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Research Article

Cancer Immunology Research

# Expanded and Activated Natural Killer Cells for Immunotherapy of Hepatocellular Carcinoma

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## Abstract

Viral infection of the liver is a major risk factor for hepatocellular carcinoma (HCC). Natural killer (NK) cells recognize virally infected and oncogenically transformed cells, suggesting a therapeutic role for NK-cell infusions in HCC. Using the K562-mb15-41BBL cell line as a stimulus, we obtained large numbers of activated NK cells from the peripheral blood of healthy donors. Expanded NK cells exerted remarkably high cytotoxicity against HCC cell lines, which was generally much higher than that of unstimulated or IL2-activated NK cells. In immunodeficient NOD/scid IL2RGnull mice engrafted with Hep3B, treatment with expanded NK cells markedly reduced tumor growth and improved overall survival. HCC cells exposed for 48 hours to 5 μmol/L of sorafenib, a kinase inhibitor currently used for HCC treatment, remained highly sensitive to expanded NK cells. HCC cell reductions of 39.2% to 53.8% caused by sorafenib in three cell lines further increased to 80.5% to 87.6% after 4 hours of culture with NK cells at a 1:1 effector-to-target ratio. NK-cell cytotoxicity persisted even in the presence of sorafenib. We found that NKG2D, an NK-cell–activating receptor, was an important mediator of anti-HCC activity. We therefore enhanced its signaling capacity with a chimeric NKG2D-CD3ζ-DAP10 receptor. This considerably increased the anti-HCC cytotoxicity of expanded NK cells *in vitro* and in immunodeficient mice. The NK expansion and activation method applied in this study has been adapted to clinical-grade conditions. Hence, these results warrant clinical testing of expanded NK-cell infusions in patients with HCC, possibly after genetic modification with NKG2D-CD3ζ-DAP10. *Cancer Immunol Res; 4(7); 574–81. ©2016 AACR.*

## Introduction

Hepatocellular carcinoma (HCC), the second most common cause of death from cancer worldwide (1), typically arises from a background of chronic liver inflammation caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (2–4). For HBV (a DNA virus), there is also evidence of viral integration, leading to expression of HBV-associated antigens in tumor cells (5–7). Early stage HCC can be cured by tumor ablation, resection, or liver transplantation, but most patients have more advanced disease at diagnosis (8, 9). For these patients and for those who relapse after liver transplant, current treatment is aimed at prolonging survival, with a median life expectancy of about 8 to 11 months (8, 9). It is clear that significant improvements in clinical outcome can come only from novel therapies capable of eluding HCC drug resistance.

Interest is growing in the use of immunotherapy for cancer, bolstered by the remarkable responses obtained with immune checkpoint inhibitors and with chimeric antigen receptor–directed T lymphocytes (10, 11). In HCC, a number of immunotherapeutic approaches have been explored, including the use of cytokines and chemokines, tumor vaccines, and PD-1 or CTLA-4 blockade (12, 13). Because HCC cells can express HBV-derived peptides, as well as oncofetal proteins and cancer-testis antigens, adoptive immunotherapy using T lymphocytes redirected with T-cell receptors (TCR) against these peptides represents an attractive possibility. Indeed, T cells expressing TCR anti-HBV envelope antigens are cytotoxic against HCC cell lines (14, 15); infusion of such cells in a patient with HCC resulted in their *in vivo* expansion and a reduction in hepatitis B surface antigen (HBsAg) without apparent liver toxicity (16). A practical constraint of this approach is that TCRs must be tailored to the patient's HLA type, which may limit patient eligibility if HLA-matched TCRs are not available. The risk of graft-versus-host disease (GvHD) or rapid rejection needs to be averted, so this approach requires the use of autologous T cells, which may be functionally impaired in patients.

Natural killer (NK) cells can recognize virally infected or transformed cells based on their expression of ligands for inhibitory and stimulatory NK receptors (17). Allogeneic NK cells have marked clinical antitumor activity in acute leukemia without causing GvHD or liver toxicity (18–21). Results of clinical trials in which cytokine-activated autologous lymphocytes containing mostly NK cells were infused in patients with HCC after ablation or resection (22, 23) indicate that this approach warrants further exploration.

Human NK cells can be activated and expanded *ex vivo* by coculture with the K562-mb15-41BBL cell line, yielding an abundance of highly cytotoxic NK cells (24, 25). In this study, we tested the capacity of these NK cells to exert cytotoxicity against HCC cells *in vitro* and in xenograft models, assessed their activity in the context of sorafenib treatment, and determined whether genetic modification of NK cells with a chimeric

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

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receptor that enhances NK activation signals could further augment anti-HCC cytotoxicity.

