NK Cells Expressing a Chimeric Activating Receptor Eliminate MDSCs and Rescue Impaired CAR-T Cell Activity against Solid Tumors

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Abstract

Solid tumors are refractory to cellular immunotherapies in part because they contain suppressive immune effectors such as myeloid-derived suppressor cells (MDSCs) that inhibit cytotoxic lymphocytes. Strategies to reverse the suppressive tumor microenvironment (TME) should also attract and activate immune effectors with antitumor activity. To address this need, we developed gene-modified natural killer (NK) cells bearing a chimeric receptor in which the activating receptor NKG2D is fused to the cytotoxic ζ-chain of the T-cell receptor (NKG2D.ζ). NKG2D.ζ-NK cells target MDSCs, which overexpress NKG2D ligands within the TME. We examined the ability of NKG2D.ζ-NK cells to eliminate MDSCs in a xenograft TME model and improve the antitumor function of tumor-directed chimeric antigen receptor (CAR)–modified T cells. We show that NKG2D.ζ-NK cells are cytotoxic against MDSCs, but spare NKG2D ligand–expressing normal tissues. NKG2D.ζ–NK cells, but not unmodified NK cells, secrete proinflammatory cytokines and chemokines in response to MDSCs at the tumor site and improve infiltration and antitumor activity of subsequently infused CAR-T cells, even in tumors for which an immunosuppressive TME is an impediment to treatment. Unlike endogenous NKG2D, NKG2D.ζ is not susceptible to TME-mediated downmodulation and thus maintains its function even within suppressive microenvironments. As clinical confirmation, NKG2D.ζ–NK cells generated from patients with neuroblastoma killed autologous intratumoral MDSCs capable of suppressing CAR-T function. A combination therapy for solid tumors that includes both NKG2D.ζ–NK cells and CAR-T cells may improve responses over therapies based on CAR-T cells alone.

Introduction

T lymphocytes can be engineered to target tumor-associated antigens by forced expression of chimeric antigen receptors (CAR; ref. 1). Although successful when directed against leukemia-associated antigens such as CD19 (2, 3), CAR-T cell therapy for solid tumors has been less effective, with best responses in patients with minimal disease (4, 5). Solid tumors recruit inhibitory cells such as myeloid-derived suppressor cells (MDSCs; ref. 6). These immature myeloid cells are a component of innate immunity and strengthen the suppressive tumor microenvironment (TME; refs. 7, 8). The frequency of circulating or intratumoral MDSCs correlates with cancer stage, disease progression, and resistance to standard chemotherapy and radiotherapy (9).

Hence, MDSCs are worth targeting in the quest to enhance CAR-T cell efficacy against solid tumors.

Natural killer (NK) cells, a lymphoid component of the innate immune system, produce MHC-unrestricted cytotoxicity and secrete proinflammatory cytokines and chemokines (10). NK cells also modulate the activity of antigen-presenting myeloid cells within lymphoid organs, and recruit and activate effector T cells at sites of inflammation (11, 12). NK cells express NKG2D, a cytotoxicity receptor that is activated by nonclassic MHC molecules expressed on cells stressed by events such as DNA damage, hypoxia, or viral infection (13). NKG2D ligands are overexpressed on several solid tumors and on tumor-infiltrating MDSCs (14). NK cells, therefore, could alter the TME in favor of an antitumor response by eliminating suppressive elements such as MDSCs. However, the NKG2D cytotoxic adapter molecule DAP10 is downregulated by suppressive molecules of the TME, such as TGFβ (15), limiting the antitumor functions of NK cells.

To overcome the repressive effect of the solid TME on NKG2D function, we used a retroviral vector to modify NK cells with a chimeric NKG2D receptor (NKG2D.ζ) comprising the extracellular domain of the native NKG2D molecule fused to the intracellular cytotoxic ζ-chain of the T-cell receptor (16). We hypothesized that primary human NK cells expressing NKG2D.ζ (NKG2D.ζ–NK cells) would maintain NKG2D.ζ expression within the suppressive TME, kill NKG2D ligand-expressing MDSCs, secrete proinflammatory cytokines and chemokines, and recruit and activate effector cells, including CAR-T cells, derived from the adaptive immune system. These benefits are not attainable from NK cells expressing the native NKG2D receptor as its functions are downmodulated in the TME. Here, we show that when NK cells

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express NKG2Dζ, immune suppression is sufficiently countered to enable tumor-specific CAR-T cells to persist within the TME and eradicate otherwise resistant tumors.

Materials and Methods

Cytokines, cell lines, and antibodies
Recombinant human interleukin (IL)2 was obtained from the National Cancer Institute Biological Resources Branch (Frederick, MD). Recombinant human IL6, GM-CSF, IL7, and IL15 were purchased from PeproTech. The human neuroblastoma cell line LAN-1 was purchased from ATCC and cultured in DMEM culture medium supplemented with 2 mmol/L L-glutamine (Gibco-BRL) and 10% FBS (HyClone). The human CML cell line K562 was purchased from ATCC and cultured in complete-RPMI culture medium composed of RPMI-1640 medium (HyClone) supplemented with 2 mmol/L L-glutamine and 10% FBS. A modified version of parental K562 cells, genetically modified to express a membrane-bound version of IL15 and 41BB ligand, K562-mb15-41BB-L, was kindly provided by Dr. Dario Campana (National University of Singapore). All cell lines were verified by either genetic or flow cytometry-based methods (LAN-1 and K562 authenticated by ATCC in 2009) and tested for Mycoplasma contamination monthly via MycoAlert (Lonza) mycoplasma enzyme detection kit (last mycoplasma testing of LAN-1, K562, and K562 parental line, and K562-mb15-41BB-L on November 2, 2018; all negative). All cell lines were used within 1 month of thawing from early-passage (<3 passages of original vial) lots.

