ORIGINAL ARTICLE

GD2-CART01 for Relapsed or Refractory High-Risk Neuroblastoma

F. Del Bufalo, B. De Angelis, I. Caruana, G. Del Baldo, M.A. De Ioris, A. Serra, A. Mastronuzzi, M.G. Cefalo, D. Pagliara, M. Amicucci, G. Li Pira, G. Leone, V. Bertaina, M. Sinibaldi, S. Di Cecca, M. Guercio, Z. Abbaszadeh, L. Iaffaldano, M. Gunetti, S. Iacovelli, R. Bugianesi, S. Macchia, M. Algeri, P. Merli, F. Galaverna, R. Abbas, M.C. Garganese, M.F. Villani, G.S. Colafati, F. Bonetti, M. Rabusin, K. Perruccio, V. Folsi, C. Quintarelli, and F. Locatelli, for the Precision Medicine Team–IRCCS Ospedale Pediatrico Bambino Gesù*

ABSTRACT

BACKGROUND

Immunotherapy with chimeric antigen receptor (CAR)–expressing T cells that target the disialoganglioside GD2 expressed on tumor cells may be a therapeutic option for patients with high-risk neuroblastoma.

METHODS

In an academic, phase 1–2 clinical trial, we enrolled patients (1 to 25 years of age) with relapsed or refractory, high-risk neuroblastoma in order to test autologous, third-generation GD2-CAR T cells expressing the inducible caspase 9 suicide gene (GD2-CART01).

RESULTS

A total of 27 children with heavily pretreated neuroblastoma (12 with refractory disease, 14 with relapsed disease, and 1 with a complete response at the end of first-line therapy) were enrolled and received GD2-CART01. No failure to generate GD2-CART01 was observed. Three dose levels were tested (3-, 6-, and 10×10^6 CAR-positive T cells per kilogram of body weight) in the phase 1 portion of the trial, and no dose-limiting toxic effects were recorded; the recommended dose for the phase 2 portion of the trial was 10×10^6 CAR-positive T cells per kilogram. Cytokine release syndrome occurred in 20 of 27 patients (74%) and was mild in 19 of 20 (95%). In 1 patient, the suicide gene was activated, with rapid elimination of GD2-CART01. GD2-targeted CAR T cells expanded in vivo and were detectable in peripheral blood in 26 of 27 patients up to 30 months after infusion (median persistence, 3 months; range, 1 to 30). Seventeen children had a response to the treatment (overall response, 63%); 9 patients had a complete response, and 8 had a partial response. Among the patients who received the recommended dose, the 3-year overall survival and event-free survival were 60% and 36%, respectively.

CONCLUSIONS

The use of GD2-CART01 was feasible and safe in treating high-risk neuroblastoma. Treatment-related toxic effects developed, and the activation of the suicide gene controlled side effects. GD2-CART01 may have a sustained antitumor effect. (Funded by the Italian Medicines Agency and others; ClinicalTrials.gov number, NCT03373097.)

The authors' full names, academic degrees, and affiliations are listed in the Appendix. Dr. Locatelli can be contacted at franco.locatelli@opbg.net or at the Department of Pediatric Hematology and Oncology and of Cell and Gene Therapy, IRCCS Ospedale Pediatrico Bambino Gesù, Piazza Sant'Onofrio, 4, 00165 Rome, Italy.

*The members of the Precision Medicine Team are listed in the Supplementary Appendix, available at NEJM.org.

Drs. Quintarelli and Locatelli contributed equally to this article.

N Engl J Med 2023;388:1284-95.
DOI: 10.1056/NEJMoa2210859

Chimeric antigen receptor (CAR) T-cell therapy is a valuable treatment for relapsed or refractory B-cell cancers.[1-3] In the field of solid tumors, however, the path to the development of effective CAR T-cell therapy has been more challenging.[4]

Neuroblastoma, the most common extracranial solid tumor in children, is responsible for 11% of all deaths from cancer in the pediatric population.[5] Almost half the patients have high-risk disease at diagnosis, and the 5-year event-free survival among these patients is 40 to 50%.[6] Children in whom first-line therapy fails have a very low probability of recovery with subsequent treatments, and they have a dismal prognosis, with a long-term survival of approximately 5 to 10%.[7]

Neuroblastoma cells express high levels of the disialoganglioside GD2, and the targeting of disialoganglioside GD2 with monoclonal antibodies has been associated with a significant increase in survival among high-risk patients, findings that indicate the sensitivity of neuroblastoma to immunotherapy and the relevance of the target antigen.[8] Few early-phase clinical trials of GD2-targeted CAR T cells have shown that the approach is feasible, and few objective responses have been reported.[9-12]

We developed a GD2-directed CAR construct incorporating two costimulatory domains — CD28 and 4-1BB.[13] In order to address potential neurotoxic effects associated with the use of our third-generation CAR T cells (GD2-CART01), we also included in the construct the gene for inducible caspase 9 (iC9) as a safety switch allowing the adoptively transferred cells to be killed if they were associated with dangerous toxic effects.[14] We report the 3-year results of our phase 1–2 clinical trial involving patients with relapsed or refractory high-risk neuroblastoma.

Methods

Trial Design and Oversight

At the IRCCS Ospedale Pediatrico Bambino Gesù in Rome, we conducted a phase 1–2 clinical trial to assess the feasibility and safety of GD2-CART01 in patients with relapsed or refractory high-risk neuroblastoma and to test the treatment efficacy of the recommended dose. The phase 1 portion of the trial was designed as a dose-finding trial, following an escalation and de-escalation schema: the first patients received GD2-CART01 at a dose of 3×10^6 CAR-positive T cells per kilogram of body weight. Two dose-escalation levels were specified — 6 and 10×10^6 CAR-positive T cells per kilogram. If the initial dose level was associated with dose-limiting toxic effects, two additional de-escalation dose levels were planned — 2 and 1×10^6 CAR-positive T cells per kilogram. Both the national competent authority of Italy and the institutional review board of IRCCS Ospedale Pediatrico Bambino Gesù approved the trial.

A Quick Take is available at NEJM.org

Patients between the ages of 1 and 25 years with a diagnosis of high-risk neuroblastoma who had relapsed disease or who had had persistent or progressive disease during first-line treatment were enrolled in the phase 1 trial. In the phase 2 trial, patients with metastatic disease and *MYCN* amplification could be enrolled after completion of first-line treatment, even if they had a complete response. Additional trial details are provided in the Supplementary Appendix and the protocol, available with the full text of this article at NEJM.org.