## Materials and Methods

### Tumor cell lines

The human HCC cell lines Hep3B, SNU-398, HepG2, SNU-449, and PLC/PRF/5 were purchased from the American Type Culture Collection (ATCC) in May–December 2012. Cell lines were maintained in either DMEM (HyClone; GE Healthcare; for Hep3B, HepG2, and PLC/PRF/5) or RPMI-1640 (Thermo Fisher; SNU-398 and -449); media were supplemented with 10% FBS (GE Healthcare) and antibiotics. Cells from early passages (fewer than 6 months from receipt or thawing) were used for all described experiments. For the visualization of injected tumor cells in immunodeficient mice, we transduced the Hep3B cell line with a murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-GFP retroviral vector (from the Vector Development and Production Shared Resource of St. Jude Children's Research Hospital, Memphis, TN) containing the firefly luciferase gene. Transduced cells were selected for their expression of GFP with a MoFlo cell sorter (Beckman Coulter). The luciferase-positive HepG2 cell line was obtained from Dr. A. Bertoletti (Duke-NUS, Singapore) in June 2012 and its origin validated by DNA fingerprinting (DSMZ). The acute leukemia cell line K562 (obtained from the ATCC in April 2014) was grown in RPMI-1640 and 10% FBS. Expression of HLA class I was tested using an antibody to human HLA-ABC conjugated to phycoerythrin (PE; G46-2.6; BD Biosciences). Expression of NKG2D ligands was tested with an NKG2D-IgFc chimeric probe (R&D) followed by a goat-anti–human IgG conjugated to PE (Southern Biotechnology Associates). Binding was visualized using an Accuri C6 flow cytometry (BD Biosciences).

### Human NK-cell expansion

Peripheral blood samples were obtained from discarded anonymized byproducts of platelet donations from healthy adult donors at the National University Hospital Blood Bank, Singapore.

Mononucleated cells were separated by centrifugation on a Lymphoprep density step (Nycomed) and washed twice in RPMI-1640. To purify primary NK cells from peripheral blood mononucleated cells, we used the NK Cell Isolation Kit from Miltenyi. To expand NK cells, we cocultured mononuclear cells and the genetically modified K562-mb15-41BBL cell line made in our laboratory as previously described (24, 25). Briefly, peripheral blood mononucleated cells ($3 \times 10^6$) were cultured in a 6-well tissue culture plate with $2 \times 10^6$ irradiated (100 Gy) K562-mb15-41BBL cells in SCGM medium (CellGenix) containing 10% FBS and 40 IU/mL human IL2 (Novartis). Every 2 to 3 days, fresh tissue culture medium and IL2 were added. After 7 days of coculture, residual T cells were removed using Dynabeads CD3 (Thermo Fisher), producing cell populations containing >90% $CD56^+$ $CD3^-$ NK cells. Expanded NK cells were maintained in SCGM with FBS, antibiotics, and 400 IU/mL IL2 for up to 9 days before the experiments.

In some experiments, an aliquot of peripheral blood mononucleated cells was used to obtain lymphocytes; T lymphocytes were activated by stimulation with Dynabeads Human T-Activator CD3/CD28 Thermo Fisher) for 7 days.

### Retrovirus production and NK-cell transduction

The RD114-pseudotyped MSCV retrovirus containing the NKG2D-CD3ζ-IRES-DAP10 construct, composed of the cDNA encoding NKG2D, the intracellular domain of CD3ζ, and DAP10, was previously described (26). To transduce NK cells, retroviral vector-conditioned medium was added to RetroNectin (Takara)-coated polypropylene tubes; after centrifugation and removal of the supernatant, expanded NK cells ($5 \times 10^5$) were added to the tubes and left at 37°C for 12 hours; fresh viral supernatant was added on 2 other successive days (26). Cells were then maintained in SCGM with FBS, antibiotics, and 400 IU/mL of IL2 until the time of the experiments, 3 to 10 days after transduction. Surface expression of NKG2D was analyzed by flow cytometry using an anti-human NKG2D antibody conjugated to PE or peridinin chlorophyll protein (PerCP; R&D).

### Cytotoxicity assays

Target cells were suspended in RPMI-1640 with 10% FBS, labeled with calcein AM, and plated into 96-well flat bottom plates (Costar). The plates were placed in an incubator for at least 4 hours to allow for cell attachment before adding NK cells. Primary and/or expanded NK cells, suspended in RPMI-1640 with 10% FBS, were then added at various effector-to-target (E:T) ratios as indicated in Results and cocultured with target cells for 4 hours. At the end of the cultures, the supernatant was removed, replaced with PBS, and the calcein AM signal was measured using an FLx800 plate reader (BioTek). In some tests, we used luciferase-labeled cells and measured cell killing with the same plate reader after adding BrightGlo (Promega) to the wells.

To block NKG2D binding to its ligands, we used a purified nonconjugated antibody to NKG2D (149810; R&D). An isotype matched nonreactive immunoglobulin (R&D) and CD56 antibody (N901; Beckman Coulter) were used as controls.

In some experiments, HCC cells were treated for 24 to 48 hours with sorafenib (Selleckchem; 2–10 μmol/L). Cells were then detached by treatment with trypsin, washed, and processed for NK-cell cytotoxicity testing.

