Ex Vivo Expansion of Highly Cytotoxic Human NK Cells by Cocultivation with Irradiated Tumor Cells for Adoptive Immunotherapy

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Abstract
Adoptive natural killer (NK) cell therapy may offer an effective treatment regimen for cancer patients whose disease is refractory to conventional therapy. NK cells can kill a wide range of tumor cells by patterned recognition of target ligands. We hypothesized that tumor targets sensitive to NK lysis would drive vigorous expansion of NK cells from human peripheral blood mononuclear cells (PBMC). Here, we provide the basis for developing a novel ex vivo expansion process. By screening class I–negative or mismatched tumor cell lines we identified a Jurkat T-lymphoblast subline termed KL-1, which was highly effective in specifically expanding NK cells. KL-1 addition to PBMC cultures achieved approximately 100-fold expansion of NK cells with nearly 90% purity, accompanied by reciprocal inhibition of T-cell growth. Marked elevations in expression of activation receptors, natural cytotoxicity receptors (NKp30, NKp44), and adhesion molecules (CD11a, ICAM-1) were associated with high tumor-lytic activity, in both in vitro and in vivo models. KL-1–mediated expansion of NK cells was contact dependent and required interactions with CD16, the Fcγ receptor on NK cells, with ligands that are expressed on B cells. Indeed, B-cell depletion during culture abrogated selective NK cell expansion, while addition of EBV-transformed B cells further augmented NK expansion to approximately 740-fold. Together, our studies define a novel method for efficient activation of human NK cells that employs KL-1–lysed tumor cells and cocultured B cells, which drive a robust expansion of potent antitumor effector cells that will be useful for clinical evaluation. Cancer Res; 73(8); 1–10. ©2012 AACR.

Introduction
Natural killer (NK) cells have been characterized as effector cells of the innate immune system with potent cytotoxic activity. As the first line of defense against tumor cells and viruses, NK cells are poised to mediate killing, without the requirement for prolonged preactivation, through patterned recognition of target ligands (1, 2). Selective engagement of activating receptors (e.g., NKp46, NKp44 and NKp30, NKG2D, DNAM-1, 2B4) in the absence of target inhibitory ligands leads to NK cell activation, expansion, and cytotoxicity (3). Clinical evidence of antitumor responses attributable to NK cells can be found in the setting of haploidentical allogeneic stem cell transplantation for leukemia where improved survival following transplant was noted among patients receiving donor NK cells which were KIR-ligand mismatched (4, 5).

A more refined approach using autologous or allogeneic NK cells for adoptive transfer has been used for treating patients with ovarian, breast, renal cell cancer, glioblastoma, and other solid tumors with occasional clinical responses (6–10). In contrast to vaccine therapy or antigen-specific adoptive T-cell therapy, identification of target tumor antigens is not required for NK cell therapy, which can be more universally applied and particularly effective for treating solid tumor malignancies that have lost expression of self-MHC as a mechanism of immune escape from T cells (11, 12). However, a major challenge to the broader application of adoptive NK cell therapy has been to develop a method for enriching and expanding the low-frequency NK cells to numbers sufficient for adoptive transfer from the peripheral blood mononuclear cell (PBMC) population (13, 14). For T cells, in vitro expansions to more than 1,000-fold can be routinely achieved by T-cell receptor (TCR) triggering using anti-CD3 monoclonal antibody (mAb) in combination with
exogenous cytokine in the presence of irradiated feeder cells (15). By contrast, NK cell expansion is dependent on pattern recognition of multiple activating and inhibitory ligands, and current strategies have circumvented a specific triggering requirement by phenotype selection of CD56+ cells and CD3 depletion of ‘contaminating’ cytokine-responsive T cells from PBMC. Although this approach has been successfully developed in a small number of clinical trials, the requirement for immunomagnetic enrichment and in vitro high-dose interleukin (IL)-2 may be prohibitive to most centers due to the cost and regulatory hurdles associated with extensive in vitro manipulation, additional regulatory approval and GMP-related infrastructure support for handling bead-based cell-separation procedures.

