Nature of tumor control by permanently and transiently-modified GD2 chimeric antigen receptor T cells in xenograft models of neuroblastoma

Nathan Singh1, Xiaojun Liu2, Jessica Hulitt3, Shuguang Jiang2, Carl H. June2,4, Stephan A. Grupp5, David M. Barrett3* and Yangbing Zhao2,4*

1Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104
2Abramson Family Cancer Research Institute, University of Pennsylvania Cancer Center, Philadelphia, PA 19104
3Division of Oncology and 5Department of Pathology, Children’s Hospital of Philadelphia, Philadelphia, PA 19104
4Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104

*Contributed equally to this work

Running Title: Permanently and transiently-modified CAR T cells for neuroblastoma

Key words: immunotherapy, chimeric antigen receptors, pediatrics, solid tumors, RNA electroporation

Support: Supported by NIH R01CA120409 (C.H.J. and Y.Z), P01CA066726 (C.H.J. and Y.Z.), and grants from the Pennsylvania Department of Health, Cookies for Kids Cancer, Solving Kids Cancer, the WW Smith Charitable Trust and the Weinberg Funds (S.A.G.)

Corresponding Author:
Nathan Singh, M.D., M.S.
Hospital of the University of Pennsylvania
Department of Medicine
100 Centrex Building
3400 Spruce Street
Philadelphia, Pennsylvania 19104
Email: nathan.singh@uphs.upenn.edu
Phone: 215-827-9554

Author contributions:
N.S., C.H.J., S.A.G., D.M.B. and Y.Z. designed the research, interpreted the data and reviewed the manuscript
N.S., X.L., S.J. and J.H. performed the research
N.S. wrote and all authors revised the manuscript

Conflicts of Interest: C.H.J. and Y.Z. have patent applications in some of the technology described in this manuscript, and S.A.G, C.H.J. and Y.Z. receive research funding from Novartis. All other authors: no conflict of interest.

Abstract Word Count: 240
Text Word Count: 4976
Figures: 6
Supplemental Figures: 1
References: 37
Abstract

Chimeric antigen receptor (CAR) therapy has begun to demonstrate success as a novel treatment modality for hematologic malignancies. The success observed thus far has been with T cells permanently-engineered to express chimeric receptors. T cells engineered using RNA electroporation represent an alternative with the potential for similar efficacy and greater safety when initially targeting novel antigens. Neuroblastoma is a common pediatric solid tumor with the potential to be targeted using immunotherapy. We performed xenograft studies in NSG mice assessing the efficacy of both permanently-modified and transiently-modified CAR T cells directed against the neuroblastoma antigen GD2 in both local and disseminated disease models. Disease response was monitored by tumor volume measurement and histologic examination, as well as in vivo bioluminescence. RNA-modified GD2 CAR T cells mediated rapid tumor destruction when delivered locally. A single infusion of lentivirally-modified GD2 CAR T cells resulted in long-term control of disseminated disease. Multiple infusions of RNA GD2 CAR T cells slowed progression of disseminated disease and improved survival, but did not result in long-term disease control. Histologic examination revealed that the transiently-modified cells were unable to penetrate significantly the tumor environment when delivered systemically, despite multiple infusions of CAR T cells. Thus, we demonstrate that RNA-modified GD2 CAR T cells can mediate effective antitumor responses in vivo, and permanently-modified cells are able to control disseminated neuroblastoma in xenograft mice. Lack of long-term disease control by RNA-engineered cells resulted from an inability to penetrate the tumor microenvironment.
Introduction

Neuroblastoma is the most common extra-cranial pediatric solid tumor. Derived from neuroendocrine tissue of the sympathetic nervous system, it accounts for 9% of cancer diagnoses and 15% of cancer deaths in children (1). Current standard of care for high-risk disease consists of chemotherapy, surgery, consolidation chemotherapy followed by stem-cell transplant, tumor-directed radiation, and antibody-based therapy. This exhaustive regimen still only yields in a 3-year event-free survival of ~45% (2).

Historically, poor tumor immunogenicity and immune evasion (3, 4) have resulted in largely disappointing T-cell therapy trials. Advances in understanding of T cell and tumor interaction, cell production techniques and engineering platforms, as well as the recognition of the need for host conditioning have recently allowed for significant improvements. Chimeric antigen receptors (CAR) are molecules composed of an extracellular antigen-binding domain derived from the immunoglobulin variable chain (scFv) linked to intracellular T-cell activating and co-stimulatory domains (5, 6). The antigen-specific scFv allows for MHC-independent tumor antigen recognition that initiates robust T-cell stimulation, even in response to previously immuno-evasive malignancies (7).