CAR-encoding retroviral vectors
The construction of the SFG-retroviral vector encoding NKG2Dζ, as shown in Supplementary Fig. S1A, was previously described (17). The SFG-retroviral vector encoding NKG2Dζ, an internal ribosomal entry site (IRES), and truncated CD19 (tCD19), was generated by subcloning NKG2Dζ from a retroviral vector (18) kindly provided by Dr. Charles L. Sentman (Dartmouth Geisel School of Medicine, Hanover, NH) into pSFG.IRES.tCD19 (19). RD114-spediotyped viral particles were produced by transient transfection in 293T cells, as previously described (20).

Expansion and retroviral transduction of human NK and T cells
Human NK cells were activated, transduced with retroviral constructs (Fig. 1A), and expanded as previously described by our laboratory (21). Briefly, peripheral blood mononuclear cells (PBMC) obtained from healthy donors under Baylor College of Medicine IRB-approved protocols, were cocultured with irradiated (100 Gy) K562-mb15-41BB-L at a 1:10 (NK cell:irradiated tumor cell) ratio in G-Rex cell culture devices (Wilson Wolf) for 4 days in Stem Cell Growth Medium (CellGenix) supplemented with 10% FBS and 500 IU/mL IL2. Cell suspensions on day 4 (containing 50%–70% expanded/activated NK cells) were transduced with SFG-based retroviral vectors, as previously described (22). The transduced cell population was then subjected to secondary expansion to generate adequate cell numbers for experiments in G-Rex devices at the same NK cell:irradiated tumor cell ratio with 100 IU/mL IL2. This 17-day human gene-modified NK cell protocol resulted in >97% pure CD56+/CD3− NK cell population with average 77.4% ± 18.2% (n = 25) of NK cells transduced with the construct of interest. For most experiments, transduced NK cells were purified to >95% by magnetic column selection of truncated CD19 selection marker–positive cells.

For production of GD2.CAR-T cells (autologous to MDSCs and NK cells), PBMCs from healthy donors were suspended in T-cell medium (TCM) consisting of RPMI-1640 supplemented with 45% Click’s Medium (Gibco-BRL), 10% FBS, and 2 mmol/L L-glutamine, and cultured in wells precoated with CD3 (OKT3, CRL-8001; ATCC) and CD28 (clone CD28.2; BD Biosciences) antibodies for activation. Human IL15 and IL7 were added on day +1, and cells underwent retroviral transduction on day +2, as previously described (22). T cells were used for experiments between days +9 to +14 after transduction, with phenotype as shown in Supplementary Fig. S1B and S1C.

Induction and enrichment of human MDSCs
Our method for ex vivo generation of human PBMC-derived MDSCs was derived from published reports (23), with slight modifications. Briefly, PBMCs were sequentially depleted of CD25+ expressing cells and CD3-expressing cells by magnetic column separation (Miltenyi Biotech). Resultant CD25−CD3− CD3+ PBMCs were plated at 4 × 10^6 cells/mL in complete-RPMI medium with human IL6 and GM-CSF (both at 20 ng/mL) onto 12-well culture plates (Sigma Corning) at 1 mL/well. Plates were incubated for 7 days with medium and cytokines being replenished on days 3 and 5. Resultant cells were harvested by gentle scraping, and MDSCs were purified by magnetic selection using CD33 magnetic microbeads (Miltenyi Biotech). Cells were analyzed by multicolor flow cytometry for CD33, CD14, CD15, HLA-DR, CD11b, CD83, and CD163 (BD Biosciences). MDSCs were defined as either monotypic (M-MDSCs; CD14+, HLA-DRlow/−), PMN-MDSCs (CD14−, CD15+, CD11b+), or early-stage MDSCs (lineage−, HLA-DRlow/+, CD33−), as per published guidelines (24). In addition to the above markers, MDSCs were stained for PD-1, PD-L2, and NKG2D ligands via an NKG2D-Fc chimera (BD Biosciences) followed by FITC-labeled anti-Fc. This pan-ligand staining approach was determined to be the most efficient way to assess NKG2D ligand expression on human MDSCs because (i) NKG2D ligand expression had not previously been reported for human MDSCs and thus simultaneous evaluation of the eight different NKG2D ligands would have been required, and (ii) we found poor reproducibility in staining patterns using individual commercially available ligand antibodies, even within the same donor.

