

***Ex Vivo* Expansion of Natural Killer Cells Using Cryopreserved Irradiated Feeder Cells**

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Abstract. *Currently, feeder cells are γ -irradiated immediately before use for the ex vivo expansion of natural killer (NK) cells from human peripheral blood. Storing irradiated feeder cells by cryopreserving them in multiple vials would be more convenient than irradiating cells each time they are needed. We compared NK cell expansion using cryopreserved-irradiated feeder cells (cryopreserved group) and freshly-irradiated feeder cells (fresh group). To expand NK cells, peripheral blood mononuclear cells were isolated and co-cultured with-100 Gy-irradiated K562 leukemia cells that had been modified to express 4-1BB ligand and membrane-bound (mb) interleukin (IL)-15 (K562-mb15-41BBL cells) for three weeks in the presence of IL-2 and IL-15. Fresh and cryopreserved K562-mb15-41BBL feeder cells expressed similar levels of 4-1BB ligand, whereas membrane-bound IL-15 expression was lower in the cryopreserved cells than in the fresh cells. The NK cell expansion rate did not differ between the two groups (980-fold vs. 1058-fold, respectively), although the mean NK cell purity was higher in the fresh-group than in the cryopreserved-group at day 14 (94.1% vs. 92.5%, respectively) and day 21 (97.1% vs. 95.4%, respectively). The NK cells from the two feeder cell groups did not differ in*

cytotoxicity against various malignant cell lines at effector-to-target ratios of 4:1, 2:1, and 1:1, or in the expression pattern of NK cell receptors [cluster of differentiation (CD)-16, natural killer group-2, member D (NKG2D), CD69, NKp30, NKp44, NKp46, and CD158b] and level of interferon- γ secretion. Our results demonstrate that cryopreserved irradiated feeder cells can be used for the ex vivo expansion of human NK cells and provide a convenient improvement on current methods.

Natural killer (NK) cells can kill cancer cells in the absence of prior stimulation and are a promising tool for cell therapy of cancer (1, 2). Given that NK cells represent a small fraction of peripheral blood mononuclear cells (PBMCs), obtaining a sufficient number of NK cells to meet clinical requirements, especially when multiple infusions are planned, is challenging. NK cell-based therapies would greatly benefit from reliable methods of producing large numbers of highly cytotoxic NK cells.

It has been reported that NK cells can be expanded upon activation by cytokines such as interleukin (IL)-2 and the binding of proteins from cell lines such as K562 and HFWT (Wilms' tumor-derived cells) to NK cell receptors such as natural killer group-2, member D (NKG2D) and NKp46 (3-5) (see http://www.nkcells.info/mw/index.php/NK_cell_expansion#2011). Lymphocytes, monocytes, umbilical cord mesenchymal cells, Epstein-Barr virus-transformed lymphoblastoid cells, HFWT cells, RPMI 8866 cells, and K562 cells have been used as feeder cells for the expansion of NK cells from PBMCs (6). Among them, K562 human leukemia cells have been used most commonly as feeder cells for NK cell expansion (5-7). Recently, remarkable rates of highly cytotoxic NK cell expansion were reported using a protocol that employs irradiated, genetically-engineered K562 cells, such as K562-based artificial antigen-presenting cells with membrane-bound IL-21 (mbIL21) and the K562

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cell line modified to express membrane-bound IL-15 (mbIL15) and 4-1BB ligand (K562-mb15-41BBL)-used in the present study (7-9).

Mouse embryonic feeder cells are commonly used for long-term culture procedures and for colony regeneration of embryonic stem cells, and are usually inactivated by irradiation and mitomycin (10). Like these mouse embryonic feeder cells, the feeder cells should be irradiated or mitotically-inactivated using mitomycin C to avoid the overgrowth of K562 cells and to ensure that no viable K562 cells are infused along with the expanded NK cells (11).

Although irradiation is the safer and more effective method, irradiators are not available in some institutes. Moreover, irradiation of feeder cells each time they are needed is a laborious procedure. The process would be easier and less time-consuming if cryopreserved irradiated feeder cells retained a high level of viability for a reasonable period of time (10). However, little is known regarding cryopreservation of genetically modified feeder cell, K562-mb15-41BBL feeder cells, particularly after γ -irradiation. In the present study, we compared the abilities of cryopreserved-irradiated and freshly-irradiated K562-mb15-41BBL cells to serve as feeder cells for NK cell expansion.

Materials and Methods

Culture and irradiation of K562-mb15-41BBL feeder cells. K562 leukemia cells that had been modified to express 4-1BB ligand and membrane-bound IL-15 (K562-mb15-41BBL cells; kindly provided by Dario Campana, St. Jude Children's Research Hospital, Memphis, TN) were used for the activation and expansion of NK cells from human PBMCs (5, 8, 12). K562-mb15-41BBL cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified incubator containing 5% CO₂. Gamma irradiation (100 Gy; 5.1 Gy/min) was applied using a Gammacell 3000 Elan irradiator (Best Theratronics, Ottawa, Canada) to prepare non-proliferating K562-mb15-41BBL feeder cells.

