

# Expansion of Highly Cytotoxic Human Natural Killer Cells for Cancer Cell Therapy

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## Abstract

**Infusions of natural killer (NK) cells are an emerging tool for cancer immunotherapy. The development of clinically applicable methods to produce large numbers of fully functional NK cells is a critical step to maximize the potential of this approach. We determined the capacity of the leukemia cell line K562 modified to express a membrane-bound form of interleukin (IL)-15 and 41BB ligand (K562-mb15-41BBL) to generate human NK cells with enhanced cytotoxicity. Seven-day coculture with irradiated K562-mb15-41BBL induced a median 21.6-fold expansion of CD56<sup>+</sup>CD3<sup>-</sup> NK cells from peripheral blood (range, 5.1- to 86.6-fold; *n* = 50), which was considerably superior to that produced by stimulation with IL-2, IL-12, IL-15, and/or IL-21 and caused no proliferation of CD3<sup>+</sup> lymphocytes. Similar expansions could also be obtained from the peripheral blood of patients with acute leukemia undergoing therapy (*n* = 11). Comparisons of the gene expression profiles of the expanded NK cells and their unstimulated or IL-2-stimulated counterparts showed marked differences. The expanded NK cells were significantly more potent than unstimulated or IL-2-stimulated NK cells against acute myeloid leukemia cells *in vitro*. They could be detected for >1 month when injected into immunodeficient mice and could eradicate leukemia in murine models of acute myeloid leukemia. We therefore adapted the K562-mb15-41BBL stimulation method to large-scale clinical-grade conditions, generating large numbers of highly cytotoxic NK cells. The results that we report here provide rationale and practical platform for clinical testing of expanded and activated NK cells for cell therapy of cancer. [Cancer Res 2009;69(9):4010-7]**

## Introduction

Natural killer (NK) cells can kill cancer cells in the absence of prior stimulation and hold considerable potential for cell-based therapies targeting human malignancies (1-4). This notion is corroborated by the observation that, among patients with leukemia undergoing hematopoietic stem cell transplantation, the antileukemic effect of the transplant was significantly greater when the donor NK cells exhibited a killer inhibitory receptor (KIR) profile that predicted a higher cytotoxicity against the leukemic cells of the recipient (3, 5-7). Moreover, allogeneic NK cells might

be beneficial when directly infused into patients, a procedure that was shown to induce clinical remission in patients with high-risk acute myeloid leukemia (AML; ref. 8). Infusions of NK cells have also been proposed as a means to improve the treatment of other cancers (9).

Because NK cells represent a small fraction of peripheral blood mononuclear cells, generating them in numbers sufficient to meet clinical requirements, especially if multiple infusions are planned, is problematic. Hence, NK cell-based therapies would greatly benefit from reliable methods to produce large numbers of fully functional NK cells *ex vivo*. Unlike T and B lymphocytes, which readily respond to a variety of stimuli, NK cells typically do not undergo sustained proliferation. Indeed, their reported proliferative responses to cytokines with or without coculture with other cells have generally been modest and of short duration in most studies (10-16).

We previously found that the K562 leukemia cell line genetically modified to express membrane-bound interleukin (IL)-15 and 41BB ligand specifically activates NK cells, drives them into the cell cycle, and allows their genetic modification (17). In this study, we determined the capacity of NK cells stimulated by contact with K562-mb15-41BBL cells to exert anti-AML cytotoxicity.

## Materials and Methods

**Cells.** The AML cell lines K562, HL-60, KG1, and U937 (American Type Culture Collection) were maintained in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (BioWhittaker). The K562-mb15-41BBL cell line was made by transducing K562 cells with constructs encoding the "membrane-bound" form of IL-15 (IL-15 + CD8 $\alpha$ ) and human 41BB ligand (both containing green fluorescent protein; ref. 17). We also transduced K562 and U937 cells with a murine stem cell virus-green fluorescent protein vector (from the St. Jude Vector Laboratory) containing the firefly luciferase gene and selected them using green fluorescent protein expression and flow cytometry.

Peripheral blood was obtained from 50 healthy adult donors. Mononuclear cells were separated on a Lymphoprep density step (Nycomed) and washed twice in RPMI 1640. Purified unstimulated NK cells were obtained by CD3 depletion followed by CD56 enrichment using the AutoMACS system (Miltenyi Biotec) or the Miltenyi NK Cell Isolation Kit. Primary leukemic cells were obtained from 9 patients with AML; peripheral blood was obtained from 11 patients with acute leukemia (8 acute lymphoblastic leukemia and 3 AML) in clinical remission after appropriate informed consent and institutional review board approval.

**NK cell activation and expansion.** Peripheral blood mononuclear cells ( $1.5 \times 10^6$ ) or purified NK cells ( $0.1 \times 10^6$ ) were incubated in a 24-well tissue culture plate with  $1 \times 10^6$  K562-mb15-41BBL cells and 10 IU/mL human IL-2 (National Cancer Institute BRB Preclinical Repository) in RPMI 1640 and 10% fetal bovine serum. We called this method NK Cell Activation and Expansion System (NKAES; Fig. 1A). Medium was exchanged every 2 days with fresh medium and IL-2. At the end of the cultures, residual T cells were removed with Dynabeads CD3 (Invitrogen).

