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 (NKG2Dζ). NKG2Dζ-NK cells target MDSCs, which overexpress NKG2D ligands within the TME. We examined the ability of NKG2Dζ-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 NKG2Dζ-NK cells are cytotoxic against MDSCs, but spare NKG2D ligand–expressing normal tissues. NKG2Dζ-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, NKG2Dζ is not susceptible to TME-mediated downmodulation and thus maintains its function even within suppressive microenvironments. As clinical confirmation, NKG2Dζ-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 NKG2Dζ-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 suppress the aggressive 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 (NKG2Dζ) 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 NKG2Dζ (NKG2Dζ-NK cells) would maintain NKG2Dζ 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...
express NKG2D, 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 ViaMycAlert (Lonza) mycoplasma enzyme detection kit (last mycoplasma testing of LAN-1, 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 the actual parental line, and K562-mb15-41BB-L on November 2, 2018; all negative). All cell lines were used within 1 month of thawing from primary culture (<3 passages of original vial) lots.

**Expansion and retroviral transduction of human NK and T cells**

Human NK cells were activated, transduced with retroviral CAR.41BB, as shown in Supplementary Fig. S1A, was previously described (17). The SFG-retroviral vector encoding NKG2D, an internal ribosomal entry site (IRES), and truncated CD19 (tCD19), was generated by subcloning NKG2D, a retroviral vector (18) kindly provided by Dr. Charles L. Sentman (Dartmouth Geisel School of Medicine, Hanover, NH) into pSFG.IRES.tCD19 (19). RD114-spedotypd viral particles were produced by transient transfection in 293T cells, as previously described (20).

**CAR-encoding retroviral vectors**

The construction of the SFG-retroviral vector encoding NKG2D in human NK cells, an internal ribosomal entry site (IRES), and truncated CD19 (tCD19), was generated by subcloning NKG2D, a retroviral vector (18) kindly provided by Dr. Charles L. Sentman (Dartmouth Geisel School of Medicine, Hanover, NH) into pSFG.IRES.tCD19 (19). RD114-spedotypd viral particles were produced by transient transfection in 293T cells, as previously described (20).

For production of CD2 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 I1.5 and I1.7 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 posttransduction, 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 Biotec). Resultant CD25-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 Biotec). Cells were analyzed by multicolor flow cytometry for CD33, CD14, CD11b, HLA-DR, CD11c, CD33, and CD14+ (BD Biosciences). MDSCs were defined as either monocytic (M-MDSCs, CD14+, HLA-DR-low/-, PMN-MDSCs (CD14+, CD15+, CD11b+), or early-stage MDSCs (lineage-, HLA-DR-low/-, CD33+), as per published guidelines (24). In addition to the above markers, MDSCs were stained for CD14, CD15, HLA-DR, HLA-DM, CD33, and CD25. The construction of the SFG-retroviral vector encoding GD2-CAR, as shown in Supplementary Fig. S1A, was previously described (17).

**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).

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**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 [⁵¹]Cr-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 × 10⁵ purified GD2.CAR-T cells were placed in 100 μL of TC1 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⁺ 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 underside 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 × 10⁶ Firefly luciferase(FLuc⁺)–expressing LAN-1 neuroblastoma cells admixed with 3 × 10⁵ 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 × 10⁶ 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⁺NK cells to reduce intratumoral MDSCs, 1 × 10⁶ unmodified or NKG2D⁺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 (CD14⁺, CD16⁻HLA-DRlow) cells were enumerated by flow cytometry. The absolute number of human MDSCs within a tumor digest was estimated per mouse (n = 5 mice/group), compared with pretreatment MDSC numbers, and presented as mean ± SEM of either experimental replicates or number of donors, as indicated.

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⁺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 vivo 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 with 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⁺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⁺ and a truncated CD19 (iCD19) 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 CD19 on CD3éCD5éCD3éNK 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). NKG2DéNK 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 NKG2DéNK cells showed no increase in cytotoxicity from wild-type K562 cells, as determined by the expression of CD19 on NK cells (90% vs. 90% 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, NKG2DéNK cells maintained NKG2D expression and cytotoxicity after exposure to the same concentrations of TGFb and soluble MICA/B (Fig. 2C and D). This lack of sensitivity to downregulation by these tumor-associated components should benefit the function of NKG2DéNK cells within the TME.

