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# CAR T Cells Administered in Combination with Lymphodepletion and PD-1 Inhibition to Patients with Neuroblastoma

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**Targeting disialoganglioside (GD2) on neuroblastoma (NB) with T cells expressing a first-generation chimeric antigen receptor (CAR) was safe, but the cells had poor expansion and long-term persistence. We developed a third-generation GD2-CAR (GD2-CAR3) and hypothesized that GD2-CAR3 T cells (CARTs) would be safe and effective. This phase 1 study enrolled relapsed or refractory NB patients in three cohorts. Cohort 1 received CART alone, cohort 2 received CARTs plus cyclophosphamide and fludarabine (Cy/Flu), and cohort 3 was treated with CARTs, Cy/Flu, and a programmed death-1 (PD-1) inhibitor. Eleven patients were treated with CARTs. The infusions were safe, and no dose-limiting toxicities occurred. CARTs were detectable in cohort 1, but the lymphodepletion induced by Cy/Flu increased circulating levels of the homeostatic cytokine interleukin (IL)-15 ($p = 0.003$) and increased CART expansion by up to 3 logs ($p = 0.03$). PD-1 inhibition did not further enhance expansion or persistence. Antitumor responses at 6 weeks were modest. We observed a striking expansion of CD45/CD33/CD11b/CD163$^+$ myeloid cells (change from baseline, $p = 0.0126$) in all patients, which may have contributed to the modest early antitumor responses; the effect of these cells merits further study. Thus, CARTs are safe, and Cy/Flu can further increase their expansion.**

## INTRODUCTION

The disialoganglioside (GD2) is universally expressed on melanoma, lung cancer, and neuroblastoma (NB) and found on few healthy tissues. Immunotherapeutic targeting of GD2 with monoclonal antibodies (ch14.18) has significantly improved event-free survival of high-risk NB patients,[1,2] and it was recently incorporated in the standard care for these patients. Nonetheless, treatment may still be ineffective and is associated with significant toxicities.[1]

An alternative immunotherapeutic approach is to express chimeric antigen receptors (CARs) targeting the same validated GD2 antigen on effector T cells.[3] CAR-based immunotherapy can combine the specificity of a monoclonal antibody (mAb) with the effector function, active biodistribution, and long-term persistence of T cells.[4] CAR T cells can produce a high rate of sustained complete remission (CR) in patients with hematologic malignancies even when the disease is advanced.[5]

In a previous study, we compared the effects of activated T cells (ATCs) and Epstein-Barr virus-specific cytotoxic T cells (EBVSTs), both of which expressed a first-generation GD2-CAR (i.e., one that lacks embedded costimulatory signaling domains) in relapsed and refractory NB patients. We found that cells were well tolerated, and 3 of 11 treated patients entered CR.[3,6] Because the infused ATCs and EBVSTs expressed genetically distinguishable first-generation CARs, we could also show that initial persistence was greater in the CAR-VST population than in CAR-ATCs. We proposed that the superiority of CAR-VSTs might be attributable to the physiologic costimulation received during engagement of their native, virus-specific TCR with the professional antigen-presenting cells (APCs) expressing viral antigens that are found in EBV-seropositive recipients. For the current clinical study, we determined whether we could compensate for the lack of physiologic costimulation in GD2-CAR ATC by substituting a next-generation CAR that incorporated its own costimulatory signals. In preclinical studies, we found that a third-generation GD2-CAR incorporating both the CD28 and the OX40 costimulatory endodomains (GD2-CAR3) provided ATC with the greatest antitumor activity,[7] and here we report the results of a clinical study using this CAR in patients with relapsed or refractory NB.

The aim of this study testing a third-generation CAR with CD28 and OX40 costimulatory endodomains was to define the feasibility, safety,

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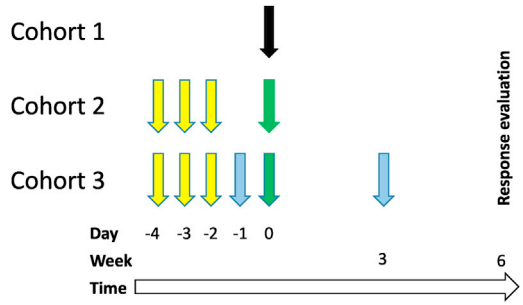


**Figure 1. Flow Chart of Clinical Trial NCT01822652**

Black arrow indicates GD2-CAR3 T cells expanded with IL-2 and administered after a freezing step. Yellow arrows indicate conditioning with cyclophosphamide 500 mg/m$^2$/dose on days −4, −3, and −2 and fludarabine 30 mg/m$^2$/dose on days −4 and −3. Green arrows indicate GD2-CAR3 T cells expanded with IL-7/15 and administered without a freezing step. Blue arrows indicate PD-1 inhibitor, pembrolizumab, given at 2 mg/kg/dose on days −1 and 21. Response to therapy evaluation was completed with 3D imaging (bone marrow testing when applicable) on week 6.

and persistence of GD2-CAR3 T cells with or without conditioning and programmed death-1 (PD-1) inhibition. Thus, we used an adaptive-design phase 1 clinical trial with three cohorts, all receiving autologous GD2-CAR3 T cells (Figure 1). In cohort 1, we infused escalating doses of GD2-CAR3 T cells alone. In cohort 2, to improve the expansion and persistence of GD2-CAR3 T cells, we gave conditioning with cyclophosphamide and fludarabine (Cy/Flu) prior to cell infusions;[8] whereas in cohort 3, to overcome the immunosuppressive effect of the tumor microenvironment, we combined Cy/Flu with two doses of PD-1 antibody.[9]

## RESULTS

### Patient Characteristics and Study Cohorts

Eleven patients (eight female, three male) with a median age of 6.5 years (range 4.1–23.6 years) with relapsed or refractory NB were enrolled, and all were infused with GD2-CAR3 T cells. All patients had active disease at the time of the infusions; all clinical characteristics are in Table 1. According to the International Neuroblastoma Staging System,[10] 1 patient had an unresectable, stage 3 pelvic tumor and 10 patients had stage 4 (metastatic) disease; bone and bone marrow were the most common metastatic sites. All patients had response to therapy assessment at week 6 (day 42) post-CAR T cell infusion (Table 1).

### Safety of Infusions

All GD2-CAR3 infusions were well tolerated, and none of the patients had any immediate reactions at the time of administration. No dose-limiting toxicities were observed. Similar numbers of febrile episodes were detected in all cohorts (2, 1, and 2 in cohorts 1, 2, and 3, respectively), and fever and neutropenia were seen in one patient from both cohorts 2 and 3. There was one episode of cytokine release syndrome (CRS) in patient 1C, but this did not exceed grade 2 and required only prophylactic antibiotics. The episode resolved spontaneously by day 7 after presentation (Table 2).