We postulated that a simplified strategy resembling in vivo conditions would lead to more robust expansion and could be achieved using unmodified NK target cell lines. The fact that allogeneic tumor lines can be a target for NK cells due to the missing self-MHC and the presence of potential activating ligands allowed us to screen patient-derived tumor lines as a feeder for expanding NK cells in vitro. Among a panel of allogeneic cell lines tested, a human T lymphoblastoid line, Jurkat, (named KL-1 for the particular strain used here) was found to trigger specific expansion of NK cells while suppressing that of T cells and achieve a population of approximately 90% activated CD3+CD56+NKG2D+ NK cells. We validate this approach and explore potential mechanisms of selective NK cell activation observed with the use of this feeder line.

Materials and Methods

Cell lines, cell culture, and PBMCs

U937 (human promonocytic leukemia line; the American Type Culture Collection, ATCC), CEM (human T lymphoblastoid line; ATCC), Jurkat, designated as KL-1 (human T lymphoblastoid line; Korean Cell Line Bank), and K562 (human erythroleukemia cell line; ATCC) were cultured in RPMI-1640 (Welgene) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Lonza) at 37°C in 5% CO₂. Human PBMCs were isolated from healthy volunteers and cancer patients with breast, colon, and advanced gastric cancer patients (Supplementary Fig. S1). The Epstein–Barr virus (EBV)-transformed lymphoblastoid B cell line (LCL) was generated from PBMCs using EBV supernatant from B95-8 cells (16). All studies involving human subjects were approved by the Institutional Review Board of Korea University Hospital with donor consent.

Expansion of NK cells

PBMCs were prepared from peripheral blood using Ficoll–Paque PLUS (GE Healthcare) and washed twice with PBS. Feeder lines were gamma-irradiated (300 Gy) and cocultured with PBMCs in RPMI-1640 media with 10% FBS plus recombinant human IL-2 (500 U/mL; rIL-2; Proleukin; Novartis). Medium was changed every 3 days with fresh IL-2. On day 5, expanded NK cells were transferred to T25 flasks or T75 flasks at a concentration of 0.5 × 10⁶ cells/mL. The number of PBMCs was assessed by staining cells with trypan blue at intervals up to 15 days in culture. CD3+ T cells or CD20+ B cells were depleted by Midi MACS (magnetic cell sorting) according to the manufacturer’s protocol (Miltenyi Biotech). The rate of NK cell expansion in PBMCs stimulated by KL-1 cells was compared with that cocultured with K562, CEM, and U937 cells. The absolute number of NK cells was calculated by multiplying the total viable number of cells by the percentage of CD56+CD3- cells determined by flow cytometry. Fold expansion was calculated by dividing the number of viable NK cells present at the end of culture by the number of viable NK cells at the beginning of culture.

Antibodies and flow cytometry

Anti-human CD3 (OKT3), CD56 (MEM188), CD19 (HB19), HLA-ABC (W6/32), CD11a (HI111), ICAM-1 (HA58), NKG2D (5C6), MICA/B (6D4), CD4 (OKT4), CXCR1 (8F1-1-4), CXCR4 (12G5), CCR7 (3D12), CD2 (RPA-2.10), CD49d (9F10), CD25 (BD6), CD16 (CB16), CD69 (FN50), and CD57 (TB01) mAbs were purchased from ebioscience. Anti-human 2B4 (clone 2-69), CD48 (YU145), Nkp44 (P44-8.1), CD4 (RPA-T4), CD2 (RPA-2.10), CD58 (IC3), CD122 (Mik-j3), CD132 (AG184), CD183 (1C6), Nkp30 (P30-15), Nkp46 (9E2), DNAM-1 (DX11), KIRNKAT2 (DX27), CD94 (HP-3D9), NKB1 (DX9), CD158a (HP-3E4), and CD158b (CH-L) mAbs were purchased from BD Pharmingen. Anti-human ULBP1 (170818), ULBP2 (165903), and ULBP3 (166510) were purchased from R&D systems. Fluoroscein isothiocyanate (FITC) conjugated and purified anti-mouse immunoglobulin G (IgG) were purchased from ebioscience. Purified anti-human CD16 (clone CB16; ebioscience), 2B4 (clone C1.7; ebioscience), and NKG2D (149810; R&D Systems) were used to block the binding of receptors from their ligands. For surface staining, the cells were stained with the indicated PE-, FITC-, APC-, or PerCP-conjugated mAbs in 100 µL of FACS buffer (BD Biosciences). Flow cytometry was carried out with a FACSCalibur and FACSCantoII (BD Biosciences) and the data were analyzed with FlowJo (Three Star) software.