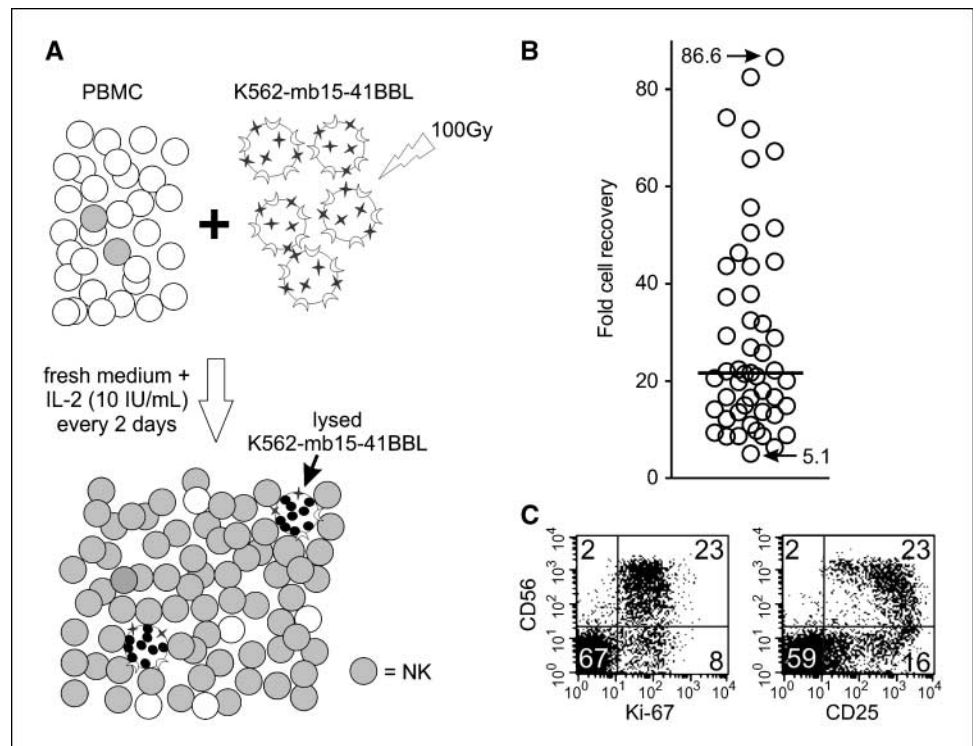
**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** Activation and expansion of NK cells from peripheral blood. **A**, schema of the NKAES method. Irradiated K562-mb15-41BBL cells are mixed with peripheral blood mononuclear cells (PBMC) at a 1:1.5 ratio. **B**, NKAES-NK cell expansion from mononuclear cells from 50 healthy donors. Fold recovery of CD56<sup>+</sup>CD3<sup>+</sup> cells after 7 days of culture relative to the number of input cells. Bar, median. **C**, Ki-67 and CD25 expression was examined after 5 days of NKAES culture, a stage at which cultures typically still contain a majority of non-NK cells as well as NK cells in their initial phase of expansion; >90% CD56<sup>+</sup> cells expressed both markers, whereas CD56<sup>-</sup> cells remained mostly Ki-67<sup>-</sup> and CD25<sup>-</sup>.



NK cell expansion stimulated by K562-mb15-41BBL cells was compared with the results of stimulation with IL-2 (10-6,000 IU/mL), IL-12 (1-10 ng/mL; R&D Systems), IL-15 (1-100 ng/mL; R&D Systems), and/or IL-21 (1-10 ng/mL; Biosource International). Cytokines were added to the cultures every other day.

**Gene expression array and cell marker studies.** Gene expression was analyzed using U133 Plus 2.0 Arrays (Affymetrix). RNA isolation, cDNA preparation, and hybridization were done according to Affymetrix protocols. Arrays were read with a laser confocal scanner (Agilent). Probe intensities were measured using the MAS 5.0 algorithm, scaled to a target intensity of 500. Probe sets called absent across all samples were excluded and signal intensities were log-transformed. Differentially expressed genes were identified with Linear Models for Microarray Analysis (18) implemented in Bioconductor (19), controlling the false discovery rate according to Benjamini and Hochberg (20). Data were deposited in National Center for Biotechnology Information Gene Expression Omnibus (GSE12198).<sup>6</sup> Cell marker expression was done by flow cytometry using the antibodies listed in Supplementary Table S1.

**In vitro cytotoxicity assays.** Target cells ( $1.5 \times 10^5$ ) were placed in 96-well U-bottomed tissue culture plates and incubated with NK cells at various effector-to-target (E:T) ratios in RPMI 1640 with 10% fetal bovine serum. Unstimulated and NKAES-NK cells were cultured with 1,000 U/mL IL-2 for 16 h before the assay; in some experiments with NKAES-NK cells, such addition of IL-2 was omitted. Cells were harvested after 4 h coculture and labeled with phycoerythrin-conjugated anti-CD13, CD15, CD34, and CD33 antibodies and FITC-conjugated anti-CD56 (Becton Dickinson) and analyzed by flow cytometry (17, 21). The numbers of target cells recovered from cultures without NK cells were used as a reference.

NKAES-NK cell cytotoxicity was also assessed in 5-day cultures, with human bone marrow-derived mesenchymal cells (MSC), supporting the viability of leukemic cells (22, 23). MSC ( $2 \times 10^4$  per well) were plated on 96-well tissue culture plates precoated with 1% gelatin. After 5 days,  $2 \times 10^5$  primary AML cells were added followed 2 h later by NKAES-NK

cells. Cultures were done in the absence of exogenous IL-2 at 37°C in 5% CO<sub>2</sub>.

**Murine models.** The lifespan of NKAES-NK cells and their antileukemic capacity was determined in 8- to 14-week-old NOD.Cg-Prkdc<sup>scid</sup>IL2rg<sup>tm1Wjl</sup>/SzJ (NOD/scid-IL2R<sup>g</sup> null) mice (The Jackson Laboratory). NKAES-NK cells ( $3 \times 10^7$ ) were injected intravenously with or without administration of 25,000 IU IL-2 intraperitoneally; their persistence was monitored with an anti-CD45 antibody (Becton Dickinson).

Luciferase-transduced K562 or U937 cells were injected intraperitoneally or intravenously ( $0.2 - 1 \times 10^6$  per mouse). T-cell depleted NKAES-NK cells were injected intraperitoneally or intravenously ( $1 - 3 \times 10^7$  per mouse) as indicated in Results. NKAES-NK cells were from the same donor when multiple injections were administered. In some experiments, multiple injections of NKAES-NK cells were given together with intraperitoneal injections of IL-2 (25,000 IU each). Injections of tissue culture medium with or without IL-2 instead of NK cells served as controls. Leukemia progression was evaluated using a Xenogen IVIS-200 system (Caliper Life Sciences) 5 min after intraperitoneal injection of a D-luciferin potassium salt solution (3 mg/mouse). Photons emitted were quantified using the Living Image 3.0 software.

**Large-scale clinical-grade NK cell activation and expansion.** Apheresis products from 12 healthy donors were collected using a COBE Spectra (Caridian) and processed following current Good Manufacturing Practice (cGMP) guidelines. After centrifugation over a density gradient (Ficoll-Paque; GE Healthcare), mononuclear cells were placed in SCGM medium (CellGenix) supplemented with 10% fetal bovine serum, 50 mg/L gentamicin sulfate (Hospira), and 10 IU/mL human IL-2 (Proleukin; Novartis) at a concentration of  $0.5 \times 10^5$  CD56<sup>+</sup>CD3<sup>+</sup> cells/mL. Irradiated (100 Gy) K562-mb15-41BBL cells (from a Master Cell Bank generated following cGMP guidelines) were added at a ratio of 1 CD56<sup>+</sup>CD3<sup>+</sup> cell to 10 K562-mb15-41BBL cells. Cells were cultured in a closed VueLife bag system (American Fluoroseal) in a humidified incubator at 37°C, 5% CO<sub>2</sub>, with an initial total culture volume of 60 mL/bag. Multiple bags were set up to accommodate different numbers of mononuclear cells.

