

**Cancer Therapy: Preclinical**

See commentary p. 3819

**Cytotoxicity of Activated Natural Killer Cells against Pediatric Solid Tumors**Duck Cho<sup>1</sup>, David R. Shook<sup>1</sup>, Noriko Shimasaki<sup>1</sup>, Yu-Hsiang Chang<sup>1</sup>, Hiroyuki Fujisaki<sup>1</sup>, and Dario Campana<sup>1,2,3</sup>**Abstract**

**Purpose:** To develop new therapies for children with solid tumors, we tested the cytotoxicity of natural killer (NK) cells expanded by coculture with K562-mb15-41BBL cells. We sought to identify the most sensitive tumor subtypes, clarify the molecular interactions regulating cytotoxicity, and determine NK antitumor potential *in vivo*.

**Experimental Design:** We tested *in vitro* cytotoxicity of expanded NK cells against cell lines representative of Ewing sarcoma (EWS;  $n = 5$ ), rhabdomyosarcoma ( $n = 4$ ), neuroblastoma ( $n = 3$ ), and osteosarcoma ( $n = 3$ ), and correlated the results with expression of inhibitory and activating NK receptor ligands. We also compared expanded and primary NK cells, determined the effects of activating receptor ligation and of chemotherapeutic drugs, and assessed the therapeutic effect of NK cell infusions in xenografts.

**Results:** In 45 experiments, EWS and rhabdomyosarcoma cell lines were remarkably sensitive to expanded NK cells, with median cytotoxicities at 1:1 effector/target ratio of 87.2% and 79.1%, respectively. Cytotoxicity was not related to levels of expression of NK receptor ligands, nor was it affected by pretreatment of target cells with daunorubicin or vincristine, but was markedly inhibited by preincubation of NK cells with a combination of antibodies against the NK-activating receptors NKGD2 and DNAM-1. Expanded NK cells were considerably more cytotoxic than unstimulated NK cells, and eradicated EWS cells engrafted in nonobese diabetic/severe combined immunodeficient Il2rnull mice.

**Conclusions:** Among pediatric solid tumors, EWS and rhabdomyosarcoma are exquisitely sensitive to expanded NK cells. The NK expansion method described here has been adapted to large-scale conditions and supports a phase I clinical study including patients with these malignancies. *Clin Cancer Res*; 16(15); 3901–9. ©2010 AACR.

Cure rates for children with acute leukemia have steadily increased, but despite aggressive treatment, nearly half of the patients with solid tumors such as Ewing sarcoma (EWS), rhabdomyosarcoma, osteosarcoma, and neuroblastoma have progressive disease (1–6). Prognosis is particularly poor for those patients with metastatic disease, at least two thirds of whom have disease progression (1–6). Outcome after recurrence is generally dismal. For patients with recurrent EWS, for example, the likelihood of long-term survival is currently <20%, and <10% if relapse occurs within 2 years (1, 7–12). Therefore, new

therapeutic approaches that bypass the cellular mechanisms of drug resistance are urgently needed, particularly for patients with high-risk features, such as metastatic or recurrent disease.

The known potential of immune cells to recognize and kill tumor cells suggests their possible role in anticancer treatment. Natural killer (NK) cells can kill allogeneic hematopoietic cells and have been administered either in the setting of hematopoietic stem cell transplantation or after nonmyeloablative immunosuppressive therapy to enhance the effect of chemotherapy in patients with acute leukemia, yielding encouraging results (13–17). NK cells can also lyse malignant nonhematopoietic cells as shown by reports indicating NK cell cytotoxicity against EWS and osteosarcoma cell lines *in vitro* (18–22), as well as neuroblastoma cell lines *in vitro* (19, 23–25) and *in vivo* (26). Preliminary clinical data suggest that donor NK cells may exert antitumor activity in children with solid tumors undergoing allogeneic hematopoietic stem cell transplant (27).

NK cell cytotoxicity relies on the balance between activating stimuli and suppressive signals, including those delivered by killer immunoglobulin-like receptors that

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### Translational Relevance

Infusions of natural killer (NK) cells are being increasingly considered as a means to improve cancer therapy. In efforts to develop new therapies for children with solid tumors, we sought to identify the tumor subtypes that were most sensitive to the cytotoxicity of NK cells expanded with a method developed in our laboratory. We found that Ewing sarcoma and rhabdomyosarcoma cells were exquisitely sensitive to these NK cells, which were considerably more cytotoxic than unstimulated NK cells and eradicated sarcoma cells engrafted in immunodeficient mice. Because of the results of this study, patients with Ewing sarcoma and rhabdomyosarcoma are now eligible for enrollment in a recently initiated clinical study testing infusions of allogeneic expanded NK cells.

recognize specific MHC class I alleles (28–30). An increase in the NK cell activation status, a decrease in suppressive signals (such in the case of altered or mismatched MHC class I), or both should result in heightened NK cell cytotoxicity. Moreover, the magnitude of NK cell cytotoxicity is directly proportional to the ratio between the number of NK cells and target cells (i.e., effector/target ratio or E:T). Thus, in the context of NK cell therapy, the infusion of large numbers of preactivated, allogeneic NK cells is predicted to achieve maximum anticancer effect. To this end, we developed a method that allows specific activation and expansion of human NK cells from peripheral blood (31–33). In the present study, we determined the cytotoxicity of NK cells generated by this method against pediatric solid tumors.

