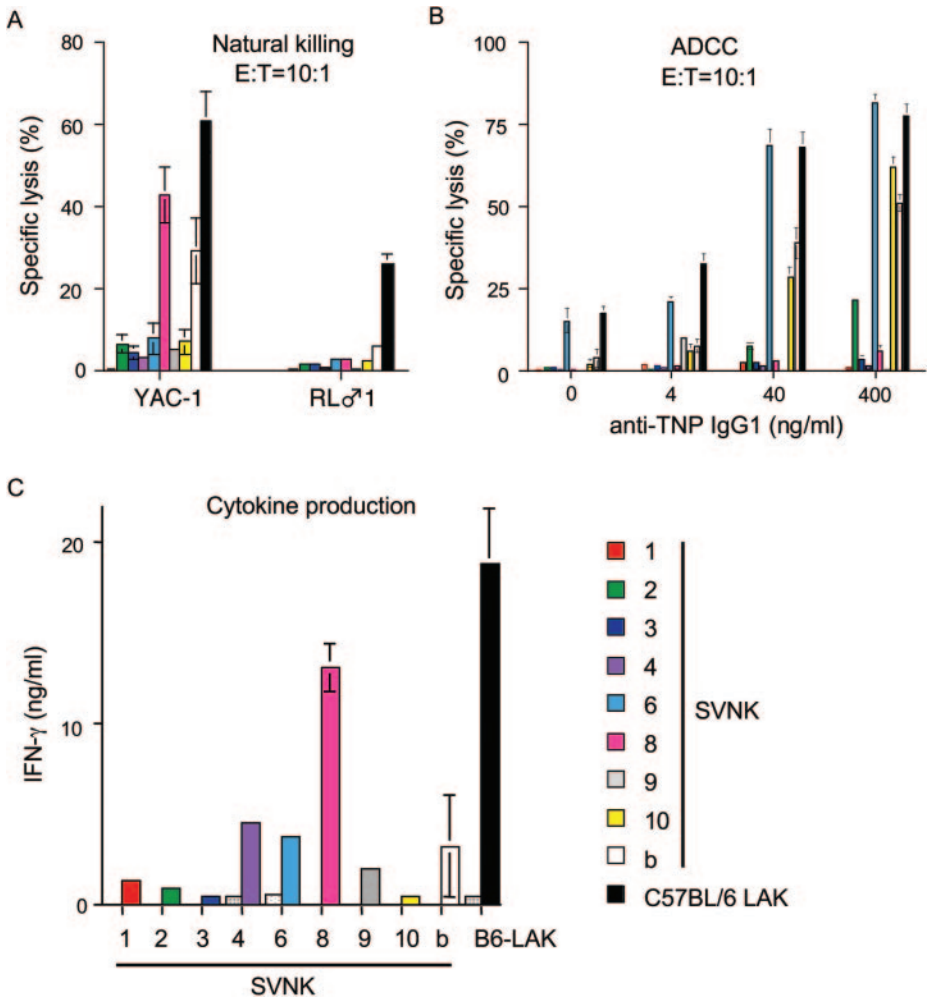

Fig. 6. **Assessment of killer activity and cytokine production of SVNK lines.** A: Natural killing activity was determined by conventional ^{51}Cr release assaying. The YAC-1 or RL♂1 cell line was co-cultured with the NK cell lines in an effector to target (E:T) ratio of 10:1 for 4 h. The mean ± SD for triplicate wells is shown. B: ADCC activity was measured by ^{51}Cr release assaying. EL-4 was chemically labeled with TNP and opsonized with various amounts of anti-TNP antibodies, and then used as a target. The mean ± SD for triplicate wells is shown. C: Cytokine production was determined by ELISA. Supernatants of NK cell lines stimulated with YAC-1 (open column) or EL-4 (dotted column) for 24 h were subjected to IFN-γ–specific sandwich ELISA. The means ± SD for duplicate wells are shown.

Table 1. **Transfection efficiency of an exogenous gene in SVNK cells.**

Transfection	DNA amount (μg)[a]					
	0.8	1	3	10	30	40
Lipofectamine	0.32[b] (88.4[c])	nd[d]	nd	nd	nd	nd
Effectene	nd	0.28 (19.9)	0.75 (nd)	0.83 (nd)	nd	nd
Electroporation	nd	nd	nd	0.87 (26.3)	3.84 (nd)	7.11 (nd)

[a]EGFP expression vector in the indicated amounts was introduced into SVNK 6 or 293T cells, and EGFP expression was determined by flow cytometric analysis after 48 h. [b]Data are expressed as transfection efficiency (%). [c]Data in parentheses indicate the transfection efficiency (%) of 293T cells as a control. [d]nd, not done.