Cryopreservation and thawing of irradiated K562-mb15-41BBL feeder cells. Freshly-prepared 100-Gy- γ -irradiated K562-mb15-41BBL cells were suspended at a density of 2×10^6 cells/ml in 90% heat-inactivated FBS and 10% dimethyl sulfoxide (DMSO). They were then split among multiple vials, which were frozen individually. The cryovials containing the cells were placed in a Nalgene Cryo 1°C Freezing Container (Thermo Fisher Scientific, Inc., Waltham, MA, USA) filled with 100% isopropyl alcohol and stored at -80°C overnight. The cryovials were transferred to the liquid phase of a nitrogen tank and kept for one to four months for experiments. For thawing, the cryopreserved irradiated K562-mb15-41BBL cells were transferred directly from the liquid nitrogen tank to a 37°C waterbath and quickly thawed with continuous shaking. The thawed cells were then suspended in RPMI-1640 medium.

Expansion of NK cells from PBMCs. All participants signed a consent form approved by the Institutional Review Board of

Chonnam National University Hwasun Hospital. PBMCs were isolated from the blood samples by density-gradient centrifugation with Ficoll-Hypaque (d=1.077 gm/ml, Lymphoprep™; Axis-Shield, Oslo, Norway) and washed in PBS. *Ex vivo* activated and expanded NK cells were generated as previously described (8). Freshly-prepared PBMCs were co-cultured with freshly-prepared (fresh group) or cryopreserved and thawed (cryopreserved group) 100-Gy- γ -irradiated K562-mb15-41BBL feeder cells in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 4 mM L-glutamine in a 24-well tissue culture plate. Every other day, the medium was replaced with fresh medium containing 10 IU/ml human IL-2. After one week, the concentration of IL-2 was increased to 100 IU/ml, and 10 IU/ml IL-15 was added to the medium. The medium was replaced every other day for an additional two weeks in both groups. Weekly repeated stimulation with K562-mb15-41BBL cells was not performed.

Flow cytometric analysis. A purity analysis of NK cell percentage and an NK cytotoxicity assay using Calcein AM-stained effector cells were performed as previously described (13). Various cancer cells were used as target cells. K562 (derived from chronic myeloid leukemia cells), which are sensitive to NK cells, RPMI-8226 (derived from human myeloma cell), Raji cells (derived from Burkitt lymphoma cell), and HL-60 (derived from acute promyelocytic leukemia) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). ES8 cells (derived from Ewing's sarcoma cell) were kindly provided by Dario Campana. The neuroblastoma cell lines (SH-SY5Y and IMR-32) and osteosarcoma cell lines (MG-63 and Saos-2) were obtained from the Korean Cell Line Bank (Seoul, Korea). The target cells and effector cells were co-cultured at effector-to-target (E:T) ratios ranging from 4:1 to 1:1 for 4 h at 37°C in a humidified incubator containing 5% CO₂. The expression of cell surface markers on the expanded NK cells was assessed using a phycoerythrin (PE)-conjugated mouse antibody to human CD16, NKG2D, CD69, NKp30, NKp44, NKp46 and CD158b (BD Pharmingen™) and a PE-conjugated mouse IgG1 (isotype control).

To detect the expression of 4-1BBL and mbIL-15 on the cell surfaces of freshly-prepared and cryopreserved K562-mb15-41BBL cells, the cells were incubated with PE-conjugated mouse anti-human CD137 ligand (4-1BBL; BD Pharmingen) or mouse anti-human IL-15, followed by PE-conjugated goat anti-mouse IgG, and analyzed cytometrically (FACSCaliber flow cytometer; BD Immunocytometry Systems, San Jose, CA, USA).

K562-mb15-41BBL cells express green fluorescent protein (GFP), therefore they can be detected easily by flow cytometry in expanded NK product. Fresh- and cryopreserved-irradiated-K562-mb15-41BBL cells were kept in culture in RPMI-1640 complete medium without PBMCs for 10 days. Viable K562-mb15-41BBL feeder cells were analyzed by flow cytometry in culture with fresh and cryopreserved irradiated K562-mb15-41BBL cells. The frequency of irradiated K562-mb15-41BBL feeder cells was analyzed in expanded NK cells at day 0, 3, 7 and 10 after culture.

The data were acquired and analyzed using the BD CellQuest™ Pro Software.

Enzyme-linked immunosorbent assay (ELISA). Interferon (IFN)- γ levels in the cell supernatants were measured using a commercially available human IFN- γ ELISA set (BD Biosciences, San Diego, CA, USA). Briefly, expanded NK cells from the fresh and cryopreserved groups were placed onto flat-bottomed 96-well microplates