## Materials and Methods

### Tumor cell lines

The EWS cell lines TC71, SK-N-MC, EW8, and A673 express the *EWS-FLI1* fusion protein (34–36); ES8 was derived at St. Jude Children's Research Hospital and confirmed to contain the t(11;22) abnormality and express the *EWS-FLI1* fusion.<sup>4</sup> The rhabdomyosarcoma cell line RH30 was derived at St. Jude Children's Research Hospital from the bone marrow of a 16.5-year-old male patient with alveolar rhabdomyosarcoma; RH36 (embryonal) and RH41 (alveolar) are extensively characterized rhabdomyosarcoma cell lines incorporated into the National Cancer Institute–supported Pediatric Preclinical Testing Program (37); TE-32 is also an established model of rhabdomyosarcoma (38, 39). The neuroblastoma cell line JF was developed at St. Jude Children's Research Hospital

from a 1-year-old female patient with stage III-C neuroblastoma;<sup>4</sup> SK-N-SH and NB1691 are established neuroblastoma cell lines (37, 40). Likewise, the cell lines U-2 OS, HOS, and MG-63 are well-known models of osteosarcoma (41–43).

The cell lines U-2 OS, HOS, and MG-63 were obtained from the American Type Culture Collection. EW8, RH36, RH41, A673, SK-N-MC, SK-N-SH, and TE-32 were provided by Dr. Peter Houghton; TC71 was provided by Dr. Stephen Skapek; and NB-1691 was provided by Dr. Andrew Davidoff (all providers at St. Jude Children's Research Hospital). ES8, RH30, and JF were available from the St. Jude Children's Research Hospital tissue repository.

The osteosarcoma cell lines U-2 OS, HOS, and MG-63 were maintained in DMEM (Cellgro) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and antibiotics. All other cells were maintained in RPMI 1640 (Invitrogen), also supplemented with 10% FBS and antibiotics. The expression of ligands for NK activating, inhibitory, and adhesion molecules was analyzed by staining with monoclonal antibodies and flow cytometry. Antibodies to human HLA-ABC (G46-2.6), MIC A/B (6D4), CD112 (R2.525), CD54 (LB-2), and CD58 (IC3) were obtained from BD Biosciences; CD155 (300907), ULBP-1 (170818), ULBP-2 (165903), ULBP-3 (166510), and CD48 (394307) were from R&D Systems; and HLA-E (3D12HLA-E) was from eBiosciences.

### NK cell activation and expansion

The K562-mb15-41BBL cell line was made as previously described (31). Briefly, K562 cells were first transduced with a construct encoding the “membrane-bound” form of interleukin (IL)-15 [IL-15 plus CD8 $\alpha$  and green fluorescent protein (GFP)]. Cells with high expression of GFP and of surface IL-15 (“K562-mb15”) were then transduced with human 4-1BB ligand. We transduced the ES8, TC71, and EW8 cell lines with a murine stem cell virus internal ribosome entry site GFP retroviral vector (from the St. Jude Vector Development and Production Shared Resource) containing the firefly luciferase gene (gift of Dr. K. Mihara, University of Hiroshima, Hiroshima, Japan). Transduced cells were selected for their expression of GFP with a MoFlo Cell Sorter (Cytomation).

Peripheral blood samples were obtained from healthy adult donors. Mononuclear cells collected from the samples by centrifugation on a Lymphoprep density step (Nycomed) were washed twice in RPMI 1640. To obtain primary NK cells, we used peripheral blood mononuclear cells and the Miltenyi NK Cell Isolation kit (Miltenyi Biotec).

Peripheral blood mononuclear cells ( $1.5 \times 10^6$ ) were incubated in a 24-well tissue culture plate with  $1 \times 10^6$  K562-mb15-41BBL cells in the presence of 10 IU/mL human IL-2 (National Cancer Institute BRB Preclinical Repository) in RPMI 1640 and 10% FBS. Medium was exchanged every 2 days with fresh medium and IL-2. After 7 days of coculture, residual T cells were removed with Dynabeads CD3 (Invitrogen).

<sup>4</sup> S. Ragsdale, St. Jude Children's Research Hospital, personal communication.

### Cytotoxicity assays

Target cells were suspended in RPMI 1640 tissue culture medium with 10% FBS, labeled with calcein AM, and plated in triplicate onto 96-well flat-bottomed plates (Costar). The plates were placed in an incubator set at 37°C and 5% CO<sub>2</sub> for 4 hours to allow for cell attachment. Expanded NK cells suspended in RPMI 1640 with 10% FBS and 100 IU/mL IL-2 were then added at various E:T ratios as indicated in Results. The plates were then centrifuged and incubated as above for various time periods as indicated in Results. Cells were then detached using trypsin plus EDTA and stained with propidium iodide. Cytotoxicity was measured with a flow cytometry–based method, enumerating the number of viable target cells (calcein AM positive, propidium iodide negative, and with light scattering properties of viable cells) in cultures with and without NK cells (32, 44).

