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

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Introduction

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); 1777–86. ©2012 AACR.

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...
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DAP10, and the intracellular domain of CD3

nucleotide was then removed from the NcoI site to make DAP10 in frame. The procedures used for virus production, gene transduction, mRNA electroporation, and analysis of chimeric receptor are described in Supplementary Methods.

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 iodide and cytotoxicity was measured by flow cytometry using FACScalor 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 I49810; 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 × 10⁶) were plated into each well of a 96-well flat bottom plate and incubated with anti-Biotin MACSbeads (Miltenyi Biotec) coated with biotin-conjugated anti-NKG2D antibody (clone 7D11; eBioscience; 10 beads for 1 NK cell) for 4 hours at 37°C. Anti-human CD107α antibody conjugated to phycoerythrin (BD Biosciences) was added at the beginning of the cultures and 1 hour later GolgiStop (0.15 μL; BD Biosciences) was added. The cells were stained with anti-human CD56 conjugated to fluoroscein 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/Fc chimera (R&D), PE-conjugated goat anti-human IgG/FC (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 × 10⁶) 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 × 10⁶ in 200 μL/well of a 96-well plate) with or without

Human NK cell expansion

Peripheral blood samples were obtained from healthy adult donors. Mononuclear cells collected by centrifugation on a Lymphoprep density step (Nycomed) were washed twice in RPMI-1640. To expand CD56+/CD3− NK cells, we cocultured peripheral blood mononuclear cells and the genetically modified K562-mb15-41BBL cell line made in our laboratory, as previously described (25, 27). In brief, peripheral blood mononuclear cells (1.5 × 10⁶) were cultured in a 24-well tissue culture plate with 1 × 10⁵ K562-mb15-41BBL cells in RPMI-1640 medium containing and 10% FBS and 10 IU/mL human IL-2 (National Cancer Institute BRB Preclinical Repository). Every 2 days the tissue culture medium was exchanged with fresh medium and IL-2. After 7 days of coculture, residual T cells were removed using Dynabeads CD3 (Invitrogen), producing cell populations containing >95% CD56+/CD3− NK cells.

Plasmids

The pMSCV-IREs-GFP, pEQ-PAM3(-E), and pRDF were obtained from the St. Jude Vector Development and Production Shared Resource (28). The cDNA encoding NKG2D, DAP10, and the intracellular domain of CD3ζ were subcloned by PCR using cDNA derived from human expanded NK cells as a template. Expression cassettes were subcloned into EcoRI sites of MSCV vector. Because NKG2D and CD3ζ are type II and type I proteins, respectively, we removed the ATG initiation codon of NKG2D and added an ATG start codon to the cDNA of the intracellular domain of CD3ζ to prepare a construct containing both proteins. NKG2D and CD3ζ were then assembled using splicing by overlapping extension by PCR (SOE-PCR). We then replaced GFP in the vector with DAP10 (containing a FLAG-tag) between the NcoI and NotI sites; 1

osteoarcoma), DU 145, PC-3, and LNCaP (prostate carcinoma), Km12L4 (colon carcinoma), SNU1 (gastric carcinoma), SW900 (lung squamous cell carcinoma), HepG2 (hepatocellular carcinoma), and MCF7 (breast carcinoma) were from the American Type Culture Collection. The rhabdomyosarcoma cell lines RH18, RH36, TE-32, and the neuroblastoma cell line SKNSH were provided by Dr. Peter Houghton (Nationwide Children’s Hospital, Columbus, OH); RH30 (rhabdomyosarcoma) was from the St. Jude Children’s Research Hospital tissue repository (11). These cell lines were characterized by the providers for molecular and/or gene expression features. Cell lines were expanded after receipt, cryopreserved and cells for experiments were obtained from recently thawed vials. Human mesenchymal cells were developed in our laboratory (26), RPMI-1640 (Invitrogen) with 10% FBS (Atlanta Biologicals) and antibiotics, was used to maintain all cell lines except U-2 OS, HOS, and MG-63, which were maintained in DMEM (Cellgro).

For the visualization of tumor cells in immunodeficient mice, U-2 OS cells were transduced with a murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-green fluorescent protein (GFP) retroviral vector (from the St. Jude Vector Development and Production Shared Resource) containing the firefly luciferase gene and selected for their expression of GFP with a FACScalor cell sorter (BD Biosciences).

