Research Article

Cancer Immunology Research

Phase I Trial of Autologous CAR T Cells Targeting NKG2D Ligands in Patients with AML/MDS and Multiple Myeloma

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Abstract

NKG2D ligands are widely expressed in solid and hematologic malignancies but absent or poorly expressed on healthy tissues. We conducted a phase I dose-escalation study to evaluate the safety and feasibility of a single infusion of NKG2D-chimeric antigen receptor (CAR) T cells, without lymphodepleting conditioning in subjects with acute myeloid leukemia/myelodysplastic syndrome or relapsed/refractory multiple myeloma. Autologous T cells were transfected with a γ-retroviral vector encoding a CAR fusing human NKG2D with the CD3ζ signaling domain. Four dose levels (1×10^6–3×10^7 total viable T cells) were evaluated. Twelve subjects were infused [7 acute myeloid leukemia (AML) and 5 multiple myeloma]. NKG2D-CAR products demonstrated a median 75% vector-driven NKG2D expression on $CD3^+$ T cells. No dose-limiting toxicities, cytokine release syndrome, or CAR T cell–related neurotoxicity was observed. No significant autoimmune reactions were noted, and none of the ≥ grade 3 adverse events were attributable to NKG2D-CAR T cells. At the single injection of low cell doses used in this trial, no objective tumor responses were observed. However, hematologic parameters transiently improved in one subject with AML at the highest dose, and cases of disease stability without further therapy or on subsequent treatments were noted. At 24 hours, the cytokine RANTES increased a median of 1.9-fold among all subjects and 5.8-fold among six AML patients. Consistent with preclinical studies, NKG2D-CAR T cell–expansion and persistence were limited. Manufactured NKG2D-CAR T cells exhibited functional activity against autologous tumor cells *in vitro*, but modifications to enhance CAR T-cell expansion and target density may be needed to boost clinical activity.

Introduction

Chimeric antigen receptor (CAR) T-cell therapy can induce remarkable clinical remissions in refractory B-cell lineage leukemias and lymphomas (1–6). However, clinical success targeting non–B-cell antigens has been limited, and novel strategies to target acute myeloid leukemia (AML), multiple myeloma (MM), and solid tumors are needed. Here, we report results from a first-in-human phase I trial using CAR-expressing autologous T cells targeted against NKG2D ligands in adult patients with AML/myelodysplastic syndrome (MDS) and relapsed/refractory myeloma. Both the CAR structure and nature of the targeted antigens were distinct from CAR approaches in previous clinical trials. Rather than incorporating single-chain fragment immunoglobulin variable regions, this CAR used the naturally occurring NKG2D receptor as the antigen-binding domain, fused to the intracellular domain of CD3ζ. A γ-retroviral vector was used to express the NKG2D-CAR, relying on endogenous Dap10 expression and colocalization to provide a costimulatory signal (7). NKG2D is an activating receptor expressed on NK cells, invariant NKT cells, γδ T cells, $CD8^+$ T cells, and a small fraction of $CD4^+$ T cells. NKG2D forms a homodimer and associates with the adaptor molecule Dap10 in the transmembrane domain to form a hexameric complex that mediates its function in T cells via recruitment of the p85 PI3-kinase and Vav-1 signaling complex (8). In its native form, NKG2D provides a costimulatory signal to T cells but relies on antigen recognition via the T-cell receptor (TCR) to deliver the primary activating signal (7). In contrast, NKG2D ligand recognition through the NKG2D-CAR mediates primary T-cell activation.

NKG2D is highly conserved and recognizes a group of eight ligands, namely, MICA, MICB, and the UL16-binding proteins (ULBP) 1–6. NKG2D ligands are upregulated in response to DNA damage, infection with certain pathogens, and importantly, malignant transformation (7). NKG2D ligand expression has been reported in a broad range of solid tumors and hematologic malignancies, including AML and MM, whereas ligands are

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generally absent on healthy tissues (9–14). Thus, NKG2D-based CAR T cells have the potential for broad oncologic applications. Murine studies by Sentman and colleagues demonstrate NKG2D-CAR T-cell efficacy in eradicating established MM, lymphoma, and ovarian cancers and the induction of autologous immunity protective against tumor rechallenge, even after NKG2D-CAR T cells were no longer detectable (15–21). NKG2D-CAR T cells have also been shown to inhibit the growth of tumors with heterogeneous NKG2D ligand expression (22). Importantly, human NKG2D-CAR T cells do not react to autologous peripheral blood mononuclear cells (PBMC) or bone marrow (BM) from healthy donors *in vitro* (16). Nevertheless, concern for ligand upregulation on healthy tissues and induction of an inflammatory feedback loop under conditions of cell stress is justified. Analogous to cytokine release syndrome (CRS), which has been observed in CAR T-cell clinical trials, dose- and strain-dependent NKG2D-CAR toxicity was associated with production of inflammatory cytokines in murine models (23, 24). Therefore, this trial was designed emphasizing safety parameters. Lymphodepleting chemotherapy, which was not required for efficacy in murine models, was not used to minimize the risk of NKG2D ligand induction on healthy tissues. Dose-escalation started at a single low dose of 1×10^6 viable T cells. In this first-in-human trial, we demonstrated the feasibility and safety of infusion of freshly manufactured autologous NKG2D-CAR T cells in AML and MM patients up to a dose of 3×10^7 T cells. Observations of transient hematologic improvement and unexpected disease stability in some patients, as well as demonstration of CAR T-cell activity against autologous tumor cells *in vitro*, support further development of this approach.

Materials and Methods

Study design and participants

This single-center phase I study was conducted as per the Declaration of Helsinki and Good Clinical Practice guidelines and approved by the FDA, the Recombinant DNA Advisory Committee and the Dana-Farber/Harvard Cancer Center institutional review board, #NCT02203825 (www.clinicaltrials.gov). All patients provided written informed consent. Eligible subjects were ≥18 years old with AML or MDS, not in remission and without standard treatment options, or relapsed/refractory MM with measurable and progressive disease after prior therapy with an immunomodulator and proteasome inhibitor. Notable exclusion criteria included central nervous system involvement, active infections, uncontrolled medical disorders, active autoimmune disease, prior disease-directed therapy within 3 weeks of T-cell infusion, and prior allogeneic stem cell transplantation (SCT), gene therapy, or adoptive T-cell therapy. Infusion criteria included absence of fever, active infection, and rapid disease progression. Products were infused in patients after administration of acetaminophen 650 mg by mouth and diphenhydramine 25 to 50 mg by mouth or intravenously. A modified Fibonacci "3 + 3" dose-escalation design of a single infusion of NKG2D-CAR T cells ranging from 1×10^6 to 3×10^7 total viable $CD3^+$ T cells across 4 dose levels was followed, with the provision to enroll at least 1 patient with AML/MDS and MM in each 3-patient cohort.

The primary endpoints included evaluating the safety and feasibility of a single intravenous infusion of freshly manufactured NKG2D-CAR T cells without lymphodepleting conditioning. Feasibility was defined by the frequency of enrolled subjects who did receive NKG2D-CAR T cells. Safety was defined by the absence of NCI CTCAE v4.0 ≥ grade 3 events or grade 2 autoimmune events with possible, probable, or definite attribution to NKG2D-CAR T cells. Secondary endpoints included progression-free survival, clinical antitumor effects, and immunologic correlatives. Objective tumor response rates were monitored according to the Myeloma IMWG Uniform Response Criteria, MDS IWG Response Criteria, and revised "Cheson" criteria for AML, respectively, with the initial response assessment at day 28 (25–28). Stable disease was not an endpoint defined for subjects with AML in the original trial design (29). To obtain samples for safety, efficacy, and immunologic analyses, patients underwent collection of plasma and peripheral blood samples at prospectively defined time points such as preinfusion, 1 and 24 hours following infusion, weekly until 28 days following infusion then monthly as relevant, as well as BM sampling and repeat imaging preinfusion, at 28 days, and 3 months as relevant.