### Xenograft model

Hep3B cells expressing luciferase were injected i.p. in NOD.Cg-$Prkdc^{scid}$ $IL2rg^{tm1Wjl}$/SzJ (NOD/scid IL2RGnull) mice (The Jackson Laboratory; $1 \times 10^6$ per mouse). NK cells were expanded for 7 days, resuspended in RPMI-1640 plus 10% FBS, and then injected i.p. ($1 \times 10^7$ cells per mouse) 7 days after HCC cells (Hep3B or HepG2) injection. One to five additional injections of NK cells were given within the following week; mice also received i.p. injections of IL2 (20,000 IU each) 3 times per week. As a control, a group of mice received tissue culture medium instead of NK cells. In another experiment, we injected NK cells transduced with NKG2D-DAP10-CD3ζ and mock-transduced NK cells, with 2 injections starting 7 days after HCC cell injection. HCC cell engraftment and progression were 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; Perkin Elmer). Photons emitted from luciferase-expression cells were quantified using the Living Image 3.0 software program. Mice were euthanized when bioluminescence reached $1 \times 10^{10}$ (Hep3B) or $1 \times 10^{11}$ photons/second (HepG2), or earlier if they showed physical signs

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warranting euthanasia. All animal experiments were performed in accordance with a protocol approved by the National University of Singapore Institutional Animal Care and Use Committee.

## Results

### Expanded activated NK cells exert high cytotoxicity against HCC cells

We determined the cytotoxic capacity of expanded NK cells from 23 healthy donors against the HCC cell lines Hep3B, SNU-398, HepG2, SNU-449, and PLC/PRF/5; we performed 110 experiments, each with multiple measurements at various E:T ratios. Expanded NK cells were highly cytotoxic (Fig. 1A; Supplementary Fig. S1). For example, at 2:1 E:T, mean (± SD) cytotoxicity at 4 hours was 89.4% ± 10.5% for Hep3B, 82.8% ± 13.9% for SNU-398, 75.2% ± 9.7% for HepG2, 63.6% ± 23.6% for SNU-449, and 70.1% ± 20.6% for PLC/PRF/5. When the assays were prolonged to 24 hours, there was high cytotoxicity even at very low E:T ratios: at 1:10, 73.9% ± 2.0% for Hep3B, 51.7% ± 4.6% for SNU-449, and 47.2% ± 8.1% for PLC/PRF/5; at 1:20, 70.7% ± 1.6%, 39.7% ± 4.7%, and 36.9% ± 8.6%, respectively (Fig. 1B). The range of cytotoxicity against HCC cells approached that measured against the leukemia cell line K562, a highly sensitive NK-cell target (Fig. 1A; Supplementary Fig. S1). This is remarkable because, contrary to K562 cells, all HCC cell lines expressed HLA-Class I molecules (Supplementary Fig. S2), which can engage inhibitory receptors on NK cells (17).

To determine if the NK-cell expansion procedure by coculture with K562-mb15-41BBL cells resulted in an increased NK-cell potency against HCC cells, we compared the cytotoxicity of expanded NK cells with that of purified NK cells from the same donors prior to stimulation. As shown in Fig. 1C, expanded NK cells were much more effective ($P < 0.0001$ for each of the 5 cell lines analyzed by $t$ test or two-way ANOVA). Similarly, expanded NK cells were also more powerful than purified NK cells stimulated overnight with 1,000 IU/mL of IL2 for all cell lines ($P < 0.0001$), with the exception of SNU-398, which was equally sensitive to expanded or IL2-stimulated NK cells (Fig. 1D).

### Anti-HCC activity of expanded NK cells in a xenograft model

To further test the anti-HCC capacity of expanded NK cells, we engrafted NOD/scid IL2RGnull mice with Hep3B cells expressing the luciferase gene. Of the 14 mice injected with Hep3B cells, 9 received treatment with expanded NK cells 7 days after tumor engraftment, whereas 5 received injections of medium alone. Tumor expansion, as measured by *in vivo* imaging, occurred at a much slower rate in mice treated with NK cells. Thus, on day 14, median tumor size relative to measurements on day 7 was 277.6% in control mice and 87.9% in NK-treated mice; on day 21, median percentages were 1,048.7% and 128.0%, respectively ($P < 0.01$ for either comparison; Fig. 2A; Supplementary Fig. S3). In agreement with these results, NK-cell treatment significantly improved overall survival (Fig. 2B; $P < 0.001$).