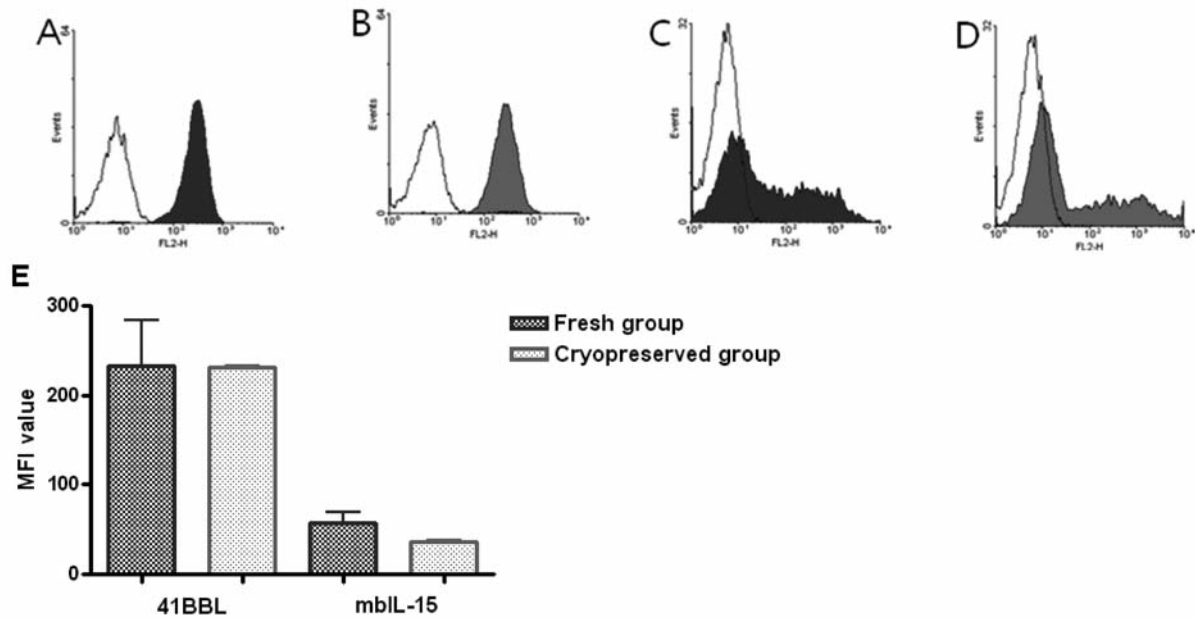


Figure 1. Expression of 4-1BB ligand (4-1BBL) and membrane-bound interleukin-15 (mbIL-15) on K562-mb15-41BBL cells was determined by flow cytometry, using a phycoerythrin (PE)-conjugated mouse antibody to human 4-1BBL, and a mouse antibody to human IL-15 and a PE-conjugated anti-mouse IgG. A: 4-1BBL expression on freshly-irradiated K562-mb15-41BBL cells (mean fluorescence intensity, MFI=232.4). B: 4-1BBL expression on cryopreserved-irradiated K562-mb15-41BBL cells (MFI=231.7). C: mbIL-15 expression on fresh K562-mb15-41BBL cells (MFI=57.2). D: mbIL-15 expression on cryopreserved K562-mb15-41BBL cells (MFI=36.6). E: Results of the expression of 4-1BBL and mbIL-15 on freshly- and cryopreserved-irradiated K562-mb15-41BBL cells. PE-conjugated antibody to mouse IgG1 was used as an isotype control. The white regions represent the isotype control.

containing K562 cells (4×10^5 /well) at an E:T ratio of 2:1 and were co-cultured overnight at 37°C in a humidified incubator containing 5% CO_2 . The cell-free culture supernatants were harvested and frozen at -80°C until analysis. The assay was performed according to the manufacturer's recommended procedure. Samples from three healthy donors were analyzed in triplicate. The concentration of IFN- γ was determined by standard curve regression analysis.

Statistics. The results are shown as means \pm SD and were analyzed for statistical significance between groups using the Mann-Whitney U-test. Values of $p < 0.05$ indicated statistical significance.

Results

Expression of 4-1BB ligand and membrane-bound IL-15 on fresh and cryopreserved K562-mb15-41BB feeder cells. The cell surface expression of 4-1BB ligand and membrane-bound IL-15 on irradiated K562-mb15-41BB cells was examined by flow cytometry and compared between fresh and thawed-cryopreserved cells. The fresh and cryopreserved-irradiated K562-mb15-41BB cells had similar levels of 4-1BB expression; the mean fluorescent intensity (MFI) of labeled fresh cells was 232.4 ± 71.7 , and that of labeled cryopreserved cells was 231.7 ± 2.8 ($p > 0.05$; Figure 1A and B). The expression of membrane-bound IL-15 was slightly lower on cryopreserved-thawed cells ($\text{MFI} = 36.6 \pm 2.3$) than on fresh cells ($\text{MFI} = 57.2 \pm 17.4$, $p > 0.05$; Figure 1C and D).

NK cell expansion rate and purity. Using the two feeder cell groups, we compared the purity and expansion rate of NK cells from PBMCs isolated from seven healthy donors. The mean NK cell purity was higher with the fresh feeder cells than with the cryopreserved feeder cells at day 14 (94.1 ± 2.4 vs. $92.5 \pm 2.3\%$, $p = 0.018$) and day 21 (97.1 ± 1.9 vs. $95.4 \pm 2.6\%$, $p = 0.018$; Figure 2A). NK cells proliferated rapidly over a period of three weeks in both groups, and the NK cell expansion rate did not differ significantly between the fresh and cryopreserved groups (Figure 2B).