In vitro T-cell suppression assay
T-cell proliferation was assessed using CellTrace Violet (Thermo Fisher) dye dilution analysis, as per manufacturer’s recommendations. Briefly, 1 × 10^5 CellTrace Violet–labeled T cells (isolated at the time of MDSC generation) were plated onto 96-well plates in the presence of plate-bound 1 μg/mL CD3 and 1 μg/mL CD28 antibodies with 50 IU/mL IL2 in the absence or presence of autologous MDSCs or peripheral blood monocytes (as a myeloid control) at 1:1, 4:1, and 8:1 T-cell:MDSC ratios. In some experiments, only the 4:1 ratio is shown as this was determined as optimal for assessment of suppression. After 4 days of coculture, T cells were labeled with CD3 antibody and assessed for cell division using CellTrace Violet dye dilution by flow cytometry. Percent suppression was calculated as follows: [(% proliferating T cells in the absence of MDSCs − % proliferating T cells in presence of MDSCs)/% proliferating T cells in the absence of MDSCs] × 100. Proliferation was defined as a percentage of T cells undergoing active division as represented by CellTrace Violet dilution peaks, as previously described (25).
Figure 1.
NKG2D-ζ-NK cells expand and kill ligand-expressing targets. A, Schematic of SFG-based retroviral vector constructs for transduction of human NK cells. B, Human NK cells were expanded as described in Materials and Methods, and the percentage of CD56⁺/CD3⁻ NK cells at the time of retroviral transduction (day 4) is shown. Expanded NK cells (red circle) purified via depletion of CD3⁺ cells were transduced with NKG2D-ζ retroviral vector or empty vector control (referred to as "unmodified"), and transduction efficiencies are shown in the inset. C, NKG2D expression on NK cells (MFI, inset) was assessed with isotype antibody as control. Nontransduced NK cells exhibited similar NKG2D expression to empty vector–transduced NK cells. *P = 0.003 versus unmodified condition. D, Expression of NKG2D (absolute MFI on the y-axis) on NK cells from each donor (n = 25) transduced with either empty vector or NKG2D-ζ construct was determined by flow cytometry. Each pair of data points connected by a line represent cells from a single donor, to confirm surface expression of our chimeric molecule after transduction. Black lines with gray block next to each group are mean MFI ± SEM. E, NKG2D-ζ–NK cell cytotoxicity against K562 and LAN-1 tumor targets in a 4-hour ⁵¹Cr-release assay. Given that K562 and LAN-1 are both NK-sensitive targets, low E:T ratios were utilized to observe differences. Experiment is representative of at least three separate determinations from n = 10 donors. *P < 0.01 versus unmodified NK cells at same E:T ratio. F, NKG2D-ζ–NK cells were expanded after transduction culture (as shown in schema), and fold expansion and cytotoxicity both pre– (day 7) and post– (day 17) secondary expansion were determined.
In vitro CAR-T chemotaxis assay

Transwell 5-μm pore inserts (Corning) for migration experiments were prepared by coating with 0.01% gelatin at 37°C overnight, followed by 3 μg of human fibronectin (Life Technologies) at 37°C for 3 hours to mimic endothelial and extracellular matrix components, as previously described (26). Briefly, 2 × 10^5 purified GD2.CAR-T cells were placed in 100 μL of TCM in the upper chambers of the precoated Transwell inserts that were then transferred into wells of a 24-well plate. Culture supernatants (400 μL) from NKG2D.ξ or unmodified NK cells cultured with autologous MDSCs or monocytes, were placed in the lower chambers of the wells. Plain medium or medium supplemented with 1 μg/mL of the T-cell recruiting chemokine, MIG, served as negative and positive controls, respectively. The plates were then incubated for 4 hours at 37°C with 5% CO₂, followed by a 10-minute incubation at 4°C to loosen any cells adhering to the undersides of the insert membranes. The fluid in the lower chambers was collected separately, and migrated cells were counted using trypan blue exclusion. The cells were analyzed for CAR expression by flow cytometry to confirm phenotype of migrated T cells.

In vivo TME model

Twelve- to 16-week-old female NSG mice were implanted subcutaneously in the dorsal right flank with 1 × 10^6 Firefly luciferase-FIPLuc–expressing LAN-1 neuroblastoma cells admixed with 3 × 10^5 ex vivo–generated MDSCs, suspended in 100 μL of basement membrane Matrigel (Corning). Matrigel basement membrane was important in keeping tumor and MDSCs confined so as to establish a localized solid TME. Ten to 14 days later, when tumors measured at least 100 mm³ by caliper measurement, mice were injected intravenously with 5 × 10^6 GD2.CAR-T cells. Tumor growth was measured twice weekly by live bioluminescence imaging using the IVIS system (IVIS, Xenogen Corporation) 10 minutes after 150 mg/kg D-luciferin (Xenogen)/mouse was injected intraperitoneally. In experiments examining the ability of NKG2D.ξ–NK cells to reduce intratumoral MDSCs, 1 × 10^7 unmodified or NKG2D.ξ–NK cells were injected intravenously when tumors measured at least 100 mm³. At the end of the experiment, tumors were harvested en bloc, digested ex vivo, and intratumoral human MDSCs (CD33^−, HLA-DR^low^ cells) were enumerated by flow cytometry. The absolute number of human MDSCs within a tumor digest was enumerated per mouse (n = 5 mice/group), compared with pretreatment MDSCs remaining per treatment group. In experiments examining the effects of NKG2D.ξ–NK cells on GD2.CAR-T cell antitumor activity, 5 × 10^6 (cell dose chosen to mitigate direct antitumor effects of NK cells) unmodified or NKG2D.ξ–NK cells were injected intravenously 3 days prior to GD2.CAR-T injection. In GD2.CAR-T cell homing experiments, CAR-T were transduced with GFP-luciferase retroviral construct prior to injection into mice bearing unmodified tumor cells (27). Mice received 5,000 IU human IL2 intraperitoneally three times per week for 3 weeks following NK cell injection to promote NK cell survival in NSG mice (28). Tumor size was measured twice weekly with calipers, and the mice were imaged for bioluminescence signal from T cells at the same time. Mice were euthanized for excessive tumor burden, as per protocol guidelines. The animal studies protocol was approved by Baylor College of Medicine Institutional Animal Care and Use Committee, and mice were treated in strict accordance with the institutional guidelines for animal care.