¹⁵³Cr-release assay

¹⁵³Cr-release assay was conducted as previously described (17). In brief, K562 and KL-1 target cells were labeled with ¹⁵³Cr (Perkin Elmer) at 50 µCi/5 × 10⁵ cells and incubated at 5 × 10⁵ cells/well with serially diluted PBMCs for 4 hours. The γ-scintillation of supernatant was measured by a γ-counter (Perkin Elmer). Percentage-specific lysis was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Proliferation assay by CFSE dilution

To determine lymphocyte proliferation, PBMCs were labeled with 5 µmol/L carboxyfluorescein succinimidyl ester (CFSE) in PBS for 10 minutes at 37°C. After washing twice with RPMI complete media, PBMCs were cocultured with irradiated KL-1 cells at 37°C with 500 U/mL IL-2. CFSE fluorescence was determined on CD56+CD3+ and CD56-CD3+ population by flow cytometry.

In vivo cytotoxicity assay

Ten- to 12-week-old female severe combined immunodeficient (SCID) mice (Jackson Lab) were maintained both at...
the University of Chicago (Chicago, IL) and Korea University (Seoul, Korea) animal housing facilities under specific pathogen-free condition. All animal experiments were approved by Institutional Animal Care and Use Committees of University of Chicago and Korea University and carried out in accordance with national and institutional guidelines. To determine the in vivo cytotoxicity of expanded NK cells, KL-1 cells were labeled with PKH26 according to the manufacturer’s instructions (Sigma). Cells were washed in medium, and $5 \times 10^6$ KL-1 cells were injected intraperitoneally (i.p.) in 250 µL PBS. After 30 minutes, $8 \times 10^6$ human expanded NK cells were injected i.p. in 800 µL PBS. After 2 days, the mice were killed and peritoneal cells were recovered. Tumor cells were distinguished by PKH26 labeling. For NK depletion, mice were injected i.v. with 100 µg anti-asialo GM1 (Wako Pure Chemical Industries Ltd., Japan) on days −2 and 0 of tumor injection.

Statistical analysis
ANOVA was conducted to compare the mean difference between the samples (PRISM). Where P value was less than 0.001, the result was considered significant (*, $P < 0.001$). All error bars represent SD.

**Result**

KL-1 feeder cells show selective expansion of NK cells from PBMCs

We postulated that expansion of NK cells from PBMCs could be achieved by in vitro exposure of PBMCs to class I-deficient or -mismatched feeder cells expressing potential NK-activating ligands and that such cells would be represented as NK target cells. To this end, we evaluated a panel of commercially available immortalized cell lines for their capacity to induce NK cell activation and expansion. These included malignancies of solid tumor origin: A549 lung carcinoma, MCF7 breast adenocarcinoma, Chang and Hela cervical adenocarcinoma, HT29 colon adenocarcinoma, MDA-MB-31 and SK-BR-3 breast adenocarcinoma, and liquid tumors known to be targets of human NK cells: K562, CEM, U937, and Jurkat/KL-1. PBMCs were cocultivated with irradiated feeder cells at 1:1 ratio in complete media with 500 U/mL IL-2 (18). Although IL-2 alone can induce proliferation of human NK cells, it failed to enrich NK cells from whole PBMC culture (Fig. 1A). Furthermore, all solid tumor lines tested failed to induce enrichment of NK cells or their significant expansion from PBMCs (Supplementary Fig. S1). However, exposure of PBMCs to irradiated NK target lines, U937, KL-1, and K562 showed enrichment of the CD56$^+$