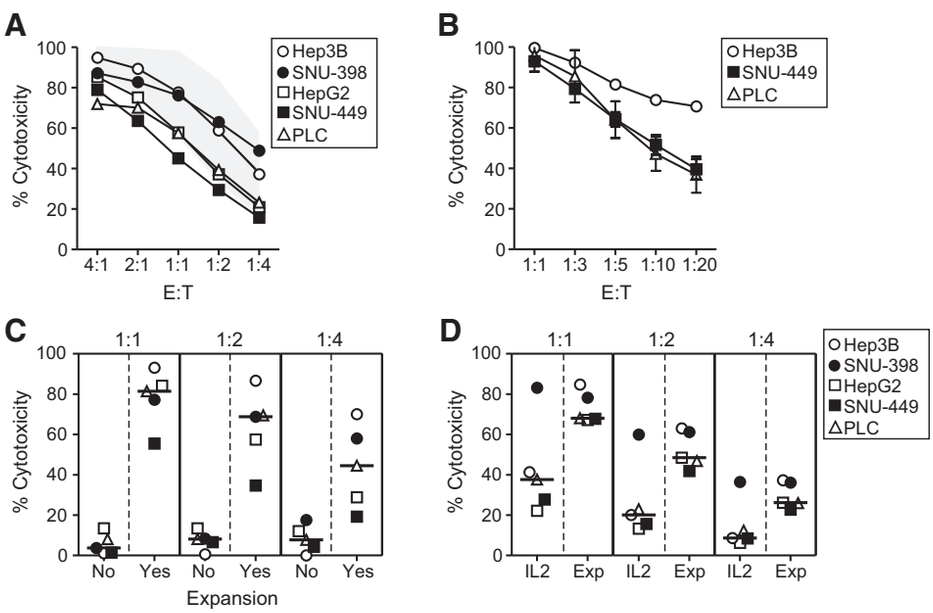


**Figure 1.**

Cytotoxicity of expanded NK cells against HCC. A, symbols represent mean percentage of cytotoxicity measured at 4 hours in experiments with Hep3B ($n = 27$), SNU-398 ($n = 22$), HepG2 ($n = 14$), SNU-449 ($n = 22$), and PLC/PRF/5 ($n = 25$) at various E:T ratios using expanded NK cells from 23 donors. Shaded area represents the range of cytotoxicity against K562 cells in 39 experiments. In each experiment, target cells cultured without NK cells were used as a reference. Results of individual experiments are shown in Supplementary Fig. S1. B, results of 24-hour cytotoxicity assays at low E:T ratio. Symbols show mean (± SD) percentage of cytotoxicity for Hep3B ($n = 9$), PLC/PRF/5 ($n = 15$), and SNU-449 ($n = 9$). C, cytotoxicity of expanded NK cells compared with that of unstimulated NK cells from the same donor in 4-hour assays at various E:T ratios. Symbols indicate mean value of triplicate measurements for each cell line. Median values are shown by horizontal bars. D, cytotoxicity of expanded NK cells compared with that of NK cells from the same donors ($n = 3$) stimulated with IL2 (1,000 IU/mL for 24 hours) in 4-hour assays. Symbols indicate the mean value of all measurements ($n = 9$) for each cell line. Median values are shown by horizontal bars.

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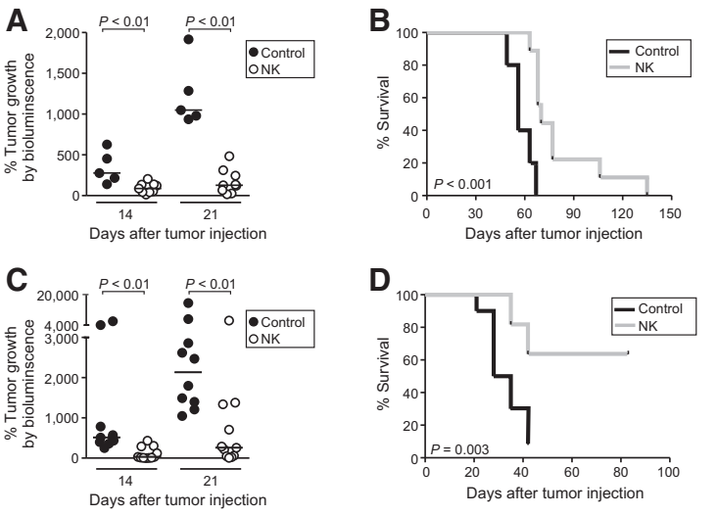


**Figure 2.**

Antitumor capacity of expanded NK cells in immunodeficient mice. A, luciferase-labeled Hep3B cells ($1 \times 10^6$) were injected i.p. in 14 NOD/scid IL2RGnull mice. Seven days later, 9 mice were treated with $1 \times 10^7$ expanded NK cells, and 5 additional injections of NK cells (from the same donor) were given within the next 7 days; 5 mice received RPMI-1640 medium only instead of NK cells. All mice received i.p. injections of IL2 (20,000 IU each) 3 times a week. Results of *in vivo* imaging of tumor growth are shown. Each symbol corresponds to the percentage of tumor growth calculated as follows: (bioluminescence value recorded on day 14 or day 21/bioluminescence value recorded on day 7) x 100. Bioluminescence was measured with a Xenogen IVIS-200 system, with imaging beginning 5 minutes after i.p. injection of D-luciferin (3 mg/mouse), and analyzed with Living Image 3.0 software. *P* values were calculated by *t* test. B, Kaplan–Meier curves indicate overall survival in control and NK-treated mice engrafted with Hep3B cells; *P* value by log-rank test is shown. C, luciferase-labeled HepG2 cells ($1 \times 10^6$) were injected i.p. in 21 NOD/scid IL2RGnull mice; 11 mice received NK cells. Cell dosages, schedule, and estimates of tumor growth were performed as described for A. D, Kaplan–Meier curves indicate overall survival in control and NK-treated mice engrafted with HepG2 cells; *P* value by log-rank test.