supplementation alone. While a permissive temperature (33°C) allowed the tsSV40LT transgene-harboring cells to actively proliferate, we observed growth arrest or remarkable cell death when cells were cultured at a non-permissive temperature, 39°C, and additionally, a decrease in the rhIL-2 concentration resulted in slower cell proliferation, suggesting that both the SV40LT protein and 1,000 IU/ml rhIL-2 were essential for vigorous cell growth. Although previous reports (*6*–*8*) described the requirement of lower doses of rhIL-2 (50–100 IU/ml) for the establishment and maintenance of these NK cell lines, our cell lines required a relatively high dose of rhIL-2 (1,000 IU/ml). We conjecture that the requirement of a higher rhIL-2 concentration by our SVNK cell lines is due to the continuous culture with 1,000 IU/ml rhIL-2, which may lead to the selection or adaptation of SVNK cells to the high IL-2-permissive cells. Long-term culture of primary cells generally results in gradual cell senescence, and it is commonly considered to be difficult to achieve cell immortalization. Karlhofer *et al*. reported their repeated failure to establish a C57BL/6 mouse–derived NK cell line (*7*). Based on these observations and our results, SV40LT and rhIL-2 may be necessary and sufficient for readily establishing murine NK cell lines.

Although eight NK cell clones and a bulk NK cell line were established, functional analysis revealed that only a few of the established NK cell clones retained one or all of the characteristic features of freshly isolated LAK cells, the other clones showing impaired or non-functional phenotypes. Among the cell lines with the characteristic

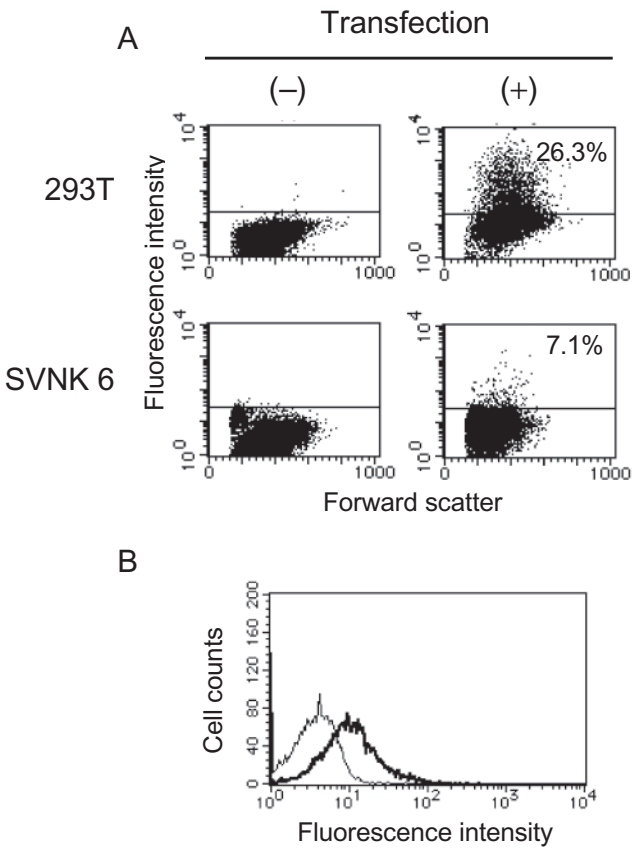

Fig. 7. **Exogenous gene expression of SVNK.** A: 2.5×10^5 SVNK 6 cells were electroporated with 40 μg of EGFP expression vector in a Gene Pulser. After 48 h, the cells were harvested and subjected to flow cytometry for EGFP expression. (–), untreated: (+), electroporated. 293T cells were employed as a control. B: Electroporated SVNK 6 cells were selected with geneticin for 1 month, and then subjected to flow cytometry (thick line). Untreated cells were used as a negative control (thin line).

functions of NK cells, SVNK 8 and b possessed higher natural killing activity against YAC-1 cells, SVNK 6, 10 and b retained higher ADCC activity, and SVNK 8 exhibited higher cytokine production in response to YAC-1 cells. Thus, each cell line with specific characteristic functions constitutes a useful tool for investigating defined functions of NK cells. Due to functional impairments, we could not exclude the possibility of cell senescence during long-term culture, and thus another explanation, such as the susceptibility of SVNK cells to a certain type of target cell or MHC class I-independent inhibition mechanism, namely the CD66a homophillic interaction reported in human NK cells and melanomas (*34*), might be the case. Since our NK cell lines exhibited heterogeneous functions, for instance, SVNK 8 showed excellent natural killing activity, while SVNK 10 exhibited insufficient natural killing activity even though its ADCC activity was notable, further studies such as cDNA subtraction cloning might provide clues regarding the determinants of NK cell functions at the molecular level.