IHC of neuroblastoma xenografts

On day 32 of in vivo experiments, animals were sacrificed, tumors were harvested, and sectioned bluntly ex vivo to separate tumor periphery (outer 1/3 of tumor volume) versus core (non-necrotic inner 2/3 of tumor volume), and n = 5 sections/tumor sample were analyzed for the presence of GD2.CAR-T and NKG2D.ξ–NK cells by H&E and human CD3 and CD57 immunostaining performed by the Human Tissue Acquisition and Pathology Core of Baylor College of Medicine. Lack of CD57 expression on infused GD2.CAR-T was confirmed by flow cytometry prior to administration. CD57 was chosen as the marker for NK cells in tumor tissue in our study because LAN-1 tumors naturally express the prototypical NK marker CD56, truncated CD19 expression was inadequate for in situ staining, and CD57 had previously been used as a marker for tissue-localized activated NK cells (28). The number of human CD3^+^ and CD57^−^ cells in representative sections of tumors from periphery versus core of the treatment groups indicated were enumerated per high-powered field at 40× magnification, and the percentage of the total number of cells enumerated within tumors found in the periphery versus core in each treatment group indicated from tumors with and without MDSCs is shown as mean ± SEM of n = 5 sections/periphery or core, n = 5 tumors/group.

Analysis of intratumoral MDSCs from patients with neuroblastoma

Tumor tissue and matched peripheral blood of neuroblastoma patients obtained in the context of a specimen/laboratory study after patient identification had been removed were thawed and analyzed for MDSC subsets by flow cytometry or utilized in in vitro assays, as described in figure legends or Results. The tissue acquisition protocol was performed after review and approval by the Baylor College of Medicine Institutional Review Board. Briefly, subjects with a diagnosis of high-risk or intermediate-risk neuroblastoma were eligible to participate. Written informed consent, or appropriate assent for participation, in accordance with the Declaration of Helsinki was obtained from each subject or subject’s guardian for procurement of patient blood and tumor tissue and for subsequent analyses of stored patient materials.

Statistical analysis

Data are presented as mean ± SEM of either experimental replicates or number of donors, as indicated. A paired two-tailed t test was used to determine significance of differences between means, with P < 0.05 indicating a significant difference. For in vitro bioluminescence, changes in tumor radiance from baseline at each time point were calculated and compared between groups using a two-sample t test. Multiple group comparisons were conducted via ANOVA via GraphPad Prism v7 software. Survival determined from the time of tumor cell injection was analyzed by Kaplan–Meier and differences in survival between groups were compared by the log-rank test.

Results

NKG2D.ξ NK cells expand and have cytotoxicity against target cells

To increase killing of NKG2D ligand–expressing MDSCs, we generated primary human NK cells stably expressing NKG2D.ξ and a truncated CD19 (tCD19) marker from a retroviral vector (Fig. 1A). NK cells were expanded from PBMCs obtained from...
normal donors, transduced with retroviral construct expressing chimeric NKG2D, then cultured for 3 additional days. Transduction efficiency, as measured by the expression of tdTomato on CD56+CD3- NK cells after the additional 3 days, was 71.3% ± 16% (n = 25 normal donors) and produced a 5.4 ± 1.1-fold increase in NKG2D expression on the NK cell surface (Fig. 1B–D). NKG2D⁺NK cells showed greater cytotoxicity (79.2% ± 5.6%, n = 10 normal donors) against wild-type K562, a highly NK cell–sensitive tumor cell line that naturally expresses NKG2D ligands, than mock vector–transduced (hereafter referred to as unmodified) NK cells (40.5% ± 2.1%) at 2:1 E:T ratio in a 4-hour cytotoxicity assay (Fig. 1E). In contrast, transgenic NKG2D⁺NK cells did not increase NK cell killing of LAN-1 neuroblastoma cells that are marginally NK sensitive, but lack NKG2D ligands. To determine if in vitro expansion affected the cytotoxic function of NKG2D⁺NK cells, we secondarily expanded transgenic NKG2D⁺NK cells for an additional 10 days (Fig. 1F, schema). As seen in Fig. 1F, NKG2D⁺NK cells expanded (120 ± 7.3-fold by day 17 of culture; n = 10 donors) similarly to unmodified and nontransduced NK cells and maintained stable cytotoxic function between days 7 and 17 of expansion. Thus, we generated and expanded high numbers of primary human NKG2D⁺expressing NK cells capable of cytotoxicity against ligand-expressing targets, even after prolonged culture.

Transgenic NKG2D⁺ is unaffected by TGFβ or soluble NKG2D ligands

Expression of the native NKG2D receptor on NK cells is downregulated by tumor-derived TGFβ and soluble NKG2D ligands, both of which are abundant in the TME (15, 29) and likely impair NK cell function in solid tumors. To determine the effect of TGFβ and soluble NKG2D ligands on NKG2D⁺ receptor expression and function, we cultured NKG2D⁺NK cells in the presence of TGFβ or the soluble NKG2D ligands, MICA and MICB, and examined NKG2D expression and NK cytotoxicity after 24, 48, and 72 hours. After exposure to TGFβ or soluble MICA/B, unmodified NK cells significantly downregulated NKG2D (MFI of 25 vs. 95 in nonexposed NK cells at 48 hours) and were less cytotoxic (20% ± 5.1% killing vs. 40% ± 3.7% killing by nonexposed NK cells at 48 hours) to NKG2D ligand–expressing K562 targets (Fig. 2A and B). In contrast, NKG2D⁺NK cells maintained NKG2D expression and cytotoxicity after exposure to the same concentrations of TGFβ and soluble MICA/B (Fig. 2C and D). This lack of sensitivity to downregulation by these tumor-associated components should benefit the function of NKG2D⁺NK cells within the TME.