Human MDSCs express NKG2D ligands and are killed by NKG2DéNK cells

To study the effects of human NK cells on autologous MDSCs, we generated human MDSCs by culture of CD3éCD50 PBMC with IL6 plus GM-CSF for 7 days, followed by CD33é 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 TGFb, IL6, IL10, and PDL-1 in amounts often greater than tumor

Figure 2.
Transgenic NKG2Dé is unaffected by TGFb or soluble NKG2D ligands. NKG2Dé or unmodified NK cells (n = 5 donors) were cultured in the presence of TGFb (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 TGFb for 24, 48, and 72 hours, as assessed by 7-AAD vital staining, was >90%. * = P < 0.001 versus non-TGFb/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 GD2 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 NK2G2D 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 NK2G2D ligands, whereas immature and mature dendritic cells expressed little, consistent with previous data (13). The neuroblastoma cell line LAN-1, subsequently used in our TME model, did not express NK2G2D ligands.

To evaluate MDSC susceptibility to killing by NK2G2D.ζ⁻NK cells, we performed both short- and long-term killing assays. Figure 3B shows enhanced killing of MDSCs by autologous NK2G2D.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 NK2G2D, as preincubation with an NK2G2D-blocking Ab reduced the cytotoxicity to levels achieved by unmodified NK cells. NK2G2D.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 NK2G2D ligand (approximately 7% of cells; MFI 11.4), were mildly susceptible to lysis by NK2G2D.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 NK2G2D.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). NK2G2D.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 NK2G2D.ζ⁻NK cells to eliminate MDSCs in patients with suppressive TMEs.

To determine whether NK2G2D.ζ⁻NK cells could control MDSC survival in long-term cultures, we cocultured NK2G2D.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, NK2G2D.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 NK2G2D.ζ⁻NK cells. Hence, NK2G2D.ζ⁻NK cells mediate potent cytotoxicity against suppressive MDSCs via their highly expressed NK2G2D ligands. In addition, through selective depletion of MDSCs in combination with immune-stimulatory cytokine secretion, NK2G2D.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 NK2G2D constructs in T lymphocytes can direct these cells to target NK2G2D ligand–expressing tumors (16, 32). However, activated T cells (ATCs) themselves upregulate NK2G2D ligands (33), with variable ligand expression intensity dependent on the T-cell activation protocol used, leading to fratricide when the chimeric NK2G2D is expressed. To determine if this off-tumor side effect occurred when the same NK2G2D.ζ was expressed in NK cells, we compared the killing of ATCs by autologous NK cells or by autologous T cells expressing our NK2G2D.ζ transgene. ATCs and NK2G2D.ζ-T cells both upregulated NK2G2D ligands during ex vivo expansion with CD3/CD28 antibodies plus IL7 and IL15, whereas NK2G2D.ζ-transduced NK cells undergoing expansion in our K562-mb15-41BB-L culture system did not (Fig. 3H). Coculture without additional stimulation of NK2G2D.ζ-T cells with autologous ATCs produced fratricide, of both the NK2G2D.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 NK2G2D.ζ⁻NK cells (Fig. 3I). These results show that NK cells expressing NK2G2D.ζ can kill autologous MDSCs while sparing other NK2G2D ligand–expressing populations, thus avoiding the fratricide seen with NK2G2D.ζ⁻expressing T cells.

**NK2G2D.ζ⁻NK cells eliminate intratumoral MDSCs and reduce tumor burden.** To determine if NK2G2D.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 NK2G2D ligand–negative LAN-1 tumor for this experiment so that the effects of NK2G2D.ζ⁻NK cells on MDSCs were not confused with their effects on the tumor cells. LAN-1 tumor cells admixed with human MDSCs were 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 NK2G2D ligand–negative tumors without MDSCs, a single infusion of 1 × 10⁷ NK2G2D.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 NK2G2D ligand expression. In mice bearing MDSC-containing tumors, 1 × 10⁷ NK2G2D.ζ⁻NK cells inhibited tumor growth (Fig. 4B), reduced NK2G2D 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 NK2G2D ligands and are only marginally sensitive to ligand-independent lysis, tumors subsequently regrew in these mice once the NK2G2D.ζ⁻NK cells had disappeared (>day 40). Thus, NK2G2D.ζ⁻NK cells can traffic to tumor sites and reduce intratumoral MDSCs but cannot themselves eradicate NK2G2D ligand–negative malignant cells in our model.
Chimeric NK Cells Reverse CAR-T Cell Impairment

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.