Certain typical surface markers of NK cells, *i.e.*, NK1.1, DX5 and Ly49, were not detected or weakly expressed on our NK cell lines. These observations, however, are consistent with those previously reported. Only Karlhofer *et al*., who established NK clones cultured in an anti–NK1.1 antibody–coated culture dish, reported NK1.1-positive NK cell clones (*7*), whereas the others were NK1.1-negative or -diminished cell lines (*6*, *8*). Recently, it was reported that the expression of DX5 (CD49b) on NK cells gradually decreased on long-term culture (*35*), and could not be detected in Quirijn's NK cell clones (*8*). Furthermore, the expression of Ly49s was also negative for all NK clones previously established (*6–8*), and hematopoietic or embryonic stem cell-derived NK cells required interaction with stromal cells to induce the expression on the NK cell surface (*36–38*). Although these observations suggest that long-term *in vitro* culture causes NK cells to lose specific antigens on their surface, including NK1.1, DX5 and Ly49s, further investigation is required to address the molecular mechanism underlying the loss of these NK antigens. We also determined the mRNA distribution of NKG2s by RT-PCR analysis, and observed non-heterogeneous expression in our NK clones except for NKG2A. Rebecca *et al*. examined the NKG2s mRNA distributions of embryonic stem cell–derived NK cells by single cell RT-PCR followed by oligo probe Southern blot analysis, and revealed that most of their clones expressed all NKG2 mRNAs (*39*). Our results support the view of Rebecca *et al.* except in the case of NKG2A. However, since these experiments were based on mRNA analysis, the protein expression of each NKG2s family on NK cell lines should be confirmed to understand the characteristics of the established cell lines in detail.

One advantage of immortalized cell lines is the ability of genetic modification by transfection. To our knowledge, the present study is the first establishment of cloned murine NK cell lines capable of exogenous gene transfer. Although conventional transfer methods, Lipofectamine 2000 and Effectene, introduced the minimum EGFP gene into our established NK cell lines, an electroporation method potentiated moderate introduction of EGFP with a relatively high concentration of the EGFP gene, and in addition, geneticin selection of EGFP-introduced cells permitted us to obtain a stable EGFP-expressing cell population. Modulation of the pulse conditions for our electroporation or other methods such as ones involving viral vectors might improve the introduction efficiency. Although a recent report described a reasonably efficient transfection procedure for primary NK cells (*40*), our current SVNK lines may serve as more useful host cells for exogenous gene expression from the point of view that SVNK lines can grow continuously, which is convenient for testing the effect of any exogenous gene for a long period of time, such as weeks or months.

In conclusion, our novel tsSV40LT-derived NK cell line panels constitute useful tools for investigating NK cell biology in detail. These cell lines were maintained for over 30 months in the presence of 1,000 IU/ml rhIL-2, and functional analyses were successfully performed. Moreover, it was verified that one NK clone could express an exogenous gene transfected.