![Figure 1. Coculture with irradiated Jurkat/KL-1 results in the enrichment and expansion of NK cells from whole PBMC cultures. PBMCs were cultured at a ratio of 1:1 without (IL-2 alone) or with irradiated KL-1, U937, K562, or CEM feeder cell lines in the presence of 500 U/mL IL-2. NK cell percentages (A) and absolute numbers (B) are shown. C, PBMCs were cultured with irradiated KL-1 feeder cells as in B and the NK cell percentage was determined every 1 to 2 days. D, NK cell expansions from PBMCs of 4 healthy donors cocultivated with irradiated KL-1 cells are shown. Results are representative of a total of 20 individuals whose PBMCs were individually evaluated for NK cell expansion and enrichment.](https://www.aacrjournals.org/cancerres/article-pdf/73/8/OF3/OF3e0044/OF3e0044.pdf)
CD3\(^-\) NK cell population to comprise 40% to 90% of the total cells within 2 weeks (Fig. 1A). Interestingly, among these cell lines, only KL-1 was able to induce robust expansion of total NK cell numbers up to 130-fold higher than baseline (starting NK cell number of \(\sim 0.7 \times 10^6\) to final of \(\sim 10 \times 10^7\); Fig. 1B). When fold expansion of NK cells in culture was monitored daily, enrichment of NK cells was apparent after day 5, reaching a maximum of 80% to 90% (CD56\(^+\)CD3\(^-\)) by 10 to 14 days (Fig. 1C). The use of KL-1 feeders and IL-2 in this protocol was then applied to PBMCs obtained from more than 20 healthy donors. Levels of NK cell enrichment and expansion among these donors were variable, but by day 14, all had achieved similarly high levels of NK cell numbers with up to 90% purity (Fig. 1D).

The difference in NK cell expansion and enrichment by feeder lines KL-1, U937, and K562 was unexpected because all these cells were killed equally well by NK cells (Supplementary Fig. S2; ref. 17). These data suggest that NK killing of tumor targets may not necessarily lead to cell proliferation and that additional stimuli must be present to trigger cell proliferation and expansion.

**KL-1 feeder cells mediate proliferation of NK cells, but not T cells, in a contact-dependent manner**

*In vitro* culture of PBMC with IL-2 alone led to the selective expansion of T cells (Fig. 2A, left), presumably due to the presence of high-affinity surface IL-2 receptors. Addition of

![Figure 2](https://example.com/figure2.png)

Figure 2. KL-1 cells promote expansion of NK cells while suppressing that of T cells. A, left, PBMCs cocultivated with irradiated KL-1 cells were assessed on day 15 for the fraction of CD56\(^-\)CD3\(^+\) T and CD56\(^+\)CD3\(^-\) NK cells; right, fraction of T and NK cells in culture was evaluated every 2 to 3 days. The percentage of T cells (CD3\(^-\)CD56\(^+\)) and NK cells (CD56\(^-\)CD3\(^+\)) in the expanding PBMC culture was determined by flow cytometry. B, PBMCs were labeled with CFSE and cocultivated without or with irradiated KL-1 cells. At day 5, the T cells (CD56\(^-\)CD3\(^+\)) and NK cells (CD56\(^-\)CD3\(^+\)) were assessed for CFSE staining. Proliferating fraction is represented by the fraction of CFSE-staining cells (MFI < \(10^3\)).

