A Chimeric Receptor with NKG2D Specificity Enhances Natural Killer Cell Activation and Killing of Tumor Cells

Yu-Hsiang Chang¹, John Connolly³, Noriko Shimasaki¹, Kousaku Mimura², Koji Kono², and Dario Campana¹

Abstract

Natural killer (NK) cells rely on surface receptors to distinguish healthy cells from cancer cells. We designed a receptor termed NKG2D-DAP10-CD3ζ that is composed of the NK cell activating molecule NKG2D plus 2 key signaling molecules, DAP10 and CD3ζ, and evaluated its capacity to promote cancer cell killing. Retroviral transduction of NKG2D-DAP10-CD3ζ markedly increased NKG2D surface expression in NK cells, which became consistently more cytotoxic than mock-transduced cells against leukemia and solid tumor cell lines. In contrast, there was no increase in cytotoxicity against nontransformed blood and mesenchymal cells. NKG2D blockade abrogated gains in cytotoxicity to cancer cells. Receptor stimulation triggered signal transduction, secretion of IFN-γ, GM-CSF, IL-13, MIP-1α, MIP-1β, CCL5, and TNF-α, and massive release of cytotoxic granules, which persisted after 48 hours of continuous stimulation. NKG2D-DAP10-CD3ζ–expressing NK cells had considerable antitumor activity in a mouse model of osteosarcoma, whereas activated NK cells were ineffective. Thus, the cytotoxic potential of NK cells against a wide spectrum of tumor subtypes could be markedly enhanced by expression of NKG2D-DAP10-CD3ζ receptors. The development of an electroporation method that permits rapid expression of the receptor in a large number of human NK cells facilitates clinical translation of this NK-based strategy for a generalized cellular therapy that may be useful to treat a wide range of cancers. Cancer Res; 73(6): 1–10. ©2012 AACR.

Introduction

Natural killer (NK) cells can recognize tumor cells as targets, a function that suggests possibilities for NK cell therapy of cancer (1). The capacity of NK cells to kill tumor cells depends on the combined effect of suppressive and stimulatory signals delivered through surface receptors. Inhibitory signals result from the interaction between NK inhibitory receptors and HLA molecules on potential target cells, whereas engagement of activating receptors by ligands expressed predominantly by virally infected and tumor cells provoke signals that ultimately cause target cell killing (1).

A key receptor for NK cell activation is NK Group 2 member D (NKG2D), a type II transmembrane-anchored C-type lectin-like protein expressed in all NK cells and in some T-cell subsets (2–4). NKG2D has multiple ligands including MHC class I chain-related A (MICA), MICB, and several UL-16-binding proteins (ULBP), which are preferentially expressed after cellular stress, infection, or DNA damage (3, 5). There is strong evidence in vitro and in animal models for an important role of NKG2D in NK cell-mediated antitumor activity (1, 4, 6–13). NKG2D is associated with DNAX-activating protein 10 (DAP10), which promotes and stabilizes its surface membrane expression (14–18). NKG2D lacks a signaling motif in its cytoplasmic domain and signal transduction upon ligation occurs via the phosphorylation of DAP10, which recruits downstream signaling effector molecules and, ultimately, cytotoxicity (14, 19).

NK cells have shown promise for immunotherapy of cancer (20–23). We reasoned that supraphysiologic activating signals should enhance NK cell antitumor capacity and hence their therapeutic usefulness. To test this idea, we designed a construct encoding a chimeric receptor containing NKG2D, DAP10, and CD3ζ (another signaling molecule known to trigger cytotoxicity in NK cells; refs. 24, 25), and expressed it into activated NK cells. We then examined their signaling profile and anticancer potential in vitro and in vivo.