In some experiments, we tested cytotoxicity using luciferase-labeled target cells. These were plated in 96-well, flat-bottomed white Viewplates (Perkin-Elmer) and exposed to NK cells as described above. At the end of the cultures, an equal volume of Promega Bright-Glo luciferase reagent (Promega) was then added to each test well, and after 5 minutes, luminescence was measured using a plate reader (Perkin-Elmer Waltham) and was analyzed with the Packard Fusion software (Packard Bioscience). Viability was calculated by comparing relative luminescent signal from control wells on each plate. All experiments were done in triplicate. Anti-NKG2D (149810; R&D Systems) and anti-DNAM-1 (DX11; BD Biosciences) antibodies were used to test the effect of these molecules on NK cell cytotoxicity.

### Murine models

ES8 cells expressing luciferase were injected i.p. in 8- to 14-week-old NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ [nonobese diabetic/severe combined immunodeficient (NOD/SCID) Il2rgnull] mice (Jackson Laboratory; 2 × 10<sup>5</sup> to 1 × 10<sup>6</sup> cells per mouse). NK cells from healthy donors were expanded for 7 days, resuspended in RPMI 1640 plus 10% FBS (1–3 × 10<sup>7</sup> cells per mouse) and then injected i.p. at the schedules indicated in the Results section. In some experiments, multiple injections of NK cells were given together with i.p. injections of IL-2 (20,000 IU each). Mice receiving tissue culture medium with or without IL-2 instead of NK cells served as controls. EWS engraftment and progression was evaluated using a Xenogen IVIS-200 system (Caliper Life Sciences), with imaging beginning 5 minutes after i.p. injection of an aqueous solution of D-luciferin potassium salt (3 mg/mouse). Photons emitted from luciferase expressing cells were quantified using the Living Image 3.0 software program. Some mice were treated with a single dose of 3.25 Gy whole-body irradiation 7 days after tumor cell injection. Irradiation was delivered by a cesium irradiator with attenuator shields to reduce radiation by ~10-fold from the source. The dose of 3.25 Gy was chosen based on previously published data about radiation sensitivity of NOD/scid Il2rgnull mice (45). If

NK cells were administered, the first infusion was done within 1 hour after the irradiation.

## Results

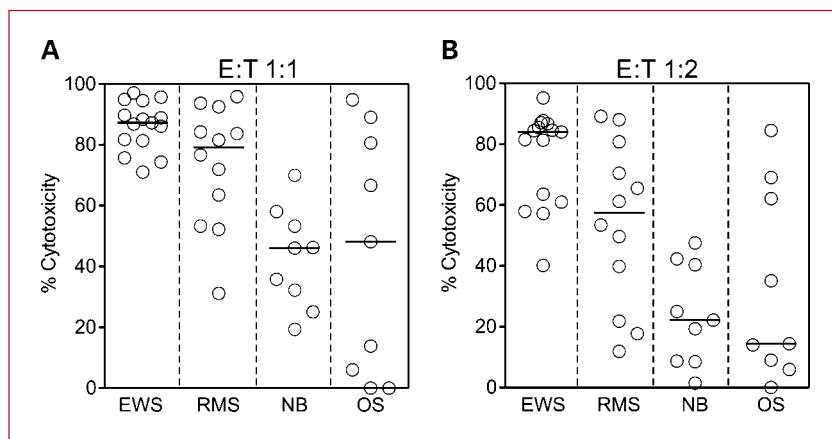
### Relative sensitivity of sarcoma and neuroblastoma cells to the cytotoxicity of expanded NK cells

To determine the relative sensitivity of cells derived from pediatric solid tumors to the cytotoxicity of expanded NK cells, we obtained NK cells from five healthy donors and stimulated them for 7 days by coculture with irradiated K562-mb15-41BBL cells. After depletion of residual T cells, we tested the cytotoxicity of the expanded NK cells against EWS (TC71, SK-N-MC, ES8, EW8, and A673), rhabdomyosarcoma (RH30, RH36, RH41, and TE32), neuroblastoma (NB1691, JF, and SK-N-SH), and osteosarcoma (U-2 OS, HOS, and MG-63) cell lines, for a total of 45 experiments. After 4 hours of culture, there was considerable heterogeneity in the degree of sensitivity to NK cell cytotoxicity among the disease-defined groups. As shown in Fig. 1A, there was a median cytotoxicity of 87.2% at a 1:1 E:T with EWS cell lines, of 79.1% for rhabdomyosarcoma cell lines, but only 46.1% and 48.1% for neuroblastoma and osteosarcoma cell lines, respectively ( $P = 0.0003$ ). At 1:2, median cytotoxicity against EWS cells continued to exceed 80% and was considerably higher than that observed in the other groups ( $P = 0.0002$ ; Fig. 1B). Thus, EWS cells are particularly sensitive to cytotoxicity of expanded NK cells. Among the other groups, susceptibility to NK cells was particularly heterogeneous. For example, median cytotoxicity at 1:1 was 89.0% for the osteosarcoma cell line HOS but 6.0% with the MG-63 cell line.