C, PBMCs were labeled with CFSE and cultured alone, with irradiated KL-1 cells in the same well, or with irradiated KL-1 cells in the overlying transwell. NK cells gated on CD56\(^+\)CD3\(^-\) fraction were assessed for CFSE staining on day 5. D, top, PBMCs were labeled with CFSE and cultured with live (nonirradiated) KL-1 cells in the same well or in overlying transwell. NK cells gated on CD3\(^-\)CD56\(^-\) fraction were assessed for CFSE staining on day 5; bottom, PBMCs were cocultivated with live (nonirradiated) KL-1 feeder cells labeled with PKH26 at various PBMC:KL-1 ratios for 24 hours. Loss of PKH26-stained cells in culture correlates with killing of KL-1 target cells. Dot plots and histograms show results of 1 representative experiment out of 5.
KL-1 to this culture shifted the bias toward activation and expansion of NK cells (CD56+CD3−) over that of T cells (CD56−CD3+). When examined over the course of 10 days, cultures exposed to irradiated KL-1 showed a marked increase in the fraction of NK cells present in culture accompanied by a concomitant decrease in the T-cell fraction (Fig. 2A, right). To understand the mechanism underlying this process, PBMCs were labeled with CFSE and cocultivated with or without irradiated KL-1 cells (Fig. 2B). In the absence of KL-1, only a modest difference in the fraction of proliferating cells was observed between NK cells and T cells in culture (left, 26.5% vs. 18.4%, respectively). However, among wells receiving irradiated KL-1, a dominant population of proliferating (CFSE-lo) CD56−CD3− NK cells was observed (up to 60% of total) that was significantly greater than the proliferating (CFSE-lo) CD3− T-cell population (3.5%; Fig. 2B, right). Therefore, the increase in the population of NK cells in KL-1–containing cultures results, in part, from an increase in proliferation of NK cells at the expense of T cells.

To further examine the nature of KL-1–mediated selective NK cell expansion, we determined whether cell-to-cell contact of KL-1 with PBMCs was required. PBMCs were labeled with CFSE and cultured in IL-2-containing media without KL-1, with KL-1 in the same well, or with KL-1 in a transwell (Fig. 2C). When KL-1 and PBMCs were cocultured in the same well, significant proliferation of CD56−CD3− NK cells was observed compared with that without KL-1 (e.g., CFSE-lo population of 43.98% vs. 29.58% without KL-1). However, this KL-1–enhanced proliferation of NK cells was no longer apparent when PBMCs and KL-1 cells were separated by a transwell (28.91%), showing a contact-dependence requirement for KL-1–mediated NK cell proliferation. A similar pattern of expansion and proliferation was observed when live KL-1 cells were used instead of irradiated KL-1 cells, suggesting that preferential expansion of NK cells over T cells might be due to the active killing of feeder cells (Fig. 2D, top). Indeed, nonirradiated KL-1 cells were efficiently lysed by NK cells, as seen by near-complete elimination of live KL-1 cells after 1 day at PBMC: KL-1 ratios of 1 or greater (Fig. 2D, bottom). These data show that proliferation and enrichment of NK cells among PBMCs by KL-1 is initiated by the lysis of KL-1 cells occurring within 24 hours from the start of culture.