Materials and Methods

Tumor cell lines

The human B-lineage acute lymphoblastic leukemia (ALL) cell lines OP-1 and REH, and the T-lineage ALL cell lines CEM-C7, Jurkat and MOLT-4 were from the St. Jude Children’s Research Hospital tissue repository; their cell marker profile was periodically tested by flow cytometry to ensure that no changes had occurred. U-2 OS, HOS, and MG-63...
Cytotoxicity and degranulation assays

Target cells were suspended in RPMI-1640 with 10% FBS, labeled with calcein AM (Sigma), and plated into 96-well flat bottom plates (Costar). Expanded NK cells, suspended in RPMI-1640 with 10% FBS and 50 IU/mL IL-2 were then added at various E:T ratios as indicated in Results, and cocultured with target cells for 4 hours. Cells were then stained with propidium ioide and cytotoxicity was measured by flow cytometry using FACSscan or Accuri flow cytometers (Becton Dickinson), enumerating the number of viable target cells (calcein AM-positive, propidium-iodide negative, and light scattering properties of viable cells; ref. 27). For adherent cell lines, the plates were placed in an incubator for at least 4 hours to allow for cell attachment before adding NK cells. At the end of the cultures, cells were detached using trypsin plus EDTA. In some experiments, NK cells were incubated with anti-NKG2D (clone 149810; R&D), anti-CD56 (BD Biosciences) or an isotype-matched nonreactive antibody for 10 minutes before coculture.

We directly tested NK cell degranulation after NKG2D stimulation with an anti-NKG2D antibody. NK cells (1×10^5) were plated into each well of a 96-well flat bottom plate and incubated with anti-Biotin MACSiBeads (Miltenyi Biotec) coated with biotin-conjugated anti-NKG2D antibody (clone 1D11; eBioscience; 10 beads for 1 NK cell) for 4 hours at 37°C. Anti-human CD107a antibody conjugated to phycoerythrin (BD Biosciences) was added at the beginning of the cultures and 1 hour later GoBiStop (0.15 μL; BD Biosciences) was added. The cells were stained with anti-human CD56 conjugated to fluo-rescein isothiocyanate (BD Biosciences) and analyzed by flow cytometry.

Expression of NKG2D ligands, phospho-protein analysis, and measurement of cytokine levels

Surface expression of NKG2D ligands was evaluated by staining with human recombinant NKG2D/Fe chimera (R&D), PE-conjugated goat anti-human IgGFc (γ; Fisher Scientific), MIC A/B (6D4, BD Biosciences), ULBP-1 (R&D) and ULBP-2 (R&D) and ULBP-3 (R&D). For phosphoprotein analysis, we cultured mock- and NKG2D-DAP10-CD3ζ-transduced expanded NK cells (8×10^5) with or without anti-NKG2D antibody and beads as described above. After 1 hour of stimulation, cell lysates were prepared using a lysis buffer containing 20 mmol/L 3-(N-morpholino) propanesulfonic acid, 2 mmol/L EGTA, 5 mmol/L EDTA, 30 mmol/L sodium fluoride, 60 mmol/L β-glycerophosphate, 20 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 1% Triton X-100, Complete Mini protease inhibitor cocktail (Roche), and 1 mmol/L dithiothreitol. After sonication, lysates were frozen at −80°C and shipped in dry ice to Kinexus for Kinex Antibody Microarray analysis. To measure cytokine/chemokine production, we cultured mock- and NKG2D-DAP10-CD3ζ expanded NK cells (1×10^5 in 200 μL/well of a 96-well plate) with or without
anti-NKG2D antibody and beads. Supernatants (120 μL) were collected after 4, 8, and 16 hours and analyzed using the Luminex human cytokine/chemokine panel I (41 human cytokines/chemokines; Merck Millipore).

**Murine models**

U-2 OS cells expressing luciferase were injected intraperitoneally in NOD.Cg-PrkdcscidIL2rgtm1Wjl/SzJ (NOD/scid IL2RG-null) mice (Jackson Laboratory; 2 × 10^6 per mouse; ref. 11). NK cells from healthy donors were expanded for 7 days, transduced with the MSCV vector containing either GFP or NKG2D-DAP10-CD3ζ suspended in RPMI-1640 plus 10% FBS (3 × 10^6 cells per mouse) and then injected intraperitoneally 7 days after U-2 OS injection. A single injection of NK cells was given together with intraperitoneal injections of IL-2 (20,000 IU each) for 4 days. As a control, a group of mice received tissue culture medium instead of NK cells. U-2 OS engraftment and progression was evaluated using a Xenogen IVIS-200 system (Caliper Life Sciences), with imaging beginning 5 minutes after intraperitoneal injection of an aequorin solution of α-luciferin potassium salt (3 mg/mouse). Photons emitted from luciferase expressing cells were quantified using the Living Image 3.0 software program. The studies were approved by the St Jude Animal Care and Use Committee.