Manufacture and Infusion of GD2-CART01

The retrovirus encoding the construct iCasp9.2A.GD2.CD28.4-1BB.zeta is approved for clinical use and has been described previously.[13] Before infusion of GD2-CART01, all the patients received the same fludarabine–cyclophosphamide lymphodepleting chemotherapy. Further details regarding the manufacture and infusion of GD2-CART01 and immune monitoring by means of flow cytometry and molecular biologic techniques are provided in the Supplementary Appendix.

Disease Assessment

Revised International Neuroblastoma Response Criteria with European International Society of Paediatric Oncology Neuroblastoma Group (SIOPEN) scoring was used to evaluate response. The first disease assessment was performed in every patient at week 6 after GD2-CART01 infusion. Both radiologic and magnetic resonance images and SIOPEN scores were evaluated in a blinded fashion by a dedicated radiologist and a nuclear radiologist.[15,16] High disease burden was defined as at least one of the following: bulky disease (defined as a mass that was ≥5 cm in the

Table 1. Characteristics of the Patients at Baseline.*

Characteristic	All Patients (N=27)
Sex — no. (%)	
Male	18 (67)
Female	9 (33)
Median age (range) — yr	6.7 (2.7–18.6)
Median no. of previous treatments (range)	3 (1–6)
Disease status at enrollment — no. (%)	
Refractory	12 (44)
Relapsed	14 (52)
No evidence of disease after NB-HR01 first-line treatment†	1 (4)
Previous treatment with anti-GD2 monoclonal antibody — no. (%)	14 (52)
MYCN status — no. (%)	
Amplification	7 (26)
Gain	5 (19)
Normal	10 (37)
Unknown	5 (19)
Site of disease involvement — no. (%)	
Bone	21 (78)
Bone marrow	12 (44)
Lymph nodes	11 (41)
Abdomen	4 (15)
Paravertebral area	7 (26)
Thorax, pleura	2 (7)
Liver	1 (4)
Result of ^{123}I-labeled MIBG scan before infusion — no. (%)‡	
MIBG score ≤7	18 (67)
MIBG score >7	9 (33)

* Baseline was the time of screening, before infusion of GD2-CART01. Percentages may not total 100 because of rounding. MIBG denotes metaiodobenzylguanidine.

† NB-HR01 first-line treatment consisted of therapy according to the protocol of the European International Society of Paediatric Oncology Neuroblastoma Group (SIOPEN) Trial for High-Risk Neuroblastoma. This therapy consisted of an induction phase involving intensive chemotherapy, surgery, high-dose chemotherapy with reinfusion of autologous stem cells, radiotherapy, differentiating treatment with isotretinoin, and immunotherapy with the anti-GD2 monoclonal antibody.

‡ MIBG scores range from 0 to 72, with higher scores indicating greater dissemination of disease.

greatest dimension), bone marrow infiltration of greater than 50%, and an MIBG (metaiodobenzylguanidine) SIOPEN score higher than 7 (on a scale of 0 to 72, with higher scores indicating greater dissemination of disease) (see the Supplementary Appendix).[17]

STATISTICAL ANALYSIS

All the characteristics of the patients and trial outcomes were described with usual summary statistics (i.e., counts and percentages for categorical factors and the mean, median, or range for continuous factors). The median follow-up was derived with Schemper's method.[18] Cox models with one or two covariates were used to derive hazard ratios with 95% confidence intervals for overall and event-free survival. The assumption of proportional hazards was assessed with the use of supremum tests.[19]

RESULTS

PATIENT CHARACTERISTICS

A total of 30 patients underwent screening and 27 were enrolled and received treatment between January 2018 and October 2021; 3 patients were excluded (2 because of rapidly progressing disease and 1 because of a psychiatric disorder) (Fig. S1 in the Supplementary Appendix). The baseline characteristics of the patients are shown in Table 1 and Table S1. Before enrollment, all the patients had had disease resistance to at least two lines of treatment (range, two to six); 1 patient in the phase 2 trial had received GD2-CART01 during complete remission at the end of first-line therapy. Thirteen patients had not received previous treatment with the GD2-directed monoclonal antibody because they did not have approved indications for its use. Of the 26 patients with relapsed or refractory disease, 24 had active, stage 4, metastatic disease at screening before treatment, including 6 who had bulky disease.

MANUFACTURING OF GD2-CART01

GD2-CART01 was manufactured successfully in all the patients. The median (±SD) total number of CAR-positive T cells at the end of production was $2\times10^9\pm1.4\times10^9$ cells per kilogram (range, 0.7 to 6.9×10^9), with a median viability of 90.5±3.7% (range, 82.2 to 96.1) and a median transduction efficiency of 72.4±9.9% (range, 50.8 to 87.3). Thus, multiple doses of cells were generated for each patient. Eleven patients received multiple

Table 2. Adverse Events in 27 Patients after the First Infusion of GD2-CART01.*

Event	Grade 1 or 2	Grade 3	Grade 4
		number of patients	
Cytokine release syndrome	19	1	0
Central neurotoxic effects	0	0	0
Peripheral neurotoxic effects or pain	6	0	0
Hematologic toxic effects			
Anemia	8	19	0
Neutropenia	0	0	27
Thrombocytopenia	1	4	19
Abnormal laboratory values			
Elevated alanine aminotransferase level, aspartate aminotransferase level, or both	13	7	0
Elevated bilirubin level	3	1	0
Electrolyte abnormalities	4	2	0
Miscellaneous			
Clostridium difficile infection	0	1	0
Rash	3	0	0
Dysuria	2	0	0
Brain hemorrhage	0	0	1

* With the exception of hematologic toxic effects, anomalies caused by the disease or by previous treatments were not included.

infusions, and each patient could receive up to four infusions. Details regarding the drug products are provided in Figures S2 and S3.

SAFETY OF INFUSION

No dose-limiting toxic effects were reported in the phase 1 trial; the recommended dose of 10×10^6 CAR-positive T cells per kilogram was determined. Details regarding all toxic effects observed are provided in Table 2 and Table S2. Cytokine release syndrome was one of the most common drug-related adverse events, observed in 20 of 27 patients (74%) after the first infusion; these events were grade 1 or 2 (as defined by Lee et al.)[20] in 19 of 20 patients (95%). Grade 3 cytokine release syndrome developed in only 1 patient after the first infusion, and it rapidly resolved after administration of tocilizumab. Serum levels of interferon-γ, interleukin-6, tumor necrosis factor α, and interleukin-10 were monitored in all the patients, and a correlation between significantly higher levels of circulating cytokines and the development and grade of cytokine release syndrome was observed (Fig. 1G).