We examined whether the variable susceptibility to NK cell cytotoxicity was significantly related to their level of HLA class I expression, but it was not (Fig. 2). We also examined the expression of ligands known to trigger NK cell activation, including MIC A/B, ULBP1, ULBP2 and ULBP3 (NKG2D ligands), and CD112 and CD155 (DNAM-1 ligands), and there was no apparent relation between the level of expression of these molecules and susceptibility to NK cell cytotoxicity (Fig. 2). Expression of other molecules such as CD54 and CD58 (adhesion molecules) and HLA-E (NKG2A/C ligand) was also not significantly related to cytotoxicity; CD48 (2B4 ligand) was not expressed in any of the cell lines (data not shown).

### Expanded NK cells are highly and specifically cytotoxic against EWS cells

A previous study reported that EWS cells were sensitive to primary NK cells (20) but the cytotoxicities observed seemed to be much lower than those that we obtained with our expanded NK cells, suggesting that expanded NK cells are more effective. To formally test this possibility, we compared the cytotoxicity of primary and expanded NK cells. As shown in Fig. 3A, expanded NK cells were far more powerful than primary NK cells. For example, mean (±SD) cytotoxicity against the TC71 cell line at 1:1



**Fig. 1.** Susceptibility of cell lines derived from pediatric solid tumors to cytotoxicity by expanded NK cells. Shown are results of 4-h cytotoxicity assays done at 1:1 E:T ratio (A) and at 1:2 E:T ratio (B). Included in the tests were cell lines derived from the EWS family of tumors (TC71, SK-N-MC, ES8, EW8, and A673), rhabdomyosarcoma (RMS; RH30, RH36, RH41, and TE32), neuroblastoma (NB; NB1691, JF, and SK-N-SH), and osteosarcoma (OS; U-2 OS, HOS, and MG-63). Each symbol represents the mean of triplicate measurements relative to control cultures with no NK cells. Horizontal bars, median values in each group.

was  $87.8\% \pm 4.5\%$  ( $n = 3$ ) with expanded NK cells compared with  $14.0\% \pm 9.3\%$  for primary NK cells ( $P < 0.001$ ). Indeed, most EWS cells seemed to be lysed after only 1 hour of coculture (Fig. 3B). To define the limits of NK cell cytotoxicity against EWS cells, we performed tests at lower E:T ratios in 4- and 24-hour cultures. In 4-hour cultures, cytotoxicities dropped to  $<50\%$  at 1:10 and  $<25\%$  at 1:20 (Fig. 4). However, when cultures were prolonged for 24 hours, cytotoxicities increased considerably; in the case of EW8,  $>70\%$  cytotoxicities could be achieved with two of the three donors at 1:20 (Fig. 4).

#### Molecular mechanisms involved in the interaction between expanded NK cells and EWS cells

A previous study showed the ligation of NKG2D and DNAM-1 in primary NK cells could decrease their cytotoxicity against EWS cells (20). Because expanded NK cells express substantially higher levels of NKG2D than primary or IL-2-activated NK cells (32), we determined whether its ligation would affect cytotoxicity. Preincubation of expanded NK cells with the anti-NKG2D antibody 149810 inhibited their cytotoxicity against the EWS cell lines ES8 ( $P = 0.011$ ,  $n = 10$ ) and EW8 ( $P = 0.0004$ ;  $n = 10$ ) at E:T ratios of 1:1 to 1:4, but did not significantly affect cytotoxicity against TC71 ( $P = 0.061$ ;  $n = 10$ ). An anti-DNAM-1 antibody (DX11) had no significant effect on the cytotoxicity against ES8 and TC71; it had a significant but overall modest protective effect on EW8 ( $P = 0.008$ ;  $n = 10$ ). However, both antibodies combined markedly reduced cytotoxicity against all three cell lines ( $P = 0.0002$  for ES8,  $P = 0.004$  for TC71, and  $P = 0.0003$  for EW8), whereas an anti-CD56 antibody, used as a control, had no discernible effect. These results (representative experiments shown in Fig. 5) point to cooperative role for NKG2D and DNAM-1 in the cytotoxicity of expanded NK cells against EWS cells.

Finally, we determined whether pretreatment of cells with chemotherapy drugs commonly used to treat EWS would have any effect on the cell expression of activating ligands and/or the susceptibility to NK cells. Expression of HLA class I, MIC A/B, ULBP1, ULBP2, CD112, and CD155

on ES8 and TC71 cells was not affected by exposure to  $LC_{50}$  concentrations of doxorubicin (10 nmol/L) for 72 hours, and the specific cytotoxicity of NK cells expanded from two donors against the two cell lines remained unchanged. Similar results were obtained with vincristine (1 nmol/L). Therefore, prior exposure to these cytotoxic drugs does not diminish their susceptibility to NK cell killing.