### Conditioning with Cy/Flu Prior to Adoptive Transfer Is Associated with Increased GD2-CAR3 T Cell Expansion

To assess the effect of Cy/Flu on the incidence of hematologic complications, we compared cohort 1 (without Cy/Flu) with cohorts 2 and 3 (with Cy/Flu) and found that, while patients in all three cohorts may develop hematologic side effects, grades 3 and 4 leukopenia, lymphopenia, neutropenia, and thrombocytopenia were seen less commonly in cohort 1 compared with cohorts 2 and 3 (grades 3 and 4 leukopenia: 0 versus 7; lymphopenia: 1 versus 7; neutropenia: 1 versus 6; and thrombocytopenia 0 versus 5; all hematologic side effects cohort 1 versus cohorts 2 and 3: $p < 0.001$, Fisher's exact test). There were no significant neurologic complications in any cohort and in particular no episodes of severe pain or other CNS-related side effects previously reported with CAR T cells targeting other antigens.[8,11] Two patients had transient, spontaneously resolving paresthesias.

At the time of study entry, patients had highly variable peripheral white blood cell counts (WBCs; median 3.91 cells × $10^3$/μL, range 2.31–6.37 cells × $10^3$/μL). After Cy/Flu, their WBC decreased (median 2.99 cells × $10^3$/μL, range 0.31–4.44 cells × $10^3$/μL; Figure S1), and patients were profoundly lymphopenic at the time of CAR T cell infusions (absolute lymphocyte count [ALC]/μL blood; cohort 1: median 1,367 [range 640–2,276] versus cohorts 2 and 3 [with lymphodepletion]: median 49 [range 9–111]) (Figure 2A). During the first 6 weeks of toxicity and response to therapy assessment, we detected significantly increased expansion of GD2-CAR3 T cells in patients in cohorts 2 and 3, as indicated by a rise in the copy number of the CAR transgene per milliliter of peripheral blood from 4 hr post-infusion to week 1 and week 4 time points ($p = 0.011$ and 0.018, respectively, Wilcoxon rank-sum test) and by the area under the curve (AUC) of copy number per milliliter over time (cohort 1: median 2,190 [range 978–8,736.1] versus cohorts 2 and 3: median 13,561.5 [range 3,100.2–184,607.5]; $p = 0.03$). The differences between cohorts diminished by week 6 (Figure 2B; Figure S2A). A CAR$^+$ T cell population could be detected by FACS analysis only in the most highly PCR-positive patients (shown for patient 2A in Figure S2B). GD2-CAR3 T cells could be detected by PCR at low levels 1 year after adoptive transfer even after most patients received salvage lymphotoxic chemotherapy after completing therapy on this study (Figure 2C; separated data for each patient, Figure S2C).

Of note, the expansion and persistence of GD2-CAR3 T cells in cohort 3, who received PD-1 blocking antibody, was no greater than that observed in cohort 2 (no PD-1). PD-1 expression on the infused cells was low on CAR T cells (CD4 subset: median 20.4%, range 18.8%–20.6%; CD8 subset: median 5.23%, range 3.38%–9.04%; Figure S3) prior to infusion. Although the level could increase after CAR T cell activation in the tumor, the low PD-1 expression at baseline may explain why the addition of PD-1 inhibition did not show any detectable change in CAR T cell expansion and survival.

### Higher Circulating IL-15 Levels Are Associated with Increased GD2-CAR3 T Cell Expansion

Next, we explored whether increased CAR T cell expansion was associated with a change in circulating levels of homeostatic cytokines.

**Table 1. Patient Characteristics and Study Cohorts**

| Cohort | Patient ID | Age (Years) | Gender | INSS[a] | Involved Sites | Dose | Cy/Flu[b] | PD-1 Inhibitor[c] |
|---|---|---|---|---|---|---|---|---|
| 1 | 1A | 23.6 | M | 4 | multifocal bone and bone marrow | $1 \times 10^7$ | no | no |
| | 1B | 5.7 | M | 4 | multifocal bone, liver, bone marrow | $1 \times 10^7$ | no | no |
| | 1C | 4.7 | F | 4 | multifocal bone, lymph node, bone marrow | $1 \times 10^8$ | no | no |
| | 1D | 22.5 | F | 4 | single skull lesion | $1 \times 10^8$ | no | no |
| 2 | 2A | 8.5 | F | 4 | single bone lesion | $1 \times 10^8$ | yes | no |
| | 2B | 7.4 | F | 4 | multifocal bone, lymph node, bone marrow | $1 \times 10^8$ | yes | no |
| | 2C | 6.5 | F | 4 | single bone lesion | $1.5 \times 10^8$ | yes | no |
| | 2D | 4.1 | F | 4 | multifocal bone and bone marrow | $1.5 \times 10^8$ | yes | no |
| 3 | 3A | 6.9 | M | 4 | multifocal bone and bone marrow | $1.5 \times 10^8$ | yes | yes |
| | 3B | 5.0 | F | 4 | multifocal bone and bone marrow | $1.5 \times 10^8$ | yes | yes |
| | 3C | 5.0 | F | 3 | single soft tissue mass | $1.5 \times 10^8$ | yes | yes |

GD2-CAR3 T cell dose calculated as $CAR^+$ T cells/$m^2$ of body surface area. INSS, International Neuroblastoma Staging System.[10]
[a]At the time of CAR T cell infusion.
[b]Cyclophosphamide 500 mg/$m^2$/dose on days −4, −3, and −2 and fludarabine 30 mg/$m^2$/dose on days −4 and −3 intravenously.
[c]PD-1 inhibitor: pembrolizumab given on days −1 and 21 at 2 mg/kg/dose intravenously.

One patient (patient 2C) was enrolled after tandem high-dose meta-iodobenzylguanidine (MIBG) and had a sustained and broad increase in multiple pro-inflammatory cytokine levels (including interleukin [IL]-15) throughout the study period. Patient 2C was therefore excluded from this analysis (Figure S4A). In cohorts 2 and 3 (receiving Cy/Flu), we detected higher IL-15 levels at the time of CAR T cell infusion than in cohort 1 (with Cy/Flu: mean 4.27 pg/mL, SD: 2.59; with Cy/Flu: mean 43.25 pg/mL, SD: 18.26; p = 0.003) (Figure 2D) and larger AUC of IL-15 over time in cohorts receiving Cy/Flu (cohort 1: mean 4.27 pg/mL, SD: 2.59; cohorts 2 and 3: mean 43.24 pg/mL, SD: 18.25; p = 0.005) (Figure 2E). No differences were detected in other homeostatic cytokine levels in patients, including IL-2 and IL-7 (data not shown). Importantly, higher circulating IL-15 levels correlated with increased GD2-CAR3 T cell expansion as judged by Spearman correlation (r = 0.796; p = 0.006) (Figure 2F). There was no generalized cytokine release associated with this treatment because serum interferon-γ (IFN-γ), IL-6, and tumor necrosis factor alpha (TNF-α) remained low (Figures S4B–S4D).