Cytotoxicity of expanded NK cells against various cancer cell lines. We examined the direct cytotoxic effects of the expanded NK cells from three healthy donors against different cancer cell lines such as K562, RPMI-8226, Raji, HL-60, SH-SY5Y, IMR-32, MG-63, Saos-2, and ES8. At an E:T ratio of 4:1, 2:1 or 1:1, no significant difference in cytotoxicity was observed between the fresh and cryopreserved feeder cell groups ($p > 0.05$; Figure 3).

Expression of receptors on expanded NK cells. The expression of the NK cell markers CD16, NKG2D, CD69, NKp30, NKp44, NKp46, and CD158b on the expanded NK cells obtained from the fresh and cryopreserved groups on day 14 was investigated by flow cytometry. Although there

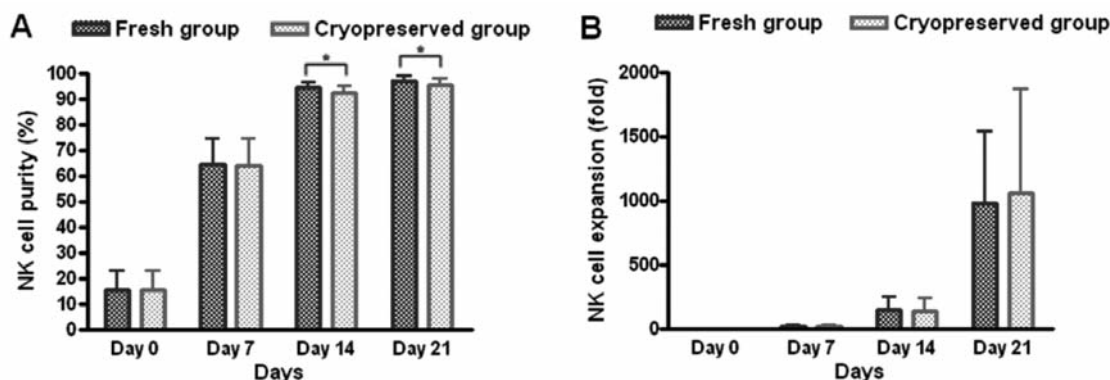


Figure 2. The purity and fold expansion of ex vivo-expanded human natural killer (NK) cells co-cultured with fresh K562-mb15-41BBL feeder cells and cryopreserved K562-mb15-41BBL feeder cells. A: Mean fold expansion of NK cells. The fold expansion rates of NK cells in the fresh group versus the cryopreserved group did not significantly differ ($p>0.05$) B: The purity of expanded NK cells was determined by flow cytometry using a fluorescein isothiocyanate-conjugated antibody to human CD3 and a phycoerythrin-CY5-conjugated antibody to human CD56. Mean NK cell purities in the fresh group versus the cryopreserved group were not significantly different on day 0 and 7, but were on day 14 and 21 ($p=0.018$).

was a slight variance among the donors, the expression of NK cell markers was similar between the two groups, in terms of both the percentage of cells expressing a specific receptor and the relative expression of each receptor analyzed (Figure 4).

IFN- γ production by expanded NK cells. The amount of IFN- γ that the expanded NK cells from three healthy donors secreted, in response to K562 cells, was assessed. The IFN- γ level, did not differ significantly between NK cells obtained with fresh feeder cells and those obtained with cryopreserved feeder cells (644.5 ± 91.4 vs. 652.1 ± 85.2 pg/ml, respectively; $p>0.05$; Figure 5).

Survival of fresh and cryopreserved irradiated K562-mb15-41BBL feeder cells and their frequency in the expanded NK cells. To study the biological effects of radiation on feeder cells in both groups, fresh and cryopreserved irradiated-K562-mb15-41BBL cells were maintained in culture in complete RPMI-1640 medium without PBMCs for 10 days. Viable fluorescein isothiocyanate (FITC)-positive K562 mb15-41BBL cells on day 0, 3, 7, and 10 were identified by flow cytometry. As shown in Figure 6A and 6C, neither freshly-irradiated nor cryopreserved-irradiated cells proliferated and only $5.18\pm0.12\%$ and $5.33\pm0.67\%$, respectively, survived at day 7 of culture. There was no significant difference regarding survival of fresh and cryopreserved irradiated feeder cells in complete RPMI-1640 medium.

Survival of irradiated feeder cells and their frequency during NK cell expansion were compared in both groups. No significant difference was observed in the frequency of FITC-positive feeder cells at culture days 0, 3, 7, and 10 during NK cell expansion between the fresh and cryopreserved groups (Figure 6B). Additionally, only

$4.76\pm4.05\%$, $1,11\pm1.92\%$ of FITC-positive feeder cells in the fresh group and $4.10\pm2.76\%$, and 0% in the cryopreserved group survived at culture days 3 and 7 of culture, respectively (Figure 6D).