#### Cytotoxicity of expanded NK cells against EWS *in vivo*

We generated a xenograft model of EWS by injecting luciferase-labeled ES8 cells in immunodeficient (NOD/scid Il2r $\gamma$  null) mice (Fig. 6). ES8 cells ( $2 \times 10^5$ ) were injected i.p. in 14 mice; in all 14, ES8 cells formed tumors that progressively expanded. All 14 mice died or were euthanized because of signs of terminal illness and/or large tumor burden; median survival in this group was 42 days. Another group of seven were injected with  $2 \times 10^5$  ES8 cells i.p. and then irradiated 7 days later with 3.25 Gy. Irradiation prolonged survival (median, 53 days;  $P = 0.0043$ ) but eventually all mice died. A third group of six mice was injected with an identical number of ES8 cells i.p. followed by five daily i.p. injections of expanded NK cells and IL-2 i.p. starting 7 days after tumor injection, a protocol previously shown to effectively eradicate leukemia in mice (32). This group also showed a significant improvement in survival (median, 54.5;  $P = 0.0263$ ), and one mouse survived disease-free for  $>100$  days. Finally, we determined whether combining irradiation with NK cell therapy would further improve outcome: mice were irradiated on day 7 posttumor engraftment with 3.25 Gy and then infused with NK cells (five daily i.p. injections of expanded NK cells and IL-2 i.p. starting  $\sim 1$  h after irradiation). Overall survival for the eight mice treated with this approach was remarkably improved ( $P < 0.0001$ ), with five of the eight mice surviving disease free for  $>100$  days.

#### Discussion

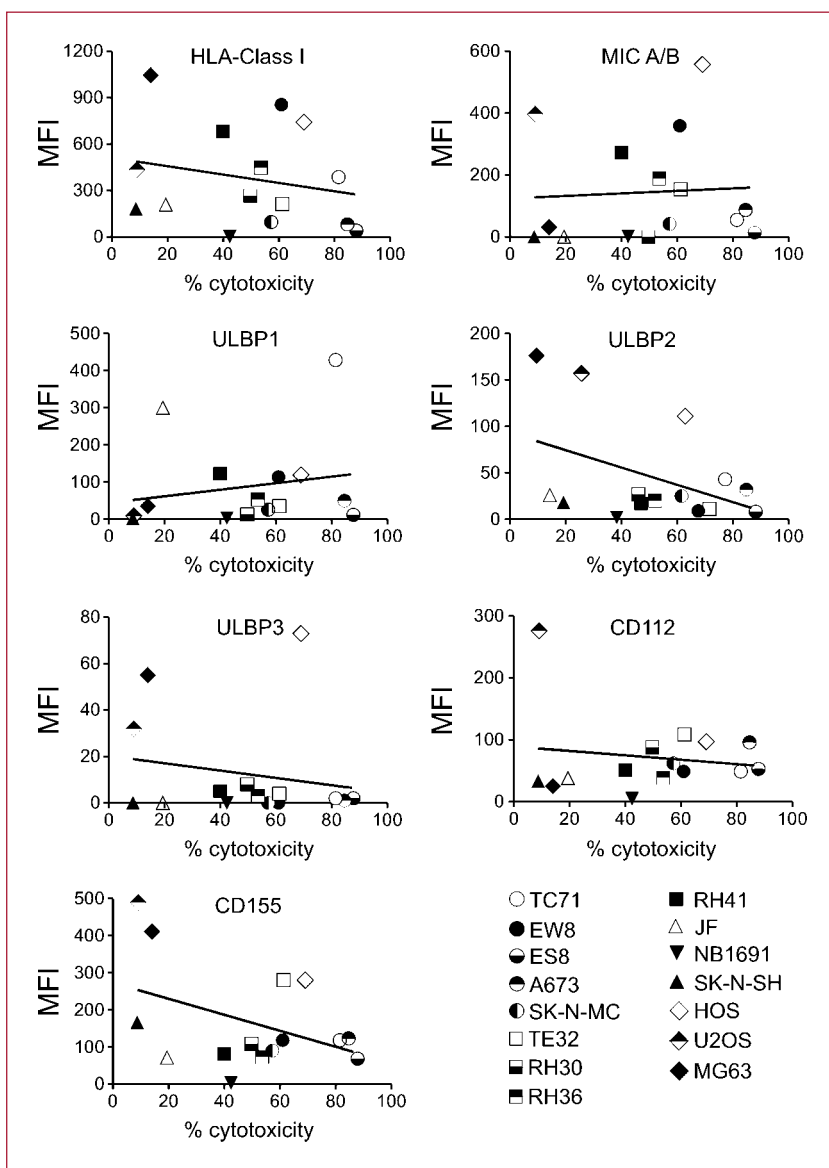
The results of this study indicate that, among pediatric solid tumors, EWS cells are exquisitely sensitive to the cytotoxicity exerted by expanded, activated NK cells.