In the most successful clinical trial of CARs to date, Porter and colleagues demonstrated complete remissions lasting >3 years in two of three adults with chronic lymphocytic leukemia (CLL) using CARs directed against the B-cell antigen CD19 (8, 9). Translating this success to acute leukemia, our group recently reported the induction of complete remissions in two children with acute lymphoblastic leukemia (ALL) using the same CD19 CAR T cells (10).

The dramatic clinical responses observed in these studies have employed T cells permanently engineered to express CARs using lentiviral vectors. This process involves \textit{ex vivo} exposure of
harvested autologous lymphocytes to self-inactivating lentiviral vector encoding a CAR, resulting in genomic integration of the CAR transgene. While >500 patient-years of data suggest that this modification is extremely unlikely to result in insertional mutagenesis in mature lymphocytes (11), these data are from adults and the increased life-span of modified cells in children raises additional theoretical safety concerns. More importantly, when targeting solid tumor antigens the risk of on-target off-tumor toxicity becomes a significant concern. Several adverse events have demonstrated the potential risks of uncontrolled CAR T cells (12, 13), and have highlighted the need for safer CAR T cells moving forward, especially in early clinical testing (14, 15). Given these considerations, we and other groups have previously reported the development of an mRNA electroporation-based approach to induce transient CAR expression (16-18). This strategy creates an efficient CAR expression system that ensures complete loss of CAR-driven T-cell activity in a predictable time frame without the need to administer other systemic agents to eliminate modified T cells. We have reported the efficacy of transiently-modified CD19 CAR T cells in a disseminated xenograft model of systemic ALL (19), and recently demonstrated enhanced efficacy of these transiently-modified cells when delivered repeatedly in an optimized dosing strategy (20). This optimized therapeutic regimen approached the antitumor responses observed with permanently-modified CD19 CAR T cells and demonstrated long-term disease control, suggesting that multiple infusions of transiently-modified CAR T cells may present an alternative to genome-modifying T-cell engineering techniques.

RNA CAR T cells have demonstrated in vitro activity (21) and in vivo efficacy in localized models of solid tumors, and have similarly shown enhanced efficacy using multiple cell infusions (17, 22). Based on these findings, as well as our own experience with RNA CAR T cells in ALL, we evaluated a CAR targeting GD2, a diasialoganglioside expressed on the surface of most neuroblastomas (1) that has already been shown to be an effective target for neuroblastoma immunotherapy (23). A single chain antibody variable region fragment (scFv) targeting GD2 was
linked to the CD3ζ and 4-1BB intracellular signaling domains and tested in localized and disseminated animal models of neuroblastoma. We demonstrate that multiple infusions of RNA GD2 CAR T cells result in effective antitumor responses to disease in vivo, and that a single low-dose infusion of permanently-modified GD2 CAR T cells results in long-term control of disseminated neuroblastoma. Multiple infusions of RNA GD2 CAR T cells are less effective at controlling disseminated disease, and our data highlight the potential mechanism underlying this lack of efficacy. Together, these data clarify the necessary components for success of transiently-modified CAR T cells in solid tumors.

Materials and Methods

T-cell expansion in vitro

Human T cells were isolated from healthy donors by the University of Pennsylvania Human Immunology Core. Cells were incubated with microbeads coated with CD3 and CD28 stimulatory antibodies (Life Technologies, Grand Island, NY, Catalog #111.32D). T cells were combined at a ratio of 1 cell : 3 beads and cultured in T-cell culture media. Beads were removed on day 7-8 of stimulation. Cells were counted, volumes measured (Multisizer 3, Beckman Coulter, Brea, California) every 48 hours. When cell growth kinetics and volume suggested cells had rested down from activation, they were cryopreserved.