After 48 and 120 h, each cell culture bag was fed by the addition of 60 mL fresh complete medium. Cells were harvested after 7 days. Residual T cells were depleted using the CliniMACS System (Miltenyi), and cells were

<sup>6</sup> <http://www.ncbi.nlm.nih.gov/geo/>

washed and resuspended for infusion in PlasmaLyte-148 (Baxter) with 0.5% human serum albumin (pharmaceutical grade). Quality control and product release testing include flow cytometry (CD56 and CD3), sterility (Gram stain and culture), endotoxin, and *Mycoplasma*.

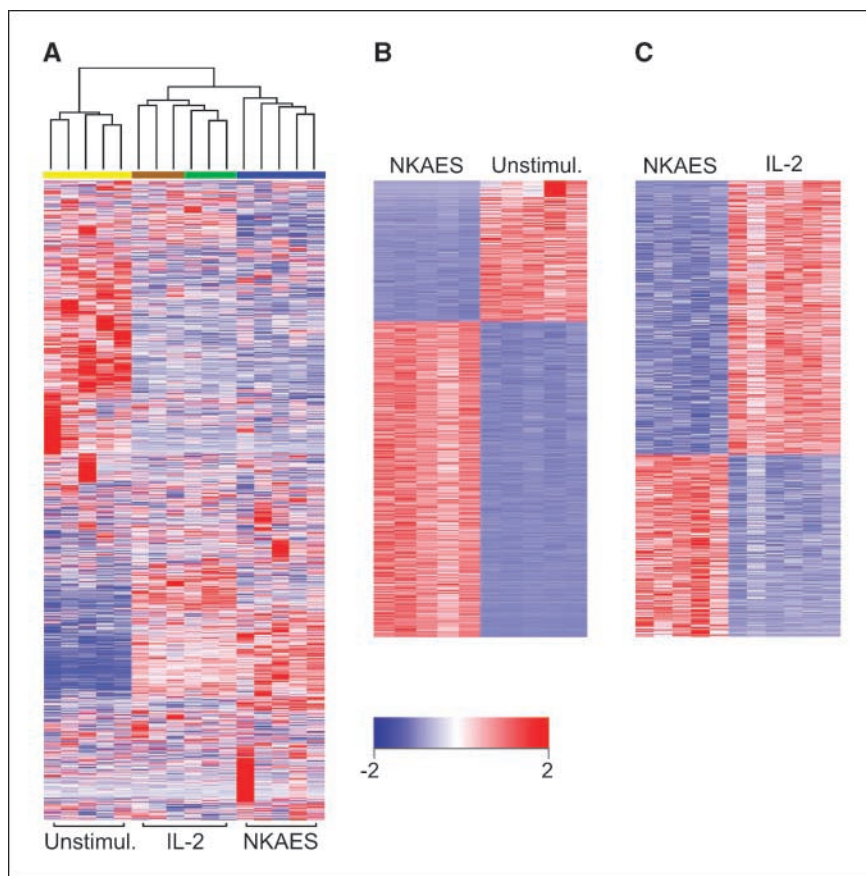
## Results

**Expansion of human NK cells.** When peripheral blood mononuclear cells from 50 healthy donors were placed in NKAES cultures, median CD56<sup>+</sup>CD3<sup>-</sup> cell recovery after 7 days was 21.6-fold (range, 5.1- to 86.6-fold) of the input cells (Fig. 1B). Expansion of CD3<sup>+</sup> T cells was minimal (median recovery, 1.2-fold; range, 0.3- to 3.7-fold). The specific stimulation of NK cells was corroborated by analysis of the expression of Ki-67, a marker of cell proliferation, and CD25, the IL-2 receptor  $\alpha$ -chain, after 5 days of culture. More than 90% CD56<sup>+</sup>CD3<sup>-</sup> cells expressed both markers, whereas CD56<sup>-</sup> cells remained mostly Ki-67<sup>-</sup> and CD25<sup>-</sup> (Fig. 1C). NK cell recovery increased to 152-fold (70- to 744-fold;  $n = 5$ ) after 14 days and 277-fold (201- to 1,459-fold) after 21 days of culture. Median percentage of CD56<sup>+</sup>CD3<sup>-</sup> cells was 62.9% (31.0-92.1%) on day 7, 90.0% (72.7-96.5%) on day 14, and 96.8% (75.6-98.0%) on day 21; that of CD3<sup>+</sup> T cells was 29.1% (4.5-64.3%), 8.3% (3.0-17.3%), and 3.1% (1.8-23.4%), respectively. K562-mb15-41BBL cells produced significantly higher NK cell expansions than K562 cells alone or K562 cells expressing either mb15 or 41BBL (data not shown; ref. 17). NKAES-NK cells maintained heterogeneous expression of KIR molecules ( $n = 3$ ; Supplementary Fig. S1A). NK cells expressing KIR molecules (KIR2DL1, KIR2DL2/3, and KIR3DL1) and those lacking these molecules expanded equally in NKAES cultures ( $n = 6$ ; Supplementary Fig. S1B).