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REFERENCES

1. Cerwenka, A., and Lanier, L.L. (2001) Natural killer cells, viruses and cancer. *Nat. Rev. Immunol.* **1**, 41–49
2. Yokoyama, W.M., and Plougastel, B.F. (2003) Immune functions encoded by the natural killer gene complex. *Nat. Rev. Immunol.* **3**, 304–316
3. Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P., and Salazar-Mather, T.P. (1999) Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* **17**, 189–220
4. Abdullah, N., Greenman, J., Pimenidou, A., Topping, K.P., and Monson, J.R. (1999) The role of monocytes and natural killer cells in mediating antibody-dependent lysis of colorectal tumour cells. *Cancer Immunol. Immunother.* **48**, 517–524
5. Dennert, G. (1980) Cloned lines of natural killer cells. *Nature* **287**, 47–49
6. Manoussaka, M., Georgiou, A., Rossiter, B., Shrestha, S., Toomey, J.A., Sivakumar, P.V., Bennett, M., Kumar, V., and Brooks, C.G. (1997) Phenotypic and functional characterization of long-lived NK cell lines of different maturational status obtained from mouse fetal liver. *J. Immunol.* **158**, 112–119
7. Karlhofer, F.M., Orihuela, M.M., and Yokoyama, W.M. (1995) Ly-49-independent natural killer (NK) cell specificity revealed by NK cell clones derived from p53-deficient mice. *J. Exp. Med.* **181**, 1785–1795
8. Vos, Q., Ortaldo, J.R., Conan-Cibotti, M., Vos, M.D., Young, H.A., Anderson, S.K., Witherspoon, K., Prager, I., Snapper, C.M., and Mond, J.J. (1998) Phenotypic and functional characterization of a panel of cytotoxic murine NK cell clones that are heterogeneous in their enhancement of Ig secretion in vitro. *Int. Immunol.* **10**, 1093–1101
9. Jha, K.K., Banga, S., Palejwala, V., and Ozer, H.L. (1998) SV40-Mediated immortalization. *Exp. Cell Res.* **245**, 1–7
10. Ohnishi, K., Daigo, M., and Tokunaga, T. (1995) SV40-adenovirus immortalized cell lines derived from mouse lymphoid dendritic cell preparations. *Immunol. Cell Biol.* **73**, 205–211
11. MacDougall, M., Thiemann, F., Ta, H., Hsu, P., Chen, L.S., and Snead, M.L. (1995) Temperature sensitive simian virus 40 large T antigen immortalization of murine odontoblast cell cultures: establishment of clonal odontoblast cell line. *Connect. Tissue Res.* **33**, 97–103
12. Andley, U.P., Rhim, J.S., Chylack, L.T., Jr., and Fleming, T.P. (1994) Propagation and immortalization of human lens epithelial cells in culture. *Invest. Ophthalmol. Vis. Sci.* **35**, 3094–3102
13. Townsend, M., Macpherson, J., Krilis, S., Reddel, R., and Symonds, G. (1993) Immortalization and characterization of human cell lines with mast cell and monocytic properties. *Br. J. Haematol.* **85**, 452–461
14. Fukaya, K., Asahi, S., Nagamori, S., Sakaguchi, M., Gao, C., Miyazaki, M., and Namba, M. (2001) Establishment of a human hepatocyte line (OUMS-29) having CYP 1A1 and 1A2 activities from fetal liver tissue by transfection of SV40 LT. *In Vitro Cell Dev. Biol. Anim.* **37**, 266–269
15. Wakkach, A., Chastre, E., Bruand, C., Cohen-Kaminsky, S., Emami, S., Gespach, C., and Berrih-Aknin, S. (2001) Phenotypic and functional characterization of human thymic stromal cell lines. *Cell. Mol. Biol. (Noisy-le-grand)* **47**, 167–178
16. Obinata, M. (1997) Conditionally immortalized cell lines with differentiated functions established from temperature-sensitive T-antigen transgenic mice. *Genes Cells* **2**, 235–244
17. Yanai, N., Suzuki, M., and Obinata, M. (1991) Hepatocyte cell lines established from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene. *Exp. Cell Res.* **197**, 50–56
18. Yanai, N., Satoh, T., Kyo, S., Abe, K., Suzuki, M., and Obinata, M. (1991) A tubule cell line established from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene. *Jpn. J. Cancer Res.* **82**, 1344–1348
19. Sugiyama, N., Tabuchi, Y., Horiuchi, T., Obinata, M., and Furusawa, M. (1993) Establishment of gastric surface mucous cell lines from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene. *Exp. Cell Res.* **209**, 382–387
20. Hosoya, K., Tetsuka, K., Nagase, K., Tomi, M., Saeki, S., Ohtsuki, S., Takanaga, H., Yanai, N., Obinata, M., Kikuchi, A., Okano, T., and Terasaki, T. (2000) Conditionally immortalized brain capillary endothelial cell lines established from a transgenic mouse harboring temperature-sensitive simian virus 40 large T-antigen gene. *AAPS PharmSci.* **2**, E27
21. Kameoka, J., Yanai, N., and Obinata, M. (1995) Bone marrow stromal cells selectively stimulate the rapid expansion of lineage-restricted myeloid progenitors. *J. Cell Physiol.* **164**, 55–64
22. Ebihara, S., Endo, S., Ito, K., Ito, Y., Akiyama, K., Obinata, M., and Takai, T. (2004) Immortalized dendritic cell line with efficient cross-priming ability established from transgenic mice harboring the temperature-sensitive SV40 large T-antigen gene. *J. Biochem.* **136**, 321–328
23. Kanehira, M., Kaifu, T., Maya, K., Kaji, M., Nakamura, A., Obinata, M., and Takai, T. Novel mast cell lines with enhanced proliferative and degranulative abilities established from temperature-sensitive SV40 large T antigen transgenic mice. submitted.
24. Zheng, N., Ye, S.L., Sun, R.X., Zhao, Y., and Tang, Z.Y. (2002) Effects of cryopreservation and phenylacetate on biological characters of adherent LAK cells from patients with hepatocellular carcinoma. *World J. Gastroenterol.* **8**, 233–236
25. Jiang, S., Persechini, P.M., Perussia, B., and Young, J.D. (1989) Resistance of cytolytic lymphocytes to perforin-mediated killing. Murine cytotoxic T lymphocytes and human natural killer cells do not contain functional soluble homologous restriction factor or other specific soluble protective factors. *J. Immunol.* **143**, 1453–1460
26. Raskovalova, T., Huang, X., Sitkovsky, M., Zacharia, L.C., Jackson, E.K., and Gorelik, E. (2005) Gs protein-coupled adenosine receptor signaling and lytic function of activated NK cells. *J. Immunol.* **175**, 4383–4391
27. Conzen, S.D., and Cole, C.N. (1995) The three transforming regions of SV40 T antigen are required for immortalization of primary mouse embryo fibroblasts. *Oncogene* **11**, 2295–2302
28. Kubota, A., Kubota, S., Lohwasser, S., Mager, D.L., and Takei, F. (1999) Diversity of NK cell receptor repertoire in adult and neonatal mice. *J. Immunol.* **163**, 212–216
29. Smith, H.R., Heusel, J.W., Mehta, I.K., Kim, S., Dorner, B.G., Naidenko, O.V., Iizuka, K., Furukawa, H., Beckman, D.L., Pingel, J.T., Scalzo, A.A., Fremont, D.H., and Yokoyama, W.M. (2002) Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc. Natl. Acad. Sci. USA* **99**, 8826–8831
30. Toyama-Sorimachi, N., Taguchi, Y., Yagita, H., Kitamura, F., Kawasaki, A., Koyasu, S., and Karasuyama, H. (2001) Mouse CD94 participates in Qa-1-mediated self recognition by NK cells and delivers inhibitory signals independent of Ly-49. *J. Immunol.* **166**, 3771–3779
31. Derre, L., Corvaisier, M., Pandolfino, M.C., Diez, E., Jotereau, F., and Gervois, N. (2002) Expression of CD94/NKG2-A on human