Generation of CAR constructs and RNA electroporation

CARs containing scFv domains directed against GD2 or CD19 linked to CD3ζ and 4-1BB intracellular signaling domains were produced as previously described (24, 25) (GD2-z construct was generously provided by Dr. Malcolm Brenner, Baylor College of Medicine, Houston, Texas). Development of constructs for RNA manufacture was performed as previously described (17). mScript RNA System (CellScript, Madison, WI, Catalog #MSC11625) was utilized to generate capped in vitro transcribed RNA, which was purified using an RNeasy Mini Kit (Qiagen, Inc.,
Valencia, CA, Catalog #74104). T cells were expanded and frozen as described above. Prior to electroporation, cells were thawed, washed three times with Opti-MEM and resuspended in Opti-MEM medium at a final concentration of 1-3x10^8 cells/mL. T cells were then mixed with transcribed mRNA at a concentration of 10μg mRNA/0.1mL T cells and electroporated in a 2mm cuvette using an ECM830 Electro Square Wave Porator (both from Harvard Apparatus BTX, Holliston, MA, Catalog #450125, #450002) 1-2 hours prior to infusion into animals. Viability post-transfection ranged from 50 – 80%, and in all cases T cells demonstrated >95% CAR expression.

**Production of lentiviral vectors and T-cell transduction**

High-titer, replication-defective lentiviral vectors were produced using 293T human embryonic kidney (HEK) cells (26). HEK293T cells were seeded at 10^7 cells per T150 tissue culture flask 24 hours before transfection. On the day of transfection, cells were treated with 7μg of pMDG.1, 18μg of pRSV.rev, 18μg of pMDLg/p.RRE packaging plasmids and 15μg of transfer plasmid in the presence of either Express-In Transfection Reagent (Open Biosystems, Lafayette, CO) or Lipofectamine 2000 transfection reagent (Life Technologies, Grand Island, NY, Catalog #11668019). Transfer plasmids containing CAR constructs were modified so that expression of the CAR was under the control of the EF-1α promoter as previously described (25). Viral supernatants were harvested 24 hours and 48 hours after transfection and concentrated by ultracentrifugation overnight at 10,500xg. 24 hours after initial stimulation, T cells were exposed to lentiviral vector at a concentration of 5-10 infectious particles per T cell, and then cultured as described above.

**CAR detection on modified cells**
Cells were washed and resuspended in FACS buffer (PBS + 1% bovine serum albumin), then incubated with biotin-labeled polyclonal goat anti-mouse F(ab)2 antibody (Jackson Immunoresearch, West Grove, PA, Catalog #115-066-072) at 4°C for 25 minutes, and then washed twice. Cells were then incubated with R-phycoerythrin (PE)-conjugated streptavidin (BD Biosciences, Franklin Lakes, NJ, Catalog #554061) for 10 minutes at 4°C, then washed twice. Flow cytometry acquisition was performed on an Accuri C6 Cytometer (BD Biosciences, Franklin Lakes, NJ, Catalog #342975, #653118). Analysis was performed using FlowJo software (Treestar, Inc, Ashland, OR).

**Mouse Xenograft studies**

6-10 week old NOD-SCID-γc−/− (NSG) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) or bred in house under an approved Institutional Animal Care and Use Committee (IACUC) protocol and maintained in pathogen-free conditions. For flank tumor studies, animals were injected subcutaneously with 2x10⁷ SY5Y human neuroblastoma cells suspended in 0.2mL Matrigel (BD Biosciences, San Jose, CA, Catalog #354234). For disseminated tumor studies, animals were injected via tail vein with 2x10⁶ SY5Y cells in 0.1mL sterile PBS. T cells were injected in 0.1mL sterile PBS. Cyclophosphamide (Baxter Healthcare, Deerfield, IL, Catalog #1001995601) was injected I.P. at 60mg/kg 24 hours prior to any cell administration following the initial infusion. Animals were monitored for signs of disease progression and overt toxicity, such as xenogeneic graft-versus-host disease, as evidenced by >10% loss in body weight, loss of fur, diarrhea, conjunctivitis and disease-related hind limb paralysis.

**Measurement of flank tumors**

Flank tumor measurements were made bi-weekly using electronic calipers (Fowler-Sylvac, Boston, MA, Catalog #54-200-777). Longest length and width measurements were recorded and
tumor volume was calculated according to the formula \((\frac{\text{width} + \text{length}}{2})^3/2\). Mice were sacrificed when tumors reached 3cm³, or when tumor burden inhibited animal activity.

**Bioluminescent imaging**

Disease burdens were monitored over time using a Xenogen IVIS bioluminescent imaging system, as previously described (19, 27).