One patient received two infusions of rimiducid (a dimerizing agent intended to induce caspase 9 and destroy the adoptively transferred GD2-CART01) after an altered state of consciousness developed; however, the diagnostic workup revealed a brain hemorrhage as the causative event. Central neurotoxic effects did not develop in any of the patients.

Severe hematologic toxic effects developed in all the patients, induced by lymphodepletion and sustained after GD2-CART01 infusion. In addition, grade 3, transient hepatic toxic effects developed in seven patients after infusion; in five of these patients, hepatic toxic effects were present before enrollment or before GD2-CART01 infusion and transiently worsened with the development of cytokine release syndrome. In one of the other two patients, a clinically relevant hepatic infiltration of disease was present and led to grade 3 toxic effects on activation of GD2-CART01. The other patient had grade 3 hepatic

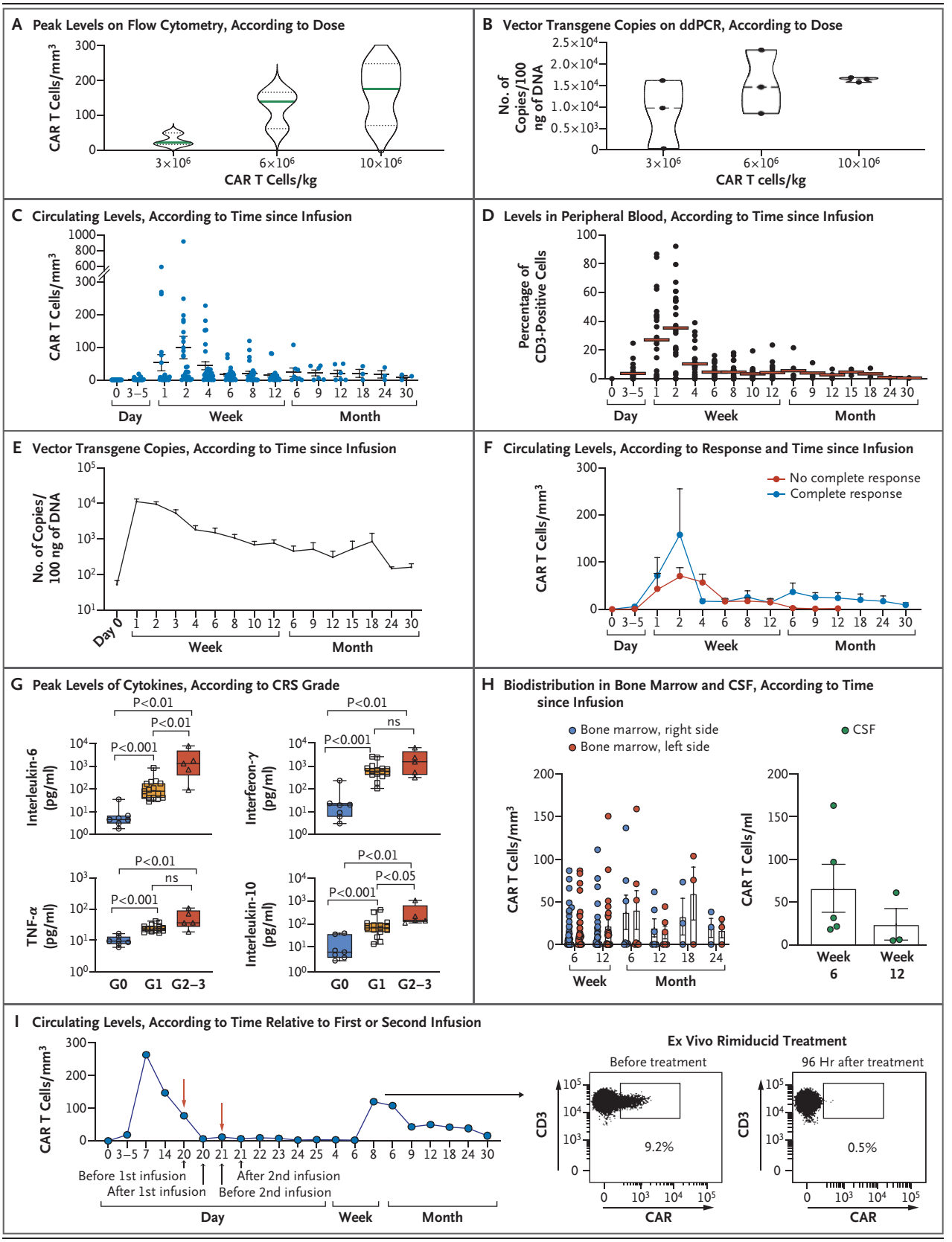


Figure 1 (facing page). Expansion, Persistence, and Biodistribution of GD2-CART01.

Shown are the peak levels of chimeric antigen receptor (CAR) T cells (GD2-CART01) in peripheral blood in each cohort of patients who received one of the three dose levels tested in the phase 1 trial, detected either by means of flow cytometry (Panel A) or by means of droplet digital polymerase-chain-reaction (PCR) (ddPCR) assay (Panel B). Data in Panels A and B are shown as violin plots; in each plot, the solid horizontal line represents the median, and the dotted horizontal lines the upper and lower quartiles. Shown also are the absolute numbers of circulating GD2-CART01 cells per cubic millimeter (Panel C) and the percentage of GD2-CART01 cells in CD3-positive cells in peripheral blood (Panel D), detected by means of flow cytometry, in the entire cohort of patients. In Panel C, each solid circle represents one patient. In Panel D, the red line represents the median, and the solid circles patients with CAR T-cell proliferation. The expansion of GD2-CART01 in the patients, evaluated by real-time PCR, is shown in Panel E. The mean (±SD) circulating GD2-CART01 in the peripheral blood in patients who had a complete response and in those who did not is shown in Panel F. The mean peak level of GD2-CART01 and the area under the curve in patients with a response were 157.95±97.35 cells per cubic millimeter and 436.0±229.0, respectively, whereas those in patients who did not have a response were 70.38±17.90 cells per cubic millimeter and 224.4±126.6, respectively. The peak serum levels of interleukin-6, interferon-γ, tumor necrosis factor α (TNF-α), and interleukin-10, stratified according to cytokine release syndrome (CRS) grade, are shown in Panel G. CRS grades range from 0 to 5 (death), with higher grades indicating more severe toxic effects. The wide biodistribution of GD2-CART01, detected by flow cytometry in the bone marrow and cerebrospinal fluid (CSF) of the patients, is shown in Panel H. In Panels G and H, the horizontal line within each box represents the median, and the lower and upper borders of each box the 25th and 75th percentiles, respectively. The kinetics of circulating GD2-CART01 in a patient receiving rimiducid (red arrows) are shown in Panel I (left side). The rapid clearance of CAR T cells 4 hours after the first administration of the dimerizing agent and the subsequent re-expansion, with long-term persistence, are shown. Rebounding GD2-CART01 cells collected at week 8 were exposed in vitro to rimiducid for 96 hours, with a remarkable decrease in the number of CAR-positive T cells (right side). In Panels C, E, F, G, and H, means ±SD (I bars) are shown.