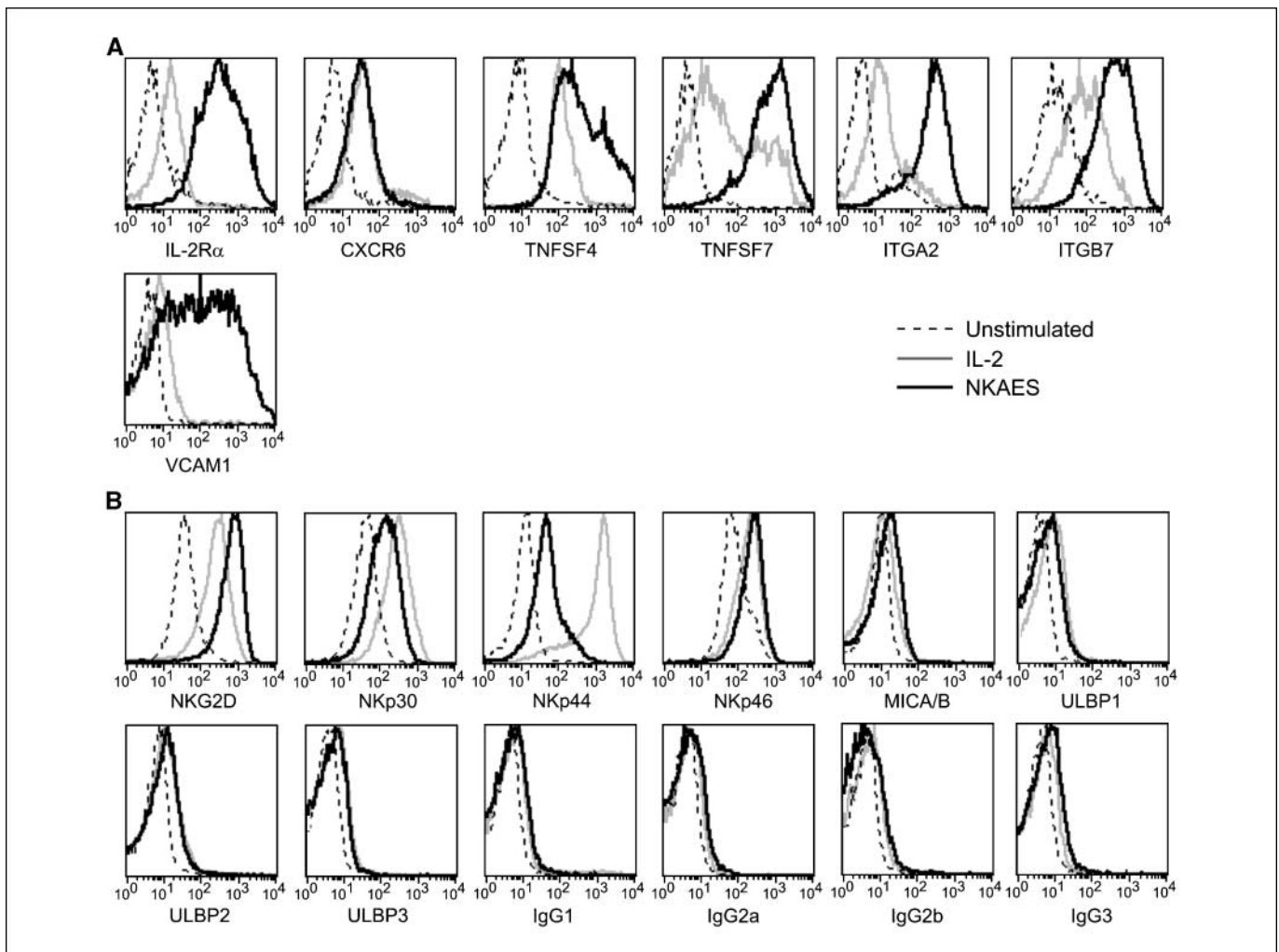
NKAES cultures could also expand NK cells from patients with acute leukemia as shown by experiments with peripheral blood obtained from 8 children with acute lymphoblastic leukemia and 3 with AML undergoing chemotherapy but in clinical remission. Median 7-day NK cell expansion was 17.3-fold (range, 3.6- to 142.5-fold), a rate similar to that observed with NK cells from healthy adult donors.

IL-2 alone at 10 IU/mL induced no significant expansion of CD56<sup>+</sup>CD3<sup>-</sup> cells (median, 1.1-fold; range, 0.2- to 4-fold;  $n = 18$ ); increasing IL-2 concentration to 100 IU-1,000 IU/mL resulted in only slightly higher recoveries (median, 2.0-fold; range, 1.0- to 3.2-fold;  $n = 7$ ). Likewise, 6,000 IU/mL IL-2 resulted in a 7-day median recovery of 1.3-fold (range, 1.1- to 1.3-fold;  $n = 4$ ). Addition of IL-15 (1-100 ng/mL) to 7-day cultures containing 10 to 20 IU/mL IL-2 did not improve NK cell expansion (median, 1.3-fold; range, 0.3- to 5.8-fold;  $n = 22$ ). We also tested the stimulatory effects of IL-12 (1-10 ng/mL;  $n = 6$ ), IL-21 (1-10 ng/mL;  $n = 6$ ), and these cytokines in various combinations with IL-2 and IL-15 ( $n = 7$ ) with no significant improvement over recoveries obtained with single stimuli.

**Genetic features of NKAES-NK cells.** We compared the global gene expression profiles of NKAES-NK cells ( $n = 5$ ) with those of unstimulated ( $n = 5$ ) and IL-2-stimulated purified CD56<sup>+</sup>CD3<sup>-</sup> NK cells ( $n = 6$ ). Unsupervised hierarchical clustering analysis correctly clustered unstimulated, IL-2-stimulated, and NKAES-NK cells in three distinct groups (Fig. 2A). The differences in gene expression profile of NKAES-NK cells and that of unstimulated or IL-2-stimulated NK cells was further corroborated by supervised clustering analyses (Fig. 2B and C).



**Figure 2.** Gene expression analysis of unstimulated, IL-2-stimulated, and NKAES-NK cells. Unstimulated NK cells ( $n = 5$ ) were studied immediately after immunomagnetic separation of CD56<sup>+</sup>CD3<sup>-</sup> cells. IL-2-stimulated cells ( $n = 6$ ) and NKAES-NK cells ( $n = 5$ ) were studied after 7 days of culture. Two concentrations of IL-2 were used: 200 IU/mL ( $n = 3$ ) and 6,000 IU/mL ( $n = 3$ ). A, unsupervised hierarchical clustering analysis resulted in three major clusters, each containing all unstimulated (*Unstimul.*), IL-2-stimulated, and NKAES-NK samples. IL-2-stimulated NK samples were in one major cluster regardless of the IL-2 concentration used (*brown line*, 200 IU/mL; *green line*, 6,000 IU/mL). B, supervised clustering analysis of NKAES-NK cells compared with unstimulated NK cells. C, supervised clustering analysis of NKAES-NK cells compared with IL-2-stimulated cells. B and C, top 1,000 differentially expressed probe sets (false discovery rate < 0.02).



**Figure 3.** Cell marker expression of unstimulated NK cells, NK cells stimulated with 6,000 IU/mL IL-2 for 7 days, and NKAES-NK cells. *A*, flow cytometry analysis of molecules differentially expressed by gene array analysis. Overlay histograms show results of one of three representative experiments. *B*, expression of natural cytotoxicity receptors (NKp30, NKp44, and NKp46), NKG2D, and its ligands (MICA/B, ULBP1, ULBP2, and ULBP3).