**Immunohistochemistry**

Histology was performed by the Pathology Core Facility at the Children’s Hospital of Philadelphia. Subcutaneous tumors and tumor-infiltrated livers were excised post-mortem, and preserved in 4% paraformaldehyde. Image analysis was performed using ImageScope software (Aperio, Vista, CA). Staining was performed on a Bond Max automated staining system with the Bond Refine polymer staining kit (both Leica Microsystems, Buffalo Grove, IL). Standard protocol was followed, with the exception of the primary antibody incubation, which was extended to 1 hour at room temperature. Anti-human CD3 antibody (Dako, Carpinteria, CA, Catalog #M7254) was used at 1:50 dilution and antigen retrieval was performed with E1 retrieval solution for 20 minutes. Slides were rinsed and dehydrated through a series of ascending concentrations of ethanol and xylene. Stained slides were then digitally scanned at 20x magnification on an Aperio OS slide scanner (Aperio, Vista, CA).

**Cell Line Identity Testing**

The SH-SY5Y (SY5Y) tumor cell line was obtained originally from the American Type Culture Collection (CRL-2266, ATCC, Manassas, Virginia) and generously donated to our laboratory by Dr. John Maris, Children’s Hospital of Philadelphia. The parent cell line was genotyped by short tandem repeat (STR) analysis (28). Cell lines were tested for mycoplasma and verified every six
months, or after any genetic modification, such as luciferase transduction, to ensure identity and mycoplasma-free status.

**Statistical considerations**

All statistical analysis was performed with Prism 4 (Graphpad Software, La Jolla, CA). For comparison among multiple groups, Kruskal-Wallis analysis was performed with Dunn Multiple Comparison tests to compare individual groups. Survival curves were compared using the log-rank test with Bonferroni correction for multiple comparisons.

**Results**

**mRNA GD2 CAR T cells mediate rapid neuroblastoma cell killing in vivo**

Subcutaneous injection of tumor cells in the mouse flank is a well-established model of solid tumor therapy in mice. To assess the antitumor efficacy of our transiently-modified GD2 CAR T cells in a solid tumor model of neuroblastoma, NOD/SCID/γc−/− (NSG) mice were injected subcutaneously with 2x10⁷ SY5Y (a GD2⁺ human neuroblastoma cell line) cells, and given two weeks to establish large flank tumors. On day 15, 5x10⁶ human T cells electroporated with RNA encoding either a GD2 or CD19 CAR bearing the 4-1BB and CD3ζ signaling domains (GD2-BB/z or CD19-BB/z) were injected into the tumor. On day 20, several mice were sacrificed and tumors were excised and probed for the presence of human T cells by immunohistochemical analysis. Intra-tumoral injection of RNA GD2 CAR T cells resulted in rapid necrosis of target neuroblastoma, with T cells diffusely present throughout the tumor environment (Figure 1A). RNA CD19 CAR T cells, on the other hand, did not alter the tumor architecture after intra-tumoral injection, and quickly diffused out of the tumor and localize to the periphery of the tissue (Figure 1B). Mice not sacrificed for histology received two additional intra-tumoral (I.T.) injections of 1.5x10⁷ cells each, and RNA GD2 CAR T cells mediated significant antitumor responses while RNA CD19 CAR T cells permitted rapid tumor growth (Figure 1C, *p* < 0.001).
Tumor expansion observed in mice treated intra-tumorally with RNA CD19 CAR T cells was similar to that in mice treated with saline ($p = 0.7912$).

**Intravenous injection of neuroblastoma cells results in a reproducible model of disseminated neuroblastoma**

Subcutaneous tumors have many anatomic and physiologic differences when compared to spontaneous intra-abdominal tumors, several of which have direct bearing on the physiology of cellular therapies. Previous studies of CAR T cells in solid tumors, such as epithelial ovarian cancer (29), have employed a peritoneal tumor model in which tumor cells are injected directly into the peritoneum to establish disease along the internal peritoneal wall, mimicking natural disease. We sought to develop a model that would more closely mimic the clinical setting of disseminated neuroblastoma in which CAR T cells are likely to be given. SY5Y neuroblastoma cells were engineered to express click beetle green luciferase (SY5Y-CBG) prior to delivery, which allowed for longitudinal observation of a disseminated tumor burden. Tumor cells were injected intravenously and animals were observed for the establishment and progression of systemic disease. Within 2 hours of cell delivery, bioluminescent signal was observed in the lungs, and then rapidly disseminated (Supplemental Figure 1). As early as day 7, disease was detected in the abdomen, and was subsequently observed in the bone marrow and lymph nodes. Necropsy revealed that this abdominal signal resulted from hepatic infiltration of neuroblastoma, causing diffuse nodular hepatic disease.