Clinical γ-retroviral vector and cell product manufacture and analysis

Construct design. The NKG2D-CAR gene (chNKG2D) was created by fusing the human cytoplasmic CD3ζ coding sequence to the full-length human NKG2D gene (*KLRK1*; ref. 30; Fig. 1A). Sequence of NKG2D CAR transgene: (CD3ζ cytoplasmic domain underscored; NKG2D gene **bold**).

ATGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCG-TACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTA-GGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGG-CCGGGACCCTGAGATGGGGGGAAAGCCGCAGAGAAGGAAG-AACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAG-ATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCG-CCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCA-GTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGG-CCCTGCCCCCTCGCGAATTC**GGGTGGATTCGTGGTCGGAGG-TCTCGACACAGCTGGGAGATGAGTGAATTTCATAATTATAACT-TGGATCTGAAGAAGAGTGATTTTTCAACACGATGGCAAAAGC-AAAGATGTCCAGTAGTCAAAAGCAAATGTAGAGAAAATGCAT-CTCCATTTTTTTTCTGCTGCTTCATCGCTGTAGCCATGGGAAT-CCGTTTCATTATTATGGTAGCAATATGGAGTGCTGTATTCCTA-AACTCATTATTCAACCAAGAAGTTCAAATTCCCTTGACCGAAA-GTTACTGTGGCCCATGTCCTAAAAACTGGATATGTTACAAAAA-TAACTGCTACCAATTTTTTGATGAGAGTAAAAACTGGTATGAG-AGCCAGGCTTCTTGTATGTCTCAAAATGCCAGCCTTCTGAAA-GTATACAGCAAAGAGGACCAGGATTTACTTAAACTGGTGAAG-TCATATCATTGGATGGGACTAGTACACATTCCAACAAATGGAT-CTTGGCAGTGGGAAGATGGCTCCATTCTCTCACCCAACCTAC-TAACAATAATTGAAATGCAGAAGGGAGACTGTGCACTCTATG-CATCGAGCTTTAAAGGCTATATAGAAAACTGTTCAACTCCAAA-TACATACATCTGCATGCAAAGGACTGTGTAA.**

NKG2D is a type II protein in which the NH_2 terminal is located intracellularly, whereas CD3ζ is a type I protein with the COOH terminal in the cytoplasm. Therefore, the CD3ζ cytoplasmic domain, containing its three ITAMs, was fused to NKG2D in reverse orientation (COOH terminal of CD3ζ to NH_2-terminal of NKG2D), as described (20), and then cloned into the Moloney murine leukemia virus-based γ-retroviral vector SFG, received from the laboratory of Dr. Gianpietro Dotti (Baylor College of Medicine) with the permission of Dr. Richard Mulligan (Harvard Medical School; ref. 31). Upon expression, the orientation of the CD3ζ chain was in an inverted orientation as part of the CAR. No signal peptide was required for CAR expression, given that NKG2D is a type II protein.

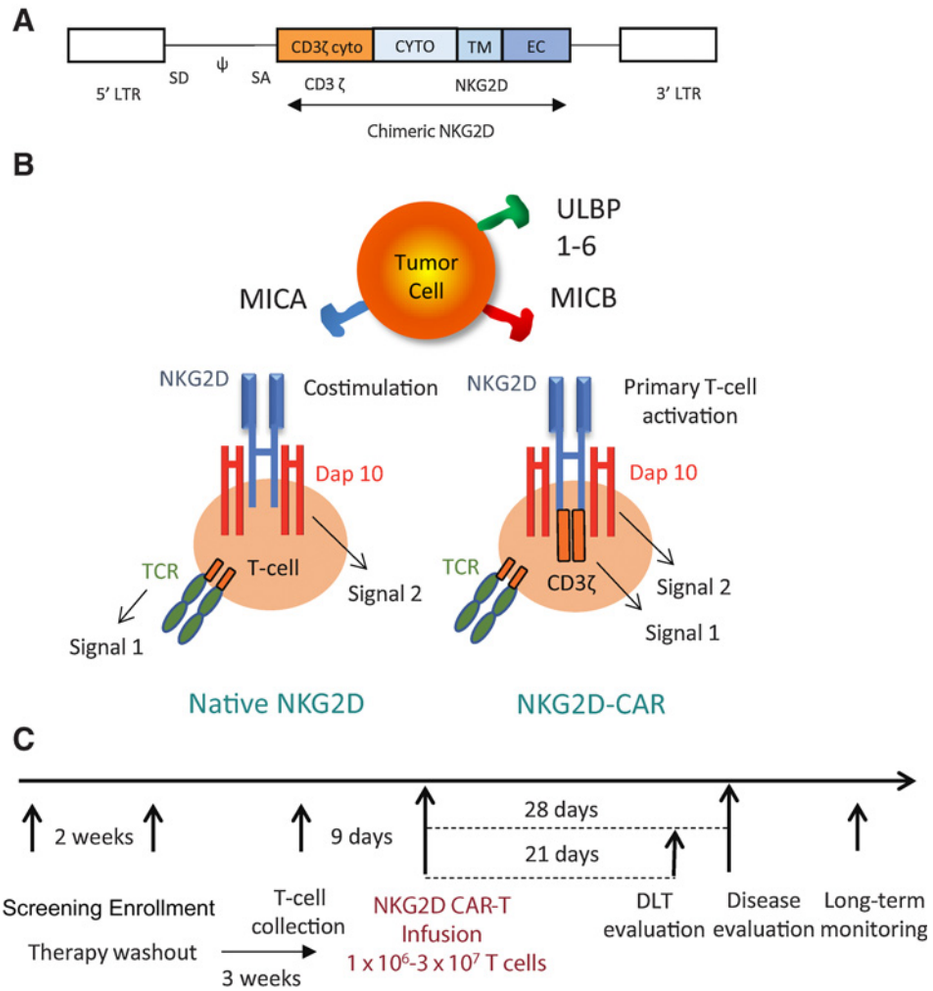


Figure 1.
NKG2D-CAR concept and trial design. **A,** Full-length NKG2D with preserved extracellular (EC), transmembrane (TM), and cytoplasmic domain (CYTO) were fused to the cytoplasmic domain of the CD3ζ chain to create the NKG2D-CAR and cloned into the SFG vector. **B,** In T cells, recognition of NKG2D ligands by native NKG2D provides costimulation via Dap10 signaling but relies on primary T-cell activation through the TCR. In NKG2D-CAR T cells, the CAR directly activates NKG2D-CAR T cells through binding of MICA, MICB, or the UL-16 binding proteins (ULBP) 1–6 on tumor cells, independent of the TCR, and receives a costimulatory signal through the preserved transmembrane interaction with Dap10. **C,** Trial schema indicates a 3-week washout period from prior therapy, 9-day manufacturing process, infusion of freshly manufactured T cells according to dose level, DLT evaluation at 21 days, and response evaluation at 28 days after infusion.

By using full-length NKG2D, colocalization with endogenously expressed Dap10 in the transmembrane domain provides a costimulatory signal. Untransduced $CD8^+$ T cells may receive a costimulatory signal via native NKG2D, but they require a primary activating signal through their TCR. In NKG2D-CAR–expressing T cells, recognition of NKG2D ligands via the CAR mediates primary T-cell activation, independent of the endogenous TCR and provides a costimulatory signal via endogenously expressed Dap10 (Fig. 1B).

Cells and cell lines. PG13 producer lines were generated under cGMP conditions by repeated transduction with transient retroviral supernatant obtained from a Phoenix-Eco packaging cell line transfected with SFG-chNKG2D vector. Clinical-grade high-titer viral supernatant was produced from a PG13 SFG-chNKG2D clone at Rimedion (Indiana University), per cGMP procedures. RPMI 8866 (95041316) and K562 (CRL-3344) were purchased from Sigma-Aldrich and P815 (TIB-64) from ATCC prior to the performance of the experiments. Cells lines were passaged at the recommended ratios and used between the passages of 3 and 30. Cultures were regularly checked for *mycoplasma* contamination by qPCR using MycoSeq (Thermo Fisher).