After excluding probe sets whose maximum expression signal was <500 and setting a false discovery rate threshold of <0.05, 5,841 probe sets were differentially expressed by  $\geq 2$ -fold in NKAES-NK and unstimulated NK cells; 2,697 were overexpressed in NKAES-NK cells and 3,144 were underexpressed. Using the same parameters, there were 915 probe sets differentially expressed by  $\geq 2$ -fold between NKAES-NK and IL-2-stimulated NK cells; 366 were overexpressed in NKAES-NK cells and 549 were underexpressed. Differentially expressed genes included genes encoding cytokines and chemokines, cytokine and chemokine receptors, adhesion molecules, and molecules involved in T-cell stimulation (Supplementary Tables S2-S5). Differential expression of some of these molecules was confirmed by staining with specific antibodies and flow cytometry (Fig. 3A). We also studied the expression of natural cytotoxicity receptors (NKp30, NKp44, and NKp46), NKG2D, and its ligands (MICA/B, ULBP1, ULBP2, and ULBP3) by flow cytometry in cells from three donors (Fig. 3B). Notable differences were the higher expression of NKG2D in NKAES-NK cells and that of NKp44 in IL-2-stimulated cells.

**Cytotoxicity of expanded NK cells against AML cells.** The cytotoxicity of NKAES-NK cells from 12 healthy donors was tested

against the AML cell lines K562, KG1, U937, and HL-60. NKAES-NK cells were consistently cytotoxic even when NK cells were outnumbered by target cells (Fig. 4A). We also tested the cytotoxicity of NKAES-NK cells from 3 patients with acute leukemia. The cytotoxicities observed were similar to those recorded with NKAES-NK cells derived from healthy donors (Supplementary Fig. S2).

We then tested NKAES-NK cell cytotoxicity against primary leukemic cells from 9 newly diagnosed patients with AML representative of the spectrum of AML subtypes (4 M1, 1 M2, 2 M4, 1 M5, and 1 M7). NK cells from 9 healthy donors were included in these studies for a total of 22 experiments. NKAES-NK cells were highly effective against primary AML cells overall. For example, mean  $\pm$  SD cytotoxicity after 4 h coculture at a 2:1 E:T ratio was  $43 \pm 18\%$  (Fig. 4B). To determine whether NKAES-NK cells were cytotoxic at very low E:T ratio in prolonged cultures, we cultured them with AML cells from 5 patients in the presence of bone marrow-derived MSC layers (to prevent spontaneous apoptosis of the AML cells). After 5 days of culture at a 1:10 E:T ratio, cytotoxicity was higher than 50% for 4 of the samples (Fig. 4C). In 2 of the 5 cases, considerable cytotoxicity (39% and 47%) were observed in cultures in which the E:T ratio was 1:100.

In the above experiments, there was no visible damage to the MSC. Nevertheless, we specifically tested the effect of NKAES-NK cells from 4 donors against allogeneic MSC in 4 h ( $n = 2$ ) or 5 day cultures ( $n = 2$ ). In all experiments, cytotoxicity was consistently <20% even at E:T ratios of 8:1. NKAES NK cells (from 2 other donors) also lacked cytotoxicity against allogeneic T cells: in assays lasting 4 to 24 h, cytotoxicity was always <20% at 8:1 or lower E:T ratios. Thus, NKAES cultures do not appear to significantly enhance the cytotoxicity of NK cells against nontransformed cells.

**Cytotoxicity of NKAES-NK cells compared with that of unstimulated and IL-2-stimulated NK cells.** Using K562, HL-60, U937, and KG1 cells as targets, we directly tested the relative cytotoxicity of NKAES-NK cells and of freshly isolated CD56<sup>+</sup>CD3 cells from the same donors ( $n = 12$ ). NKAES-NK cells were significantly more cytotoxic than their unstimulated counterparts regardless of the E:T ratio (Fig. 5A).

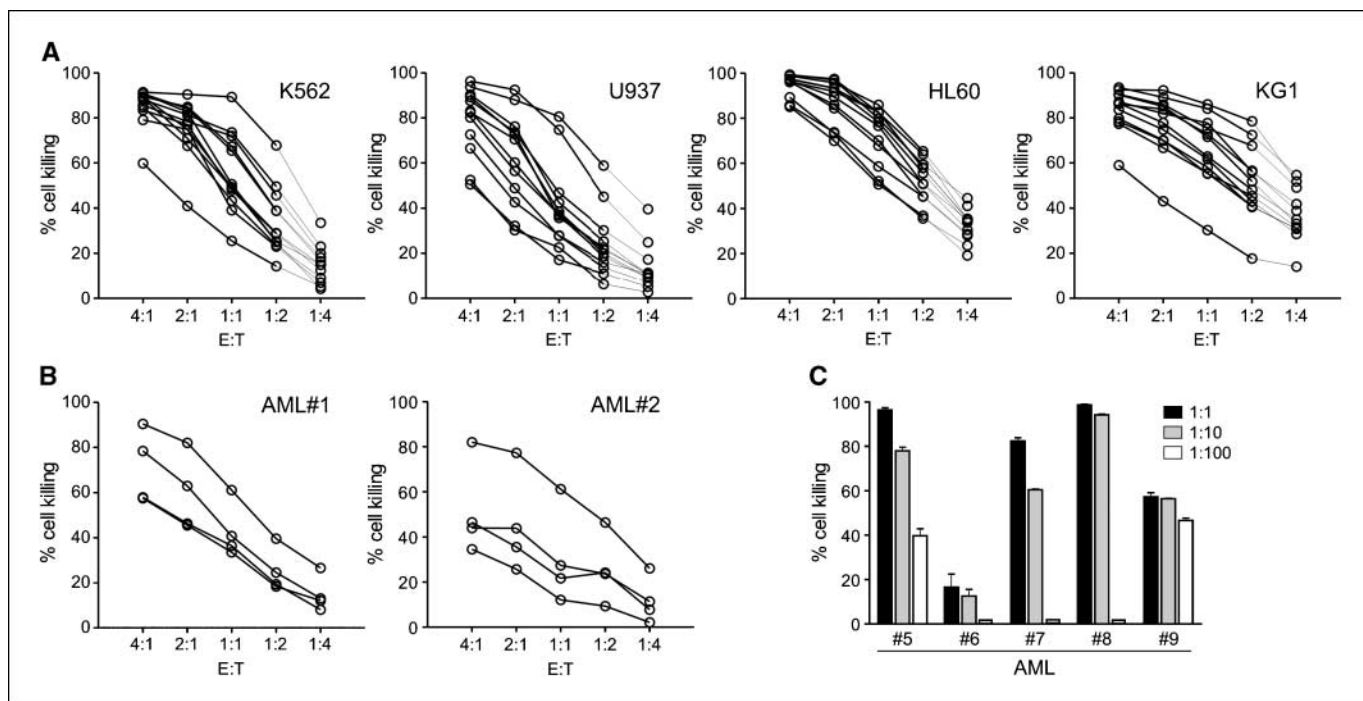
Early studies had indicated that IL-2 enhances the cytotoxicity of NK cells, with maximum effects achieved within 18 h incubation (10, 24). Moreover, infusion of allogeneic NK cells exposed to 1,000 IU/mL IL-2 overnight have been shown to exert antileukemic activity in AML patients (8). We therefore exposed unstimulated and NKAES-NK cells to 1,000 IU/mL IL-2 for 16 h and then compared their cytotoxicity against AML cell lines. NKAES-NK cells were more powerful overall (Fig. 5B). We next performed similar comparisons with NK cells stimulated for 7 days with 6,000 IU/mL IL-2. Increase IL-2 stimulation appeared to enhance cytotoxicity of NK cells, but median cytotoxicities produced by NKAES-NK cells remained the highest at all E:T ratios and were significantly higher at 1:2 and 1:4 E:T (Fig. 5C). Finally, we determined whether exposure of NKAES-NK to 1,000 IU/mL IL-2 for 16 h was important to achieve the high cytotoxicities observed. In experiments with NKAES-NK cells from 3 donors tested against K562, HL-60, KG1,