G, 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 ATC fold change at day 0 and 3 cocultures.
CXCL8 (IL8) were also produced, but there was no significant response to autologous MDSCs (Fig. 5A). Large amounts of chemokines were produced by supernatants derived from unmodified CAR-T cells revealed CXCR1 (binds CXCL8), CCR2 (binds CCL2), and CCR5 (binds CCL3, CCL5). Analysis of chemokine receptor expression on second-generation GD2.CAR-T cells revealed CXCR1 (binds CXCL8), CCR2 (binds CCL2), and CCR5 (binds CCL3, 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 tumors allowed subsequently infused GD2.CAR-T cells to localize effectively to tumor sites, with bioluminescence in 5 of 5 tumors containing MDSCs, CAR-T cells localized poorly: only 1 of 5 mice showed bioluminescent signal on day 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, pretreatment 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 the core of tumors containing MDSCs, suggesting TME suppression of CAR-T infiltration. However, pretreatment of tumors containing MDSCs with NKG2D.ζ-NK cells secrete chemokines that recruit GD2.CAR-T cells
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 produce 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, CCL5), 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 tumors allowed subsequent 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). In contrast, pretreatment of tumors containing MDSCs with 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).

When tumors reached a volume of ≈100 mm^3 (day 10), 5 × 10^6 NKG2D.ζ-NK cells were infused, followed 3 days later (day 13) by infusion of 5 × 10^6 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, pretreatment of tumors containing MDSCs with NKG2D.ζ-NK cells 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).

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NKG2Dζ-NK cells increased the fraction of intratumoral CAR-T cells (70% ± 13%) within the tumor core. Equal numbers of NKG2Dζ-NK cells were observed within both peripheral and core samples from MDSC-positive and MDSC-negative tumors (Supplementary Fig. S5), suggesting the ability of NK cells to traffic well within tumors despite the presence of MDSCs.

**Elimination of MDSCs increases antitumor activity of GD2-CAR-T cells**

To determine if the activities of NKG2Dζ-NK cells described above enhance the antitumor function of CAR-T cells, we treated mice bearing subcutaneous, luciferase-labeled neuroblastoma containing MDSCs with GD2.CAR-T cells preceded by NKG2Dζ-NK cells, in a similar set-up to experiments in Fig. 5C. As seen in Fig. 6A and B, a single injection of 5 × 10^6 NKG2Dζ-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). NKG2Dζ-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 NKG2Dζ-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 (NKG2Dζ-NK cells) are directly cytotoxic to autologous MDSCs, thus eliminating MDSCs from tumors. In addition, NKG2Dζ-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 PDL-1 and PDL-2, 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 may also enhance checkpoint inhibition. Second, elimination of MDSCs should also decrease other MDSC-associated effects, including neovascularization via their expression of VEGF, production of immunosuppressive metabolic products such as PGE2 and adenosine, and establishment of tumor-supportive stroma via their expression of iNOS, FGF, and matrix metalloprotei-

nases (8). In short, the ability of NKG2D-ζ-NK cells to eliminate MDSCs alters the TME in multiple ways that should improve antitumor immunity.

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
signaling adaptor molecule for enhanced surface expression of NKG2D, in T cells showed activity against NKG2D ligand–overexpressing tumors, but at the cost of fratricide in vitro and lethal toxicity in mice (16, 32, 33). Using our standard T-cell activation/expansion protocol (22), we also observed upregulation of NKG2D ligands, leading to fratricide in T cells expressing NKG2D. When NKG2D– T cells engage NKG2D ligands expressed on normal tissues, they will not receive the physiologic NK cell–directed inhibitory inputs that would safely regulate this potent and unopposed chimeric receptor activity. By contrast, when NKG2D is expressed on NK cells, they are able to recognize inhibitory NK cell ligands such as self-MHC expressed on healthy self-tissues, counteracting otherwise unopposed positive signals from NKG2D ligands. Thus, an NK cell platform for NKG2D enhancement may limit toxicity while taking advantage of the cytotoxic and immune-modulatory potential of the receptor-ligand system.