Manufacturing process. Autologous PBMCs were obtained by peripheral venipuncture (~120 mL) or nonmobilized apheresis guided by an absolute lymphocyte count (ALC) threshold of 0.6K cells/μL, followed by Ficoll-isolation (GE Healthcare). Cell manufacturing occurred over 9 days in the Dana-Farber Cell Manipulation Core Facility under cGMP conditions (Fig. 1C). Fresh PBMCs were seeded at 1×10^6 cells/mL with interleukin 2 (IL2; 100 IU/mL; Proleukin, Bayer HealthCare) and OKT3 (40 ng/mL; MACS GMP CD3 pure, Miltenyi Biotec) for 48 hours in X-Vivo 15 medium (Lonza) supplemented with 5% human AB serum (Gemini Bio-products) and 2 mmol/L L-glutamine (Lonza) in T75 cm^2 cell culture flasks (Nunc). Activated cells were then transduced twice with clinical-grade SFG-chNKG2D retrovirus supernatant in retronectin-coated [Retronectin (GMP), Takara BIO Inc.] 24-well plates (Cellstar Greiner Bio-one) in the presence of IL2 (100 IU/mL) on days −6 and −5. Eighteen to 24 hours after the last transduction, cells were transferred into G-Rex flasks (Wilson Wolf) in complete X-Vivo 15 supplemented with IL2 (100 IU/mL). Cells were fed with additional fresh complete X-Vivo 15 with IL2 (100 IU/mL) on day −2, and transduced T cells were harvested after approximately 48 additional hours of culture. Cells were resuspended in Plasmalyte (Baxter) supplemented

with 1% human Albumin (Grifols), counted using trypan blue, and infused fresh. Release criteria included ≥70% viability, ≥5% $CD8^+$ T-cell transduction efficiency, negative testing for endotoxin (<5 EU/kg/hour), negative gram stain (analyzed on day of release), and ≤5 vector copy number (VCN)/cell and negative PCR testing for *mycoplasma* (a sample of cells was obtained on day −1 prior to harvest for analysis and analyzed by qPCR using MycoSEQ (Thermo Fisher). The manufacturing process is comprehensively detailed separately (32).

Flow cytometry of manufactured products. Products were characterized using standard flow cytometry techniques to determine surface expression of T-, B-, NK, myeloid, and plasma cell and T-cell differentiation markers with 4 panels including combinations of the following fluorophore-conjugated anti-human monoclonal antibodies (mAb; Supplementary Table S1): (i) NKG2D expression: CD3, CD4, CD8, CD314 (NKG2D); (ii) myeloid cells: CD34, CD14, CD33, CD11b; (iii) T-, B-, and NK cells: CD45, CD3, CD4, CD8, CD19, CD16, CD56; (iv) plasma cells: CD38, CD138, CD3, CD19. Cells were acquired on a BD FACSCanto flow cytometer and analyzed using BD FACSDiva Version 6.1.3 (BD Biosciences). Native NKG2D has high expression and is indistinguishable by surface staining from NKG2D-CAR expression on $CD8^+$ T cells (7). We, therefore, established a threshold of NKG2D surface fluorescence intensity, exceeded by only 5% of $CD8^+$ T cells in a mock-treated autologous T-cell culture and measured transduction efficiency based on the percentage of $CD8^+$ T cells exceeding this threshold for each patient. Transduction efficiency in $CD4^+$ T cells was measured relative to isotype controls (Supplementary Fig. S1). Aliquots of end-of-manufacturing samples were cryopreserved and subsequently stained with the following anti-human mAbs to determine the T-cell differentiation state of the infused product using standard staining techniques: CD45RO, CD3, CD4, CD8, CCR7, CD314, acquired on a BD FACSCanto flow cytometer and analyzed using FACSDiva Version 8.0.1 (BD Biosciences).

Standard extracellular protein staining techniques. Staining on NKG2D-CART cell products was performed directly after harvest from culture for release testing and on thawed, cryopreserved product for T-cell differentiation markers. For staining of patient samples, whole blood was incubated in RBC lysis buffer (eBioscience). All cells were then washed twice in PBS ± 2% FCS, and patient samples and fresh final product incubated with Fc receptor block (Miltenyi) at 4°C prior to staining. After incubation with relevant monoclonal antibodies (Supplementary Table S1) at 4°C, cells were washed with PBS ± 2% FCS and, if relevant, were fixed using Cytofix (BD) prior to analysis.

VCN and replication competent retrovirus (RCR) testing. To determine VCN, the retroviral DNA sequence was monitored by qPCR in samples of total DNA using primers and a labeled probe (Integrated DNA Technologies) specific for the fusion region of the NKG2D CAR construct. One million cells were processed for DNA extraction using Blood and Cell Culture DNA kit (Qiagen) and 100 ng of DNA was used in each reaction, using SsoAdvanced Universal Probes Supermix (Bio-Rad) on a StepOnePlus Real-Time PCR System (Applied Biosystems) with rapid temperature ramp speed in a validated assay. The primer and probe sequences were as follows: forward primer (5′-GCCACCAAGGACACCTAC-3′), reverse primer (5′-CTCATCTCCCAGCTGTGTC-3′), probe (5′-[FAM]-AATTCGGGTGGATTCGTGGTCGG-[BHQ]-3′). The probe was labeled at the 5′ end, as indicated, with 6-FAM, and 3′ Black Hole Quencher (IDT).

To determine copy number per unit DNA, a 7-point standard curve was generated consisting of 10–1 × 10^6 copies of SFG. NKG2D-CAR plasmid DNA spiked into 100 ng nontransduced genomic DNA. Each data point (sample, standard curve) was obtained in quadruplicate. Pass/fail parameters were based on prestablished acceptance ranges for assay performance derived from qualification studies and included r^2 values, amplification efficiency, absence of potential amplification in healthy donor DNA, and ability to accurately quantify known amounts of plasmid DNA spiked into genomic DNA within a predefined result range. The following formula was used to control for outlier CT values among quadruplicates: $Z = \frac{|\text{average Ct} - \text{sample Ct}|}{\text{Standard deviation}}$. Data points with a Z value > 1.1 were discarded (33). The lower limit of quantification for the assay was 30 copies/100 ng DNA. Copy number per cell was determined based on the following formula: average VCN/cell = (copies/ng DNA in the reaction) × (total ng DNA/cell number).

For RCR testing, DNA was isolated from the 1 × 10^6 NKG2D CAR product T cells, as well as 15 mL of T-cell supernatant from CAR T-cell product cultures at day −1 as described above. Primers (IDT) used were GALV5'II: 5′-ACCACAGGCGACAGACTTTTT-3′ and GALV3'II: 5′-TGAGACAGCCTCTCTTTTAGTCCT-3′. PCR-certified water and PG13 DNA were used as negative and positive controls (500 bp for the GALV gene amplification), respectively, and cells and cell supernatant from NKG2D CAR T-cell cultures were evaluated for the presence of this gene. PCR reactions were run under the following conditions: 95°C denaturation, 65°C annealing, and 72°C extension for a total of 35 cycles. Amplified samples were analyzed on a 1% agarose gel in 1XTAE SyberSafe (Thermo Fisher Scientific) and visualized under UV light. Samples were then evaluated for the presence or absence of a 500-bp band, characteristic of GALV protein (34).

NKG2D-CAR T-cell function by enzyme-linked immunosorbent assay (ELISA). Concurrent with NKG2D-CAR product release and infusion, *in vitro* functional activity was assessed by coculture of 100,000 clinical-grade NKG2D-CAR T cells with 100,000 autologous patient PBMCs or BM cells (cryopreserved prior to infusion and involved by underlying AML or MM) or with healthy donor PBMCs, RPMI 8866, K562, or P815 cells at a 1:1 ratio for 24 hours. Cells were cultured in complete X-Vivo media (Lonza) in 96-well round bottom plates (Corning). NKG2D-CAR T cells or mock-treated T cells were incubated with purified anti-human CD314 (NKG2D, 20 μg/mL, clone 1D11; BD) or isotype antibody (20 μg/mL) for 15 minutes prior to coculture. Cell-free media were harvested and concentrations of IFNγ determined by ELISA (IFNγ Duoset ELISA, R&D Systems).