and U937, there were no significant differences with and without the additional incubation with IL-2 (data not shown).

**Cytotoxicity of NKAES-NK cells *in vivo*.** NKAES-NK cells injected intravenously into immunodeficient mice were detectable in peripheral blood after 4 days, representing  $0.78 \pm 0.52\%$  or  $10.1 \pm 6.7/\text{mm}^3$  (mean  $\pm$  SD;  $n = 6$ ) of mononuclear cells, but decreased to <0.1% ( $1.5/\text{mm}^3$ ) on day 10 and became undetectable thereafter. By contrast, when injection of NKAES-NK cells was accompanied by intraperitoneal injection of IL-2 (25,000 IU; daily during the first week and once every 2 days in the following weeks), the percentage of NK cells was much higher:  $6.2 \pm 4.1\%$  on day 4,  $7.0 \pm 2.5\%$  on day 17, and  $8.4 \pm 3.9\%$  on day 24 ( $106.2 \pm 69.3/\text{mm}^3$ ,  $179.3 \pm 65.0/\text{mm}^3$ , and  $215.5 \pm 98.9/\text{mm}^3$ , respectively). They were still detectable on day 31 at  $\sim 0.1\%$  and became undetectable on day 38.

We then generated murine models of AML by injecting luciferase-labeled AML cell lines in immunodeficient (NOD/scid-IL2Rgnull) mice. K562 and U937 cells ( $0.2-1 \times 10^6$ ) promptly engrafted in these mice regardless of whether they were injected intraperitoneally or intravenously. In initial experiments, we injected K562 intraperitoneally or intravenously followed by intraperitoneal injection of  $1 \times 10^7$  NKAES-NK cells 1 day later. Results were compared with those for mice injected with K562 cells in the same way but not treated with NK cells. In all of the mice injected with NKAES-NK cells, there was a marked suppression of leukemic cell growth and/or a decrease in the leukemic cell burden, although the growth of AML cells eventually progressed (Supplementary Fig. S3).

Mice engrafted with either K562 or U937 cells were also treated with multiple injections of NKAES-NK cells and IL-2. As illustrated in Fig. 6 and Supplementary Fig. S4, disease progression in mice treated with NK cells was either substantially delayed or completely



**Figure 4.** Antileukemic activity of NKAES-NK cells against AML cells. **A**, 4 h cytotoxicity against leukemic cell lines with NK cells from 12 donors. Mean of four measurements at each E:T ratio. **B**, 4 h cytotoxicity of NKAES-NK cells from 4 donors against AML cells from 2 patients. **C**, cytotoxicity against AML cells from 5 patients after 5 d of culture on MSC. Mean  $\pm$  SD cell killing at the indicated E:T ratios in triplicate cultures.

abrogated, with several mice achieving long-term remissions and no apparent signs of xenogeneic graft-versus-host disease. Finally, we tested whether initiation of NKAES-NK cell therapy 11 days after injecting mice with AML cells would still result in an antileukemic effect. NKAES-NK cells had a significantly antileukemic effect also with this schedule, although they did not eradicate leukemia (Supplementary Fig. S5). These results confirm the antileukemic capacity of NKAES-NK cells and suggest that multiple infusions of NK cells are likely to be required to maximize their therapeutic effect.