### M2 Macrophage-like Myeloid Subset CD45/CD33/CD11b/CD163$^+$ Expands in Peripheral Blood of Patients Treated with GD2-CAR3 T cells Irrespective of Mononuclear Cell Depletion with Cy/Flu

We also analyzed the effects of treatment on the phenotype and kinetics of myeloid subsets during the initial 6-week follow-up period in all patients. The change in the absolute monocyte count (AMC) is shown in Figure S5. We examined whether the change in monocytes correlated with an increase in myeloid subsets with potential immunoinhibitory properties. Although there was no change in the CD45/CD11b/CD33$^+$, CD15/human leukocyte antigen (HLA)-DR$^-$ myeloid-derived suppressor cell-like (MDSC-like) subsets before or after CAR T cell infusions (Figures 3A and 3B), we detected a marked increase in cells with an M2-associated phenotype (CD45/CD33/CD11b/CD163$^+$) in all patients in all cohorts after infusion of CAR T cells (one detailed representative in Figure 3C; all are summarized in Figures 3D and 3E). At baseline (within 96 hr prior to CAR T cell infusion), the percentage of cells in the CD45/CD33/CD11b/CD163$^+$ myeloid subset was low (approximately 0.01%); however, the percentage of this population increased significantly after CAR T cell infusions irrespective of cohort (week 1: median 3.15%, range 1.97%–13.27%; week 2: median 4.41%, range 0.77%–14.79%; week 4: median 1.78%, range 0.01%–9.73%; week 6: median 0.825%, range 0.01%–4.23%; p = 0.0126, Wilcoxon signed rank test, p values reflect comparison with baseline) (Figure 3D). Independent of Cy/Flu or PD-1 inhibition, a striking expansion of the absolute number of these myeloid cells was also detected, exceeding 100 cells/μL in some patients (week 1: median 9.86 cells/μL, range 2.41–56.68 cells/μL; week 2: median 22.4 cells/μL, range 2.76–152.23 cells/μL; week 4: median 6.89 cells/μL, range 0.04–42.13 cells/μL; p = 0.0126, Wilcoxon signed rank test, p values reflect comparison with baseline) (Figure 3E). The appearance of CD45/CD33/CD11b/CD163$^+$ myeloid subset in the peripheral blood thus occurs independent of changes in the MDSC-like population or of administration of Cy/Flu.

### Clinical Responses after GD2-CAR3 T Cell Infusions

We measured disease burden pre- and post-infusion as detailed in the Materials and Methods, and response to therapy was evaluated according the International Neuroblastoma Response Criteria and Curie scoring.[10] Clinical responses are summarized in Table 3. Of the 11 evaluable patients, 6 had progressive disease (PD) and 5 had stable disease at 6-week follow-up. For patients who received more than one infusion, the best response (measured 6 weeks after each infusion) was seen after the first infusion. Among patients with stable disease, patient 3A achieved a CR after his salvage regimen. After two re-infusions of GD2-CAR3 T cells, patient 3C had a small residual pelvic mass that was resectable after therapy and the patient remains in CR. Additional response to therapy evaluation by comparing the number of

**Table 2. Safety Assessment of GD2-CAR3 T Cells**

| | Cohort 1 | | | | Cohort 2 | | | | Cohort 3 | | | | All Cohorts | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Grade | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Inflammation Related | | | | | | | | | | | | | | | | |
| Fever | 1 | 1 | | | | 1 | | | 2 | | | | 3 | 2 | | |
| Fever and neutropenia | | | | | | | 1 | | | | 1 | | | | 2 | |
| CRS | 1 | | | | | | | | | | | | 1 | | | |
| Total | 2 | 1 | | | | 1 | 1 | | 2 | | 1 | | 4 | 2 | 2 | |
| Hematologic | | | | | | | | | | | | | | | | |
| Anemia | 2 | | 1 | | 1 | 1 | 2 | | 1 | 1 | 1 | | 4 | | 4 | |
| Leukopenia | | 1 | | | | | 1 | 3 | | | 2 | 1 | | | 3 | 4 |
| Lymphopenia | | 1 | 1 | | | | | 4 | | | | 3 | | | 1 | 7 |
| Neutropenia | 1 | | | | | | 2 | 2 | | | 1 | 1 | 1 | | 3 | 3 |
| Thrombocytopenia | 1 | | | | | | 2 | 2 | | | | 1 | 1 | | 2 | 3 |
| Total | 4 | 2 | 2 | 0 | 1 | 1 | 7 | 11 | 1 | 1 | 4 | 6 | 6 | | 13 | 17 |
| Neurologic | | | | | | | | | | | | | | | | |
| Arthralgia | | 1 | | | | | | | | | | | | 1 | | |
| Extremity pain | | | 2 | | | | | | | | | | | | 2 | |
| Paraesthesia | 2 | | | | | | | | | | | | 2 | | | |
| Lymph node pain | | 1 | | | | | | | | | | | | 1 | | |
| Facial pain | | 1 | | | | | | | | | | | | 1 | | |
| Headache | 1 | | | | 1 | | | | | | | | 2 | | | |
| Back pain | 1 | | | | 1 | | | | | | | | 2 | | | |
| Bone pain | | 1 | | | | | | | | | | | | 1 | | |
| Abdominal pain | | | | | 2 | | | | | | | | 2 | | | |
| Pelvis pain | | | | | 1 | | | | | | | | 1 | | | |
| Neck pain | | | | | 1 | | | | | | | | 1 | | | |
| Total | 4 | 4 | 2 | | 6 | | | | | | | | 10 | 4 | 2 | |

Grading according to the Common Terminology Criteria for Adverse Events version 4.0 (CTCAEv4). CRS, cytokine release syndrome.

MIBG avid lesions before and after infusions was available for eight patients. Curie scores increased in four patients, remained stable in one patient, and improved in three patients (Figure 4A). Lastly, we evaluated the long-term survival of patients treated with GD2-CAR3 T cells. The median survival for all patients was 506 days, with seven patients alive in long-term follow-up. All four patients in cohort 1 progressed and died at 32–506 days (median 230 days). By contrast, six of seven patients in the lymphodepleted treatment cohorts (2 and 3) survive at >265 to >724 days (median survival [MS] not reached), and two of these subjects (in cohort 3) achieved CR as described earlier. Survival curves for all patients and for patients in cohort 1 versus cohorts 2 and 3 are shown in Figures 4B and 4C, respectively.