Analysis of patient samples following NKG2D-CAR T-cell infusion

NKG2D CAR transgene and RCR detection by PCR. To quantify NKG2D-CAR transgenes and RCR present in peripheral blood (PB) and/or BM, genomic DNA was extracted from cryopreserved PB or BM aspirate samples using QIAamp DNA blood extraction kits (Qiagen), quantified by spectrophotometer, and stored at −80C. qPCR analysis on genomic DNA samples and data analysis was performed as described for VCN and RCR detection on the

NKG2D-CAR product, with the sets of primers and probe used for each assay detailed below.

NKG2D CAR transgene detection: Forward primer (5′-GCCACCAAGGACACCTAC-3′), reverse primer (5′-CTCATCTCCCAGCTGTGTC-3′), probe (5′-[FAM]-AATTCGGGTGGATTCGTGGTCGG-[BHQ]-3′). Probe was labeled at the 5′ end, as indicated, with 6-FAM, and 3′ Black Hole Quencher (IDT).

RCR detection: Forward primer (5′-TCCGGAGACCATCAGTATCT-3′), reverse primer (5′-GGATAGTAATAGATGCGAGGAATCA-3′), probe (5′-[FAM]-CCCTTGCCTCTCCACCTCAGTTT-[BHQ]-3′). Probe was labeled at the 5′ end, as indicated, with 6-FAM, and 3′ Black Hole Quencher (IDT).

Circulating NKG2D-CAR T cells and NKG2D ligand expression by flow cytometry. Standard whole blood flow cytometry techniques with combinations of the following anti-human mAbs were used to monitor kinetics of NKG2D-CAR T cells in PB and BM, and NKG2D ligand expression on tumor cells: NKG2D, CD3, CD4, CD8, CD117, CD123, CD34, CD138, CD38, CD45, HLA-DR, CD33, MICA, MICB, ULBP1, ULBP2/5/6, ULBP3, and ULBP4 (Supplementary Table S1). Cells were acquired on an LSRFortessa (BD Biosciences), and analysis performed using FlowJo software V10 (Treestar). Specific fluorescence indices (SFI) were calculated by dividing the median fluorescence intensity obtained with specific monoclonal antibodies by that of isotype controls.

ELISAs for soluble MICA and MICB. Soluble MICA and MICB in cell-free patient plasma were measured by ELISA using human MICA and MICB ELISA kits as per the manufacturer's instructions (Thermo Scientific).

Analysis of cytokine secretion. To evaluate cytokines, 20 μL of patient plasma was diluted 1:1 with assay buffer and processed according to the manufacturer's instructions for the MILLIPEX MAP Human Cytokine/Chemokine Bead Panel (Millipore) at baseline and several time points after infusion for the following 41 cytokines: sCD40L, EGF, Eotaxin/CCL11, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFNα2, IFNγ, IL1α, IL1β, IL1ra, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12 (p40), IL12 (p70), IL13, IL15, IL17A, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGFα, TNFα, TNFβ, and VEGF. Samples were run on the Bio-Plex 200 Suspension Array System (Bio-Rad). Standard curves were generated using human cytokine standards provided by the manufacturer (Millipore). Bio-Plex Manager V6.0 was used to analyze the data (Bio-Rad).

Statistical analysis

The maximum-tolerated dose (MTD) was defined as the highest T-cell dose at which $\leq$1 of 6 patients developed a dose-limiting toxicity (DLT). DLTs were defined as $\geq$grade 3 toxicities not directly attributable to underlying disease or $\geq$grade 2 autoimmune toxicity per NCI CTCAE v4.0, occurring within 21 days, at least possibly related to T-cell infusion and not controllable to $\leq$grade 1 within 72 hours. Patient and clinical characteristics were summarized as median and range for continuous variables and numbers and percentages for categorical variables. All reported toxicities, regardless of attribution, were summarized by type, maximum grade, and sorted by number of patients experiencing the toxicity. The maximum grade consolidates reports of a given toxicity for each patient over time. Overall survival (OS) was defined from the time of infusion to death or censored at the last known alive date. Spearman correction coefficient was used to summarize the correlation between ALC at time of infusion and maximum detected transgene level. Two-sample *t* tests were used to compare IFNγ production under specific conditions with nominal *P* values reported; $P < 0.05$ was considered significant. Analyses were performed using SAS Version 9.4 (SAS Institute).

Results

Patient characteristics

Fourteen patients consented. One patient died prior to initiating screening, and one enrolled patient became ineligible due to rapid disease progression prior to cell collection. Twelve patients had autologous T cells collected, received NKG2D-CAR T cells, and were evaluable. Median age was 70 years (range, 44–79 years), and 7 subjects had AML and 5 had MM. The median percentage of BM blasts on core biopsy in subjects with AML was 50% (range, 15%–60%), with a median of 1 prior therapy (range, 0–4). Four AML patients had disease secondary to MDS, 3 had complex cytogenetics, 3 had TP53 mutations, 2 had FLT3 mutations (1 ITD, 1 TKD). All myeloma patients had undergone $\geq$5 therapies, including $\geq$1 autologous SCT. Three subjects had plasmacytomas with little marrow involvement. Median serum M-spike was 1.75 g/dL (range, 0–3.16 g/dL), and median urine M-spike was 256 mg/24 hours (range, 0–3,969 mg/24 hours; Table 1; Supplementary Table S2).

Feasibility of manufacturing

Autologous PBMCs were obtained via peripheral venipuncture ($n = 5$) or nonmobilized apheresis ($n = 7$). All patients who underwent collection had CAR T-cell products successfully manufactured from initial fresh PBMCs and received NKG2D-CAR T cells at the protocol-specified doses. The $CD4^+/CD8^+$ composition was variable, with a median $CD8^+$ T cells of 36% (range, 7–89%). Products were predominantly comprised of $CD45RO^+CCR7^-$ T-effector memory (Tem) and $CD45RO^+CCR7^+$ central memory (Tcm) cells, with a median $CD8^+$ Tem of 51% (range, 39–73%) and $CD8^+$ Tcm of 36% (range 4–59%). The median VCN of the NKG2D-CAR transgene in the infused product was 0.61 copies/cell (range, 0.37–1.86), and the median transduction efficiency was 66% (range, 15%–87%) on $CD8^+$ T cells and 92% (range, 74%–98%) on $CD4^+$ T cells. Median NKG2D-CAR expression in the entire $CD3^+$ compartment was 75% (range, 55%–90%), resulting in median transduced T-cell doses of 7.38×10^5, 2.15×10^6, 6.92×10^6, and 2.45×10^7 in the respective dose cohorts (Table 2). AML blasts and $CD38^+CD138^+$ plasma cells were efficiently eliminated during the manufacturing process (median, $\leq$0.1% of live cells). In a single instance, $CD19^+$ B cells persisted in both mock and transduced cultures (16% of live cells), expressed NKG2D after transduction, and were included in the infused product. This did not result in any clinical toxicities; workup, including an extensive leukemia/lymphoma flow panel, and viral testing was negative. Of the 12 patients, 11 received fresh NKG2D-CAR product immediately after harvest from manufacturing. For 1 patient, the product was stored at 4°C until resolution of respiratory symptoms, subsequently met viability criteria ($\geq$70%), and was administered within 48 hours.

Safety and clinical outcomes

Products were infused inpatient after administration of acetaminophen and diphenhydramine without prior lymphodepletion.

Table 1. Patient characteristics

Characteristic	No. (%) of patients ($n = 12$)
Age, years	
Median	70
Range	44–79
Sex	
Male	9 (75%)
Female	3 (25%)
Disease type	
AML	7 (58%)
MM	5 (42%)
AML characteristics	
FLT-3 ITD	1
TP53 mutation	3
FLT-3 TKD	1
Complex cytogenetics	3
Secondary AML	4
Disease burden	
AML, marrow blasts (core), Median	50%
Range	15%–60%
AML, # with peripheral blasts	4 (57%)
AML, % peripheral blasts, Median	8%
Range	0%–58%
MM, marrow plasma cells, Range	<5%–80%
MM, FLC mg/dL, Median	1,524
Range	0–2,615
MM, serum M-protein, Median	1.75 g/dL
Range	0–3.16 g/dL
MM, urine M-protein, Median	256 mg/24 h
Range	0–3969 mg/24 h
MM, # with plasmacytomas	3 (60%)
# Prior regimens	
AML, Median	1
Range	0–4
MM	All ≥5
MM, prior ASCT	All ≥1
ALC, K/μL, Median	0.58
Range	0.09–2.37

Abbreviations: ALC, absolute lymphocyte count; AML, acute myeloid leukemia; ASCT, autologous stem cell transplant; FLC, free light chain ratio; ITD, internal tandem duplication; MM, multiple myeloma; MDS, myelodysplastic syndrome; TKD, tyrosine kinase domain.