Unlike wild-type NKG2D, transgenic NKG2D– expression and activity were not sensitive to downmodulation by TGFB or soluble NKG2D ligands, allowing improved function in the TME. Native NKG2D relies solely on the intracytoplasmic adaptor DAP10 for mediating its cytolytic activity in human NK cells (41). TGFB1 and soluble NKG2D ligands both decrease DAP10 gene transcription and protein activity, and thus reduce NKG2D function in endogenous NK cells (42, 43). In contrast, transgenic NKG2D does not rely on DAP10-based signaling for its activity, because signaling occurs through the Fas–chain. Thus, this construct provides a stable cytolytic pathway capable of circumventing TME-mediated downregulation of native NKG2D activity. A previous study expressing a chimeric NKG2D molecule that incorporated DAP10 reported enhanced NK cytotoxicity compared with NKG2D alone in vitro against a variety of human cancer cell lines as well as in a xenograft model of osteosarcoma (44). However, this report did not address the susceptibility of this complex to downmodulation by TGFB or soluble NKG2D ligands, or whether these NK cells had activity against MDSCs.

NKG2D–NK cells countered immunosuppression mediated by MDSCs leading to enhanced CAR-T cell tumor infiltration and expansion at tumor sites, CAR-T functions that are impaired in patients with solid tumors (45). Unlike the GD2–CAR T-cells in our model, NKG2D–NK cells homed effectively to MDSC-engulfed tumors and released an array of chemokines that increased T-cell infiltration of tumor. Unlike pharmacologic strategies aimed at enhancing leukocyte trafficking, including administration of lymphoatkin or TNFα (46), our approach does not require continuous cytokine administration. In fact, the ability of chimeric NKG2D to augment NK immune function specifically within the immunosuppressive TME provides for the local release of chemotactic factors, reflecting a more homeostatic method by which to increase CAR-T infiltration. Once there, CAR-T cells should meet an environment favorably modified by NKG2D–NK cell–mediated elimination of MDSCs and production of proinflammatory cytokines. Indeed, elimination of MDSCs from a GD2– tumor xenograft enhanced the activity of GD2–CAR T-cells in our model, including T-cell survival and intratumoral expansion. Given the suppressive effects of MDSCs in neuroblastoma (47, 48), the model shows how reversal of an MDSC-mediated suppressive microenvironment can improve antitumor functions of effector T cells.

Clinical neuroblastoma contains intense infiltrates of MDSCs (49), which are not included in tumor xenograft models currently used to study human cell therapeutics. Our data suggest that coinoculation of tumors with suppressive components (such as MDSCs) can model TME-mediated suppression of CAR-T activity against solid tumors and provides a method by which to understand and counter immunosuppression. Although NSG mice lack a complete immune system in which to examine the effects of multiple endogenous immune components, our ability to engraft exogenous components (e.g., human MDSCs) within our TME model provides the possibility of simulating different immunosuppressive aspects of the solid TME. In fact, further model development utilizing human inhibitory macrophages and regulatory T cells (Treg) as additional suppressive components of the TME is currently under way in our laboratory. In summary, we describe an approach to reverse the suppressive TME using engineered human NK cells. We have shown that generation and expansion of our NK cell product is feasible and that NKG2D–NK cells have antitumor activity within a suppressive solid TME without toxicity to normal NKG2D ligand–expressing tissues. Hence, the elimination of suppressive MDSCs by NKG2D–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, Viracayte, and Sanofi. C.M. Rooney has ownership interest in Viracayte 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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Acknowledgments
The authors would like to thank Dr. Charles L. Sentman (Dartmouth Geisel School of Medicine, Hanover, NH) for providing the retroviral vector encoding the initial NKG2Dζ–. This work was supported, in part, by the St. Baldrick’s Foundation Fellowship Award (R. Parihar), the Conquer Cancer Foundation Young Investigator Award (R. Parihar), Hyundai Hope on Wheels Foundation (R. Parihar), the American Cancer Society (R. Parihar), and Alex’s Lemonade Stand Foundation. In addition, the authors acknowledge the support of Lisa Rollins at the Center for Cell and Gene Therapy and the Human Tissue Acquisition and Pathology Core of Baylor College of Medicine. We thank Malcolm K. Brenner for expert editorial input.

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Received August 21, 2018; revised November 5, 2018; accepted January 11, 2019; published first January 16, 2019.
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