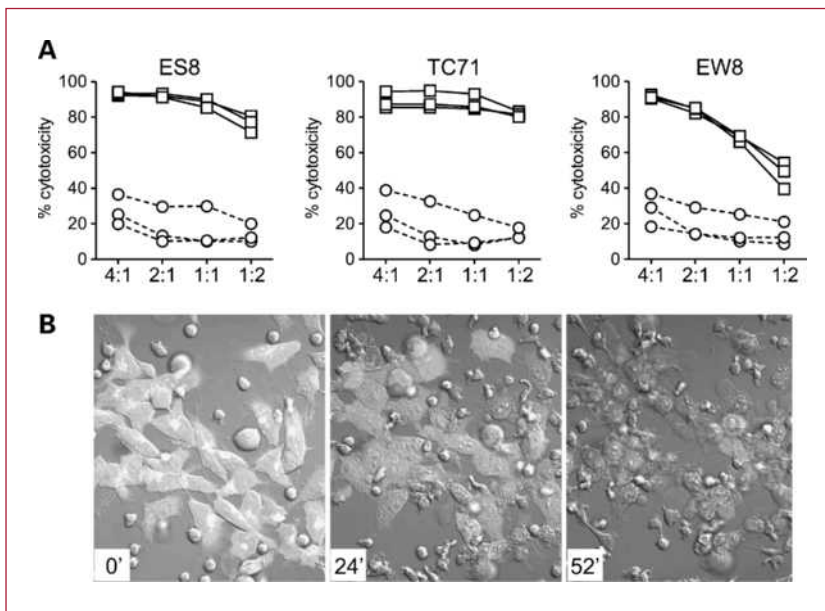
Expanded NK cells could kill nearly all EWS cells within 4 hours at a 1:1 ratio and still be considerably cytotoxic at a 1:10 or lower ratios if cultures were prolonged for 24 hours. Expanded NK cells had much more powerful anti-EWS capacity than primary, nonexpanded, NK cells. The anti-EWS effect of NK cells was also seen in experiments with immunodeficient mice bearing EWS tumors, in which NK cell infusions produced durable remissions. The interaction between NKG2D and DNAM-1 on the surface of NK cells with their ligands on tumor cells seems to be critical for NK cell cytotoxicity against EWS cells, as interference with these molecules considerably reduced cell killing. However, the key ligands remain to be identified.

The results with EWS cells recall those that we previously obtained with expanded NK cells against acute

myeloid leukemia cells (32), suggesting that EWS and acute myeloid leukemia are the main targets for NK cell therapy of cancer in children. Most cell lines derived from other cancer cell types were also sensitive to expanded NK cell cytotoxicity. For example, rhabdomyosarcoma cells were as sensitive as EWS at a 1:1 E:T ratio but less sensitive when the proportion of NK cells was reduced. The sensitivity of osteosarcoma cells varied widely. The molecular mechanisms underlying these differences remain to be elucidated but cannot be attributed to the levels of HLA class I expression in the tumor cells alone. Neuroblastoma cell lines were significantly less sensitive than EWS cells to expanded NK cells, suggesting that neuroblastoma should have a lower priority among pediatric solid tumors eligible for NK cell therapy. NK cell cytotoxicity against neuroblastoma cells might be

**Fig. 2.** Relation between susceptibility to expanded NK cell cytotoxicity and expression of ligands for NK cell inhibitory or activating receptors in pediatric solid tumor cell lines. For each surface molecule, the mean fluorescence intensity (MFI; mean of triplicate measurements) measured in each cell line is plotted on the Y axis, with the percent cytotoxicity measured in 4-h assays at 1:1 E:T ratio plotted on the X axis. Lines in each plot, linear regression analyses.  $R^2$  values were 0.05 for HLA-class I, 0.004 for MIC A/B, 0.19 for ULBP1, 0.19 for ULBP2, 0.03 for ULBP3, 0.06 for CD112, and 0.18 for CD155.