**Results**

**Chimeric receptor design and expression in expanded NK cells**

We expanded human NK cells from peripheral blood mononuclear cells, prepared a cDNA library and cloned the genes encoding NKG2D, DAP10, and CD3ζ. We then inserted the construct containing the 3 genes into a MSCV retroviral vector and used it to transduce expanded activated NK cells (Fig. 1A).

We first determined whether retroviral transduction of the construct resulted in gains of NKG2D expression as compared with cells transduced with an MSCV vector containing only GFP. In experiments with expanded NK cells from 21 donors (>98% CD56+ CD3− after T-cell depletion), median percentage of GFP-positive cells after transduction with the GFP vector (mock) was 80% (range 67–96%). Transduction with the NKG2D-DAP10-CD3ζ construct in NK cells from the same donors resulted in a marked increase in NKG2D expression (P < 0.0001; Fig. 1B). We compared the results of NKG2D-DAP10-CD3ζ transduction to those obtained after transduction of a NKG2D-CD3ζ lacking DAP10 in experiments with NK cells from 6 donors. As shown in Fig. 1C, NKG2D expression was consistently higher when DAP10 was present in the construct (P = 0.0027), in agreement with previous reports indicating that DAP10 supports NKG2D expression (14–17).

To ensure that all components of the receptor were expressed, we used a construct containing DAP10 with a FLAG-tag. As shown in Fig. 1D, NK cells transduced with NKG2D-DAP10-CD3ζ expressed DAP10. By Western blotting with an antibody detecting phospho-(pY83)-CD3ζ, we showed that these cells expressed a chimeric protein containing CD3ζ in addition to endogenous CD3ζ (Fig. 1E). Thus, the 3 components of the NKG2D-DAP10-CD3ζ receptor can be effectively expressed in human NK cells.

**NKG2D-DAP10-CD3ζ receptors increase the antitumor cytotoxicity of activated NK cells**

NK cells expanded and activated after coculture with the K562-mb15-41BBL cell line exert cytotoxicity, which is much higher than that of primary or IL-2-stimulated NK cells (11, 27). We determined whether expression of NKG2D-DAP10-CD3ζ receptors in these cells could further improve their antitumor cytotoxicity. For this purpose, we targeted a broad panel of tumor cell lines originating from T-cell ALL (CEM-C7, MOLT-4, Jurkat) and B-cell ALL (REH, OP-1), osteosarcoma (U-2 OS, MG-36, HOS), prostate carcinoma (DU 145, PC-3, LNCaP), rhabdomyosarcoma (RH18, RH30, TE32, RH36), neuroblastoma (SKNSh), Ewing sarcoma (TC71), colon carcinoma (KM12L4), gastric carcinoma (SNU1), lung squamous cell carcinoma (SW900), hepatoma (HepG2), and breast carcinoma (MCF7). We conducted 4-hour cytotoxicity assays with NK cells expanded from 14 donors at 1:1 or 1:2 effector:target (E:T) ratios for a total of 65 experiments. For each cell line, we first determined the E:T ratio that would produce sub-maximal levels of cytotoxicity and then tested the gains produced by transducing NK cells with NKG2D-DAP10-CD3ζ genes from the same donors transduced with a vector containing GFP alone were used as a control. As shown in Fig. 2A and B, expression of the NKG2D-DAP10-CD3ζ receptor significantly increased overall cytotoxicity against both leukemic and solid tumor cell lines (P < 0.0001). Gains in cytotoxicity were particularly evident in the ALL cell lines REH, MOLT4, and CEM-C7, in the osteosarcoma cell lines U-2 OS, MG-36, HOS, in the prostate carcinoma cell lines DU 145 and PC-3, and in the rhabdomyosarcoma cell line RH36 (Fig. 2C). In contrast, the B-lineage ALL cell line OP-1 remained relatively refractory to NK cells despite NKG2D-DAP10-CD3ζ receptor expression (Fig. 2A).

We determined whether expression of NKG2D-DAP10-CD3ζ receptors also increased the cytotoxicity of expanded NK cells against nontransformed cells, such as allogeneic peripheral blood mononuclear cells and bone marrow-derived mesenchymal cells. As shown in Fig. 2D, cytotoxicity remained below 20% at 1:1 ratio, regardless of whether NK cells were transduced with the receptor or with GFP (Fig. 2D). These results indicate that expression of NKG2D-DAP10-CD3ζ receptors can markedly enhance NK cell cytotoxicity against cancer cells without significantly increasing their activity against nontumor cells.