To determine whether expanding NK cells can kill K562-mb15-41BBL feeder cells, the survival rate of irradiated feeder cells in both types of expanded NK cell group was compared with that of irradiated feeder cells in complete RPMI-1640 medium. The survival rate of FITC-positive feeder cells was significantly lower in expanded NK cells than in complete RPMI-1640 medium group ($3.0\pm3.2\%$ vs. $80.4\pm3.5\%$ at culture day 3, $p=0.028$; $0.6\pm1.4\%$ vs. $5.3\pm0.4\%$, respectively, at culture day 7, $p=0.028$).

Discussion

The effect of cryopreservation on the expression of genetically-engineered molecules on K562 cells was examined. The surface expression of 4-1BBL on K562-mb15-41BBL cells was not altered by cryopreservation, whereas the expression of membrane-bound IL-15 was slightly lower on cryopreserved-thawed K562-mb15-41BBL feeder cells, compared with fresh cells. However, no statistically significant difference was observed between cryopreserved and freshly-irradiated feeder cells, suggesting that the expression of genetically-engineered molecules in feeder cells is not severely affected by cryopreservation.

The abilities of cryopreserved- and freshly-irradiated K562-mb15-41BBL cells to serve as feeder cells for NK cell expansion were compared. Although the purity of expanded NK cells was slightly higher with fresh feeder cells than with cryopreserved cells at days 14 and 21, no difference was found between the fresh and cryopreserved groups in terms of the NK cell expansion rate, cytotoxicity towards various