## DISCUSSION

We report a phase 1 study of autologous T cells expressing a third-generation GD2-specific CAR conducted with adaptive trial design to evaluate the stepwise addition of Cy/Flu and PD-1 inhibition to CAR T cells in three consecutive relapsed or refractory NB patient cohorts. GD2-CAR3 T cell infusions were administered with or without Cy/Flu conditioning and two peri-infusion doses of PD-1 antibody to NB patients and were well tolerated in all cohorts. We found that Cy/Flu increases the expansion of GD2-CAR3 T cells after adoptive transfer, an effect that is not further modified by addition of peri-infusion PD-1 blockade. We detected striking expansion of circulating CD45/CD33/CD11b/CD163$^{+}$ M2 macrophage-like myeloid cells in all patients after CAR T cell infusions irrespective of lymphodepletion. Five of 11 patients had stable disease at 6-week response assessment, and patients went on to individualized salvage chemotherapy. All four patients in cohort 1 progressed and died at 32–506 days (median 230 days), whereas six of seven patients in the lymphodepleted treatment cohorts (2 and 3) survive at >265 to >724 days (MS not reached), and two of these subjects (both in cohort 3) achieved CR.

Our evaluation of GD2-CAR3 T cells showed safety, even when the cells expand and persist in vivo. Although administration of Cy/Flu increased grades 3 and 4 hematologic side effects, and two patients

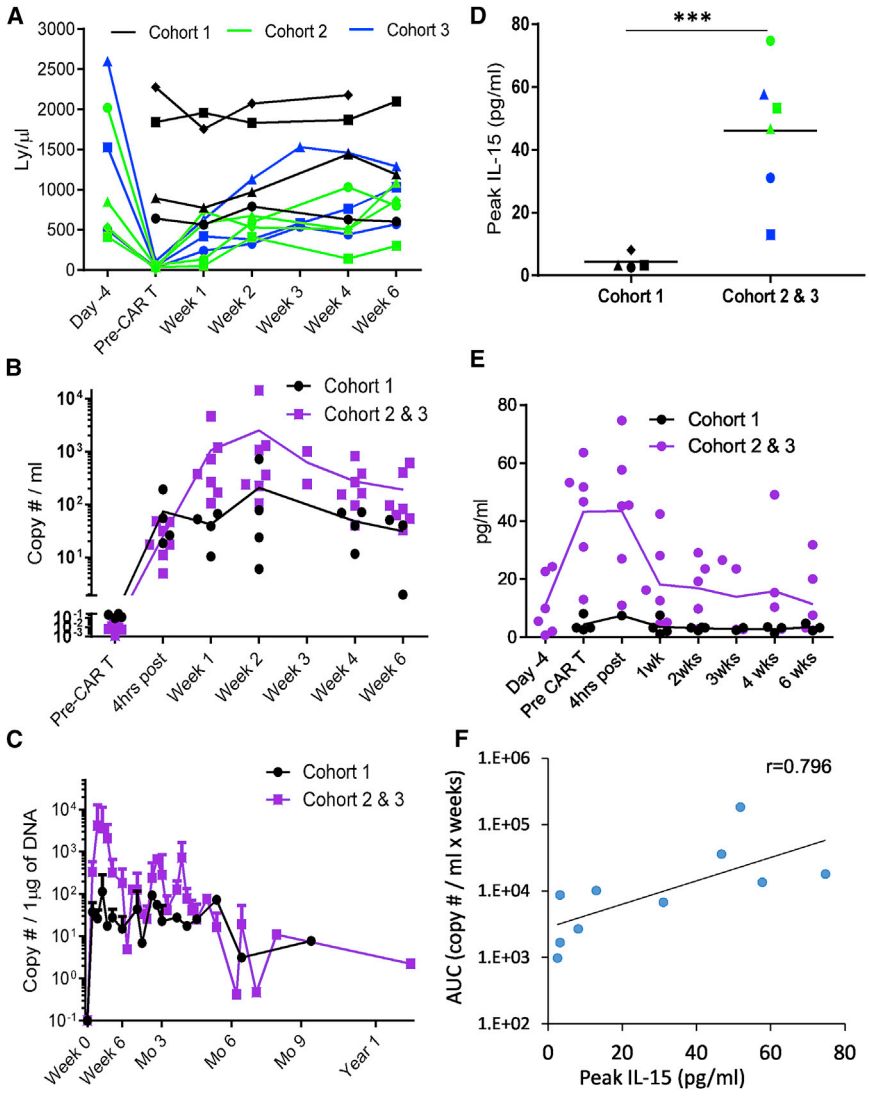


**Figure 2. Expansion and Persistence of GD2-CAR3 T Cells after Adoptive Transfer**

Peripheral blood evaluation of patients infused with GD2-CAR3 T cells at indicated time points. (A) Absolute lymphocyte count (Ly/µL). (B) Expansion of GD2-CAR3 T cells in patients in cohort 1 (black) or cohorts 2 and 3 (purple) prior to week 6 response to therapy evaluation by real-time PCR; transgene copy numbers per milliliter. Lines represent median. (C) Long-term persistence of GD2-CAR3 T cells in patients treated in cohort 1 (black) or cohorts 2 and 3 (purple). (D) Peak IL-15 levels (pg/mL) in patients detected on the day of CAR T cell infusions with (cohorts 2 and 3) and without lymphodepletion (cohort 1) measured by Luminex assay. Mean with SD. (E) Peripheral blood IL-15 levels (pg/mL) in patients in cohort 1 (black) or cohorts 2 and 3 (purple). Line represents median. (F) Association of area under the curve (AUC) of GD2-CAR3 T cells' expansion until response to therapy evaluation at week 6 and peak IL-15 levels. (A and D) Patients in cohort 1 are represented in black, cohort 2 in green, and cohort 3 in blue; each patient per cohort is represented by a different shape. ***$p < 0.001$, t test.

developed febrile neutropenia, this was an anticipated and manageable consequence of the drugs' myelosuppressive effects.