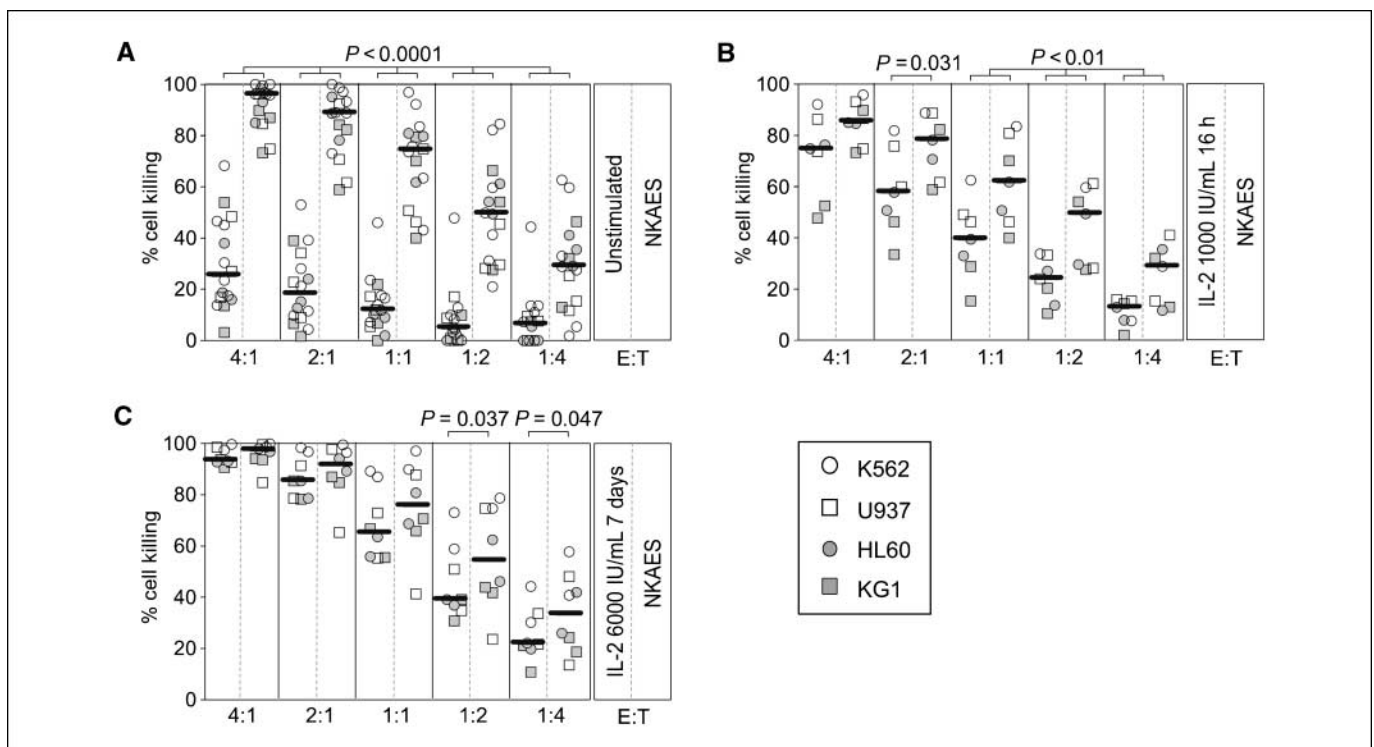
**Generation of clinical-grade expanded NK cells.** We developed a Master Cell Bank of K562-mb15-41BBL cells following cGMP guidelines. We used these cells to stimulate the expansion of NK cells from apheresis products under clinical-grade large-scale conditions. Expansion of CD56<sup>+</sup>CD3<sup>-</sup> cells ranged from 33- to 141-fold (median, 90.5-fold;  $n = 12$ ) after 7 days of culture (Supplementary Table S6). On day 7, CD56<sup>+</sup>CD3<sup>-</sup> cells represented 83.1% (range, 72.9-85.9%) of the resulting cell population; CD56<sup>+</sup>CD3<sup>+</sup> NK/T cells were 3.2% (1.7-9.5%) and CD56<sup>-</sup>CD3<sup>+</sup> T lymphocytes were 9.1% (5.6-13.5%; Supplementary Table S6); the remaining cells were B cells and monocytes. Therefore, the overall NK cell yield in these experiments was higher than that observed in small-scale experiments (Fig. 1B). Comparative experiments (data not shown) indicated that this difference was attributable to the use of SCGM tissue culture medium, instead of RPMI 1640, in the large-scale expansions, a medium that appears to be well suited to support NK cell growth (25). The resulting NK cells were indistinguishable from NK cells expanded under the original culture conditions; their cytotoxicity against the AML cell lines K562, U937, HL-60, and KG1 was within the range of the cytotoxicities observed with

the cells obtained with small-scale NKAES cultures (data not shown). Thus, large numbers of highly cytotoxic NK cells can be generated under conditions that meet clinical and regulatory requirements for cell therapy.