toxic effects 3 weeks after infusion of GD2-CART01, after resolution of cytokine release syndrome; these toxic effects were attributed to other hepatotoxic drugs that she was receiving. Other adverse events are listed in Table 2. The side-effect profile of multiple infusions was similar to the side-effect profile of a single infusion, as detailed in Table S3.

GD2-CART01 IN VIVO EXPANSION AND PERSISTENCE

GD2-CART01 cells were detected in vivo by means of flow cytometry in 26 of 27 patients (96%). The correlation between the dose levels tested in phase 1 and the peak GD2-CART01 level in peripheral blood, evaluated by means of both flow cytometry and droplet digital polymerase-chain-reaction (PCR) assay, is shown in Figure 1A and 1B. Overall, peak expansion was observed in the second week after infusion on flow cytometry and was anticipated by almost 1 week with a droplet digital PCR signal. GD2-CART01 persisted for at least 3 months in 75% of the evaluable patients and for more than 2 years in 2 children (median persistence, 3 months; range, 1 to 30). In patients who received a second infusion, assessment of GD2-CART01 persistence after the first dose was terminated at the time of the second dose infusion, regardless of the circulating levels detected (Fig. 1C, 1D, and 1E), with a magnitude of expansion expressed as an area under the curve of 329.8±165.7.

Expansion of GD2-CART01 in patients who had a response to treatment and those who did not have a response to treatment is shown in Figure 1F. After expansion, a predominance of CD8-positive CAR-positive cells was detected in peripheral blood and was maintained at all time points, although CD4-positive CAR-positive cells expanded and were detectable as well (Fig. S4).

For both CD4-positive CAR-positive and CD8-positive CAR-positive cells, the phenotype showed a strong prevalence of central and effector memory subpopulations, with a prolonged persistence of central memory CD4-positive cells (Fig. S5). GD2-CART01 cells were detected in bone marrow up to 2 years after infusion (Fig. 1H), and they were detected in the cerebrospinal fluid in five patients up to 12 weeks after infusion. We did not observe any difference in the bone marrow infiltration by GD2-CART01 between patients with a response (i.e., six patients who had a complete response in the bone marrow) and those without a response (i.e., six patients who did not have a complete response in the bone marrow and three patients who had relapse in the bone marrow after infusion) (Fig. S6). Moreover, GD2-CART01 cells that persisted long term did not show a relevant expression of exhaustion markers. At the end of production, GD2-CART01 cells in the four patients with low in vivo expansion (peak of expansion <5% in

peripheral blood) were characterized by a significantly higher proportion of terminally differentiated CD4-positive effector memory cells re-expressing CD45RA than the products of patients with higher in vivo expansion (Fig. S7).

Finally, we evaluated the kinetics of GD2-CART01 over time in the patient who received rimiducid, and we observed a sharp decrease in the level of circulating GD2-CART01 4 hours after infusion of the dimerizing agent. This decrease was sustained after the second infusion (Fig. 1I). After 6 weeks, we detected a new expansion of GD2-CART01, with levels that remained elevated at subsequent follow-up and were still detectable 30 months after infusion. The patient continued to have a complete response.

We then evaluated whether GD2-CART01 cells that re-expanded could derive from a subpopulation of cells that had lost sensitivity to rimiducid, functionality of the iC9 suicide gene, or both. We exposed GD2-CART01 cells obtained from the patient at the time of re-expansion (i.e., at week 8) to rimiducid in vitro and evaluated the levels of residual cells after 96 hours. As shown in Figure 1I and Figure S8, we observed that the percentage of CAR-positive T cells decreased to 0.5%, whereas in the absence of rimiducid the percentage remained at 9.2%. These findings indicate that GD2-CART01 cells persisting in vivo long term after rimiducid infusion were still responding to the dimerizing agent.

CLINICAL RESPONSES AFTER GD2-CART01 INFUSION AND OUTCOMES

Six weeks after infusion of GD2-CART01, 9 of 27 patients (33%) had a complete response (8 patients) or maintained a complete response (1 patient) (Table S4). A description of 2 patients with MIBG-negative lesions at screening is provided in the Supplementary Results section in the Supplementary Appendix. With a median follow-up of 1.7 years (interquartile range, 1.2 to 2.6), a complete response was maintained in 5 of 9 patients (56%) (Fig. 2A). All 9 patients had a complete response after the first infusion and did not receive further treatments. Two of the 4 patients with disease that relapsed had been enrolled in the phase 1 trial and had received low doses of GD2-CART01 (3- and 6×10^6 CAR-positive T cells per kilogram). Of the remaining patients, 8 of 27 patients (30%) had a partial response, 5 of 27 patients (19%) had stable disease, and 5 of 27 patients (19%) did not have a response. A total of 17 of 27 patients (63%) had an objective response.