In terms of CAR T cell-associated toxicities, only one patient developed CRS, which resolved spontaneously without corticosteroid or anti-IL-6 treatment. CRS was not observed even in patients in whom CAR T cells in peripheral blood expanded comparably with successful CD19-CAR T cell studies.[8,12] Because CRS can be associated with response to therapy,[8,11] it is possible that its incidence will increase if more potent GD2-CAR therapies are developed. Alternatively, the prevalence and severity of CRS may be more dependent on the cell population targeted than on the effector cells per se. Thus, CD19-specific effector cells target normal and malignant B cell populations that serve as antigen-presenting cells and express an array of costimulatory molecules that enhance T cell activation.[13] By contrast, the lack of costimulatory molecules on NB[14] may limit T cell activation in response to antigen and reduce the likelihood of CRS. Additionally, the tumor microenvironment in NB contains myeloid cells, particularly M2 polarized tumor-associated macrophages (M2 TAMs), with immunosuppressive properties[15] that may further diminish T cell activation and reduce the release of pro-inflammatory cytokines. Moreover, we observed no CNS toxicities of the type associated with CD19-CAR T cell infusions, which may occur independently of CRS. The mechanisms that underlie these neurotoxicities are poorly understood but have been asserted to be exacerbated by Cy/Flu.[5] In our study, seven patients received Cy/Flu without CNS-related side effects, notwithstanding substantial expansion of CAR T cells in peripheral blood.[8,12] Thus, the neurotoxicities of the Cy/Flu combination, like CRS, may be CAR target specific rather than generalizable for the combination of lymphodepleting drugs and subsequent CAR T cell engraftment.

Of note, targeting GD2 with CAR T cells did not produce the painful peripheral neurotoxicity that is often associated with administration of GD2-specific monoclonal antibodies (mAbs).[1,2] Instead, the incidence and severity of pain-related side effects after GD2-CAR3 T cells remained as low as in our previous reports, even though GD2-CAR3 T cells exhibited greater expansion.[3] The frequent and severe pain noted in patients receiving GD2-specific mAbs[1,2] is attributed to the presence of moderate levels of the targeted antigen within pain receptors on sensory nerves and within the dorsal horns of the spine.[16] The mechanisms by which GD2 mAbs initiate pain remain controversial, although complement fixation is thought to contribute.[17]

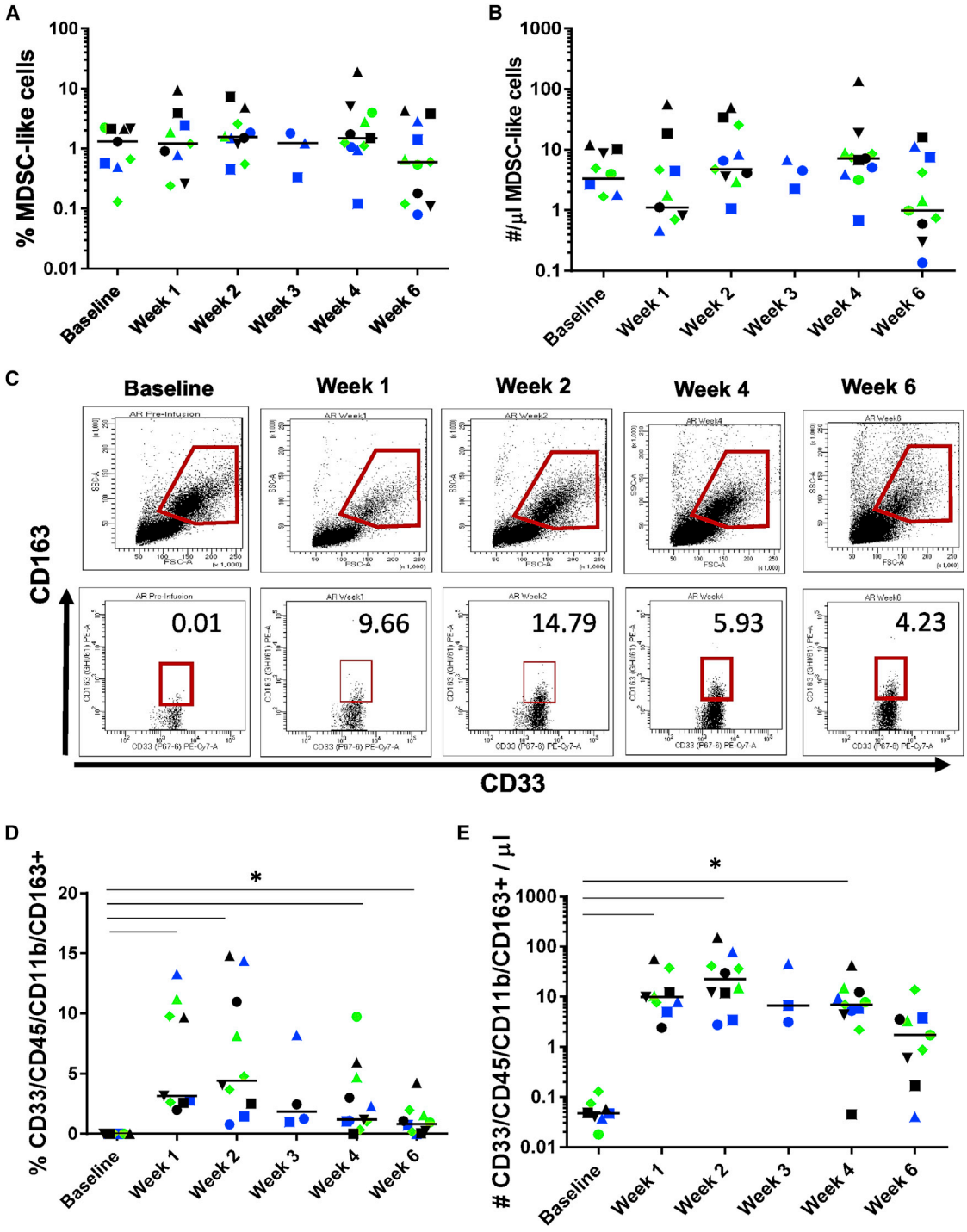


**Figure 3. Change of Peripheral Blood Myeloid Subsets after GD2-CAR3 T Cell Infusion**

Peripheral blood myeloid subset composition was analyzed with multiparametric flow cytometry at indicated time points. (A) Kinetics of CD45/CD33$^{+}$, HLA-DR/CD15$^{-}$ peripheral blood myeloid-derived suppressor cell-like (MDSC-like) subset as percentage of peripheral blood monocytes. (B) Absolute number of MDSC-like myeloid subset in peripheral blood per microliter. (C) Appearance of CD45/CD33/CD11b/CD163$^{+}$ myeloid subset in a representative patient after GD2-CAR3 T cell infusion. (D) Kinetics of CD45/CD33/CD11b/CD163$^{+}$ peripheral blood myeloid cell subset as percentage of peripheral blood monocytes. (E) Absolute number of CD45/CD33/CD11b/CD163$^{+}$ peripheral blood myeloid cell subset per microliter. Patients in cohort 1 are represented by black shapes, cohort 2 by green shapes, and cohort 3 by blue shapes. Each shape and color combination represents a patient. *$p < 0.05$, t test.