**NK cytotoxicity is triggered by ligation of NKG2D-DAP10-CD3ζ receptors**

We analyzed the relation between NKG2D-DAP10-CD3ζ-mediated cytotoxicity and expression of NKG2D ligands on target cells. To this end, we used a human recombinant NKG2D/Ig Fc reagent to measure the collective expression of all NKG2D ligands. The cell line OP-1 did not show any labeling with NKG2D/Ig Fc, and also gave a negative staining with antibodies to MICA/B, ULBP-1, ULBP-2, and ULBP-3, thus explaining its resistance to NK cell killing regardless of whether these expressed NKG2D-DAP10-CD3ζ or not. All the remaining...
cell lines studied were labeled by NKG2D/Ig Fc but we found no significant relation between level of overall NKG2D ligand expression and NKG2D-DAP10-CD3ζ receptor-mediated cytotoxicity (Fig. 3A). Nontransformed bone marrow-derived mesenchymal cells and peripheral blood monocytes had a relatively weak staining with NKG2D/Ig Fc, and most peripheral blood lymphocytes had no staining at all.

To ascertain whether the increase in cytotoxicity produced by transduction of the NKG2D-DAP10-CD3ζ receptor was directly related to receptor stimulation, we used an anti-NKG2D blocking antibody (clone 149810; ref. 11). In experiments with the U-2 OS osteosarcoma cell line, preincubation of NK cells with the antibody markedly inhibited NK cytotoxicity and abrogated the gains achieved by NKG2D-DAP10-CD3ζ receptor transduction (Fig. 3B). Conversely, direct stimulation of the receptor by an anti-NKG2D agonistic antibody (clone 1D11; ref. 29) provoked massive lysosomal granule exocytosis, as detected by CD107a expression (30); this was significantly higher than that achieved by NKG2D stimulation of mock-transduced NK cells ($P < 0.001$; Fig. 3C and D).

Engagement of NKG2D-DAP10-CD3ζ triggers signal transduction, cytokine secretion, and sustained stimulation

To further understand the signaling properties of NKG2D-DAP10-CD3ζ and the differences from the signals triggered by endogenous NKG2D, we stimulated mock- and NKG2D-DAP10-CD3ζ–transduced activated NK cells with the anti-NKG2D agonistic antibody for 1 hour and analyzed cell lysates with the Kinex antibody microarray, which contains 809 anti-phosphoprotein antibodies. As shown in Fig. 4A, the phospho-protein profile of NKG2D-DAP10-CD3ζ–expressing NK cells was substantially different from that of mock-transduced NK cells. Particularly prominent after NKG2D-DAP10-CD3ζ stimulation was the phosphorylation of the CREB1 transcription factor, known to promote activation and proliferation of T and B cells (31), of TBK1, a serine-threonine protein kinase and NF-$\kappa$B activator with prosurvival roles (32), and of ACK1, a tyrosine-protein and serine/threonine-protein kinase, which regulates AKT (33), a key effector of DAP10 signaling (34).

To determine whether NKG2D-DAP10-CD3ζ–signaling resulted in an increased cytokine/chemokine secretion, we
stimulated receptor- or mock-transduced NK cells from 3 donors with the biotin-labeled anti-NKG2D agonistic antibody and anti-biotin beads and measured cytokine/chemokine levels in the supernatants after 4, 8, and 16 hours. As shown in Fig. 4B and Supplementary Fig. S1, engagement of NKG2D-DAP10-CD3ζ caused a marked increase in IFN-γ, GM-CSF, IL-13, MIP-1α, MIP-1β, CCL5, and TNF-α production (P < 0.01 by 2-way ANOVA for all comparisons). For these 7 factors, levels were also significantly higher when NKG2D-stimulated cells (either mock- or NKG2D-DAP10-CD3ζ–transduced) were compared with the same cells cultured without antibody (Supplementary Table S1). Levels of the other cytokines/chemokines measured [IL-1α, IL-1β, IL-6, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-15, IL-17, sCD40L, EGF, eotaxin, FGF-2, FGF-2, Flt-3 ligand, fractalkine, G-CSF, GRO, IFN-α2, IP-10, MCP-1, MCP-3, MDC, PDGF-DD, PDGF-BB, TGFα, TNF-β, and VEGF] were not significantly different between mock- and NKG2D-DAP10-CD3ζ–transduced NK cells, regardless of NKG2D stimulation (Supplementary Table S1).