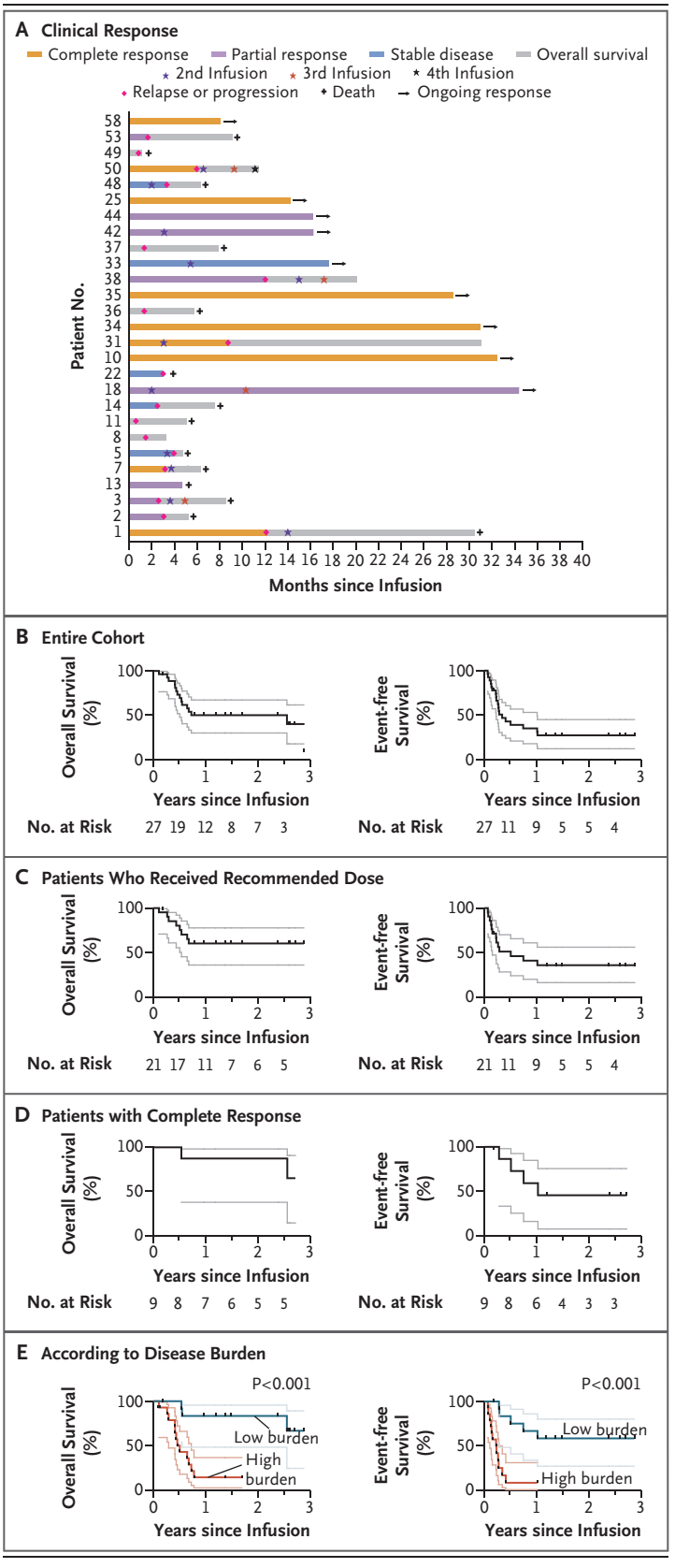
In three patients (Patients 18, 42, and 44), we observed a peculiar clinical course. All three patients had a reduction in MIBG-positive lesions that was consistent with a partial response after the first infusion (Fig. 2A). Two of them (Patients 18 and 42) received additional infusions of GD2-CART01; one then had stable disease, and a deeper partial response developed in the other. In the subsequent follow-up, the three patients had persistently stable disease in the long term, although no other therapy was administered. All three patients had a negative positron-emission tomographic scan. The durability of these remissions will be informative.

In the entire cohort, the MIBG score decreased from a mean of 13 (range, 0 to 72) to 8 (range, 0 to 38). We observed a relevant decrease in tumor size and MIBG avidity in bulky tumor masses in Patient 3 (Fig. 3A) and Patient 13 (Fig. 3B). In the whole cohort, 3-year overall survival was 40% (Fig. 2B) and event-free survival was 27%; these percentages were 60% and 36%, respectively, among patients who received GD2-CART01 at the recommended dose (10×10^6 CAR-positive T cells per kilogram) (Fig. 2C). Among the patients who had a complete response after the first infusion, at 3 years, the overall survival was 66% (Fig. 2D) and event-free survival was 50%. Previous treatment with the anti-GD2 monoclonal antibody did not affect the outcome (Fig. S9).

Patients with low disease burden had significantly longer survival than those with a higher disease burden. At 3 years, overall survival was 67% among the patients with low disease burden, as compared with 0% among those with high disease burden ($P<0.001$) (Fig. 2E); event-free survival was 58% and 0%, respectively ($P<0.001$). Similarly, in the univariate analysis, high disease burden was the only variable associated with lower event-free survival (hazard ratio for the earliest of no response, disease relapse or progression, last contact, or death from any cause, 5.7; 95% confidence interval, 2.0 to 16.6; $P=0.001$). None of the patients with disease that relapsed or progressed after treatment had a concomitant re-expansion of GD2-CART01 despite the increased antigen stimulation.

Figure 2. Long-Term Outcomes after GD2-CART01 Infusion.

Panel A shows a swimmer plot of the response to GD2-CART01 over time in each enrolled patient. Panel B shows the 3-year overall survival among the entire cohort of patients, calculated from the date of GD2-CART01 infusion to the date of last contact or death from any cause, as well as the 3-year event-free survival among the entire cohort of patients, calculated from the date of GD2-CART01 infusion to the earliest of the following events: no response, disease relapse or progression, last contact, or death from any cause. Panel C shows the 3-year overall survival and the 3-year event-free survival among the patients who received the recommended dose of GD2-CART01 (10×10^6 cells per kilogram of body weight). Panel D shows the overall survival and the event-free survival among patients who had a complete response. Panel E shows the overall survival and the event-free survival among patients with a high disease burden as compared with those with a low disease burden. In the Kaplan–Meier curves in Panels B through E, tick marks indicate censored data.



GD2 EXPRESSION

We evaluated the possible treatment-induced alteration of GD2 antigen expression by means of flow cytometric analysis of bone marrow samples obtained before infusion and after treatment failure and of tumor-biopsy specimens obtained at relapse. None of the patients with disease that relapsed had an absence of GD2 expression, and we did not observe a decrease in its expression level (Fig. S10).