These results were confirmed in experiments performed with NOD/scid IL2RGnull mice engrafted with HepG2 cells. Significantly slower tumor growth and improved survival were observed (Fig. 2C and D; Supplementary Fig. S3).

### Interaction between expanded NK cells and sorafenib

Sorafenib is an important component of the contemporary treatment of advanced HCC (27). In initial experiments, we found that the $IC_{50}$ of sorafenib after 48 hours was 4.27 μmol/L for Hep3B, 7.74 μmol/L for SNU-449, and 6.81 μmol/L for PLC/PRF/5 (Supplementary Fig. S4A). To compare the relative cytotoxicity of sorafenib and NK cells, and to determine whether prior exposure to sorafenib would affect NK-cell activity, we treated the 3 cell lines with 5 μmol/L of sorafenib for 48 hours, washed the drug away, and added NK cells to the cultures for another 4 hours. As shown in Fig. 3A, NK cells dramatically increased the anti-HCC effects of sorafenib. When directly compared with cells cultured for 48 hours with the sorafenib vehicle only (DMSO 0.1% v/v), the NK sensitivity of cells exposed to sorafenib remained essentially unchanged in SNU-449 and was only slightly lower in Hep3B and PLC/PRF/5 (Supplementary Fig. S4B).

To determine whether NK-cell cytotoxicity was impaired by sorafenib, we performed experiments in which sorafenib and NK cells were added simultaneously to cultures of PLC/PRF/5 cells. The presence of NK cells in these cultures, even at a low E:T ratio, significantly increased sorafenib cytotoxicity. For example, mean (± SD) 24-hour cytotoxicity with 5 μmol/L of sorafenib increased from 48.8% ± 12.6% to 67.9% ± 5.1% with NK cells at 1:10 E:T and to 96.2% ± 3.3% with NK cells at 1:1 E:T ($n = 6$; $P < 0.01$ for either comparison; Fig. 3B). Notably, the presence of sorafenib at either 5 μmol/L or 10 μmol/L did not affect the capacity of NK cells to exert cytotoxicity (Supplementary Fig. S4C).

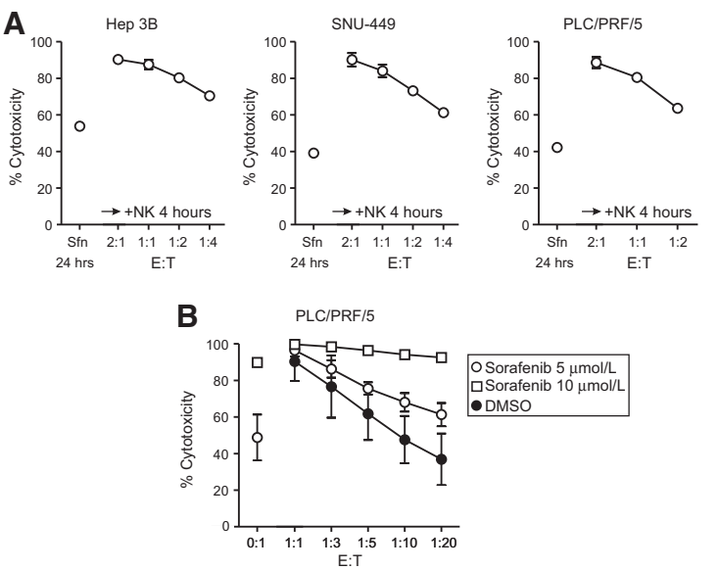


**Figure 3.**

Cytotoxicity of expanded NK cells against HCC cells exposed to sorafenib. A, sequential cytotoxicity by sorafenib ("Sfn") and expanded NK cells. HCC cells were cultured with 5 μmol/L of sorafenib. After 48 hours, the number of viable cells was compared with that of cells cultured with DMSO (0.05% v/v) used as vehicle. Cells were then washed, and expanded NK cells were added to determine 4-hour cytotoxicity. Symbols indicate mean (± SD) percentage of cytotoxicity relative to cells cultured with DMSO and not exposed to NK cells; data are from experiments with NK cells from 3 donors, with 3 measurements each. B, cytotoxicity exerted by sorafenib and expanded NK cells. Sorafenib (5 or 10 μmol/L) or DMSO (0.1% v/v) vehicle and expanded NK cells were added to HCC cells; HCC cell viability was measured after 24 hours. Symbols indicate mean (± SD) percentage of cytotoxicity relative to cells cultured with DMSO and not exposed to NK cells; data are from experiments with NK cells from 2 donors, with 3 measurements each.