**Ligand expression profile of KL-1 cells**

To evaluate whether the contact-dependence requirement for KL-1 was associated with expression of known NK activation ligands, we surveyed KL-1 for surface expression of a panel of ligands, known to bind to NK receptors through homo- or
Figure 4. Exposure to KL-1 cells upregulates activating receptors NK cells and increases their cytolytic function against K562 and KL-1 tumor targets. A, following 11 days of KL-1-mediated NK expansion, the surface expression of various receptors was assessed by multicolor flow cytometry as specified and compared with resting NK cells (day 11 vs. 0). For each receptor, analysis was made on CD56+ CD3- subsets within the whole population. Representative data (n = 5) showing comparative phenotyping of NK cells on day 0 and day 11 are shown (Supplementary Fig. S3). B, expression of activating and adhesion receptors was compared between NK cells cultured with or without KL-1 in the presence of IL-2. Histograms show results of 1 representative experiment out of 5. C, in vitro cytolytic activity of NK expanded cultures is assessed by 51Cr release assay. NK targets, K562 and KL-1 cells, were labeled with Sodium Chromate51 and admixed with NK expanded culture (PBMC+KL-1) or PBMC alone at effector-to-target ratios of 20:1 to 0.7:1. Specific lysis was determined as described in Materials and Methods. The mean ± SD cell killing at the indicated E:T ratios in triplicate cultures is shown. D, in vivo cytolytic activity of NK cells is shown; 5 × 10^6 KL-1 tumor cells were labeled with PKH and injected i.p. into 10- to 12-week-old female SCID mice together with 8 × 10^6 human expanded NK cells (hNK). Mice received 100 μg of anti-asialo-GM1 to deplete endogenous NK cells on day –2 and day 0 before injection of cells. After 2 days, the mice were killed and peritoneal cells were recovered. Three groups of mice were treated receiving: NK cells but no tumor, tumor but no NK cells, and both tumor and NK cells. Extent of tumor killing was assessed by comparing percentage of PKH+ cells recovered from peritoneal lavage, with or without NK cell transfer. Results are representatives of a minimum of 5 independent experiments.
heterotypic interactions (Fig. 3A). We found that KL-1 had elevated surface expression of ULBP1, CD48, CD2, CD58, and ICAM-1; however, antibody blockade of these molecules during in vitro culture did not influence KL-1–mediated expansion of NK cells (data not shown, Fig. 3B). Blockade of their counter-receptors on NK cells: NKG2D (for ULBP2, MICA/B) and 2B4 (CD48) also failed to affect in vitro expansion (Fig. 3B). However, blockade of the low-affinity FcR, FcyIIIR (CD16), on the NK cell surface led to a significant reduction in fraction of NK cells generated; conversely, the use of anti-CD16 antibody, when cross-linked on a plate, led to enhanced NK cell yield among PBMC in the absence of KL-1 (Fig. 3C), suggesting a role for CD16 ligation in KL-1–mediated expansion of NK cells.

**Exposure to KL-1 upregulates multiple activating and adhesion receptors on NK cells and is associated with strong tumor lytic capacity both in vitro and in vivo**

We examined the surface levels of a panel of NK receptors expressed on KL-1 cultured NK cells. Among the receptors tested, activated NK cells showed significant upregulation of ICAM-1, CD11a, CD48, CD2, CD49d, and CD58 adhesion receptors; NKp90, NKp44, 2B4, DNAM-1, and NKGD2 activating receptors; and CD25 and CD69 activation markers (Fig. 4A). No substantial changes were observed in the expression levels of CD11b, NKp46, CD122, CD132, CD181, and CD57. Slight upregulation was detected on CD183, CD184, and CCR7 chemokine receptors and KIRNKAT2, CD94, NKB1, CD158a, and CD158b inhibitory receptors. Interestingly, the level of CD16 was significantly downregulated on activated NK cells compared with that on resting cells. The changes in surface expression described here are representative of 4 other healthy donors and 6 patient donors (Supplementary Fig. S3 and S4). When NK cells obtained at the end of coculture with irradiated KL-1 feeder were compared with that cultured in IL-2 alone, we found additional elevation of CD11a, CD2, and NKp44 expression (Fig. 4B). Activated NK cells showed enhanced lytic function against 2 leukemia tumor targets, K562 and KL-1, as measured by an in vitro 51Cr-release assay (Fig. 4C). Even at the effectortarget ratio of 0.7:1, NK cells were able to lyse K562 and KL-1 cells approximately at 35% and 80%, respectively. In contrast, PBMCs cultured with IL-2 in the absence of KL-1 feeder were compared with that cultured in IL-2 alone, we found additional elevation of CD11a, CD2, and NKp44 expression (Fig. 4B). Activated NK cells showed enhanced lytic function against 2 leukemia tumor targets, K562 and KL-1, as measured by an in vitro 51Cr-release assay (Fig. 4C). Even at the effectortarget ratio of 0.7:1, NK cells were able to lyse K562 and KL-1 cells approximately at 35% and 80%, respectively. In contrast, PBMCs cultured with IL-2 in the absence of KL-1 feeders exhibit poor killing of NK targets. These data suggest that NK cells cultured with KL-1 feeder is feasible