No infusional toxicity, CRS, severe autoimmunity (specifically, no colitis or pneumonitis), CAR T cell–related encephalopathy syndrome (CRES), or death was observed. One hundred twenty adverse events (AE) were reported: 18 (15%) grade 3 and 8 (7%) grade 4 (Supplementary Table S3). Of the grade ≥3 AEs, none were attributed to the NKG2D-CAR T cells but, rather, to underlying disease progression or infection. No DLTs were observed. Patient 8 (MM) had cryoglobulinemia preceding CAR T-cell infusion and experienced complications of hyperviscosity syndrome with acute hearing loss and bilateral intracochlear bleeds, despite plasmapheresis (35, 36). Events related to progressive plasmacytomas included cord compression, biliary obstruction and gastric erosion, and bleeding. None of the observed cases of parainfluenza (day 19, patient 1, AML), influenza A (day 24, patient 6, MM), and gram-negative biliary sepsis (day 29, patient 3, MM with prior peripancreatic plasmacytomas) prompted significant inflammatory or autoimmune symptoms besides fever. Patient 4 (MM) experienced new upper respiratory symptoms on the day of planned infusion. Out of concern for NKG2D ligand upregulation in bronchial epithelial cells under oxidative stress (37), cell infusion was delayed within a predefined 48-hour window until symptoms had resolved and a negative infectious workup was confirmed. No subsequent respiratory complications occurred. Two patients, at dose level 4, developed a self-limited grade 1 follicular erythematous rash between 1 and 3 months after NKG2D CAR T-cell infusion. In patient 12 (AML), this was deemed probably related to NKG2D-CAR T cells given concurrent improvements in hematologic parameters. PB NKG2D-CAR transgene levels at that time were detectable at low levels. Skin biopsy revealed nonspecific, mild spongiotic and interface dermatitis, with a very mild lymphocytic infiltrate. The rash resolved without systemic or topical steroids before additional skin sampling for qPCR detection of NKG2D-CAR sequences could be obtained, and the NKG2D-CAR transgene became undetectable in the PB thereafter.

Objective clinical responses to NKG2D-CAR T-cell therapy alone, defined in the study protocol, were not seen, and all subjects received subsequent therapy (Fig. 2A). Three subjects (2 MM and 1 AML) started within 28 days of NKG2D-CAR T-cell infusion. Median OS was 4.7 months (range, 1.2–24.9+ months), with survival rates of 75% and 42% at 3 and 6 months, respectively. Two subjects remained alive at 16.8 months and 24.9 months, respectively. Patients 3, 4, and 8 (MM) exhibited disease stability for over 1 year on alternative therapies. Likewise, patient 5 (AML), maintained stable disease for 6 months on an IDH-1 inhibitor trial despite a mutated allele frequency of <5% IDH and 54% p53 mutation burden at CAR T-cell infusion. Patient 7 (AML, dose level 3), with 50% BM blasts and a p53 mutation with allele burden of 80% at infusion, experienced resolution of B-symptoms, transient decrease in transfusion requirements, and had a 4.4-month survival without further therapy (Fig. 2A). Lastly, patient 12 (AML, dose level 4) experienced improvement in hematologic parameters without further interventions (Fig. 2B), concurrent with development of a grade 1 skin rash and met the definition of "stable disease" at 3 months as defined by recent AML guidelines (29). He remained clinically stable for 6 months without further therapy and was alive at last follow-up on alternative treatment.

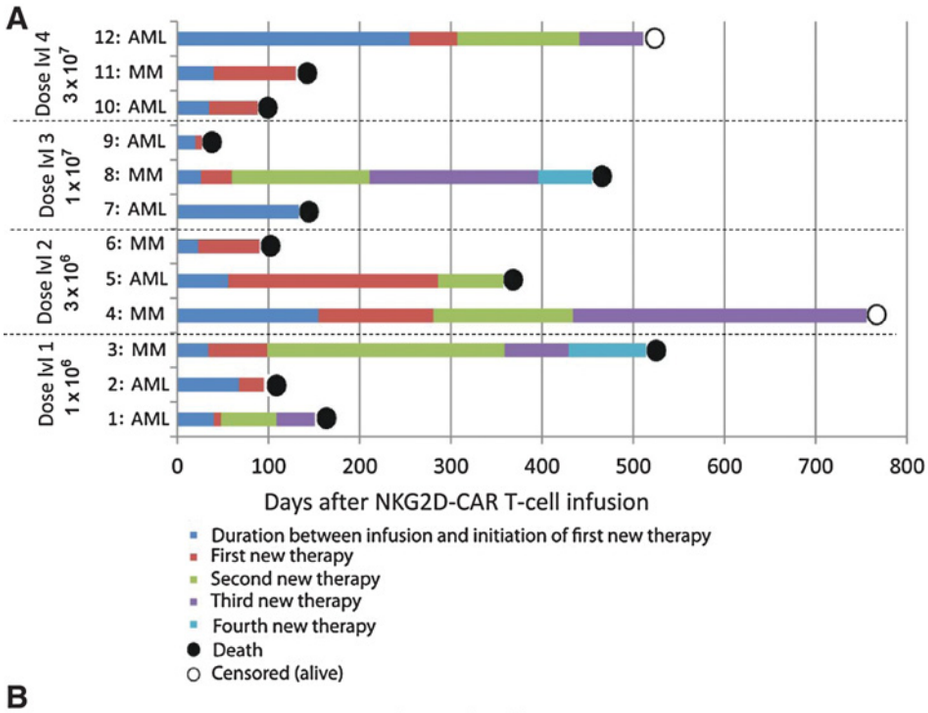
Analysis of patient T-cell phenotypes, cytokines, and tumor ligand expression following infusion

NKG2D-CAR T cells were transiently detected in 4 patients by qPCR in PB (AML, dose levels 1 and 4; MM, dose levels 1 and 2; Fig. 3A), whereas analysis of BM at day 28 was negative for the NKG2D-CAR transgene in all evaluable patients ($n = 9$). The highest transgene level was observed in patient 3 (MM, dose level

Table 2. Cell product characteristics

Cell composition	Median	Range
$CD8^+$ (among $CD3^+$ cells)	36%	7%–89%
Effector memory (among $CD8^+$ cells)	51%	39%–73%
Effector memory (among $CD4^+$ cells)	48%	32%–76%
Central memory (among $CD8^+$ cells)	36%	4%–59%
Central memory (among $CD4^+$ cells)	34%	20%–53%
Vector copy number/cell	0.61	0.37–1.86
NKG2D CAR^+ (among $CD8^+$)	66%	15%–87%
NKG2D CAR^+ (among $CD4^+$)	92%	74%–98%
NKG2D CAR^+ (among $CD3^+$)	75%	55%–90%
# of transduced viable T cells infused		
Dose level 1	7.38 × 10e5	7.35–7.95
Dose level 2	2.15 × 10e6	1.64–2.67
Dose level 3	6.92 × 10e6	6.31–7.54
Dose level 4	2.45 × 10e7	2.28–2.70

NOTE: Effector memory ($CD45RO^+$, $CCR7^-$), central memory ($CD45RO^+$, $CCR7^+$).