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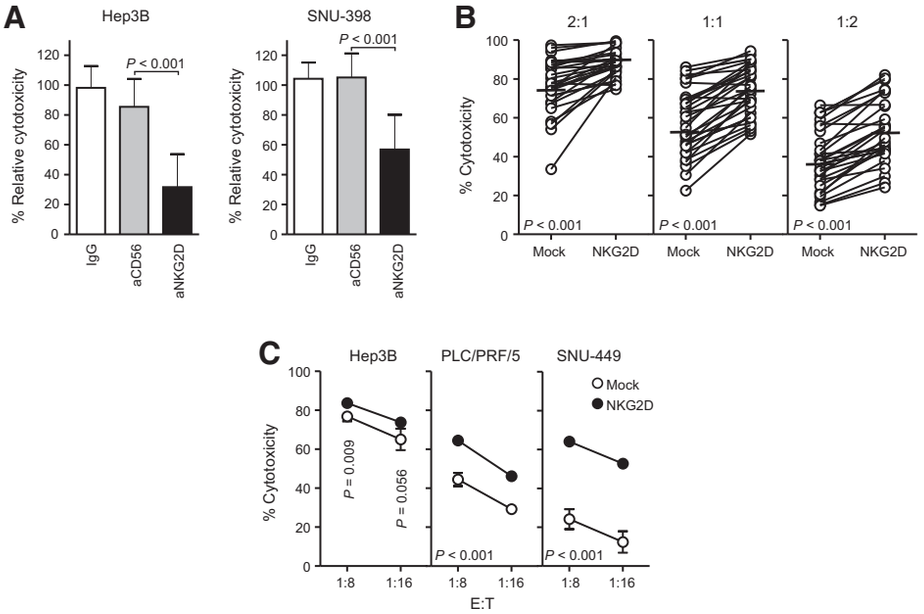


**Figure 4.**

NKG2D is an important determinant of NK-cell cytotoxicity against HCC. A, anti-NKG2D, anti-CD56, or nonreactive mouse IgG were added to cocultures of expanded NK cells and HCC cells. Bars show mean (± SD) percentage of 4-hour cytotoxicity relative to cocultures without antibody (Hep3B, $n = 9$ at 2:1 and 1:1 E:T; SNU-398, $n = 18$ at 2:1, 1:1, and 1:2 E:T). B, cytotoxicity of expanded NK cells transduced with NKG2D-CD3ζ-DAP10 ("NKG2D") compared with mock-transduced cells. Symbols represent mean 4-hour cytotoxicity (of triplicate measurements) relative to cultures without NK cells (31 experiments using NK cells from 8 donors). Horizontal bars indicate median values. *P* values were calculated by paired *t* test. C, cytotoxicity of expanded NK cells transduced with NKG2D-CD3ζ-DAP10 compared with mock-transduced cells after 24 hours of culture. Symbols represent mean (± SD) cytotoxicity of 3 measurements with NK cells from 1 donor. *P* values were calculated by *t* test.