Human MDSCs express NKG2D ligands and are killed by NKG2D⁺NK cells

To study the effects of human NK cells on autologous MDSCs, we generated human MDSCs by culture of CD3⁻CD25⁺ PBMC with IL6 plus GM-CSF for 7 days, followed by CD33⁺ selection, as described in Materials and Methods. The phenotypic characterization of these MDSCs and confirmation of their suppressive capacity are shown in Supplementary Fig. S2. Routinely, our ex vivo–generated MDSCs contained monocytic (M)-MDSC and early(e)-MDSC subsets, with few (average <1%) polymorphonuclear (PMN)-MDSCs (Supplementary Fig. S2A), roughly reflecting the subset composition reported in patients with solid tumors (9, 30). The MDSCs expressed the suppressive factors TGFβ, IL6, IL10, and PD1-1 in amounts often greater than tumor

Figure 2.

Transgenic NKG2D⁺ is unaffected by TGFβ or soluble NKG2D ligands. NKG2D⁺ or unmodified NK cells (n = 5 donors) were cultured in the presence of TGFβ (5 ng/mL; A, B) or the soluble NKG2D ligands MICA and MICB (C, D) for 24, 48, and 72 hours. NKG2D⁺ receptor expression was determined by flow cytometry, and NK cytotoxicity against K562 targets was assessed in a 4-hour Cr-release assay at an 5:1 E:T ratio using 48-hour exposed NK cells. Viability of transduced NK cells after exposure to TGFβ for 24, 48, and 72 hours, as assessed by 7-AAD vital staining, was >90%. *P < 0.001 versus non-TGFβ/MICA-treated NK groups at same time points.
cells [Supplementary Fig. S2B and S2C] and suppressed proliferation and cytokine secretion by autologous T cells stimulated with plate-bound CD3/CD28 antibodies [Supplementary Fig. S2D and S2E] and by second-generation CD2 CAR-T cells encoding 4-1BB and CD3-ζ endodomains stimulated with the GD2+ tumor line LAN-1 [Supplementary Fig. S2F and S2G]. As seen in Fig. 3A, MDSCs expressed as much or more NKG2D ligand than the positive control tumor line K562 (ligand MFI of 78.2 vs. 29.7, respectively). Freshly isolated peripheral blood T cells did not express NKG2D ligands, whereas immature and mature dendritic cells expressed little, consistent with previous data [13]. The neuroblastoma cell line LAN-1, subsequently used in our in vivo TME model, did not express NKG2D ligands.

To evaluate MDSC susceptibility to killing by NKG2D.ζ–NK cells, we performed both short- and long-term killing assays. Figure 3B shows enhanced killing of MDSCs by autologous NKG2D.ζ–NK cells compared with unmodified NK cells (35% ± 5.5% vs. 8% ± 2.4% cytotoxicity, respectively, at an E/T ratio of 5:1) in a 4-hour chromium-release assay. MDSC killing was dependent on NKG2D. as preincubation with an NKG2D-blocking Ab reduced the cytotoxicity to levels achieved by unmodified NK cells. NKG2D.ζ–NK cells mediated no cytotoxicity against other autologous immune cells such as freshly isolated monocytes, monocyte-derived mature dendritic cells, T cells, or B cells (Fig. 3C). Only immature dendritic cells, which expressed little NKG2D ligand (approximately 7% of cells; MFI 11.4), were mildly susceptible to lysis by NKG2D.ζ–NK cells (4.2% ± 1.7% lysis at an E/T ratio of 20:1). As confirmation of the clinical applicability of our approach, we assessed whether NKG2D.ζ–NK cells generated from patient PBMCs were able to kill highly suppressive MDSCs isolated from the patient's tumor. Tumor samples obtained from two patients with high-risk neuroblastoma at the time of first biopsy/resection contained M-MDSCs (Fig. 3D). NKG2D.ζ–NK cells generated from patient PBMCs (harvested and frozen at time of tumor sampling) mediated significant cytotoxicity in vitro against M-MDSCs purified from patient tumors, whereas unmodified patient NK cells did not (Fig. 3E). These results provide further clinical evidence for the capacity of NKG2D.ζ–NK cells to eliminate MDSCs in patients with suppressive TMEs.

To determine whether NKG2D.ζ–NK cells could control MDSC survival in long-term cultures, we cocultured NKG2D.ζ–NK cells with autologous MDSCs at a 1:1 ratio for 7 days in the presence of low-dose IL2 to maintain NK survival and quantified each cell type by flow cytometry every 2 days. As shown in Fig. 3F, NKG2D.ζ–NK cells expanded in cocultures (mean 9.5 ± 0.7-fold increase) with a concomitant reduction in MDSCs (mean 81.3 ± 9.4-fold decrease), whereas unmodified NK cells failed to expand or eliminate MDSCs. NK cells cultured alone or with autologous monocyte controls did not expand (0.8 ± 0.1-fold change). As seen in Fig. 3G, NK cell expansion and MDSC reduction correlated with a shift in the culture cytokine milieu from one that is immune-suppressive (more IL6 and IL10; less IFNγ and TNFα) in cocultures containing unmodified NK cells, to one that is immune stimulatory and enhances CAR-T antitumor function (less IL6 and IL10; more IFNγ and TNFα) in cocultures containing NKG2D.ζ–NK cells. Hence, NKG2D.ζ–NK cells mediate potent cytotoxicity against suppressive MDSCs via their highly expressed NKG2D ligands. In addition, through selective depletion of MDSCs in combination with immune-stimulatory cytokine secretion, NKG2D.ζ–NK cells skew the cytokine microenvironment to one that can support CAR-T effector functions [31].