**Table 3. Response to Therapy after Infusion with GD2-CAR3 T Cells**

| Cohort | Patient ID | Response at 6 Weeks | Number of Infusions | Response after Repeat Infusion | Best Response | Long-Term Outcome | Time to Follow-up (Days) |
|---|---|---|---|---|---|---|---|
| 1 | 1A | stable disease | 1 | | stable disease | DOD | 172 |
| | 1B | PD | 1 | | PD | DOD | 288 |
| | 1C | PD | 1 | | PD | DOD | 32 |
| | 1D | stable disease | 2 | PD | stable disease | DOD | 506 |
| 2 | 2A | PD | 1 | | PD | AWD | 724 |
| | 2B | PD | 1 | | PD | DOD | 265 |
| | 2C | stable disease | 2 | PD | stable disease | AWD | 710 |
| | 2D | PD | 1 | | PD | AWD | 656 |
| 3 | 3A | stable disease | 2 | stable disease | stable disease | CR | 535 |
| | 3B | PD | 1 | | PD | AWD | 524 |
| | 3C | stable disease | 3 | stable disease | stable disease | CR | 500 |

AWD, alive with disease; DOD, died of disease; PD, progressive disease.

The lack of toxicity from CAR T cells may therefore be attributed to lack of CAR-expressing cell access to the pain receptors or to lack of the required effector mechanisms. It therefore remains a concern that increasing CAR T cell expansion or modifying T cell trafficking or effector activity will concomitantly trigger severe neurogenic pain; our inclusion of an inducible suicide gene (inducible caspase-9 [iC9]) in this and future GD2-CAR constructs is intended to allow us to mitigate the consequences should such neurotoxicity develop.[18]

Our study confirmed that lymphodepletion can substantially increase the in vivo expansion of the infused cells.[8,19–21] Administration of conditioning therapy has multiple effects, by reducing the patient's lymphoid cell pool, increasing homeostatic cytokines, and ameliorating the tumor inhibitory microenvironment.[20,22,23] We confirmed the beneficial effects of lymphodepletion on CAR T cell engraftment and found that increased peripheral blood IL-15 levels after Cy/Flu correlate with GD2-CAR3 T cell expansion. The superior expansion of CAR T cells after Cy/Flu may also have been facilitated by the lack of cryopreservation prior to cell infusion or the substitution of IL-7/15 for IL-2 during manufacture, a modification made primarily to accelerate T cell expansion[24] for patients in cohorts 2 and 3. The phenotype of the infused cells was unaffected by this change (Figure S6), which may be the result of tonic signaling associated with GD2-CARs.[25] The benefits of lymphodepletion that have previously been reported for second-generation CAR T cells can therefore also be obtained for third-generation CAR T cells in which both CD28 and OX40 costimulatory domains are present.[20,22] Positive effect of IL-15 on the expansion of CAR T cells reported here and shown by others may be utilized to enhance adoptive cell therapies by systemic IL-15 or IL-15 superagonist administration; however, careful dosing of the effector cells and IL-15 or its superagonist will be important to avoid toxicities.[26–28]

PD-1 could be detected on GD2-CAR3 T cells, and GD2-CAR3 T cells produce IFN-γ after CAR-mediated activation, which can upregulate PD ligand-1 (PD-L1) expression on NB cells (Figure S7). Given that inhibiting the deleterious PD-1–PD-L1 interaction can improve the antitumor activity of CARs,[9,29] we administered two doses of the PD-1 inhibitor pembrolizumab to patients in cohort 3 on days −1 and +21 of GD2-CAR3 T cell infusions, although combining GD2-CAR3 T cells with PD-1 inhibitor was feasible and safe. In our study, pembrolizumab had no measurable effect on CAR T cell expansion, persistence, or circulating cytokine levels. This contrasts with data in some preclinical models and in a recent case report of a patient treated for lymphoma, in which the combination of CAR T cells with PD-1 inhibition improved the antitumor activity of CAR T cells.[30–32] Beneficial effects on CAR T persistence and expansion from checkpoint blockade may depend on the timing and duration of PD-1 inhibition or may require the presence of a greater number of tumor neoantigens (stimulating CAR T cells through their native TCRs) than are present in most pediatric malignancies including NB. Although it was only in the PD-1 cohort in whom we observed CRs after salvage therapy, no conclusions can yet be drawn from such limited observations in such a heterogeneous group of patients. The continued testing of checkpoint inhibitors in conjunction with CAR T cells in more patients and in different cancers will demonstrate whether survival benefit can indeed be obtained, even after limited exposure to checkpoint inhibition.

One of our most striking observations was the consistent increase in the circulating CD45/CD33/CD11B/CD163$^{+}$ myeloid subset following GD2-CAR3 T cell infusion. This effect was independent of lymphodepletion and has not been a general observation after administration of CAR T cells with other specificities. Inhibitory myeloid cells are increasingly being recognized as important obstacles to successful immunotherapy,[33–35] and the presence of myeloid-derived suppressor cells (MDSCs) or tumor-associated macrophages (TAMs) can worsen the prognosis of patients with cancer, including NB, and can limit the benefits of immunomodulation,[36,37] and targeting MDSCs in preclinical NB models can have a significant antitumor effect.[38] The hemoglobin-scavenger receptor CD163 is an important