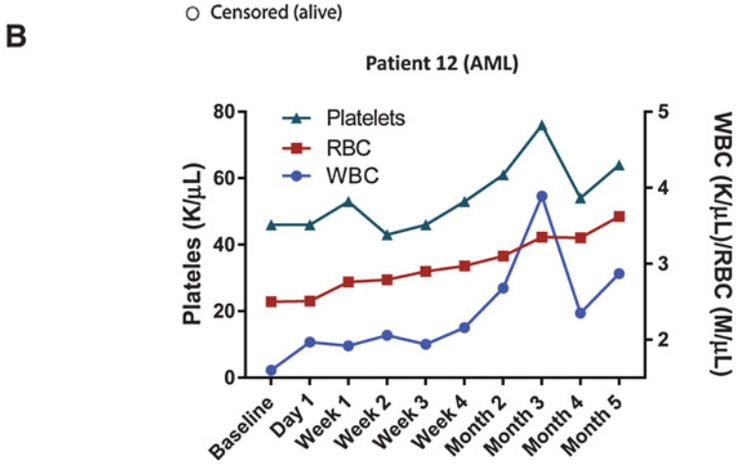


Figure 2.
Patient outcomes. **A**, Swimmer's plot indicating survival and time of subsequent therapies. **B**, Trajectory of PB counts in patient 12 (AML) after NKG2D-CAR T-cell infusion.

1), with the lowest ALC at infusion (0.08K cells/μL) (Fig. 3B), and a trend toward an inverse correlation between ALC and transgene detection was noted (correlation efficient –0.54, $P = 0.07$). Surface immunophenotyping of circulating lymphocyte subsets revealed no significant NKG2D-CAR T-cell expansion, measured by percentage of $NKG2D^+$ cells among $CD4^+$ T cells or absolute numbers of $NKG2D^+CD8^+$ or $NKG2D^+CD4^+$ T cells (Fig. 3C), but was limited by lack of a CAR-specific surface marker.

Plasma cytokines were monitored by Luminex. Except for 1 AML patient with undetectable RANTES concentrations (patient 09, dose level 3), all evaluated patients ($n = 11$) demonstrated an increase in RANTES within 3 weeks of NKG2D-CAR T-cell infusion (Fig. 3D). At 24 hours, the median fold-increase was 1.8 (range, 0–865) among all patients, with a median fold-increase of 5.8 (range, 1.64–865) among the 6 AML patients with detectable concentrations.

NKG2D ligand expression on patient AML blasts or MM plasma cells derived from pretreatment BM samples was evaluated by flow cytometry. Although ligand expression was relatively low, all 10 evaluable patients demonstrated an SFI >1.0 for at least one ligand. Consistent with other studies (14), coexpression of multiple ligands was observed (Fig. 4A). Soluble MICA in plasma was detected in 10 and MICB in 4 of 11 assayed patients prior to NKG2D-CAR T-cell infusion (Fig. 4B).

NKG2D-CAR T cells from all tested products ($n = 11$) produced IFNγ during coculture with NKG2D ligand–expressing cell lines RPMI8886 and K562, but not the ligand-negative line P815. In select patients, paired samples were available to assess *in vitro* function of NKG2D-CAR T-cell products against autologous tumor ($n = 4$). Coculture resulted in significant IFNγ production by NKG2D-CAR T cells in response to autologous tumor-containing PBMCs or BM but not to ligand-negative cell lines or healthy donor PBMCs. This effect was NKG2D-specific, as it was abrogated

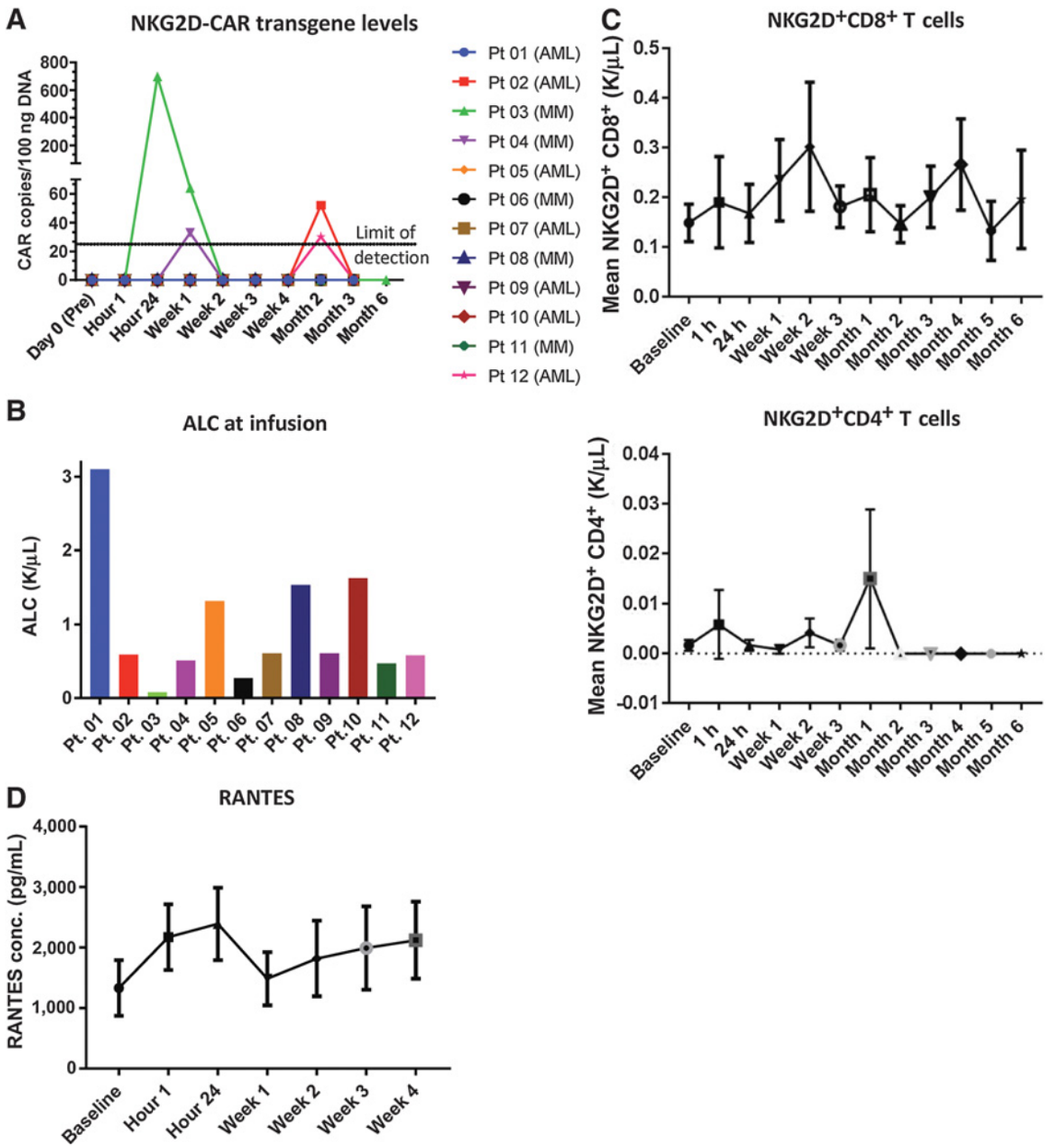


Figure 3.

Detection and bioactivity of NKG2D-CAR T cells in PB. **A**, NKG2D-CAR DNA at time points indicated on the horizontal axis as measured by qPCR. **B**, ALCs at time of infusion. Color scheme for patients is identical in **A** and **B**. **C**, Monitoring of absolute numbers of NKG2D$^+$ CD8$^+$ and CD4$^+$ T cells using flow cytometry ($n = 12$, mean ± SEM). **D**, RANTES detected ($n = 11$, mean ± SEM).

by preincubation of CAR T cells with an NKG2D-blocking mAb but not isotype control (Fig. 5).