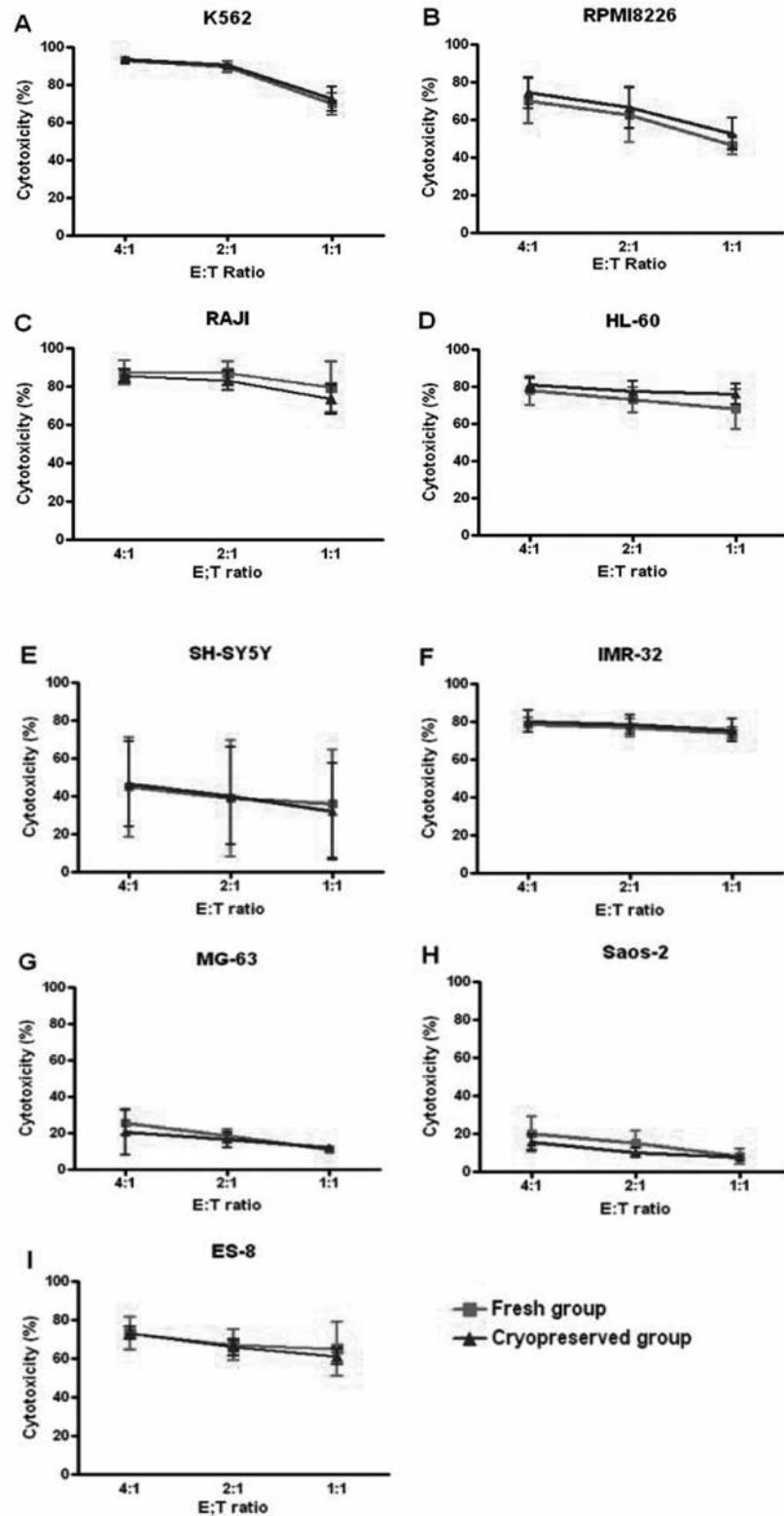


Figure 3. Cytotoxicity of natural killer (NK) cells expanded using fresh K562-mb15-41BBL feeder cells and cryopreserved K562-mb15-41BBL feeder cells. The cytotoxicity of expanded NK cells towards K562 (A) RPMI-8226, (B) ES8, (C) RAJI, (D) HL-60, (E) SH-SY5Y, (F) IMR-32, (G) MG-63, (H) Saos-2, and (I) ES8 cell lines were measured at effector:target ratios of 4:1, 2:1, and 1:1 using a flow cytometry-based method. The results presented are the means \pm SD of samples from three donors. All samples were tested in triplicate.

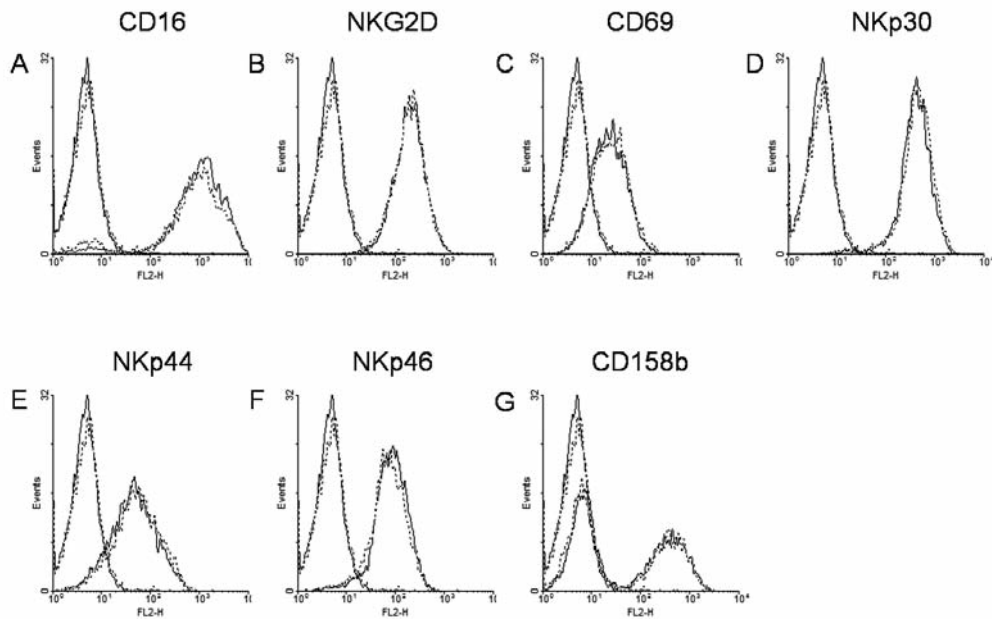


Figure 4. Expression of surface receptors on natural killer (NK) cells expanded using fresh K562-mb15-41BBL feeder cells and cryopreserved K562-mb15-41BBL feeder cells. Phycoerythrin (PE)-conjugated antibody to human CD16 (A), natural killer group-2, member D (NKG2D) (B), CD69 (C), NKp30 (D), NKp44 (E), NKp46 (F), and CD158b (G) antibodies were used to analyze the NK cell receptors on day 14. PE-conjugated antibody to mouse IgG1 antibody was used as an isotype control. Representative plots show receptor expression on NK cells expanded from the same donor. The peak at the extreme left of each figure shows the isotype control, and the peaks located to the right in each figure represent a NK cell marker. Solid lines, NK cells co-cultured with fresh K562-mb15-41BBL feeder cells; dotted lines, NK cells co-cultured with cryopreserved K562-mb15-41BBL feeder cells.

malignant cell lines, expression of NK cell receptors (CD16, NKG2D, CD69, NKp30, NKp44, NKp46, and CD158b), and levels of IFN- γ secretion.

In the present study, the cytotoxicity against the malignant Ewing sarcoma cell line and neuroblastoma cell lines was considerably high; however, osteosarcoma cell lines were less sensitive, as shown in Figure 3. The present findings seem to be consistent with a previous report (11). No significant difference was found in cytotoxicity at an E:T ratio of 4:1, 2:1, or 1:1 between the fresh and cryopreserved groups in all cell lines tested.

Cryopreservation can lead to cellular damage. Owing to its high membrane permeability, DMSO has been used extensively as a cryoprotectant for various cells and tissues. The most common cryoprotectant solution used for various kinds of stem cells consists of 10% DMSO in 10-90% fetal bovine serum (FBS) (14-17). In the present study, K562-mb15-41BBL cells were cryopreserved in 90% FBS with 10% DMSO as a cryoprotective agent.