To further explore the mechanisms underlying the enhancement of cytotoxicity triggered by the NKG2D-DAP10-CD3ζ receptors, we conducted immunofluorescence imaging studies using the U-2 OS cell line as a target. In experiments with NK cells from 3 donors, those expressing the NKG2D-DAP10-CD3ζ receptors produced clear increases in target cell apoptosis when compared with mock-transfected cells (11.7 ± 2.9 apoptotic cells/0.07 mm² vs. 3.3 ± 0.6 apoptotic cells/0.07 mm²; P = 0.033; Supplementary Movie). These gains could not be attributed to an increase in cell speed movement or cell track displacement length, which were similar for receptor- and mock-transduced NK cells: 0.027 ± 0.01 m/s versus 0.027 ± 0.01 m/s, and 18.1 ± 10.1 m/s versus 17.5 ± 6.7 m/s, respectively.

Continuous stimulation via NKG2D ligation may result in a hyporesponsive status (1). To test the anergy-inducing potential of NKG2D-DAP10-CD3ζ signaling as compared with that of endogenous NKG2D, we cultured mock- and NKG2D-DAP10-CD3ζ–transduced NK cells with the anti-NKG2D agonistic antibody and monitored exocytosis of lytic granules with CD107a staining over 48 hours. Mock-transduced NK cells were unable to degranulate after 24 or 48 hours of stimulation. By contrast, a substantial proportion of NKG2D-DAP10-CD3ζ–transduced NK cells were CD107a-positive 24 and 48 hours after continuous NKG2D ligation (Fig. 4C). Hence, NK cells bearing the receptor are capable of exerting cytotoxicity even after prolonged engagement of NKG2D.

Figure 2. Expression of NKG2D-DAP10-CD3ζ receptors increases tumor cell killing by activated NK cells. A, percentage of cytotoxicity of mock- and NKG2D-DAP10-CD3ζ–transduced NK cells against leukemia cell lines (CEM-C7, MOLT-4, Jurkat, REH, and OP-1), and solid tumor-derived cell lines (U-2 OS, MG-36, HOS, DU 145, PC-3, LNCaP, RH18, RH30, TE32, RH36, SKNSH, TC71, Knu12/L4, SNJ1, SW900, HepG2, and MC2). A total of 65 experiments were conducted using NK cells expanded from 14 donors at an E:T of 1:1 or 1:2; cell killing was measured after 4 hours of coculture. B, flow cytometric dot plots illustrate the assay used to measure cell killing. Results with one leukemia cell line (REH, top row) and one osteosarcoma cell line (U-2 OS, bottom row) are shown. Tumor cells were either cultured alone (left), with mock-transduced NK cells (middle), or with NK cells transduced with the NKG2D-DAP10-CD3ζ receptor. Residual viable target cells are in the bottom right region of each panel. C, percentage of cytotoxicity of mock- and NKG2D-DAP10-CD3ζ–transduced NK cells against selected tumor cell lines. D, percentage of cytotoxicity of mock- and NKG2D-DAP10-CD3ζ–transduced NK cells from three donors against nontransformed peripheral blood mononucleated cells (PBMC) and bone-marrow-derived mesenchymal stromal cells (MSC); P > 0.05.
Cytotoxicity of NK cells expressing NKG2D-DAP10-CD3ζ in xenografts

To compare the antitumor capacity of NK cells expressing NKG2D-DAP10-CD3ζ to that of mock-transduced cells in vivo, we generated a xenograft model of osteosarcoma by injecting luciferase-labeled U-2 OS cells (2 × 10^5) intraperitoneally in 12 immunodeficient (NOD/scid-IL2Rγnull) mice (Fig. 5). In 4 mice without treatment, U-2 OS tumors progressively expanded. Another 4 mice were injected with 2 × 10^5 U-2 OS intraperitoneally and then a single intraperitoneal injection of mock-transduced NK cells (3 × 10^6), followed by 4 daily IL-2 intraperitoneal injection; U-2 OS tumors in this group also expanded. A third group of 4 mice was injected with an identical number of U-2 OS intraperitoneally and a single intraperitoneal injection of mock-transduced NK cells transduced with the NKG2D-DAP10-CD3ζ construct (3 × 10^6), followed by 4 daily IL-2 intraperitoneal injection. Seven days after the NK cells were injected, the average signal intensity decreased dramatically and overall tumor burden remained significantly lower to that measured in mice treated with mock-transduced NK cells (P = 0.0028 by 2-way ANOVA; Fig. 5).