Discussion

Given the potential to target any of the eight NKG2D ligands found in various hematologic and solid malignancies, the efficacy in murine models (16–19, 22), and the feasibility of manufacturing in preclinical studies, we embarked on this first-in-human trial to assess the safety and feasibility of NKG2D-CAR T cells in subjects with AML/MDS and MM. We demonstrated safety without identifying an MTD at cell doses ranging 1×10^6 to 3×10^7, when administered as a single infusion without prior lymphodepleting therapy and showed feasibility of manufacturing and administration without cryopreservation. We did not observe a robust efficacy signal in this initial phase I study. However, the NKG2D-CAR approach is still in early stages of clinical development and has several unique features and strengths: unlike

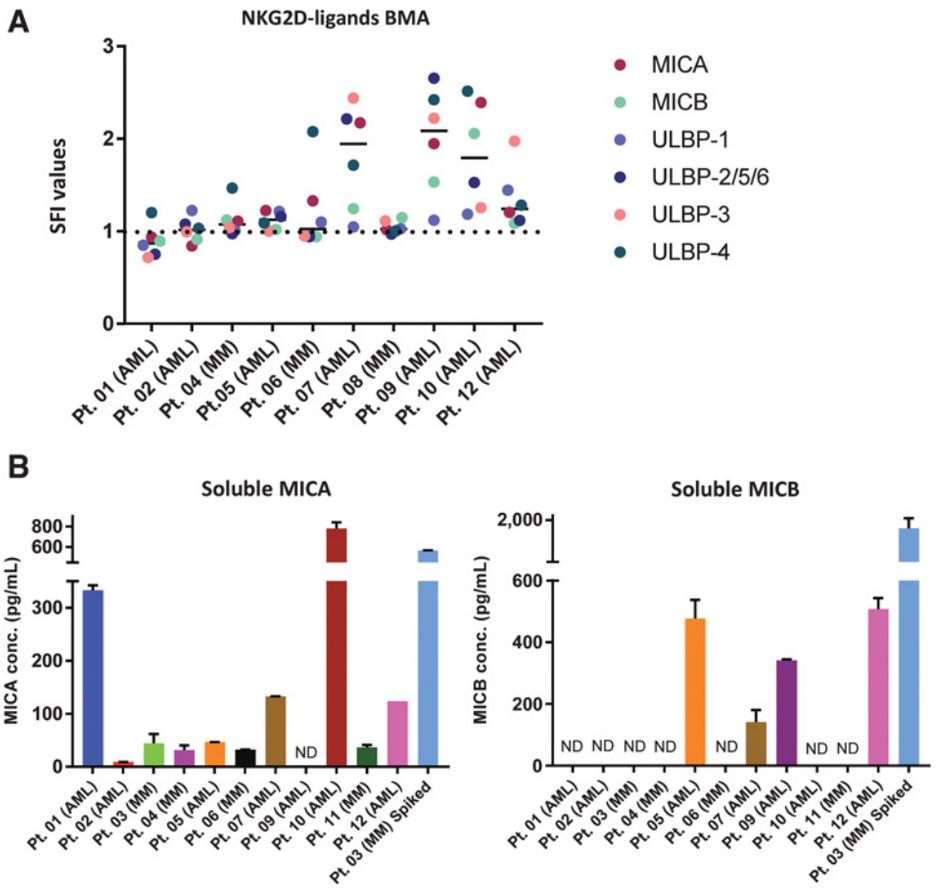


Figure 4.
NKG2D-ligand expression and soluble ligands. **A**, Surface NKG2D ligand detection in AML blasts (7 AML patients) and MM cells (3 MM patients) in bone marrow aspirates (BMA) at baseline measured by flow cytometry (SFI>1 indicates ligand expression exceeding isotype). AML blasts: live/CD45^{dim} cells expressing patient-specific AML markers including CD117, CD123, CD33, and HLA-DR. MM cells: live/CD138^{+}CD38^{+} cells. **B,** Detection of soluble MICA and MICB in patient plasma at baseline using ELISA. Plasma of patient 3 (MM) was separately spiked with soluble MICA and MICB as an internal positive control. ND: not detectable.

antigens, which are lineage-restricted to a subset of cancers, NKG2D ligands are surface-expressed in more than 15 different malignancies, including colon, breast, and lung cancers, AML, MM, Ewing sarcoma, and neuroblastoma (11). Thus, if clinical efficacy can be achieved with a reasonable safety profile, NKG2D-CAR therapy would have an impact across many malignancies with significant medical need. Reactivity against eight, often concurrently expressed, ligands might counteract tumor evasion via clonal selection, mutations, and alternative splicing (38).

Given minimal surface expression of NKG2D ligands in normal tissues, long-term toxicities, such as B-cell aplasia (39) or myeloablation, seen with other CAR strategies targeting AML are not expected (40), and no grade $\geq$3 toxicities or autoimmune events related to NKG2D-CAR therapy were observed in this study. However, the inducible nature of NKG2D ligands raises concern for potential "on-target, off-tumor" toxicity and a positive feedback loop mediated by NKG2D-CAR T cells in the setting of systemic inflammation. Toxicity resembling clinical CRS at high cell doses was observed and exacerbated by cyclophosphamide in syngeneic mouse models (23, 24). Therefore, lymphodepleting chemotherapy has been omitted pending a first-in-human safety experience, which is provided by this study. However, we evaluated preinfusion ALC as a surrogate for the degree of preexisting "lymphodepletion," and all patients with detectable NKG2D-CAR T cells had an ALC $\leq$0.59 K/μL at infusion. Additional studies are warranted to assess the impact of lymphodepletion strategies on NKG2D ligand surface expression on healthy tissues and incorporation into future studies. NKG2D-CAR T cells were detectable in four patients and did not mediate any clinically relevant toxicity, specifically, no gastrointestinal autoimmune toxicity despite concerns for MICA/B expression on intestinal epithelial cells (41, 42). Several patients developed systemic infections after infusion, which did not trigger any NKG2D-CAR T-cell expansion or toxicity. Although dose escalation started low, the final cohort of 3×10^7 cells approached doses at which toxicity and efficacy have been observed in CD19-directed CAR trials ($0.5–1 \times 10^6$/kg; refs. 4, 43). Two patients in the highest dose cohort experienced a self-resolving skin rash within 1 to 3 months after infusion, one of which was deemed probably related to CAR T cells, although a skin biopsy was nondiagnostic.

Feasibility of manufacturing and infusion of NKG2D-CAR T cells from patients with AML or MM without cryopreservation was consistently demonstrated, including with cells derived from whole blood draws. Patient blasts and CD138^{+}CD38^{+} plasma cells were efficiently eliminated during manufacturing. In a single instance, CD19^{+} B cells persisted and were transduced, similar to a reported phenomenon of leukemic B-cell transduction with a CAR (44). This did not result in clinical sequelae but raises consideration of a T-cell selection step during manufacturing.

Although the study met its primary endpoints, objective clinical efficacy was not demonstrated. However, 1 patient at the highest dose had blood count improvements and clinical stability