Previous studies have reported that expression of chimeric NKG2D constructs in T lymphocytes can direct these cells to target NKG2D ligand–expressing tumors [16, 32]. However, activated T cells (ATCs) themselves upregulate NKG2D ligands [33], with variable ligand expression intensity dependent on the T-cell activation protocol used, leading to fratricide when the chimeric NKG2D is expressed. To determine if this off-tumor side effect occurred when the same NKG2D.ζ was expressed in NK cells, we compared the killing of ATCs by autologous NK cells or by autologous T cells expressing our NKG2D.ζ transgene. ATCs and NKG2D.ζ-T cells both upregulated NKG2D ligands during ex vivo expansion with CD3/CD28 antibodies plus IL7 and IL15, whereas NKG2D.ζ-transduced NK cells undergoing expansion in our K562-mb15–41BB-I culture system did not (Fig. 3H).

Coculture without additional stimulation of NKG2D.ζ–T cells with autologous ATCs produced fratricide, of both the NKG2D.ζ effector T cells (35 ± 7.2% decrease in cell number) and the nontransduced ATC targets (98% ± 11.5% decrease in cell number; n = 3). By contrast, ATC numbers were unaffected by coculture with autologous NKG2D.ζ–NK cells (Fig. 3I). These results show that NK cells expressing NKG2D.ζ can kill autologous MDSCs while sparing other NKG2D ligand–expressing populations, thus avoiding the fratricide seen with NKG2D.ζ–expressing T cells.

NKG2D.ζ–NK cells eliminate intratumoral MDSCs and reduce tumor burden.

To determine if NKG2D.ζ–NK cells could eliminate MDSCs from tumor sites in vivo, we created an MDSC-containing TME in a xenograft model of neuroblastoma. We chose NKG2D ligand–negative LAN-1 tumor for this experiment so that the effects of NKG2D.ζ–NK cells on MDSCs were not confused with their effects on the tumor cells. LAN-1 tumor cells admixed with human M-MDSCs inoculated subcutaneously in NSG mice. These animals had increases in the suppressive cytokines IL10 (10-fold vs. tumor alone) and TGFβ (2.6-fold vs. tumor alone) in circulation by day 16 as compared with animals bearing tumors initiated without MDSCs, and the resultant tumors grew more rapidly due to increased neovascularization and tumor-associated stroma [Supplementary Fig. S3A–S3D], consistent with clinical reports of MDSC-dense tumors [34]. As seen in Fig. 4A, in mice bearing NKG2D ligand–negative tumors without MDSCs, a single infusion of 1 × 10⁸ NKG2D.ζ–NK cells resulted in a small delay in tumor growth but eventual progression, suggesting that the LAN-1 tumor itself (a marginally NK-sensitive target) can be killed at higher NK cell doses independent of NKG2D ligand expression. In mice bearing MDSC-containing tumors, 1 × 10⁹ NKG2D.ζ–NK cells inhibited tumor growth (Fig. 4B), reduced NKG2D ligand–expressing intratumoral MDSCs with only 8.7% ± 3.5% of the input MDSCs remaining (Fig. 4C), and prolonged mouse survival (median survival of 73 days vs. 29 days after unmodified NK cells; Fig. 4D). Because LAN-1 tumor cells do not express NKG2D ligands and are only marginally sensitive to ligand-independent lysis, tumors subsequently regrew in these mice once the NKG2D.ζ–NK cells had disappeared (>day 40). Thus, NKG2D.ζ–NK cells can traffic to tumor sites and reduce intratumoral MDSCs but cannot themselves eradicate NKG2D ligand–negative malignant cells in our model.
Figure 3.

Human MDSCs express ligands for NKG2D and are killed by NKG2D.ξ-NK cells. A. NKG2D ligand expression on human MDSCs by flow cytometry. Immature dendritic cells (iDC) and mature DCs (mDC) were used as myeloid controls. T cells activated with CD3 and CD28 mAbs plus 100 IU/mL IL2 for 24 hours were used as lymphocyte control. LAN-1 and K562 cells were used as negative and positive controls, respectively. MFI of NKG2D ligand expression in parenthesis. Representative data from single donor (of n = 25 normal donors). Isotype control for NKG2D staining routinely fell within the first log. B. NKG2D.ξ-NK cell cytotoxicity against autologous MDSCs as targets in a 4-hour 51Cr-release assay. In some wells of the cytotoxicity assay, a blocking mAb to NKG2D was added. Representative data from triplicate samples per data point from a single donor (of n = 25 normal donors) are shown. *, P < 0.01 versus unmodified NK cells at same E:T ratio. C. In the same experiment as B, the same batch of NKG2D.ξ-NK cells were analyzed for cytotoxicity against autologous B cells, monocytes, monocyte-derived iDC and mDC, and activated T cells (n = 10 donors examined). D. M-MDSC frequency by flow cytometry from neuroblastoma tumor samples obtained from high-risk patients, as described in Materials and Methods. E. Cytotoxicity by NKG2D.ξ-NK cells derived from patient PBMC (harvested and frozen at the time of tumor sampling) against autologous tumor-derived MDSCs in a 4-hour 51Cr-release assay. Data shown are from triplicate samples per data point at a 10:1 E:T ratio. *, P < 0.001 versus unmodified NK cells from the same donor. F. NKG2D ligand expression was determined for activated T cells (ATC) expressing NKG2D.ξ and NKG2D.ξ-NK cells. Expression of NKG2D ligands on nontransduced ATCs as control for T-cell activation. I, NKG2D.ξ-NK cells or NKG2D.ξ-T cells were cocultured with autologous ATCs at 1:1 ratio and fold change in the number of each cell type from the start of coculture was determined by flow cytometry at indicated time points. #, P < 0.001 versus corresponding cytokine in cocultures with unmodified NK cells.
CXCL8 (IL8) were also produced, but there was no significant response to autologous MDSCs (Fig. 5A). Large amounts of MIP-1α (10-fold increase), and CCL5 (RANTES; 10-fold increase) were seen in Fig. 5C, GD2.CAR-T cells injected alone on day 13 after tumor inoculation (without preadministration of NKG2D.ζ–NK cells) into mice bearing tumors devoid of MDSCs localized effectively to subcutaneous tumors in the flank (4 of 5 mice showed bioluminescent signal on days 14 and 18; Fig. 5C). There was a 10.5 ± 0.8-fold increase in bioluminescent signal on day 18, with CAR-T cell bioluminescence remaining above baseline levels for the duration of the experiment (Fig. 5D). However, in tumors containing MDSCs, CAR-T cells localized poorly: only 1 of 5 mice exhibited bioluminescent signal (Fig. 5C), with only a 1.02 ± 0.1-fold increase in bioluminescent signal on day 18 and bioluminescence falling below preinjection levels within 10 days after injection (Fig. 5D). In contrast, preadministration of NKG2D.ζ–NK cells on day 10 into mice bearing MDSC-containing tumors allowed subsequently infused GD2.CAR-T cells to localize effectively to tumor sites, with bioluminescence in 5 of 5 mice at the tumor site and a 10.9 ± 0.2-fold increase in bioluminescent signal on day 18, within 5 days of injection (Fig. 5D).