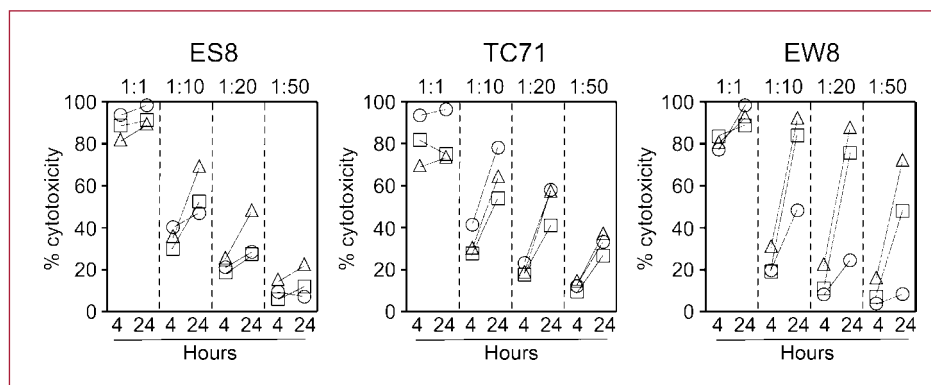


**Fig. 3.** Cytotoxicity of expanded NK cells against EWS cells. A, cytotoxicity of expanded NK cells ( $\square$ ) compared with that of primary NK cells ( $\circ$ ) against the EWS cell lines ES8, TC71 and EW8. Results obtained from three different donors at the E:T ratios indicated are shown. Each symbol is the mean of triplicate measurements relative to control cultures with no NK cells. B, live cell confocal photograph of the ES8 cell line (labeled with GFP) cultured with expanded NK cells at a 2:1 E:T ratio. The time of culture is shown in the lower left corner of each photograph. Microscopy was done with a Nikon TE2000E2 microscope equipped with a Nikon C1Si confocal using 488 and 561 nm DPSS lasers for excitation. Temperature was maintained at  $-37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  using an environmental control chamber. Images were acquired with a Nikon  $\times 40$  1.3 NA DIC objective every 20 s for 2 h using the Nikon EZC1 software. Full movie is in Supplementary Material.

considerably enhanced by directing NK cells with antibodies reacting with neuroblastoma cell markers (46). Moreover, Altwater et al. (47) using the method described here to expand NK cells showed that cytotoxicity could be considerably enhanced by transducing NK cells with a chimeric signaling receptor recognizing the neuroblastoma cell marker GD2.

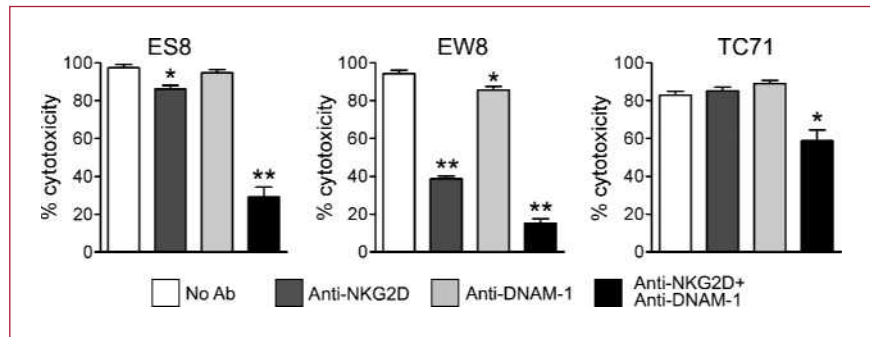
Expanded NK cells resulted in a substantial antitumor effect in mice engrafted with EWS cells, apparently achieving disease eradication in some animals. In our experiments, we also administered IL-2, as we previously observed that this cytokine significantly prolongs the survival of NK cells in immunodeficient mice (32). Moreover, administration of IL-2 is often included in clinical NK cell infusions (17, 48). We also administered multi-

ple infusions of NK cells, because in preliminary experiments, single infusions of NK cells had antitumor activity but did not eradicate the disease. As a caveat, the xenografts that we included in our experiments are not orthotopic models of EWS, and NK cells might not migrate effectively to tissues where EWS tumor cells typically reside, resulting in suboptimal E:T ratios at the tumor site. However, we suggest that NK cell infusions might be more effective against tumor cells metastasizing to the bone marrow and lymphoid organs (tissues where NK cells thrive), rather than against primary tumor masses. Thus, infusions of allogeneic expanded NK cells should enhance the effects of chemotherapy followed by autologous hematopoietic cell transplantation, while reducing the risk of disease recurrence from carryover



**Fig. 4.** Cytotoxicity of expanded NK cells against EWS cell lines at low E:T ratios. Shown are percentage of cytotoxicity (relative to cultures with no NK cells) in cocultures lasting 4 and 24 h at different E:T ratios. Symbols are mean of triplicate measurements; each NK donor is indicated by a different symbol. In the case of the 4-h measurements, SDs for all cell lines and all donors were  $<4\%$  at 1:1,  $<5\%$  at 1:10, and  $<7\%$  at 1:20 and at 1:50; for 24-h measurements, they were  $<1\%$  at 1:1,  $<5\%$  at 1:10,  $<6\%$  at 1:20, and  $<10\%$  at 1:50.