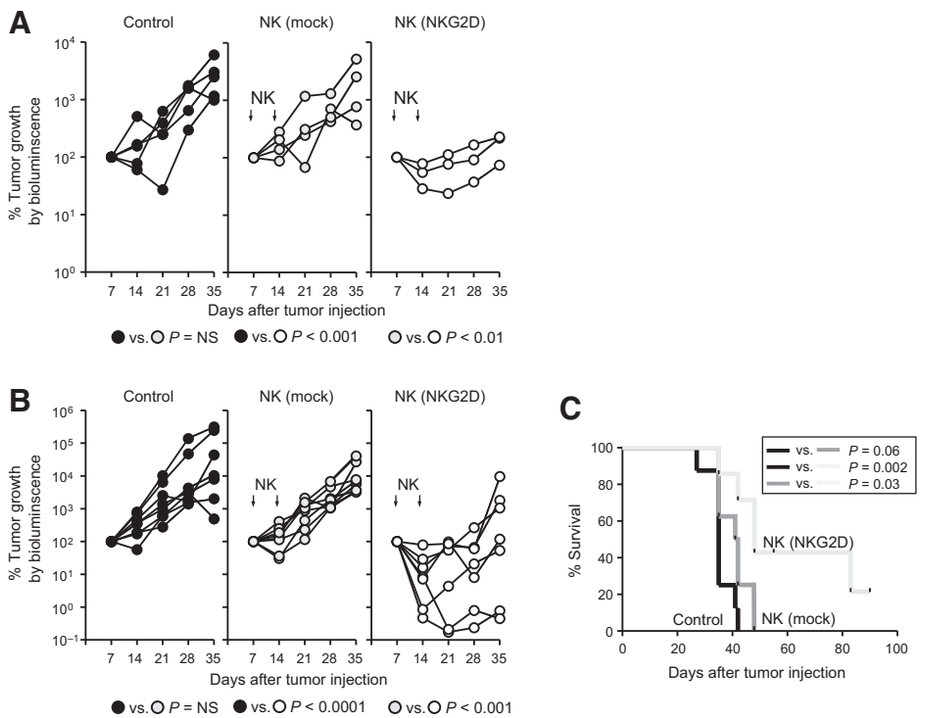


**Figure 5.**

Antitumor capacity of NKG2D-CD3ζ-DAP10–transduced NK cells in immunodeficient mice engrafted with HCC cells. A, luciferase-labeled Hep3B cells ($1 \times 10^6$) were injected i.p. in 7 NOD/scid IL2RGnull mice. Seven days later, 4 mice were treated with expanded mock-transduced NK cells and 3 mice with NKG2D-CD3ζ-DAP10–transduced NK cells ($1 \times 10^7$ NK cells per mouse); an additional injection of NK cells from the same donor was given 1 week later. All mice received i.p. injections of IL2 (20,000 IU each) 3 times a week. Bioluminescence was measured with a Xenogen IVIS-200 system, with imaging beginning 5 minutes after i.p. injection of D-luciferin (3 mg/mouse), and analyzed with Living Image 3.0 software. Tumor growth in each mouse was calculated as follows: (bioluminescence value recorded on days 14–35/bioluminescence value recorded on day 7) x 100 (*P* value by *t* test); live imaging is shown in Supplementary Fig. S9A. B, identical experiments were performed with immunodeficient mice engrafted with luciferase-labeled HepG2 cells; live imaging is shown in Supplementary Fig. S9B. C, Kaplan–Meier curves indicate overall survival in mice engrafted with HepG2 cells untreated (control) or treated with either mock-transduced NK cells or NK cells transduced with NKG2D-CD3ζ-DAP10 receptors; *P* value calculated by log-rank test. NS, not statistically significant.

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**Expression of NKG2D-CD3ζ-DAP10 augments NK-cell cytotoxicity against HCC**

Among the activating cell surface receptors that regulate NK-cell function, NKG2D plays a key role (17, 28, 29). HCC cells expressed ligands for NKG2D as shown by binding to a NKG2D-IgFc chimeric probe (Supplementary Fig. S5). However, 24-hour incubation of expanded NK cells with supernatant collected from 3- to 5-day cultures of the 5 HCC cell lines used did not affect anti-NKG2D staining, suggesting that soluble NKG2D ligands did not diminish NKG2D expression (Supplementary Fig. S6), contrary to what had been previously reported in other settings (30).

We found that addition of a blocking NKG2D antibody to cocultures of expanded NK cells with the HCC cell lines Hep3B and SNU-398 significantly inhibited cytotoxicity (Fig. 4A). Having identified NKG2D as an important determinant of anti-HCC activity, we tested whether increasing its signaling capacity would augment NK-cell killing of HCC targets. Thus, we transduced expanded NK cells with a chimeric receptor containing NKG2D together with its signaling adaptor DAP10 and the additional CD3ζ signaling domain, previously designed in our laboratory (26). Transduction of expanded NK cells with the receptor significantly increased NKG2D surface expression (Supplementary Fig. S7). As shown in Fig. 4B, NK cells expressing the NKG2D-CD3ζ-DAP10 receptor had considerably higher cytotoxicity than mock-transduced NK cells in 4-hour cytotoxicity assays: Median cytotoxicity was 75.5% for cells expressing NKG2D-CD3ζ-DAP10 versus 59.1% for mock-transduced cells at 1:1 E:T ($P < 0.001$). The advantage of the genetically modified NK cells extended to assays performed at low E:T ratios (1:8 and 1:16) for 24 hours (Fig. 4C). Expression of NKG2D-CD3ζ-DAP10 on NK cells, however, did not increase their cytotoxicity against autologous resting lymphocytes. Cell killing of autologous activated T cells was slightly increased, although this amount remained much lower than that measured against HCC cell lines in parallel tests (Supplementary Fig. S8).

Finally, we tested whether NK cells expressing the NKG2D-CD3ζ-DAP10 receptor were also more powerful than mock-transduced cells in immunodeficient mice engrafted with either Hep3B or HepG2 cells. In these experiments, only two infusions of NK cells were administered, in contrast with six infusions in the experiments with expanded NK cells shown in Fig. 2 and Supplementary Fig. S3. In mice engrafted with the Hep3B cell line, two injections of expanded NK cells were insufficient to significantly affect tumor growth (Fig. 5A; Supplementary Fig. S9A). In contrast, tumor growth was considerably reduced after injection of the NKG2D-modified NK cells from the same donors. Although none of the 5 untreated mice survived beyond day 70, 2 of the 4 mice treated with mock-transduced NK cells and 2 of 3 treated with NKG2D-NK cells survived longer (106 and 135 days for mock, 106 and 225 days for NKG2D). The superiority of NKG2D NK cells was shown in another set of experiments with a larger number of mice engrafted with the HepG2 cell line, in which two infusions of NKG2D-NK cells reduced tumor growth and improved survival (Fig. 5B and C; Supplementary Fig. S9B), whereas mock-NK cells did not.

## Discussion

The results of this study demonstrate that NK cells activated and expanded by coculture with K562-mb15-41BBL cells can effectively kill HCC cells. The range of cytotoxicity approached that observed in assays with K562, a leukemia cell line regarded as the gold-standard target for NK-cell cytotoxicity testing. As we previously observed in studies with other tumor types (25, 31), expanded NK cells were more powerful than non-stimulated and IL2-stimulated cells from the same donors, and were effective even at low E:T ratios. HCC killing was also measurable in immunodeficient mice engrafted with HCC cells; treatment with expanded NK cells reduced tumor expansion and lengthened overall survival. High cytotoxicity against HCC cannot be explained by lack of inhibitory killer immunoglobulin-like receptor (KIR) engagement: In contrast with K562, all HCC cell lines studied expressed HLA class I molecules that can bind to KIR on the surface of NK cells (17). However, interaction of the activating receptor NKG2D with its ligands is likely to be an important mechanism underlying the anti-HCC capacity of expanded NK cells: NKG2D blockade reduced cytotoxicity, and enforced expression of the NKG2D-CD3ζ-DAP10 chimeric receptor further increased anti-HCC activity *in vitro* and *in vivo*.