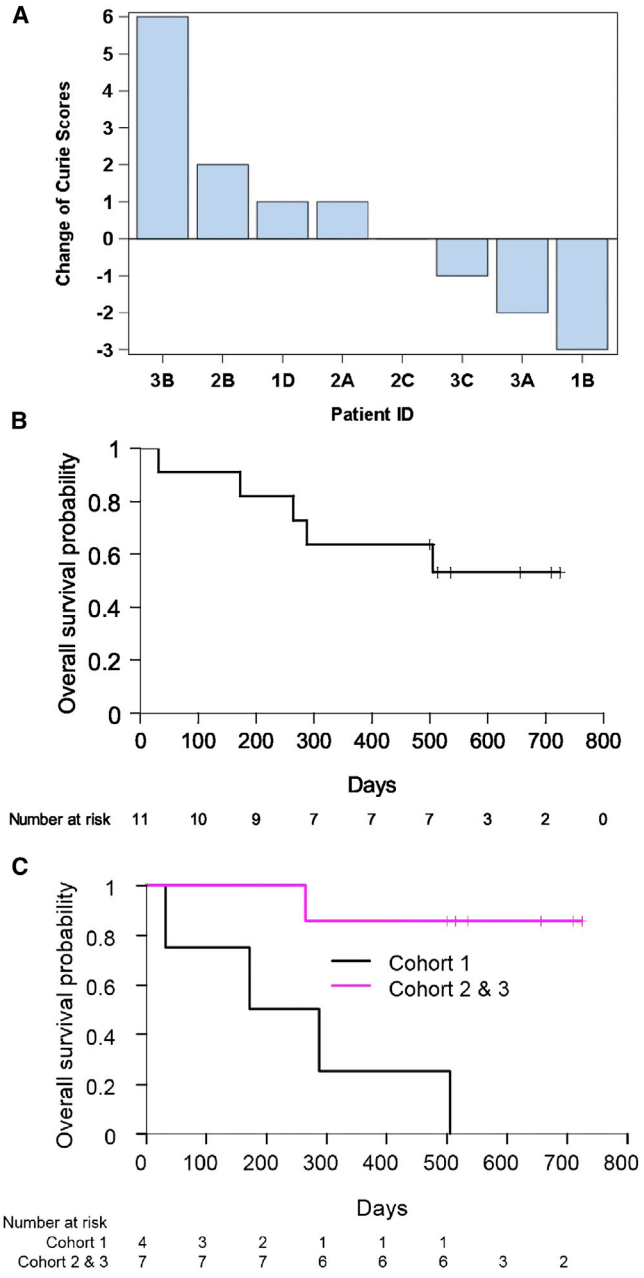


**Figure 4. Outcome of Patients Treated with GD2-CAR3 T Cells**

(A) Waterfall plots of the difference in Curie scores before and after therapy. (B) Kaplan-Meier survival curves of all patients. (C) Separated survival curves for patients in cohort 1 (black) and cohorts 2 and 3 (both shown in pink).

surface marker of M2 macrophages, and the presence of M2 markers on TAMs is associated with poor outcome in patients with NB[15] and in many other human cancers as well. The adverse effects of M2 TAMs are likely mediated by the immune-inhibitory and anti-inflammatory cytokines they secrete.[36] It is not feasible to sequentially sample tumor biopsies before and during CAR T cell treatment to confirm that the dynamic changes in M2 macrophage-like myeloid cells in peripheral blood were matched by equivalent changes in resident M2 TAMs. Nonetheless, the robust and consistent increase in the numbers of M2 macrophage-like myeloid cell numbers in peripheral blood we observe suggests that the infusion of GD2-CAR3 T cells provokes a response from the tumor that recruits marrow-derived monocytes and polarizes them to an M2 phenotype. If these M2 macrophage-like myeloid cells are then attracted to the tumor site and augment the M2 TAM population, it may be beneficial to develop countermeasures to this immune evasion strategy.[39]

Thus, our third-generation GD2-CAR3 T cells were detected at higher levels in NB patients compared with first-generation GD2-CAR T cells; this advantage can be further and safely increased by Cy/Flu treatment prior to infusion, resulting in significant, but transient, expansion of CAR T cells. Disappointingly, the immediate antitumor effects of the GD2-CAR3 T cells were no greater than observed in our earlier study of first-generation GD2-CAR T plus first-generation GD2-CAR EBVST,[3,6] and the study was not powered to show a significant benefit to survival from increased CAR T cell expansion in cohort 1 versus cohorts 2 and 3. Because of the heterogeneity of patients' prior treatment, their disease status at study entry and the individualized salvage regimens they received post-study mean that we cannot identify which components contributed to these apparent differences in longer-term outcome.

In the 10 years since we developed the GD2-CAR3 used here, we and others have shown in preclinical studies that further modifications to the antigen binding exodomain, the CAR spacer region, and the costimulatory endodomains may substantially augment the in vivo functionality of GD2-CAR T cells.[25,29,40,41] We anticipate that a combination of these modifications together with countermeasures to preexisting and evolving tumor immune evasion strategies, including the dynamic evolution we detected in the myeloid compartment, will produce both more immediate tumor responses and superior outcomes in future clinical studies.

## MATERIALS AND METHODS

### Study Design

We conducted a phase 1 study with an adaptive design in which we gave escalating doses of autologous ATCs expressing a GD2-specific CAR incorporating the 14g2a single-chain variable fragment and the CD28 and OX40 costimulatory endodomains (GD2-CAR3)[7] to patients with relapsed or refractory NB. GD2-CAR3 T cells were administered to three study cohorts (Figure 1). In cohort 1, four consecutive patients were enrolled; two patients received $1 \times 10^7$ and an additional two patients received $1 \times 10^8$ GD2-CAR3 T cells intravenously. In cohort 2, four consecutive patients were enrolled; two patients received $1 \times 10^8$, and an additional two patients received $1.5 \times 10^8$ GD2-CAR3 T cells intravenously. Patients also received Cy/Flu with cyclophosphamide at 500 mg/m$^2$/dose on days −4, −3, and −2 and fludarabine 30 mg/m$^2$/dose on days −4 and −3 prior to CAR T cell administration. In cohort 3, three consecutive patients were enrolled receiving $1.5 \times 10^8$ GD2-CAR3 T cells intravenously.

Patients received Cy/Flu as in cohort 2 and were given the PD-1 inhibitor pembrolizumab on days −1 and +21 at 2 mg/kg/dose for each dose. The detection in preclinical studies of high levels of PD-1 on GD2 CAR T cells and of PDL1 on IFN-γ-exposed neuroblasts provided the rationale for the addition of PD-1 inhibition in this cohort (Figure S7). Clinical evaluation and laboratory testing were performed on day 0 and at weeks 1, 2, 4, and 6 post-infusion for all cohorts. In addition, patients receiving Cy/Flu were evaluated on day −4, and patients receiving pembrolizumab were evaluated at week 3. All patients were included in primary and safety analyses. The trial is registered at clinicaltrials.gov (NCT01822652).

### Patients

Eligible patients had evaluable relapsed or refractory NB at the time of cell infusion. Patients had to have adequate organ function, and they were ineligible if human anti-mouse antibodies were detected or if they had a history of hypersensitivity to murine proteins. Patients must have completed previous therapy including other investigational treatments. Patients with a history of cardiomegaly, bilateral pulmonary infiltrates, and airway obstruction causing tumors were excluded, as were patients with HIV seropositivity or Lansky-Karnofsky score of less than 60%. Patients assigned to receive Cy/Flu or pembrolizumab were excluded if they had a previous history of sensitivity to these drugs. Patients receiving pembrolizumab had to have normal thyroid function tests. All participants and/or their legal guardians provided written informed consent/assent upon enrollment and prior to administration of the cells.

### Clinical Grade Vector Production

GD2-CAR3 was generated as previously described by Pulè et al.[7] To produce the clinical grade vector, the sequence of the iC9 safety switch[18] was cloned in frame, 5′ of the GD2-CAR3 gene in the SFG retroviral backbone (iC9.GD2-CAR3), and a producer cell line was picked by using PG-13 cells (ATCC CRL-10686) based on the highest-titer clone. The transgene integration was confirmed with sequencing, and the producer cell clone was validated under Good Manufacturing Practice guidelines. The final viral supplement was stored at −80°C and tested prior to release for clinical testing.