There are two conventional slow-freezing methods: controlled-rate freezing (CRF), which uses a controlled-rate freezer, and uncontrolled-rate freezing (URF), which uses a passive cooling device such as a small cryocontainer filled with isopropyl alcohol. In some studies, the URF method

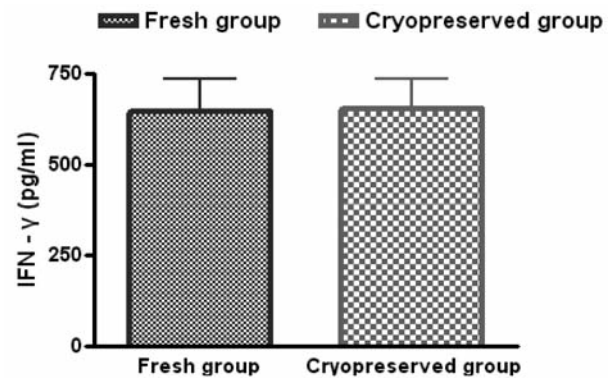


Figure 5. Levels of interferon (IFN)- γ secreted by expanded NK cells in response to K562 leukemia cells. Cell-free culture supernatants were harvested at 24 h, and the IFN- γ content was analyzed by ELISA. The results presented are the means \pm SD of samples from three donors. * p <0.05.

was comparable to the CRF method and provided an attractive alternative method for the cryopreservation of peripheral blood progenitor cells (18-20). However, in other studies, the recovery and viability rates were lower for PBMCs cryopreserved using the URF method compared with the CRF method (21, 22). In the present study, the simpler,