MULTIPLE INFUSIONS

A total of 11 patients received additional infusions; the clinical indications for multiple infusions are described in the Supplementary Appendix. Overall, 16 additional infusions were administered (Table S3). The side-effect profile of additional infusions was similar to that reported with first infusions. As shown in Figure S11, GD2-CART01 cells expanded after infusion in most of the patients, with kinetics similar to those of the first infusions, but with lower peaks. Among the patients who received additional infusions, we observed three complete responses (two in patients who received GD2-CART01 to consolidate a complete response and one in a patient who had relapsed disease after having had a complete response with the first dose) and three partial responses.

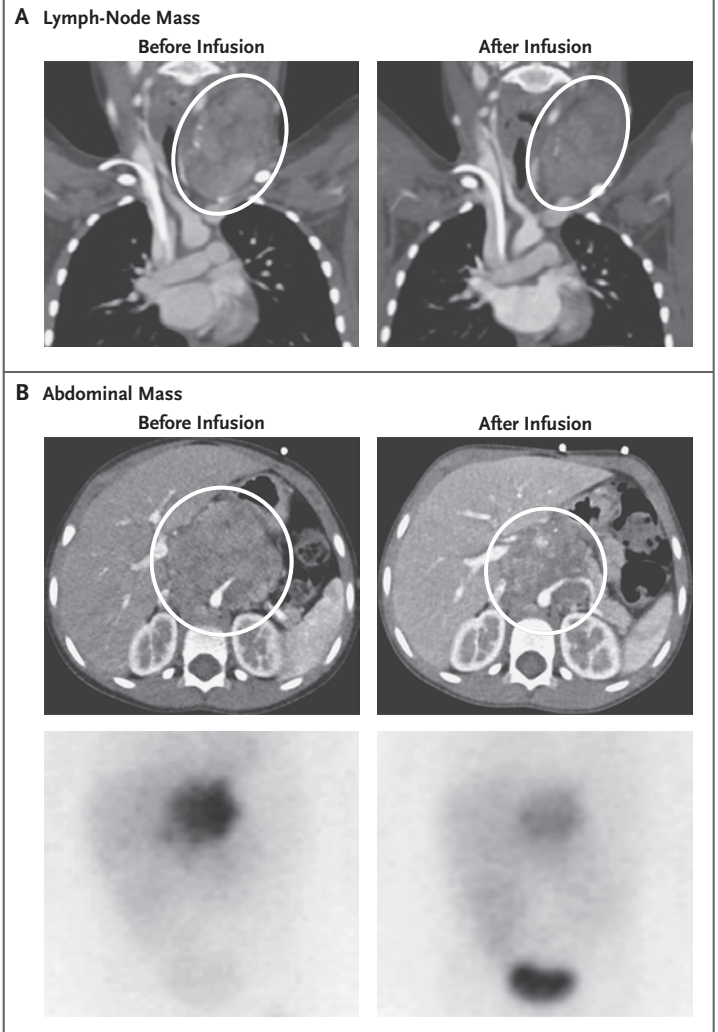


Figure 3. Radiologic Response in Two Patients with Bulky Disease.

Panel A shows a computed tomographic (CT) scan of bulky disease in cervical and supraclavicular lymph nodes in Patient 3 before and after infusion. The maximum lesion diameter decreased from 88 mm to 58 mm (a decrease of 34%). Panel B shows CT scans (upper images) and the relative metaiodobenzylguanidine (MIBG) scans (lower images) of a bulky abdominal mass in Patient 13, before and after infusion, with a decrease in the maximum lesion diameter from 88 mm to 48 mm (a decrease of 46%) and a strong decrease in MIBG avidity.

DISCUSSION

Our trial showed that GD2-CART01 cells expanded to high levels in vivo. These cells also induced an immune-related response that was manifested in part by mild cytokine release syndrome in most of the patients and by sustained clinical responses, with a complete response in 33% of the patients and 3-year event-free survival of 36% among children who received the recommended dose.

The manufacturing of GD2-CART01 was successful for all the enrolled patients, and we generated multiple doses so that we were able to administer multiple infusions. Although the induction of an immune response against the murine single-chain variable fragment has been described and remains a possible limitation to the efficacy of multiple infusions of CAR T cells,[21,22] our experience and that of others show that some patients may benefit from multiple infusions, especially if these infusions are preceded by lymphodepletion.[21] This could be an important strategy for the treatment of solid tumors because it would counteract the dysfunctional phenotype induced in CAR T cells by persistent antigen presentation in the context of a highly immune-suppressive microenvironment.[23]

In our trial, all except one patient had expansion of GD2-CART01 in peripheral blood. GD2-CART01 cells had a wide biodistribution, and 6 weeks after infusion they were detected in the cerebrospinal fluid in five patients. None of these patients had current or a history of central nervous system (CNS) disease infiltration, and none had central neurotoxic effects. Circulation of CAR T cells in the cerebrospinal fluid has been described in CD19-targeting constructs, regardless of the presence of CNS disease.[24,25] An increased permeability of the blood–brain barrier in these patients, intrinsic features of GD2-CART01, or both may have induced the circulation and homing of the cells.

Although manageable, the side-effect profile of this type of immunotherapy is not negligible. Cytokine release syndrome developed in most patients; this condition is well recognized in patients with hematologic cancer and less common in patients with solid tumors.[11,12] As already reported with other CAR constructs and immunotherapy approaches,[20] this complication correlated with the peak serum levels of the cytokines interleukin-6, interferon-γ, tumor necrosis factor α, and interleukin-10; in particular, levels of interleukin-6 and interleukin-10 also correlated with the grade of severity. In our patients, cytokine release syndrome was mostly grade 1 or 2 and reversible in all cases. One patient received rimiducid, although the event that trig-

gered its use was considered by the clinical study team to be unrelated to the CAR T cells. The use of rimiducid resulted in the rapid elimination of circulating GD2-CART01. The re-expansion of GD2-CART01 that was observed at subsequent evaluations suggests that mostly activated gene-modified cells are preferentially eliminated on activation of the suicide gene, as previously reported.[26] This approach led to an effective control of the toxic effects without completely eliminating the CAR T cells. Residual GD2-CART01 cells maintain a functionally active iC9 gene and remain sensitive to rimiducid.

Hematologic toxic effects, which were thought by the investigators to be mainly induced by the lymphodepleting chemotherapy and sustained by GD2-CART01, were another relevant adverse event that was reported in every patient. The heavy burden of previous treatment received by the patients and the common disease involvement in the bone marrow may also have had an impact on these toxic effects.