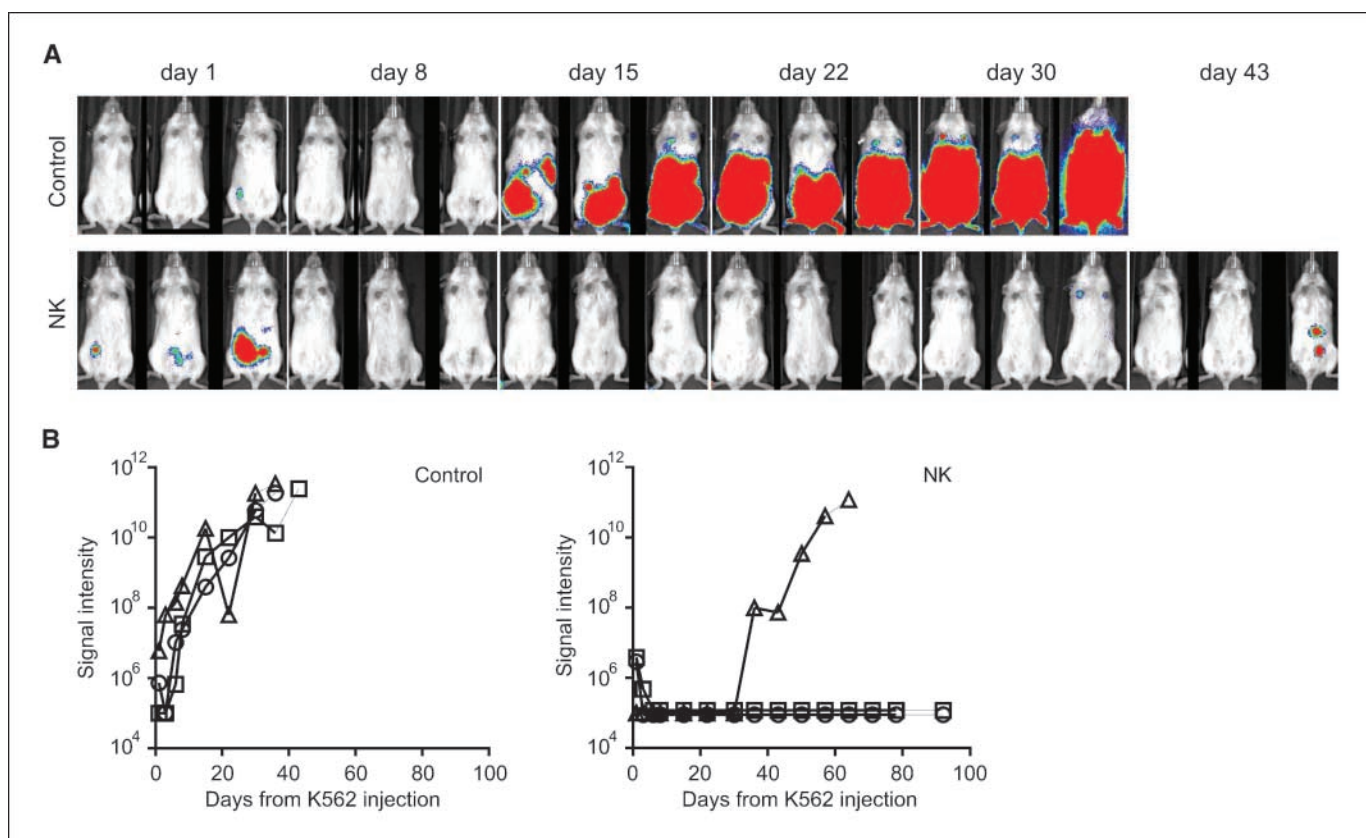
## Discussion

We found that K562-mb15-41BBL cells induce sustained and specific proliferation of human NK cells. NK cell expansion was observed in all donors tested, including patients with acute leukemia undergoing therapy, with no apparent proliferative advantage of any particular NK cell subset. Gene expression of NKAES-NK cells was markedly different than that of unstimulated and IL-2-stimulated cells not only in regards to their expression of cell proliferation-associated genes but also in that of molecules that might regulate NK cell function and their interaction with other cell types. NKAES-NK cells had powerful cytotoxicity against AML cell lines and AML cells from patients and were more potent than unstimulated or IL-2-activated NK cells from the same donors. Based on these findings, and on the effectiveness of NKAES-NK cells in murine models of AML, we developed a Master Cell Bank of K562-mb15-41BBL cells under cGMP guidelines and showed that large-scale expansion and activation of human NK cells for clinical studies was feasible, producing expansions of CD56<sup>+</sup>CD3<sup>-</sup> cells that were even higher than those observed in the initial small-scale experiments while maintaining high anti-AML cytotoxicity.

IL-2 can induce proliferative responses in human NK cells, but only a minor fraction sustains continued growth (10, 26, 27). Conceivably, some NK cell subsets might be more responsive as



**Figure 5.** Antileukemic activity of NKAES-NK cells compared with that of unstimulated and IL-2-stimulated NK cells. *A*, cytotoxicity of unstimulated and NKAES-NK cells (from the same donors;  $n = 12$ ) was tested against leukemic cell lines. Mean of two measurements after 4 h culture. *Bars*, median. Similar comparisons done between NKAES-NK cells and NK cells stimulated with 1,000 IU/mL IL-2 for 16 h (from 3 donors; *B*) or 6,000 IU/mL IL-2 for 7 d (from 2 donors; *C*). NK cells from the same donors were tested in each comparison.



**Figure 6.** Antileukemic activity of NKAES-NK cells *in vivo*. NOD/scid-IL2R $\gamma$  mice ( $n = 6$ ) were injected with K562 cells expressing luciferase ( $2 \times 10^5$ ) intraperitoneally. Then, NKAES-NK cells ( $1 \times 10^7$ ) from the same donor were injected every 2 days intraperitoneally in 3 mice, from days 1 to 11 after K562 injection. All 6 mice received 25,000 IU IL-2 intraperitoneally daily for 3 weeks. **A**, leukemia cell growth was visualized through luciferin injection and Xenogen imaging (ventral is shown). Leukemia progressed in all 3 mice not treated with NK cells (*top*) and mice were euthanized between days 37 and 44. By contrast, leukemia progression was not apparent in 2 of the 3 mice receiving NK cells, which remain alive and leukemia-free 8 months after the beginning of the experiment; in a third mouse, leukemia became detectable on day 43 (the mouse was euthanized on day 70; *bottom*). **B**, signal intensities (photons/s) detected in control mice (*left*) and NK-treated mice (*right*).

suggested by early reports of up to 50-fold expansion after culture with IL-2 for 2 weeks of a NK subset that adheres to plastic (28–31). It is unclear, however, whether some CD3<sup>+</sup> cells might have had, at least in part, contributed to the increased cell numbers (29, 30). More recently, anti-CD3 and IL-2 reportedly induced 190-fold NK expansions after 21 days from the blood of healthy individuals (32) and, surprisingly, 1,600-fold expansions after 20 days from that of patients with myeloma (25). However, the cytotoxicity of these cells against K562 cells was <10% at 1:1 E:T (25), a ratio at which NKAES-NK cells from healthy donors or leukemia patients had a median cytotoxicity of 69% cells. Our results with IL-2 alone or in combination with other cytokines are in line with those of earlier reports (10, 26, 27, 33). Indeed, most investigators have indicated that sustained expansions of CD56<sup>+</sup>CD3<sup>-</sup> cells require additional signals (14, 16), such as the presence of B-lymphoblastoid cells (26, 34, 35). B-lymphoblastoid cells, however, also induce vigorous expansions of T lymphocytes, whereas NKAES cultures do not stimulate T-cell proliferation. In the setting of allogeneic NK cell therapy, this could be an important practical advantage as it would facilitate the complete removal of residual T cells at the end of the cultures (to avoid the risk of graft-versus-host disease). Because K562-mb15-41BBL cells are lethally irradiated before culture and they are lysed by the expanding NK cells, the risk of infusing viable K562-mb15-41BBL is negligible. Nevertheless, we have incorporated safeguards in our clinical protocol. We prepare cultures of irradiated

K562-mb15-41BBL cells and monitor their growth and DNA synthesis rate. We also test for the presence of viable K562-mb15-41BBL cells at the end of the culture by flow cytometry using green fluorescent protein as a marker. The clinical product is released only if there is no cell growth and no viable of K562-mb15-41BBL cell at the end of the cultures.

Most patients with AML respond to initial treatment and achieve remission, but occult resistant leukemia persists in approximately half of the patients, leading to overt (and usually fatal) relapse (36, 37). NK cell infusions have shown to be clinically effective in patients with high-risk AML (8); they are being considered for the therapy of other hematologic malignancies (9, 38). Conceivably, NK cell therapy will be most powerful when the number of NK cells infused is sufficiently high to produce a high E:T ratio. In our murine models of AML, multiple injections of NKAES-derived cells were required to eradicate leukemia and achieve long-term remissions. The number of NK cells that can be generated with the method that we describe should meet the requirement for a high E:T ratio, particularly in the setting of minimal residual disease, and allow multiple NK cell infusions. We found that administration of IL-2 significantly prolonged the survival of NKAES-NK cells in immunodeficient mice. It is possible that other cytokines not yet available for clinical studies, such as IL-15, might prove to be superior for this purpose. Of note, it was shown in clinical studies that lymphodepletion of the recipients, a procedure essential to

ensure prolonged engraftment of the infused cells (39), resulted in high levels of serum IL-15 (8).

Although infusion of allogeneic unstimulated or IL-2-stimulated NK cells has been proven to be safe, with no significant graft-versus-host disease detected, the safety of NKAES-NK cell infusions must be established. To this end, we have begun a phase I dose-escalation clinical study of haploidentical NKAES-NK cells in patients with refractory leukemia. In addition to AML and other hematologic malignancies, some solid tumors should also be susceptible to NK cell cytotoxicity (9). Therefore, patients with these malignancies could also be eligible for clinical studies of NK cell therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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