**Expansion of NK cells by KL-1 can be augmented using allogeneic B-cell lines**

To evaluate the contribution of specific lymphocyte subsets in PBMCs to promote KL-1–mediated proliferation of NK cells, B cells or T cells were depleted before KL-1 coculture. As shown in Fig. 5A, T-cell–depleted PBMCs (PBMC-T) was not found to affect KL-1–mediated NK cell expansion. However, depletion of B cells (PBMC-B) resulted in a marked decrease in the proliferating NK cell fraction, suggesting that the presence of B cells was critical in the use of KL-1 feeder for NK expansion (Fig. 5A). To extend these findings, we postulated that immobilized B cell lines (e.g., EBV-transformed human B cells; lymphoblastoid cell line, LCL) might augment additional signals for in vitro expansion. Addition of irradiated allogeneic LCL together with the KL-1 feeder resulted in more than 740-fold expansion of the NK cell population among PBMCs (Fig. 5B). When various ratios of KL-1:L were evaluated, an optimized ratio of 0.5 to 0.5 (or 1:1) was identified as the combination yielding the highest percentage of NK cells with the greatest cell number within 13 days of culture (Fig. 5B).

**Expansion of NK cells from patients with cancer using KL-1 feeder is feasible**

To evaluate the feasibility of expanding NK cells for clinical use, we collected PBMCs from patients with breast (3), colorectal (4), and gastric cancer (Supplementary Fig. S5; ref. 6). Greater than 100-fold expansion of CD56+ CD3– NK cells was routinely achieved with the final product averaging approximately 70% NK cells (Fig. 6). Expansion data for 20 healthy donors and 5 patients are provided in Supplementary Fig. S6. KL-1–expanded NK cells from these patients showed superior lytic activity, showing that the culture method using KL-1 provides a simple and effective strategy that can be easily transferred into the clinical setting.

**Discussion**

In this study, we developed a novel 1-step strategy using irradiated KL-1 feeder to generate a highly enriched and expanded population of NK cells from PBMCs that can be easily translated for clinical use with minimal resource and cost. In contrast to conventional approaches that use a cell-separation process (e.g., immunomagnetic beads, to deplete CD3+ T cells and enrich for CD56+ cells), our strategy requires only that PBMCs be cocultured with an irradiated allogeneic T-cell lymphoblast line, KL-1, and moderate-dose IL-2 (500 U/mL) to achieve significant levels of expansion within 2 weeks. When LCLs were added, cells underwent expansion up to 740-fold, while retaining NK cell purity. Using this approach, a 1-time blood draw of 150 mL yielding a starting population of approximately 150 million PBMCs, containing almost 15 million NK cells, could be used to generate 10 billion NK cells, which is within the dosing range used in clinical studies of NK cell therapy (8, 10, 13, 20). Among the panel of NK targets tested as potential in vitro stimulator/feeder lines, KL-1, was the only line capable of
simultaneously enriching NK cells and expanding cell numbers. KL-1–mediated expansion led to robust NK cell activation, upregulating surface expression of activation receptors (NKG2D, CD94, DNAM-1, and 2B4), natural cytotoxicity receptors (NKp30 and NKp44), and adhesion molecules (CD11a and ICAM-1), and enhancing lytic activity both in vitro and in vivo. In addition, KL-1–activated cells upregulated CD183, CD184, and CCR7 chemokine receptors, which can facilitate recruitment of transferred NK cells into tumor tissues. Expansion of NK cells following KL-1 exposure occurred at the expense of a declining T-cell population. The combination of an absent TCR trigger and availability of NK activating ligands on KL-1 in the presence of IL-2 likely contribute to the dominant CD56+CD3− cell population expanded in culture.