In order to maintain tumor control, it is essential that CAR T cells persist over time. This aspect has also been underlined in the pioneering work by Louis and colleagues,[10] who found a significant correlation between persistence longer than 6 weeks and longer time to disease progression.[27] In contrast to patients in previous studies, all our patients had persistence of CAR T cells for more than 6 weeks, possibly because of the incorporation of two costimulatory domains in the construct and the use of interleukin-7 and interleukin-15 in the manufacturing of GD2-CART01.[13,28]

We observed a high incidence of response among our patients with refractory disease, a finding that indicates that GD2-CART01 has therapeutic potential against high-risk refractory or relapsed neuroblastoma. One third of our patients had a complete response, and three patients who were classified as having had a partial response owing to the persistence of MIBG-positive lesions had no evidence of recurrent disease 18 months or more after infusion, without receiving any further treatment aside from additional doses of GD2-CART01. Bone marrow was one of the sites with the best response to GD2-CART01. In some patients, we also observed a response in nonbulky soft-tissue tumor masses and bone metastasis. In our cohort of patients, the most challenging disease location was the lymph nodes.

Although the trial was not powered to detect differences between subgroups of patients, we identified disease burden at infusion as being predictive of response to treatment. We did not observe a difference in response and in long-term outcomes between patients with refractory disease and those with relapsed disease, and the previous use of anti-GD2 monoclonal antibodies did not influence outcomes. In contrast to observations with other target antigens such as CD19, CD22, and GPC2, we did not observe a loss of GD2 expression on relapsed tumor cells after loss of response to the treatment, as shown by the mean fluorescence intensity of the antigen at relapse.[29-31] These findings, coupled with the lack of re-expansion of GD2-CART01 on relapse or progression of the disease, suggest that in neuroblastoma, mechanisms of resistance other than antigen loss and short persistence of CAR T cells are responsible for treatment failure.

We recently reported the considerable negative effect exerted by polymorphonuclear myeloid-derived suppressor cells on the cytotoxic activity of GD2-CART01 in an initial cohort of patients.[32] We hypothesize that other complex immune-suppressive mechanisms — either preexisting or induced by the inflammatory storm produced by activated GD2-CART01 and differing from cell exhaustion — may contribute to the impairment of antitumor activity in this type of immunotherapy.

Our findings suggest that GD2-CART01 may induce sustained eradication of disease in a proportion of patients with relapsed or refractory neuroblastoma. Studies are under way to assess the role of GD2-CART01 in the multimodal treatment of high-risk neuroblastoma.

Supported by grants from the Italian Medicines Agency–Ricerca Indipendente (AIFA-2016-02364631, to Dr. Locatelli), the Italian Ministry of Health (grant 5×1000, to Drs. Del Bufalo, De Angelis, and Quintarelli, and CAR-T Italia Project RCR-2019-23669115, to Dr. Locatelli); Associazione Italiana per la Ricerca sul Cancro (AIRC) (special grant "5×mille" ID 9962 and an investigator grant, to Dr. Locatelli, and My First AIRC grant ID 25106, to Dr. Quintarelli); Innovative Medicines Initiative 2 Joint Undertaking (grant agreement number 116026, to Dr. Locatelli); Lazio Innova Project Immunotherapy Approaches (Codice Unico di Progetto [CUP] code E82F20000200002, to Dr. Locatelli), project CAR for Sarcoma (CUP code E82F20000240002, to Dr. Locatelli); Fondazione Neuroblastoma (CAR T-cell project, to Dr. Locatelli); Italian Piano Nazionale di Ripresa e Resilienza CN3 (National Center for Gene Therapy and Drugs based on

RNA Technology, to Dr. Locatelli); and Life-Science Hub–Terapie Avanzate (to Dr. Locatelli). Bellicum Pharmaceuticals donated rimiducid for the trial.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

A data sharing statement provided by the authors is available with the full text of this article at NEJM.org.

We thank all the patients and their parents for participating in the trial and Dr. Malcolm K. Brenner for his remarkable mentorship and his essential support in the setup of the project.

APPENDIX

The authors' full names and academic degrees are as follows: Francesca Del Bufalo, M.D., Biagio De Angelis, Ph.D., Ignazio Caruana, Ph.D., Giada Del Baldo, M.D., Maria A. De Ioris, M.D., Ph.D., Annalisa Serra, M.D., Angela Mastronuzzi, M.D., Ph.D., Maria G. Cefalo, M.D., Daria Pagliara, M.D., Matteo Amicucci, M.S.N., Giuseppina Li Pira, Ph.D., Giovanna Leone, M.D., Valentina Bertaina, Ph.D., Matilde Sinibaldi, Ph.D., Stefano Di Cecca, Ph.D., Marika Guercio, Ph.D., Zeinab Abbaszadeh, Ph.D., Laura Iaffaldano, Ph.D., Monica Gunetti, Ph.D., Stefano Iacovelli, Ph.D., Rossana Bugianesi, Ph.D., Stefania Macchia, Ph.D., Mattia Algeri, M.D., Pietro Merli, M.D., Federica Galaverna, M.D., Ph.D., Rachid Abbas, Ph.D., Maria C. Garganese, M.D., Maria F. Villani, M.D., Giovanna S. Colafati, M.D., Federico Bonetti, M.D., Marco Rabusin, M.D., Katia Perruccio, M.D., Veronica Folsi, M.D., Concetta Quintarelli, Ph.D., and Franco Locatelli, M.D., Ph.D.

The authors' affiliations are as follows: the Department of Pediatric Hematology and Oncology and of Cell and Gene Therapy (F.D.B., B.D.A., I.C., G.D.B., M.A.D.I., A.S., A.M., M.G.C., D.P., M. Amicucci, G.L.P., V.B., M.S., S.D.C., M. Guercio, Z.A., L.I., M. Algeri, P.M., F.G., C.Q., F.L.), the Transfusion Unit, Department of Laboratories (G.L.), Officina Farmaceutica, Good Manufacturing Practice Facility (M. Gunetti, S.I., R.B., S.M.), and the Nuclear Medicine Unit (M.C.G., M.F.V.), Department of Imaging (G.S.C.), IRCCS Ospedale Pediatrico Bambino Gesù, and the Department of Life Sciences and Public Health, Catholic University of the Sacred Heart (F.L.), Rome, the Pediatric Hematology and Oncology Unit, IRCCS Policlinico San Matteo, Pavia (F.B.), the Pediatric Hematology–Oncology Unit, Institute for Maternal and Child Health, IRCCS "Burlo Garofolo," Trieste (M.R.), Pediatric Oncology Hematology, Mother and Child Health Department, Santa Maria della Misericordia Hospital, Perugia (K.P.), the Pediatric Hematology–Oncology Unit, Ospedale dei Bambini, Azienda Socio Sanitaria Territoriale Spedali Civili Brescia, Brescia (V.F.), and the Department of Clinical Medicine and Surgery, University of Naples Federico II, Naples (C.Q.) — all in Italy; and INSERM, Centre d'Investigation Clinique 1418 (CIC1418) Epidémiologie Clinique, Paris (R.A.).