T lymphocytes is induced by IL-12: implications for adoptive immunotherapy. *J. Immunol.* **168**, 4864–4870
32. Lohwasser, S., Kubota, A., Salcedo, M., Lian, R.H., and Takei, F. (2001) The non-classical MHC class I molecule Qa-1(b) inhibits classical MHC class I-restricted cytotoxicity of cytotoxic T lymphocytes. *Int. Immunol.* **13**, 321–327
33. Lohwasser, S., Hande, P., Mager, D.L., and Takei, F. (1999) Cloning of murine NKG2A, B and C: second family of C-type lectin receptors on murine NK cells. *Eur. J. Immunol.* **29**, 755–761
34. Markel, G., Lieberman, N., Katz, G., Arnon, T.I., Lotem, M., Drize, O., Blumberg, R.S., Bar-Haim, E., Mader, R., Eisenbach, L., and Mandelboim, O. (2002) CD66a interactions between human melanoma and NK cells: a novel class I MHC-independent inhibitory mechanism of cytotoxicity. *J. Immunol.* **168**, 2803–2810
35. Arase, H., Saito, T., Phillips, J.H., and Lanier, L.L. (2001) Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). *J. Immunol.* **167**, 1141–1144
36. Williams, N.S., Klem, J., Puzanov, I.J., Sivakumar, P.V., Bennett, M., and Kumar, V. (1999) Differentiation of NK1.1+, Ly49+ NK cells from flt3+ multipotent marrow progenitor cells. *J. Immunol.* **163**, 2648–2656
37. Roth, C., Carlyle, J.R., Takizawa, H., and Raulet, D.H. (2000) Clonal acquisition of inhibitory Ly49 receptors on developing NK cells is successively restricted and regulated by stromal class I MHC. *Immunity* **13**, 143–153
38. Williams, N.S., Kubota, A., Bennett, M., Kumar, V., and Takei, F. (2000) Clonal analysis of NK cell development from bone marrow progenitors in vitro: orderly acquisition of receptor gene expression. *Eur. J. Immunol.* **30**, 2074–2082
39. Lian, R.H., Maeda, M., Lohwasser, S., Delcommenne, M., Nakano, T., Vance, R.E., Raulet, D.H., and Takei, F. (2002) Orderly and nonstochastic acquisition of CD94/NKG2 receptors by developing NK cells derived from embryonic stem cells in vitro. *J. Immunol.* **168**, 4980–4987
40. Goffinet, C., and Keppler, O.T. (2006) Efficient nonviral gene delivery into primary lymphocytes from rats and mice. *FASEB J.* **20**, 500–502