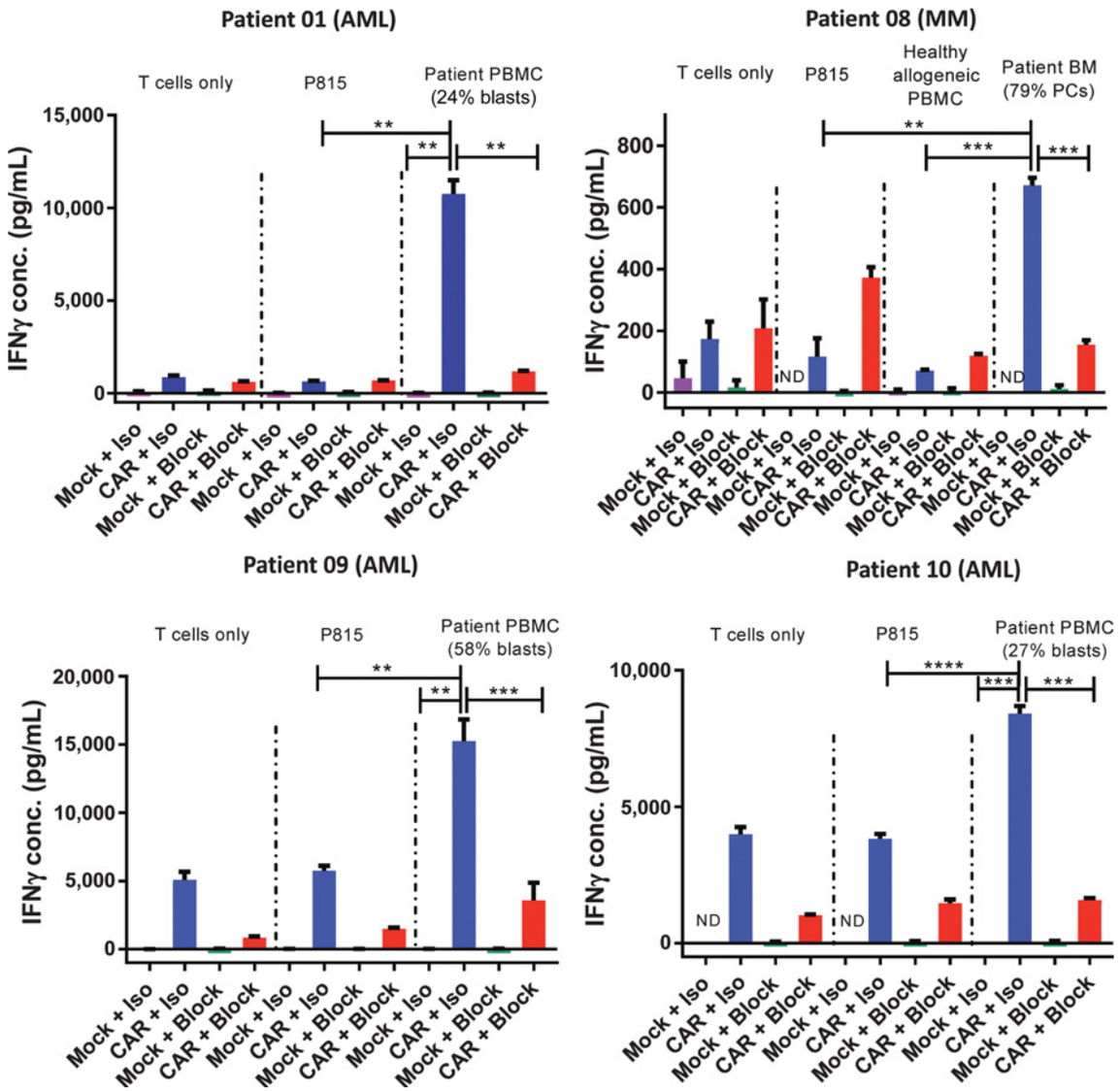


Figure 5.

Functional activity of NKG2D-CAR T cells against autologous tumor. NKG2D-CAR T cells or mock-treated autologous T cells were cultured at a 1:1 ratio with autologous patient PBMCs containing AML blasts, patient BM aspirate involved by MM, ligand-negative cell lines (P815), or healthy allogeneic donor PBMCs. IFNγ in the supernatants was measured by ELISA (**, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). T cells were incubated with an NKG2D-blocking monoclonal antibody or isotype control antibody prior to addition of target cells as indicated.

without additional therapy for months, and several patients experienced unexpected disease stability on subsequent therapies. Our correlative studies interrogated several aspects amenable to optimization in future trials. In most patients, significant NKG2D-CAR T-cell expansion and persistence were not observed. This is consistent with murine models, where repeat infusions are required for complete tumor eradication (15). Owing to the conserved nature of NKG2D, NKG2D-CAR T cells are not immunogenic. Therefore, multiple infusions and higher cell doses commensurate with other CAR trials are now being explored (NCT 03018405; ref. 45).

Although no costimulatory domain was incorporated in the CAR construct, the use of full-length NKG2D endowed the NKG2D-CAR with a costimulatory signal via endogenously expressed Dap10, which is required for NKG2D surface expression. Although Dap10 was not rate limiting to NKG2D-CAR expression, its contribution to CAR T-cell efficacy has not been established and inclusion of additional costimulatory moieties, such as 4-1BB or CD28, might significantly enhance NKG2D-CAR T-cell kinetics and efficacy. Our current manufacturing method yielded predominantly a Tem profile, characteristic of cytotoxic effector function, but limited capacity to self-renew and expand *in vivo*. Modifications to induce differentiation toward a Tcm/T-memory stem cell (Tscm) profile (46), inclusion of 4-1BB to promote outgrowth of $CD8^+$ Tcm subsets and CAR expansion via distinct metabolic pathways (47), or controlling the CD4/CD8

ratio may enhance future efficacy. Inclusion of a suicide mechanism and extracellular marker to directly identify NKG2D-CAR T cells in PB and tissues could further be beneficial in subsequent trials.

Antigen exposure is an additional factor for CAR T-cell expansion, and high disease burden is predictive of exponential expansion and associated CRS (39, 48). The reported NKG2D ligand expression in AML varies in the literature (14, 49–53). We found that expression on AML blasts and evaluable myeloma cells in our enrolled patients was low relative to CD19 expression in ALL. This may critically affect expansion, particularly because NKG2D-CAR T cells lack an alternative antigen source from nonmalignant cells. NKG2D ligand cleavage, a known process of immune evasion, may have affected surface ligand expression because all patients had detectable plasma concentrations of either soluble MICA, MICB, or both. In contrast, soluble ligands are unlikely to have impaired NKG2D-CAR T-cell function, given that *in vitro* NKG2D-CAR–mediated cytotoxicity was not inhibited by comparable concentrations of soluble MICA (20). Although additional strategies are required to promote NKG2D-CAR T-cell expansion *in vivo*, our *in vitro* studies evaluating the NKG2D-CAR T-cell product in coculture with autologous tumor cells demonstrated that ligand expression is sufficient to induce robust, tumor-specific, and NKG2D-mediated IFNγ. Increases in RANTES early after infusion are likely derived from the CAR T cells, suggesting an element of NKG2D ligand–specific activation (54). However, pharmacologic strategies to selectively enhance NKG2D ligand expression in malignant tissues and basing eligibility on NKG2D ligand expression may be warranted in future studies (52, 55–59).

In summary, our findings represent a critical assessment of the safety and feasibility of NKG2D-CAR T-cell administration in humans. Although expansion and persistence of CAR T cells was limited, no safety concerns were raised with detectable NKG2D-CAR T cells in several subjects, despite clinical conditions of infection and systemic cell stress in which ligand upregulation in healthy tissues might have triggered toxicity. Our findings identify several mechanisms by which therapeutic efficacy might be achieved in subsequent studies and pave the way for further development of this approach.

Disclosure of Potential Conflicts of Interest

S.H. Baumeister reports receiving a commercial research grant from SBIR awarded to Celdara Medical LLC. H. Trebeden-Negre has immediate family members with ownership interest in BlueBirdBio. J. Reder is cofounder, director, and CEO of and has ownership interest in Celdara Medical, LLC. C.L. Sentman reports receiving commercial research funding from Celdara Medical, Celyad SA, and Javelin Oncology; and has ownership interest in and is a consultant/advisory board member for Celyad SA and Celdara Medical. D.E. Gilham has ownership interest in Celyad. R.M. Stone reports receiving commercial research funding from Novartis, Agios, Arog, Lilly, and AbbVie and is a consultant/advisory board member for Astellas, Amfgen, Argenix, Macrogneics, Jansen, Juno, Ono, Roche, Sumitomo, Arog, Novartis, Jazz, Pfizer, AbbVie, Agios, Actinium, and Celgene. R. Soiffer is on the supervisory board at Kiadis and Nmdp Be the Match, is a consultant/advisory board member for Gilead and Juno, and has provided expert testimony for Pfizer. G. Dranoff is Global Head, Immune-Oncology at Novartis. J. Ritz reports receiving commercial research funding from Celdara Medical LLC, and Celyad SA. S. Nikiforow reports receiving a commercial research grant from SBIR awarded to Celdara Medical. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.H. Baumeister, J. Murad, L. Werner, J.K. Gicobi, A. Schmucker, D.E. Gilham, F.F. Lehmann, D.S. Neuberg, R. Soiffer, J. Ritz

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Other (performed or supervised clinical laboratory processing of CAR T cells): H. Daley

Other (clinician, direct patient care on study, education to staff and coordination of clinical trial at our site): I. Galinsky

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