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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-PrkdΔcicld−/−Il2rgtm1Wjl/SzJ (NOD/scid IL2RG-null) mice (Jackson Laboratory; 2 × 106 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 × 106 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 aqueous solution of β-luciferin potassium salt (3 mg/mouse). Photons emitted from luciferase-expression 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 construct 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 (SK-N-SH), Ewing sarcoma (TC71), colon carcinoma (Km12L4), gastric carcinoma (SNU1), lung squamous cell carcinoma (SW900), hepatoma (HepG2), and breast carcinoma (MC7). 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 cells 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 NK2D/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 phosphoprotein 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-κB 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-1ra, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-12 (p40), IL-15, IL-17, sCD40L, EGF, eotaxin, FGF-2, Flt-3 ligand, fractalkine, G-CSF, GRO, IFN-α2, IFN-γ, GM-CSF, IFN-β, 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 mm/s versus 0.027 ± 0.01 mm/s, and 18.1 ± 10.1 μm versus 17.5 ± 6.7 μm, 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.
Cytotoxicity of NK cells expressing NKG2D-DAP10-CD3z

To compare the antitumor capacity of NK cells expressing NKG2D-DAP10-CD3z to that of mock-transduced cells in vivo, we generated a xenograft model of osteosarcoma by injecting luciferase-labeled U-2 OS cells (2 x 10⁵/C2) intraperitoneally in 12 immunodeficient (NOD/scid-IL2Rgnull) mice (Fig. 5). In 4 mice without treatment, U-2 OS tumors progressively expanded. Another 4 mice were injected with 2 x 10⁵ U-2 OS intraperitoneally and then a single intraperitoneal injection of mock-transduced NK cells (3 x 10⁶) 7 days later, 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 NK cells transduced with the NKG2D-DAP10-CD3z construct (3 x 10⁶), 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-CD3z 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-CD3z receptor could be expressed by this method, we produced mRNA encoding NKG2D-CD3z 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

Figure 3. Relation between NKG2D-DAP10-CD3z ligation and increased cytotoxicity. A, relation between levels of NKG2D ligand (NKG2DL) expression and the increase in cytotoxicity caused by NKG2D-DAP10-CD3z receptor expression. Mean fluorescence intensity (MFI) of NKG2DL expression after staining cells with a human recombinant NKG2D/Ig Fc is shown on the y axis. Cytotoxicities obtained with mock- and NKG2D-DAP10-CD3z-transduced NK cells (from three or more donors) were compared for each cell line. The median gain in cytotoxicity value of 43% was used to divide the cell lines into two groups (P > 0.05). B, pre-incubation of NK cells with an inhibitory anti-NKG2D antibody (clone 149810; R&D) abrogated the gains in cytotoxicity produced by the expression of NKG2D-DAP10-CD3z. Mock- and NKG2D-DAP10-CD3z-transduced NK cells were incubated with anti-NKG2D, anti-CD56, or an isotype-matched nonreactive antibody for 10 minutes; 4-hour cytotoxicity against the U-2 OS cell line at 1:1 ratio was tested. Bars represent mean (±SD) of triplicate measurements. C, incubation of NK cells with a biotin-conjugated anti-NKG2D agonistic antibody (clone 1D11; eBioscience) and anti-biotin beads (MACSiBeads; Miltenyi Biotec) induced degranulation, which was significantly higher in NK cells expressing NKG2D-DAP10-CD3z. Percentage of CD56+ cells from six donors expressing CD107a after 4 hours of anti-NKG2D stimulation is shown. D, flow cytometric dot plots illustrating CD107a expression on mock- or NKG2D-DAP10-CD3z-transduced CD56+ cells.
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

Figure 4. NKG2D-DAP10-CD3ζ signaling and its cellular consequences. A, mock- and NKG2D-DAP10-CD3ζ-transduced NK cells were incubated with a biotin-conjugated anti-NKG2D agonistic antibody (clone 1D11; eBioscience) and anti-biotin beads (MACSiBeads; Miltenyi Biotec) for 1 hour and cell lysates were analyzed by Kinex Antibody Microarrays (Kinexus). Of 809 phosphoprotein antibodies tested, shown are those whose signals had a Z-ratio ≥ 1 and a % error range ≤ 50. Bars indicate percentage signal change in NK cells expressing NKG2D-DAP10-CD3ζ as compared with the normalized intensity in mock-transduced NK cells. B, mock- and NKG2D-DAP10-CD3ζ-transduced NK cells from 3 donors were incubated with a biotin-conjugated anti-NKG2D agonistic antibody (clone 1D11; eBioscience) and anti-biotin beads (MACSiBeads; Miltenyi Biotec). Concentration of IFN-γ and GM-CSF in the supernatants collected 4, 8, and 16 hours after initiation of stimulation was measured by Luminex (Merck Millipore). Data of the remaining cytokines/chemokines measured is in Supplementary Fig. S1 and Table S1. C, degranulation in mock- and NKG2D-DAP10-CD3ζ-transduced NK cells after continuous stimulation with anti-NKG2D. NK cells were incubated with anti-NKG2D and beads as described in A. After 4, 24, and 48 hours, expression of CD107a in CD56+ cells was measured by flow cytometry. Results from experiments with NK cells from two donors are shown.
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 × 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 × 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 α-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 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.
receptor (48). We found that expression of our receptor did not significantly increase 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 non-transplant 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-CD3ζ 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-CD3ζ-NK cells should also be tested in the setting of infectious diseases.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

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