Expression of NKG2D-DAP10-CD3ζ by electroporation

Although effective, gene expression by retroviral transduction presents considerable practical constraints for large-scale clinical application. We previously found that electroporation of mRNA results in highly efficient expression of functional receptors in NK cells, and that this method can be adapted to a clinical grade protocol for genetic engineering of large cell numbers (35). To determine whether the NKG2D-DAP10-CD3ζ receptor could be expressed by this method, we produced mRNA encoding NKG2D-CD3ζ and DAP10, electroporated them into expanded NK cells, and determined NKG2D expression 24 hours later. As shown in Fig. 6A, electroporation resulted in high NKG2D expression. NK cells electroporated with the receptor were markedly more cytotoxic against the U-2 OS cell line than mock-electroporated NK cells (Fig. 6B).

Discussion

The NKG2D activating receptor is central to the capacity of NK cells to sense cellular stress and lyse virally infected and tumor cells (1, 4, 6, 7, 9–13). In this study, we found that expression of an activating receptor with the binding
specificity of NKG2D and the combined signaling capacities of DAP10 and CD3ζ could considerably enhance the cytotoxicity of activated NK cells against leukemias and solid tumors. The cytotoxicity of NK cells expressing NKG2D-DAP10-CD3ζ receptors was directly triggered by engagement of NKG2D; receptor expression did not significantly increase cytotoxicity against nontransformed cells with low or no NKG2D ligand expression, or against leukemic cells lacking NKG2D ligands. Although most of our experiments relied on retroviral transfection of the receptor, we also developed a method to efficiently express it by electroporation, thus greatly facilitating its clinical application for cell therapy of cancer (35).

The configuration of our receptor allows for signal transduction by both DAP10 and CD3ζ and differs from the typical chimeric-antigen receptors, which contain only 1 signaling molecule, or a stimulatory plus a costimulatory molecule in tandem (36). In line with previous reports indicating that DAP10 promotes NKG2D expression on the surface membrane (14–17), we found that expression of the NKG2D-CD3ζ construct was significantly improved by concomitant expression of DAP10. Other investigators reported that a receptor coupling NKG2D and CD3ζ could be expressed in T lymphocytes and enhanced their cytotoxicity against lymphoma (37), myeloma (38), ovarian cancer (39), and Ewing’s sarcoma cells (40). Whether expression of DAP10 would increase NKG2D-CD3ζ expression also in T lymphocytes and/or increase their cytotoxicity remains to be determined.

NKG2D ligands are widely expressed among cancer cells (41, 42). Indeed, NKG2D-DAP10-CD3ζ-receptor signaling augmented the cytotoxicity of activated NK cells against a wide spectrum of tumor cell targets. However, there was considerable heterogeneity in the degree of response, with cell lines derived from ALL, osteosarcoma, prostate carcinoma and rhabdomyosarcoma most prominently revealing the enhanced cytotoxicity caused by the receptor. We suggest that these tumor types should have priority for inclusion in clinical trials of this approach. The magnitude of the increase that we observed (more than twice cells killed within 4 hours in some cases) is particularly noteworthy considering that the NK cells included in our studies were activated and can exert cytotoxicities that are already significantly higher than those of primary and IL-2 activated NK cells (27). Thus, the cytotoxic capacity of activated NK cells is not maximal and can be further enhanced by boosting activating signals. The gains in NK-mediated...
antitumor activity were also evident in experiments with immunodeficient mice engrafted with osteosarcoma cells, where NK cells expressing NKG2D-DAP10-CD3ζ receptors produced marked tumor reductions while mock-transduced activated NK cells were ineffective. Although the possibility that tumor cell subsets can escape NKG2D-DAP10-CD3ζ-mediated cytotoxicity cannot be ruled out, we think that the failure of NK cells to completely eradicate the tumor was most likely due to the fact NK cells were infused only once, and that IL-2 administration (which is critical for the survival and expansion of the activated NK cells in mice; ref. 27) was limited to 4 days.