### CAR T Cell Manufacturing

iC9.GD2-CAR3 T cells were generated using peripheral blood mononuclear cells (PBMCs) from patients first simulated with CD3 and CD28 (Miltenyi Biotec) mAbs with recombinant human IL-2 (100 U/mL; Prometheus) or IL-7 and IL-15 (10 ng/mL and 5 ng/mL, respectively; R&D Systems) on day 1 and transduced with retroviral particles encoding the GD2-CAR3 construct in 24-well, RetroNectin-coated plates (Takara Bio) on day 3. Next, T cells were washed and replated on day 5, and expanded until numbers were sufficient for testing and patient infusions. Patients in cohort 1 received T cells expanded with IL-2, which was substituted by IL-7 and IL-15 for patients in cohorts 2 and 3.[24] CAR T cell viability, CD4/CD8, and naive memory/effector subset composition were largely unaffected, but in vitro cytotoxicity of the $GD2^{+}$ NB cell line was increased after this change (Figure S6).

### Real-Time PCR to Assess CAR T Cell Expansion and Persistence

Evaluation of CAR T cell persistence was assessed by calculating the copy number of the CAR transgene after extracting genomic DNA from PBMCs with QIAamp DNA Blood Minikit (QIAGEN) according to the manufacturer's manual and measuring the transgene copy number with RT-PCR using primers (forward: 5′-GCTGCACCA ACTGTATCCATCTT-3′; reverse: 3′-GGTCCAGACTGCTGAAG CT-5′) and probe (5′-CACCCGACCCACCACC-3′) sequences (Applied Biosystems) in the ABI Prism 7700 Sequence Detector (PerkinElmer).[3] The copy number was normalized to 1 μg of DNA of PBMCs in the patient samples. Lymphodepleting chemotherapy with Cy/Flu decreases total WBC, resulting in an artificial increase of the transgene copy number measured by PCR when normalized to DNA only; therefore, for the 6 weeks following lymphodepletion, we instead show transgene copy number per milliliter of peripheral blood. AUC of CAR T cell persistence was calculated from copy number per milliliter over time.

#### *Detection of CAR T Cells in Peripheral Blood of Patients*

T cells expressing our GD2-CAR3 were detected from peripheral using 1A7 idiotypic antibody as previously described by Rossig et al.[42]

### Multiplex Assay for Cytokine Levels

Serum cytokine levels were measured with Milliplex MAP magnetic-bead-based multi-analyte panel (EMD Millipore) on the Luminex 200 system (Luminex) with the xPONENT (Luminex) software according to the manufacturer's manual.

### Immunophenotyping of GD2-CAR3 T Cells and Peripheral Blood Myeloid Cells

Peripheral blood monocyte subsets were assessed using phycoerythrin (PE), FITC, allophycocyanin, allophycocyanin-Cy7 or PE-Cy7, V500-C, or Pacific Blue-conjugated antibodies specific to CD45 (2D1), CD163 (GHI/61), CD33 (P67.6), CD11b (D12), HLA-DR (L243), CD14 (MδP9), CD15 (80H5), and PD-1 (PD1-3) from BD Biosciences; the region analyzed was first defined, followed by gating in populations on single color/side scatterplots, and subset composition was calculated using Boolean methodology after gating. PD-1 expression of GD2-CAR3 T cells was tested with EH12.1 clone (BD Horizon), and PD-L1 expression of NB cells was detected with MIH1 clone (BD Pharmingen) in preclinical studies.

### Clinical Response Assessment

Response to therapy was evaluated by 3D imaging (computed tomography [CT] or magnetic resonance imaging [MRI]), MIBG scan, and bone marrow evaluation 6 weeks after initial infusion, according to the International Neuroblastoma Response Criteria.[10] MIBG-positive lesions were quantified by Curie scoring in the eight patients for whom pre- and post-infusion imaging were available.[43] Bone marrow was evaluated by standard pathologic testing.

### Outcomes

The primary objective of this study was to evaluate the safety of GD2-CAR3 T cells in patients with relapsed or refractory NB. Secondary

objectives were to determine their in vivo persistence, antitumor response rate, and effect on peripheral blood lymphoid and myeloid cells. Adverse events were monitored according to the NIH Common Terminology Criteria for Adverse Events (CTCAE) version 4 (https://ctep.cancer.gov).

### Statistical Analysis

All patients were included in primary and safety analyses. Descriptive statistics were used to describe phenotypic data and T cell expansion. Plots of growth curves demonstrating measurements over time within patients were generated to visualize patterns of immune reconstitution. AUCs were calculated using trapezoidal rule for CAR T cell frequencies over time for every patient. Comparisons were made between groups using Wilcoxon rank-sum test or t test, whichever was appropriate, for continuous variables and the Fisher exact test for categorical variables. Correlation analysis was performed using Spearman's rank correlation coefficient. Survival data were analyzed by the Kaplan-Meier method. Overall survival (OS) was calculated from the time of the first GD2-CAR3 T cell infusion to death from any cause; observations were censored at the date of last follow-up. Changes from baseline to follow-up measures were compared using the Wilcoxon signed rank test. Statistics were computed using GraphPad Prism 6 (GraphPad Software), SAS 9.4 and R 3.3.2. Differences were considered significant when $p < 0.05$.

### Study Approval

This study was approved by the US Food and Drug Administration, the Recombinant DNA Advisory Committee of the NIH, and the Protocol Review Committee, Institutional Biosafety Committee and Institutional Review Board of Baylor College of Medicine. The study was conducted in accordance with Declaration of Helsinki principles. All participants and/or their legal guardians provided written informed consent/assent upon enrollment and prior to administration of the cells.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.05.012.

## AUTHOR CONTRIBUTIONS

A.H., C.U.L., B.S., G.D., H.E.H., C.M.R., and M.K.B. designed the clinical trial. A.H., C.U.L. and M.K.B. wrote the clinical protocol. A.H. and C.U.L. were the principal investigators of the study. H.E.H. and M.K.B. were the investigational new drug (IND) sponsors. CAR T cell manufacturing was performed by B.S., B.M., and H.Z. Flow cytometry was done by B.S., B.M., and A.D., and qPCR was performed by O.D., supervised by A.H. and B.S., and directed by A.G. H.T. performed the experiments for PDL-1 and PD-1 expression. A.H. and M.K.B. wrote the manuscript, and all authors reviewed, interpreted, and discussed the results. A.H., M.F.W., and H.L. performed statistical analysis. N.M. and A.H. analyzed the MIBG images. B.G. was responsible for regulatory approvals. A.H. and C.U.L. enrolled and managed the patients.

## CONFLICTS OF INTEREST

A.H., M.K.B., C.M.R., H.E.H., B.S., and G.D. have patent applications in the field of gene-modified T cell therapy for cancer.

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