Correlative clinical studies have implicated NK cells in HCC immunosurveillance. Thus, in patients with early-stage HCC, an immune gene signature associated with T-cell and NK-cell infiltration in resected HCC tissue was predictive of better survival (32). Toll-like receptor 3 expression that correlated with the degree of NK-cell infiltration was also a favorable predictor (33). Conversely, loss of the NKG2D ligand ULBP-1 in HCC cells was associated with shorter recurrence-free survival (34). In mice, injection of a hepatotropic adeno-associated virus that delivered IL15 and its receptor expanded the number of NK cells in the liver and prolonged survival in a liver metastatic murine HCC model, whereas depletion of NK cells eliminated the therapeutic effect (35). These data suggest that NK cells are an important component of the HCC microenvironment and might contribute to the control tumor progression. In line with this concept, it was previously reported that NK cells obtained from donor liver perfusates were significantly more cytotoxic against HepG2 cells than peripheral blood NK cells (36). The cytotoxicity against HepG2 cells exerted by our expanded peripheral blood NK cells (mean, 75.2% at 2:1 E:T in 4-hour assays) exceeded that reported for liver NK cells at the same E:T (<60%), even when liver NK cells were stimulated by 4 days of culture with IL2 (36). Besides their use outside the liver transplant context, peripheral blood NK cells would have a practical advantage in a clinical setting. By coculture with K562-mb15-41BBL cells, peripheral blood NK cells can be reliably propagated from cryopreserved apheresis products, thus facilitating their collection and infusion scheduling.

Clinical studies have suggested that infusions of immune cells may be beneficial as adjuvant therapy in patients with HCC. To this end, Cui and colleagues (22) reported results of a study in which cytokine-activated autologous lymphocytes were administered to patients with HCC after tumor ablation with radiofrequency; median progression-free survival in a control group receiving ablation was 12 months, whereas it had not been reached at the time of the analysis for the group of patients receiving ablation plus cell therapy. Lee and colleagues (23) infused a similar cell product in patients with HCC after ablation or surgical resection; the median time of recurrence-free survival was 44 months in patients receiving lymphocyte infusions and 30 months in the control group. Preclinical studies *in vitro* and in animal models suggest that much of the antitumor activity in such

cell products is mediated by NK cells (36, 37). Based on our results, the antitumor potential of expanded NK cells, particularly when armed with NKG2D-CD3ζ-DAP10 receptors, should be much higher than that of cytokine-activated lymphocytes. Importantly, the method used in our study to expand NK cells is robust and has already been adapted to clinical-grade conditions (25, 38).

Sorafenib has become part of the contemporary treatment arsenal for advanced HCC (8, 9, 27). Sorafenib reportedly inhibits cytotoxicity and IFNγ production of both resting and activated human NK cells (39). In mice, sorafenib reduced the number of NK cells and their activity against tumor cells (40). However, others have noted that short-term administration of sorafenib activates hepatic NK cells through activation of tumor-associated macrophages (41) and that sorafenib inhibits the shedding of major histocompatibility complex class I–related chain A (MICA), an NKG2D ligand (42). We found that expanded NK cells considerably enhanced the antitumor cytotoxicity of sorafenib and that NK-cell cytotoxicity appeared to be unaffected regardless of whether sorafenib was added to the cultures, suggesting that a combination of sorafenib plus expanded NK cells could have additive antitumor effects in HCC.

Despite advances in therapy, the prognosis of patients with advanced HCC remains dire. The results of this study suggest that infusions of expanded NK cells with expression of NKG2D-CD3ζ-DAP10 receptors could be a useful addition to current treatment options, such as sorafenib and experimental therapies based on TCR-directed T cells. NK cells expanded with the method described in this study are being infused systemically in clinical trials for hematologic and nonhematologic malignancies (25, 38). In HCC, intra-arterial delivery of expanded NKG2D-CD3ζ-DAP10$^{+}$ NK cells might also be explored (43). In addition to their antitumor activity, NK cells might exert antiviral activity (4, 44, 45). Although the precise role of NK cells in this context requires further clarification (4, 46), NK and NKT cells from liver allograft perfusates, infused after liver transplantation, reduced HCV RNA serum titers (47), suggesting that the application of expanded NK cells in this context is worthy of further study.

## Disclosure of Potential Conflicts of Interest

D. Campana reports having an ownership interest (including patents) in Nkarta. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** D. Campana
**Development of methodology:** T. Kamiya, Y.-H. Chang
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** T. Kamiya
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** T. Kamiya, D. Campana
**Writing, review, and/or revision of the manuscript:** T. Kamiya, D. Campana
**Study supervision:** D. Campana

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