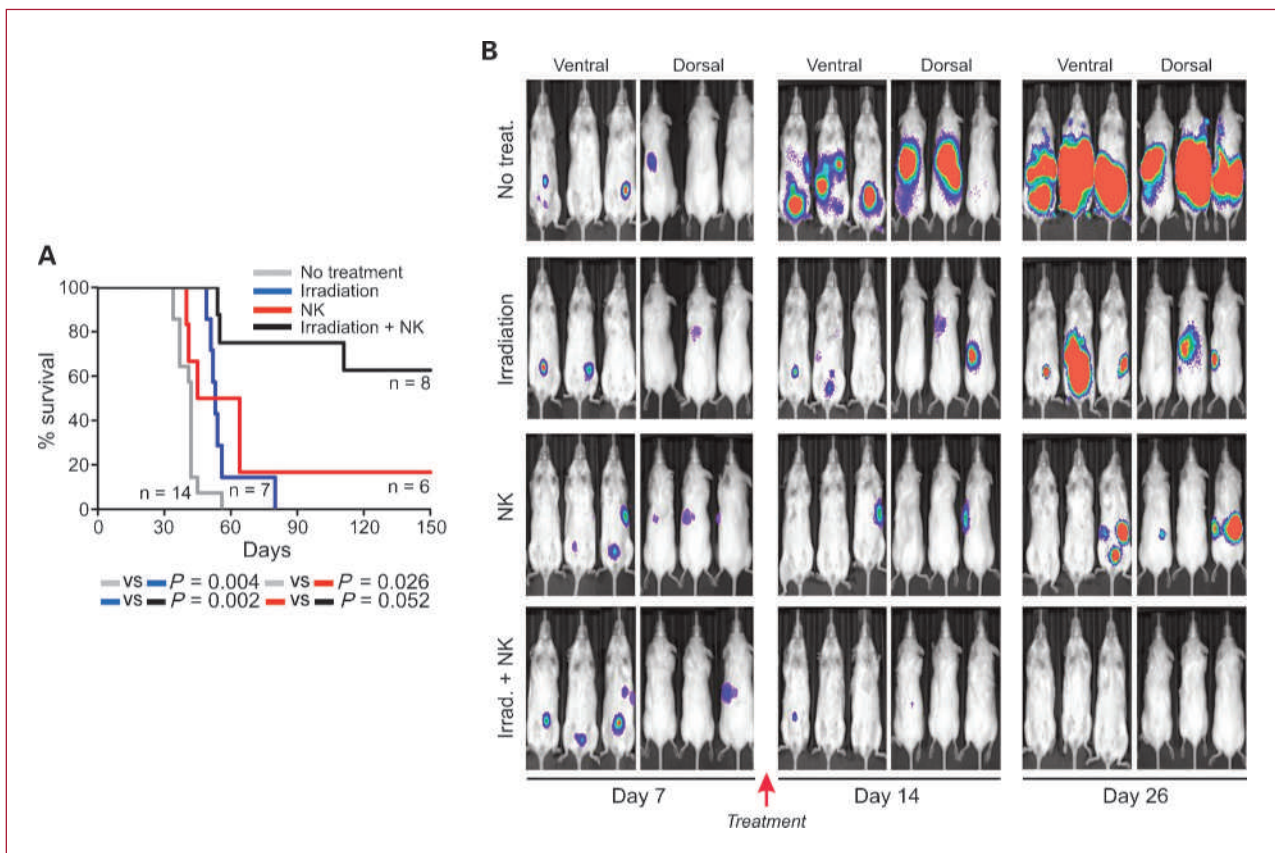
**Fig. 5.** Effect of ligating NK-activating receptors on the cytotoxicity of expanded NK cells against EWS cell lines. Cocultures of EWS with NK cells were done after incubating NK cells for 20 min with anti-NKG2D and anti-DNAM-1 antibodies (Ab), alone or in combination. Columns, mean percent cytotoxicity of triplicate measurements (relative to control cultures with no NK cells); bars, SD. \*,  $P < 0.01$ ; \*\*,  $P < 0.0001$  by  $t$  test.



tumor cells infused with the autograft. Alternatively, NK cells could be directly injected into the tumor site after surgical removal, in efforts to deplete the margins of residual tumor cells.

To be effective, NK cell infusions must achieve E:T ratios estimated to exert substantial tumor cytorreduction. In this regard, the capacity of our method to specifically expand NK cells to large numbers in addition to activate them, is particularly useful. Seven-day cultures, such as those used in this study, should yield sufficient NK cells

for single infusions. Additional stimulation with K562-mb15-41BBL cells and extended cultures can dramatically increase the number of NK cells obtained, which should be adequate for multiple infusions. The NK expansion method described here has now been adapted to large scale, clinical grade, conditions, and a clinical protocol testing the feasibility and toxicity of this approach is now open. Because of the results of this study, eligibility includes patients with EWS and rhabdomyosarcoma.



**Fig. 6.** Antitumor capacity of expanded NK cells *in vivo*. A, NOD/scid Il2rgnull were injected with  $2 \times 10^5$  ES8 i.p. Seven days later, mice were either irradiated with 3.25 Gy, treated with  $1 \times 10^7$  expanded NK cells and 20,000 IU IL-2 i.p. daily injections for 5 d with or without prior irradiation, or left nontreated. Kaplan-Meier curves indicate the survival of each group of mice;  $P$  values in comparisons between groups by log-rank test are shown. B, Xenogen imaging of ES8-luciferase tumors in four groups of three mice each. Mice received the treatment described in A or no treatment.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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