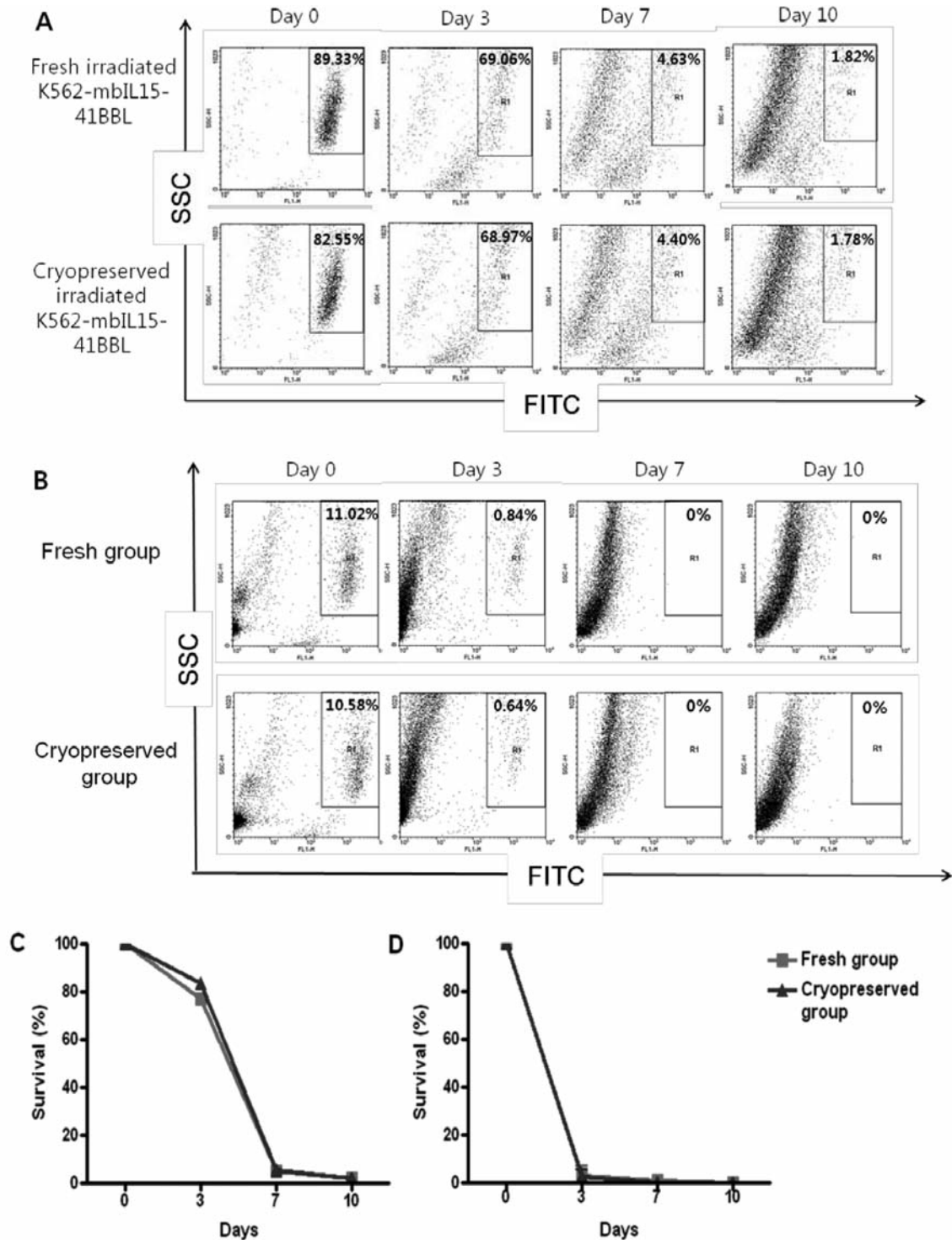


Figure 6. Survival of the fresh and cryopreserved irradiated K562-mb15-41BBL feeder cells and their frequency in the expanded natural killer (NK) cells. A and C: Survival of K562-mb15-41BBL cells was analyzed by flow cytometry. Fresh and cryopreserved irradiated feeder cells did not proliferate after culture. B: K562-mb15-41BBL cell gating in the expanded NK cells was established by gating K562-mb15-41BBL fluorescein isothiocyanate (FITC)-positive cells alone. The experiments were K562-mb15-41BBL cell gating on days 0, 3, 7, and 10 of NK cell culture using fresh K562-mb15-41BBL and cryopreserved K562-mb15-41BBL feeder cells. No significant difference was observed in the frequency of FITC-positive feeder cells between the expanded NK cell groups. D: Survival of FITC-positive feeder cells in both expanded NK cell groups was compared. Less than 5% of FITC-positive feeder cells survived at day 3 of NK cell expansion. The experiments were performed on cells from three donors, and all samples were tested in triplicate. Representative results are shown.

time-saving, and less expensive URF method was used for the cryopreservation of K562-mb15-41BBL cells.

The cryovials containing K562-mb15-41BBL feeder cells were cryopreserved in a liquid nitrogen tank for up to four months. No differences were found between the fresh and cryopreserved cells in terms of the quantity and function of the expanded NK cells. Further investigation may be necessary to determine whether a longer period, such as one year, of K562-mb15-41BBL feeder cell cryopreservation affects the expansion of NK cells from human PBMCs.

Mouse embryonic feeder cells are commonly used for long-term culture procedures and colony regeneration of embryonic stem cells, and are usually inactivated by irradiation and mitomycin (10). Similarly to these mouse embryonic feeder cells, the feeder cells should be irradiated or mitotically-inactivated using mitomycin C to avoid the overgrowth of K562 cells and to ensure that no viable K562 cells are infused along with the expanded NK cells (11). To avoid the overgrowth of feeder cells, they are irradiated at various doses of 30-100 Gy (5, 23-25), which is considered safe and effective. Similarly to other reports using 100 Gy for irradiation of K562 feeder cells (7), in the present study 100 Gy of radiation was used for feeder cell treatment. The biological effects of irradiation on K562-mb15-41BBL feeder cells in both groups were compared. As expected, both fresh- and cryopreserved-irradiated cells did not proliferate and only $5.18 \pm 0.12\%$ and $5.33 \pm 0.67\%$, respectively, survived at day 7 of culture.

To investigate whether expanding NK cells can kill K562-mb15-41BBL cells, the survival rate of irradiated feeder cells between cells during NK cell expansion and cells maintained only in complete medium was compared. The survival rate of FITC-positive K562-mb15-41BBL feeder cells was remarkably lower in expanded NK cells than feeder cells in complete RPMI-1640 medium, at culture on day 3 ($3.0 \pm 3.2\%$ vs. $80.4 \pm 3.5\%$, respectively; $p=0.028$). As expected, expanding NK cells themselves kill irradiated- K562-mb15-41BBL cells, because K562 is the cell line most sensitive to NK cells.

The release criterion of clinical grade NK cell products set by Lapteva *et al.* (26) is a $<0.1\%$ frequency of FITC-positive cells in NK products. They reported the frequency of FITC-positive cells in NK products to be $0.01-0.07\%$ at day 10 of culture. In our study, the frequency of GFP-positive cells in expanded NK cells was 0% in both the fresh group ($n=3$) and in the cryopreserved group ($n=3$), at day 10 of culture, a finding that satisfied the release criterion of $<0.1\%$. These results were expected because genetically-modified K562-mb15-41BBL cells received 100 Gy of radiation and are highly susceptible to killing by proliferating NK cells in culture. Furthermore, these results were expected because weekly-repeated stimulation with K562-mb15-41BBL cells was not performed and because our protocol used a very low ratio ($0.08:1$) of irradiated K562-mb15-41BBL cells to PBMCs, in

contrast to other protocols; 100-Gy-irradiated K562-mb15-41BBL cells seeded at a 10:1 ratio of K562-mb15-41BBL to NK cells (26), and 100 Gy-irradiated K562-based genetically engineered artificial antigen-presenting cells with mbIL21 were co-cultured with PBMCs at a ratio of 2:1 (7).

Although irradiation is a safer and more effective method than mitomycin C treatment, irradiators are not available at some institutes, and the machine usually resides outside of a good manufacturing practice (GMP) facility, although an institute may have irradiators. In that situation, the use of cryopreserved irradiated feeder cells in multiple vials would be more convenient than irradiating cells each time they are needed. Moreover, it would be useful for control of infection in a GMP facility.

In the present study, we showed that cryopreserved-irradiated feeder cells retained a similar function for one to four months compared with freshly-irradiated feeder cells. Our results suggest that cryopreserved-irradiated feeder cells can be used for the *ex vivo* expansion of human NK cells, providing a convenient improvement on current methods, especially when a γ -irradiator is not available.

Acknowledgements

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