To determine if NKG2D.ζ–NK cells can recruit T cells modified with a tumor-specific CAR to tumor sites containing MDSCs, we cocultured NKG2D.ζ–NK cells with autologous MDSCs and analyzed culture supernatants for chemokines by multiplex ELISA. Compared with unmodified NK cells, NKG2D.ζ–NK cells produced significantly greater CCL5 (RANTES; 10-fold increase), CCL3 (MIP-1α; 2-fold increase), and CCL22 (MDC; 5-fold increase) in response to autologous MDSCs (Fig. 5A). Large amounts of CXCL8 (IL8) were also produced, but there was no significant difference from the production by unmodified NK cells. Analysis of chemokine receptor expression on second-generation GD2.CAR-T cells revealed CXCR1 (binds CXCL8), CCR2 (binds CCL2), CCR5 (binds CCL3), and CCR4 (binds CCL5; see Supplementary Fig. S1C). These GD2.CAR-T cells were assayed for chemotaxis to supernatants derived from unmodified or NKG2D.ζ–NK cells cocultured with autologous MDSCs. Supernatants from NKG2D.ζ–NK cells containing cocultures induced chemotaxis of 41.1% ± 5.5% of GD2.CAR-T cells (Fig. 5B), whereas supernatants from unmodified NK cells induced chemotaxis no greater than produced by medium (14.9% ± 6.4% vs. 17.3% ± 1.9%, respectively). Chemotaxis was not induced by supernatants from unmodified or NKG2D.ζ–NK cells cocultured with monocytes. Thus, following their encounter with MDSCs, NKG2D.ζ–NK cells secrete chemokines that recruit CAR-T cells in vitro.

NKG2D.ζ–NK cells improve GD2.CAR-T cell trafficking to tumor sites

To determine the effects of the MDSC-induced, NKG2D.ζ–NK cell chemokines on CAR-T cell recruitment in vivo, we used our MDSC-containing TME xenograft model (see Fig. 4). When tumors reached a volume of ~100 mm³ (day 10), 5 × 10⁶ NKG2D.ζ–NK cells were infused, followed 3 days later (day 13) by infusion of 5 × 10⁶ luciferase gene–transduced GD2.CAR-T cells. Tumor localization and expansion of GD2.CAR-T cells were measured over time via live-animal bioluminescence imaging. As seen in Fig. 5C, GD2.CAR-T cells injected alone on day 13 after tumor inoculation (without preadministration of NKG2D.ζ–NK cells) into mice bearing tumors devoid of MDSCs localized effectively to subcutaneous tumors in the flank (4 of 5 mice showed bioluminescent signal on days 14 and 18; Fig. 5C). There was a 10.5 ± 0.8-fold increase in bioluminescent signal on day 18, with CAR-T cell bioluminescence remaining above baseline levels for the duration of the experiment (Fig. 5D). However, in tumors containing MDSCs, CAR-T cells localized poorly: only 1 of 5 mice exhibited bioluminescent signal (Fig. 5C), with only a 1.02 ± 0.1-fold increase in bioluminescent signal on day 18 and bioluminescence falling below preinjection levels within 10 days after injection (Fig. 5D). In contrast, preadministration of NKG2D.ζ–NK cells on day 10 into mice bearing MDSC-containing tumors allowed subsequently infused GD2.CAR-T cells to localize effectively to tumor sites, with bioluminescence in 5 of 5 mice at the tumor site and a 10.9 ± 0.2-fold increase in bioluminescent signal on day 18, within 5 days of injection (Fig. 5D).