We identified several activation ligands expressed by KL-1, including ULBP1, CD48, CD2, CD58 (LFA-3), and ICAM-1; however, antibody blockade to these molecules or their counterreceptors during in vitro culture, did not appear to influence KL-1–mediated expansion of NK cells. Surprisingly, antibody blockade of CD16 (FcγRIII) led to a significant decrease in NK cell proliferation. Downmodulation of surface expression of CD16 in KL-1–activated NK cells (Fig. 4A) is consistent with a model where CD16 may be actively engaged with a ligand expressed on target/feeder cells. Recently, CD16 has been identified as a lysis receptor mediating NK cytotoxicity against an EBV-transformed 721.221, 293 EBNA, and a melanoma tumor line (21). Its target ligand, however, has not been defined nor does it appear to be expressed on KL-1 cells because...
binding of CD16-Ig to KL-1 cells was not detected (data not shown). Rather, the ligand for CD16 appears to be expressed on cocultured B cells because B cell–depleted cultures fail to support KL-1–mediated NK cell expansion. Consistent with these results, we confirmed that the LCL line used in our studies express the CD16 ligand (data not shown) and led to markedly enhanced NK cell proliferation. B cells are also known to interact with NK cells in the murine model, through other receptor–ligand interactions including CD48-2B4 and CD40-CD40L (22, 23). In our model, it appears that an initiating event, that is, activation of NK cells through ligands on KL-1, possibly CD16L, is required for endogenous B cells to facilitate NK cell expansion and/or survival.

Past and current efforts to expand NK cells for adoptive therapy include in vitro exposure of patient autologous PBMC to γ-chain receptor cytokines such as IL-2 or IL-15 to enhance cytotoxicity (24, 25); however, in the absence of other signals the use of cytokines alone often fails to maintain long-term proliferation, even at relatively high doses (e.g., 1,000 U/mL IL-2; refs. 26–28). In some studies, addition of anti-CD3 or PMA/ionomycin led to NK cell enrichment and expansion, although the mechanism of triggering TCR signals to augment NK growth is unclear and generally leads to a subdominant population of NK cells admixed with T cells in the final product (27–29). The use of feeder lines was found to be necessary in providing additional growth-initiating signals for NK cells (30–32). The most commonly studied feeder line is the class I–negative leukemic line, K562, which, in unmodified form, yields modest NK cell expansion (33, 34). Genetically modifying K562 to express 4-1BB ligand and MIC-A together with soluble IL-15 led to a 200-fold increase in cell number but obtained a final product containing only 56% NK cells (35). By engineering expression of membrane-bound IL-15 and 4-1BBL, a 150-fold expansion of NK cells was achieved after 15 days of culture with 90% purity (26). Significant cytotoxicity to several AML lines was observed, accompanied by minor elevations in the natural killer cell number; these results are comparable with the cytolytic activity and enhanced NK function (20). Addition of membrane-bound IL-15 and 4-1BBL, a 150-fold increase in cell number but obtained a final product containing only 56% NK cells (35). By engineering expression of membrane-bound IL-15 and 4-1BBL, a 150-fold expansion of NK cells was achieved after 15 days of culture with 90% purity (26). Significant cytotoxicity to several AML lines was observed, accompanied by minor elevations in the natural killer cell number; these results are comparable with the cytolytic activity and enhanced NK function (20).

By comparison, the strategy reported here using genetically unmodified KL-1 led to an approximately 130-fold increase in NK cell expansion within 14 days. Our protocol yielded NK cells of approximately 90% purity with significant upregulation of NKp44 and NKp30 corresponding to high cytolytic activity and enhanced NK function (20). Addition of irradiated B cells led to approximately 740-fold increase in NK cell number; these results are comparable with the genetically modified approaches described earlier. A recent report describes the use of K562 cells expressing membrane-bound IL-21 capable of yielding prodigious expansion of NK cells (35, 37). Given the experience with K562, which alone yields only modest results, genetic modification of KL-1 using vectors such as mb-IL-21 and 4-1BBL, will likely further enhance its capacity to activate and expand NK cells in vitro.

In practice, most clinical trials to date have required cell selection (CD3/CD56−) in addition to a feeder-assisted expansion. These selection strategies incur added cost and resources including the need for trained personnel and specialized facilities to carry out clinical-grade magnetic separations. The method described in this report requires only minimal in vitro manipulation, does not include the use of genetically modified products and no pre-expansion selection/enrichment process. By obviating the requirement for genetic manipulation or immunomagnetic selection, KL-1 provides a straightforward, cost-effective, high-yield strategy for generating requisite numbers of functionally robust NK cells for adoptive cellular therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.A. Lim, T-J. Kim, J.G. Choi, C-O. Yun, K-M. Lee
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