REFERENCES

1. June CH, Sadelain M. Chimeric antigen receptor therapy. N Engl J Med 2018;379:64-73.
2. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med 2018;378:439-48.
3. Pasquini MC, Hu Z-H, Curran K, et al. Real-world evidence of tisagenlecleucel for pediatric acute lymphoblastic leukemia and non-Hodgkin lymphoma. Blood Adv 2020;4:5414-24.
4. Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nat Rev Clin Oncol 2020;17:147-67.
5. Richards RM, Sotillo E, Majzner RG. CAR T cell therapy for neuroblastoma. Front Immunol 2018;9:2380.
6. Ladenstein R, Pötschger U, Valteau-Couanet D, et al. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. Lancet Oncol 2018;19:1617-29.
7. London WB, Bagatell R, Weigel BJ, et al. Historical time to disease progression and progression-free survival in patients with recurrent/refractory neuroblastoma treated in the modern era on Children's Oncology Group early-phase trials. Cancer 2017;123:4914-23.
8. Yu AL, Gilman AL, Ozkaynak MF, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med 2010;363:1324-34.
9. Pule MA, Savoldo B, Myers GD, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 2008;14:1264-70.
10. Louis CU, Savoldo B, Dotti G, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood 2011;118:6050-6.
11. Heczey A, Louis CU, Savoldo B, et al. CAR T cells administered in combination with lymphodepletion and PD-1 inhibition to patients with neuroblastoma. Mol Ther 2017;25:2214-24.
12. Straathof K, Flutter B, Wallace R, et al. Antitumor activity without on-target off-tumor toxicity of GD2-chimeric antigen receptor T cells in patients with neuroblastoma. Sci Transl Med 2020;12(571):eabd6169.
13. Quintarelli C, Orlando D, Boffa I, et al. Choice of costimulatory domains and of cytokines determines CAR T-cell activity in neuroblastoma. Oncoimmunology 2018;7(6):e1433518.
14. Di Stasi A, Tey S-K, Dotti G, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. N Engl J Med 2011;365:1673-83.
15. Park JR, Bagatell R, Cohn SL, et al. Revisions to the international neuroblastoma response criteria: a consensus statement from the National Cancer Institute Clinical Trials Planning Meeting. J Clin Oncol 2017;35:2580-7.
16. Ladenstein R, Lambert B, Pötschger U, et al. Validation of the mIBG skeletal SIOPEN scoring method in two independent high-risk neuroblastoma populations: the SIOPEN/HR-NBL1 and COG-A3973 trials. Eur J Nucl Med Mol Imaging 2018;45:292-305.
17. Lewington V, Lambert B, Poetschger U, et al. ^{123}I-mIBG scintigraphy in neuroblastoma: development of a SIOPEN semiquantitative reporting, method by an international panel. Eur J Nucl Med Mol Imaging 2017;44:234-41.
18. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. Control Clin Trials 1996;17:343-6.
19. Borgan Ø, Zhang Y. Using cumulative sums of martingale residuals for model checking in nested case-control studies. Biometrics 2015;71:696-703.
20. Lee DW, Gardner R, Porter DL, et al. Current concepts in the diagnosis and management of cytokine release syndrome. Blood 2014;124:188-95.
21. Gauthier J, Bezerra ED, Hirayama AV, et al. Factors associated with outcomes after a second CD19-targeted CAR T-cell infusion for refractory B-cell malignancies. Blood 2021;137:323-35.
22. Hege KM, Bergsland EK, Fisher GA, et al. Safety, tumor trafficking and immunogenicity of chimeric antigen receptor (CAR)-T cells specific for TAG-72 in colorectal cancer. J Immunother Cancer 2017;5:22.
23. Poorebrahim M, Melief J, Pico de Coaña Y, Wickström SL, Cid-Arregui A, Kiessling R. Counteracting CAR T cell dysfunction. Oncogene 2021;40:421-35.
24. Abramson JS, McGree B, Noyes S, et al.

Anti-CD19 CAR T cells in CNS diffuse large-B-cell lymphoma. N Engl J Med 2017;377:783-4.
25. Mueller KT, Maude SL, Porter DL, et al. Cellular kinetics of CTL019 in relapsed/refractory B-cell acute lymphoblastic leukemia and chronic lymphocytic leukemia. Blood 2017;130:2317-25.
26. Zhou X, Dotti G, Krance RA, et al. Inducible caspase-9 suicide gene controls adverse effects from alloreplete T cells after haploidentical stem cell transplantation. Blood 2015;125:4103-13.
27. Pulsipher MA, Han X, Maude SL, et al. Next-generation sequencing of minimal residual disease for predicting relapse after tisagenlecleucel in children and young adults with acute lymphoblastic leukemia. Blood Cancer Discov 2022;3:66-81.
28. Xu Y, Zhang M, Ramos CA, et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. Blood 2014;123:3750-9.
29. Majzner RG, Rietberg SP, Sotillo E, et al. Tuning the antigen density requirement for CAR T-cell activity. Cancer Discov 2020;10:702-23.
30. Fry TJ, Shah NN, Orentas RJ, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. Nat Med 2018;24:20-8.
31. Heitzeneder S, Bosse KR, Zhu Z, et al. GPC2-CAR T cells tuned for low antigen density mediate potent activity against neuroblastoma without toxicity. Cancer Cell 2022;40(1):53-69.e9.
32. Tumino N, Weber G, Besi F, et al. Polymorphonuclear myeloid-derived suppressor cells impair the anti-tumor efficacy of GD2.CAR T-cells in patients with neuroblastoma. J Hematol Oncol 2021;14:191.