To determine if NKG2D.ζ–NK cells could promote GD2.CAR-T infiltration into the tumor bed, we compared the frequency of human GD2.CAR-T and human NK cells in the tumor periphery and the tumor core by IHC (Supplementary Fig. S4A and S4B). In tumors without MDSCs, 89% ± 11% of the total T cells in the tumor had infiltrated into the tumor core. In contrast, a much smaller fraction (39% ± 16%) infiltrated into the core of tumors containing MDSCs, suggesting TME suppression of CAR-T infiltration. However, pretreatment of tumors containing MDSCs with...
versus medium; the same GD2.CAR-T cell injection was preceded 3 days earlier by a MDSC depletion with only 26.8% of the input MDSCs remaining) resulted in no significant tumor regression or prolongation of survival in mice bearing xenografts containing human MDSCs. A single infusion of \(10^6\) NKG2D.ζ-NK cells (that had no direct antitumor effect by themselves within the other arm of the same experiment; see Fig. 6A and B), the antitumor activity of the GD2.CAR-T cells in mice bearing MDSC-containing tumors was restored to the level observed in mice whose tumors lacked MDSCs (Fig. 6C). NKG2D.ζ-NK cells preinjection also improved the overall survival of the mice with MDSC-containing tumors to a median 120 days with durable cure in 2 of 5 mice (Fig. 6D). Taken together, our results suggest that NKG2D.ζ-NK cells not only eliminate MDSCs from the TME, but also recruit CAR-T cells to intratumoral sites, which facilitates antitumor efficacy.

**Discussion**

We have developed a TME-disrupting approach that eliminates MDSCs and rescues MDSC-mediated impairment of tumor-directed CAR-T cells. We show that when coimplanted with a neuroblastoma cell line, human MDSCs both enhance tumor growth and suppress the infiltration, expansion, and antitumor efficacy of tumor-specific CAR-T cells. In this model, NK cells bearing a chimeric version of the activating receptor NKG2D (NKG2D.ζ-NK cells) are directly cytotoxic to autologous MDSCs, thus eliminating MDSCs from tumors. In addition, NKG2D.ζ-NK cells secrete proinflammatory cytokines and chemokines in response to MDSCs at the tumor site, improving CAR-T cell...
infiltration and function, and resulting in tumor regression and prolonged survival compared with treatment with CAR-T cells alone. Our cell therapy approach utilizes an engineered innate immune effector that targets the TME and shows potential to enhance efficacy of combination immune-based therapies for solid tumors.

NKG2D-ζ-NK cells directly killed highly suppressive MDSCs generated in vitro as well as those from patient tumors. NKG2D-ζ-NK cells also secreted cytokines that favored immune activation in response to MDSCs. Unmodified NK cells were unable to mediate these effects. The ability of NKG2D-ζ-NK cells to eliminate MDSCs from the TME should have several beneficial effects for antitumor immunity. First, as MDSCs express suppressive cytokines such as TGFβ and the checkpoint ligands PD-L1 and PD-L2, elimination of MDSCs should help relieve the suppression of endogenous T-cell responses and potentiate the activity of adoptive T-cell therapies. Given that high baseline numbers of MDSCs have been reported as a biomarker of poor response in the context of trials with the checkpoint inhibitors ipilimumab and pembrolizumab (35, 36), elimination of MDSCs by NKG2D-ζ-NK cells could not be achieved by previous methods that target MDSCs. Previous strategies for modulation of MDSCs within the TME have included use of agents that block single functions such as secretion of nitric oxide (37) or expression of checkpoint molecules (38); induce MDSC differentiation such as with all-transretinoic acid (39); or eliminate MDSCs such as with the cytotoxic agents doxorubicin or cyclophosphamide (40). The MDSC-eliminating effects were dependent on continued administration of the agents, with a rapid rebound in MDSCs after discontinuation. Moreover, many of these agents have off-target toxicities that include damage to endogenous tumor-specific T cells. In contrast, NKG2D-ζ-NK cells produce prolonged and specific elimination of MDSCs with the potential to kill MDSCs that are recruited to the tumor from the bone marrow, while continually secreting cytokines and chemokines which, respectively, alter TME suppression and recruit and activate tumor-specific T cells. Thus, NKG2D-ζ-NK cells exert a prolonged combination of simultaneous immune-modulatory effects that enhance antitumor immune function in ways that could not be achieved by previous methods that target MDSCs.

We observed no toxicity against normal hematopoietic cells when NKG2D-ζ was expressed in autologous human NK cells. Previous studies overexpressing an NKG2D-ζ receptor containing costimulatory endodomains (e.g., CD28 or 41BB) and DAP10, a
NK cells had activity against MDSCs. In engineered human immune systems, NK cell–mediated elimination of MDSCs from a GD2-fl tumor model developed by Cytocodes (22) demonstrated increased NK cell antitumor activity within a suppressive solid TME without toxicity to normal NKG2D ligand–expressing tissues. Hence, the elimination of suppressive MDSCs by NKG2D-Cz–NK cells may safely enhance adoptive cellular immunotherapy for neuroblastoma and for many other tumors that are supported and protected by MDSCs.

**Disclosure of Potential Conflicts of Interest**

R. Parihar is a consultant/advisory board member for GT Biopharma. S. Gottschalk reports receiving a commercial research grant from Tessa Therapeutics; has ownership interest in patents and patent applications in the field of cell and gene therapy for cancer; and is a consultant/advisory board member for Immatics, Vizacayte, and Sanofi. C.M. Rooney has ownership interest in Vizacayte and Marker Therapeutics, and is a consultant/advisory board member for Cell Medica, Bluebird Bio, Conkwest Plc, Harvard Medical School, Tessa Therapeutics, and Cell Genix GMBH. No potential conflicts of interest were disclosed by the other authors.

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NK Cells Expressing a Chimeric Activating Receptor Eliminate MDSCs and Rescue Impaired CAR-T Cell Activity against Solid Tumors

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