In our study, there was no clear relation between levels of NKG2D-ligand expression and susceptibility to NKG2D-DAP10-CD3ζ–bearing NK cells, suggesting that other signaling activating or inhibitory signal interactions may influence the degree of cell killing. It has also been shown that the pattern of NKG2D-ligand partitioning in the target cell membrane, and the degree of ligand shedding can play a role in triggering cytotoxicity (43–45). Gains in cytotoxicity brought about by NKG2D-DAP10-CD3ζ–receptor expression were dependent on its signaling, as an antagonist anti-NKG2D antibody abrogated them. It is thought that persistent stimulation of NK cells may result in suppression of NK cell cytotoxic function (46, 47). Indeed, mock-transduced NK cells were unable to degranulate after 24 hours of continuous stimulation. However, a considerable proportion of NK cells expressing NKG2D-DAP10-CD3ζ receptors were CD107a positive even after 48 hours of stimulation, indicating that the combined DAP10 and CD3ζ signals do not accelerate the occurrence of hyporesponsiveness; on the contrary, they significantly prolong NK cell function. The NKG2D receptor has been shown to contribute to autoimmunity but pathologic responses against normal tissues could be attributed to the fraction of CD8 T lymphocytes expressing this receptor. 

Figure 5. Antitumor capacity of NKG2D-DAP10-CD3ζ-transduced NK cells in a xenograft model of osteosarcoma. Luciferase-labeled U-2 OS cells (2 x 10⁶) were injected intraperitoneally in 12 immunodeficient (NOD/scid-IL2Rγnull) mice. Control mice (No NK; n = 4) received no treatment (top row); the remaining 8 mice received a single intraperitoneal injection of either mock-transduced (Mock, middle row) or NKG2D-DAP10-CD3ζ-transduced 3 x 10⁶ NK cells (NKG2D-DAP10-CD3ζ, bottom row), followed by four daily IL-2 intraperitoneal injection. Photoluminescence signals were measured at weekly intervals with a Xenogen IVIS-200 system (Caliper Life Sciences), with imaging beginning 5 minutes after intraperitoneal injection of an aqueous solution of β-luciferin potassium salt (3 mg/mouse). Right graph shows mean (±SD) measurements of photons/second quantified using the Living Image 3.0 software program (analyzed by two-way ANOVA).

Figure 6. Expression of NKG2D-DAP10-CD3ζ by electroporation. A, flow cytometric analysis of NKG2D expression in activated CD56+ CD3- NK cells 24 hours after electroporation with NKG2D-DAP10-CD3ζ and DAP10 mRNA (NKG2D-DAP10-CD3ζ) or no mRNA (mock). B, killing of U-2 OS cells after four-hour coculture with NK cells electroporated with NKG2D-CD3ζ and DAP10 mRNA or mock-electroporated at the indicated E:T ratios. Each symbol corresponds to mean (±SD) of three cocultures; P value at each E:T ratio by t-test is shown.
NKG2D Receptor Enhances NK Cell Killing of Tumors

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

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Incidentally infected patients (48, 49) may have marked increases in tumor cytotoxicity against nontransformed peripheral blood lymphocytes or bone marrow-derived mesenchymal cells. For clinical application, this potential problem should be prevented by careful depletion of T cells from the NK cell product together with transient expression of the receptor by electroporation.

It is well established that donor NK cell alloreactivity suppresses leukemia relapse after allogeneic hematopoietic stem cell transplantation (20, 21). Infusion of NK cells in a nontransplant setting has shown promise in some studies (22, 23), and hence this approach is being actively pursued at several centers using either freshly purified or activated NK cells. The method that we described here offers a new way to increase the antitumor efficacy of NK cell therapy and to widen its application. Stimulation via the NKG2D-DAP10-CD3ε receptor also resulted in a marked increase in cytokine/chemokine secretion. Thus, NK-derived GM-CSF, IFN-γ, and TNF-α promote monocyte differentiation, macrophage activation and dendritic cell maturation (1, 49, 50). Whether these cellular effects would amplify the antitumor response in vivo is unclear but they should be important during immune responses against pathogens, suggesting that infusion of NKG2D-DAP10-CD3ε-NK cells should also be tested in the setting of infectious diseases.

References


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