**Permanently-modified GD2 CAR T cells mediate regression of disseminated neuroblastoma**

We first tested the efficacy of permanently-modified GD2 CAR T cells in our disseminated model of neuroblastoma. CAR T cells were engineered using a well-established lentiviral vector transduction technique (9, 25). NSG mice were given $2 \times 10^6$ SY5Y-CBG cells via tail vein and given 1-2 weeks to establish a significant disease burden ($>10^6$ photons/sec/cm$^2$/sr), after which
time CAR T cells were delivered systemically. Animals were imaged twice weekly to monitor disease burden. A single infusion of as few as 10⁶ permanently-modified GD2 CAR T cells was sufficient to suppress and control disseminated neuroblastoma long-term (Figure 2A, \( p < 0.001 \)), while tumor growth progressed comparably after systemically-delivered control CD19 CAR T cells and saline \( (p = 0.8872) \). Not surprisingly, the disease control observed had a significant impact on the survival of animals treated with GD2 CAR T cells (Figure 2B, \( p = 0.0257 \)). Interestingly, all mortality in animals treated with GD2 CAR T cells resulted from xenogeneic GVHD, with 63% of GVHD mortality occurring in disease remission and the remaining 37% with minimal disease burdens \( (<2\times10^7 \text{ photons/sec/cm}^2/\text{sr}) \).

**Multiple infusions of RNA GD2 CAR T cells delay disease progression, but do not eradicate disseminated neuroblastoma**

We next used this disseminated model system to assess the efficacy of RNA GD2 CAR T cells against neuroblastoma. Disseminated disease was established as described, and transiently-modified T cells were delivered intravenously. Based on our experience with ALL (20), we undertook a multiple infusion strategy using RNA-electroporated T cells. Mice were treated initially with 5x10⁶ cells on day 8, and 1.5x10⁷ on days 22 and 29 after tumor implantation. We observed a transient decline in disease burden after the initial infusion, and modest disease stabilization with the next two infusions (Figure 3A). While this strategy significantly slowed disease progression \( (p = 0.0032) \), it was unable to control disease long-term. This delayed progression did, however, prolong animal survival (Figure 3B, \( p = 0.0128 \)). Again, animals treated with CD19 CAR T cells were statistically similar in both disease progression \( (p = 0.4175) \) and overall survival \( (p = 0.9834) \) to those treated with saline.

Visual representations of disease burden and response to therapy highlight the variations in antitumor activity between transiently- and permanently-modified CAR T cells (Figure 4).
Animals treated with lentivirally-modified GD2 CAR T cells demonstrate rapid loss of bioluminescent signal with maintained disease suppression long-term. Interestingly, these animals occasionally have focal disease recurrence, which is lost on subsequent evaluation. This likely represents the long-term, antitumor T-cell memory of permanently-modified CAR T cells. As was demonstrated in the quantitative bioluminescent graph (Figure 3B), animals treated with GD2 RNA CAR T cells have an initial decline in disease burden after the first cell infusion, followed by transient stabilizations with the subsequent two infusions.

**Antigen-targeted RNA CAR T cells are unable to penetrate the tumor microenvironment**

To better understand the dynamics of the T cell:tumor interaction in our disseminated solid tumor model, we examined tumor sites using immunohistochemical analysis. Animals were given the standard 1-2 weeks to establish disease after I.V. tumor injection, and then given either a single infusion of lentivirally-modified CAR T cells or three infusions of RNA CAR T cells, each separated by one week as in studies described previously. Animals were sacrificed on the day indicated, and the excised livers were sent for histopathological examination and immunohistochemical staining for human CD3. Animals receiving permanently-modified GD2 CAR T cells had rapid infiltration of CAR T cells into tumor sites by day 3, and demonstrated a significant T-cell presence in all tumor sites by day 5 (Figure 5A). Permanently-modified control CD19 CAR T cells did not demonstrate any significant tumor infiltration (Figure 5B).

Alternatively, by day 3 after infusion of the first dose of RNA-modified GD2 CAR T cells there was minimal infiltration of these CAR T cells into the tumor site, and this T-cell presence had largely dissipated by day 6. 24 hours after the second infusion of RNA GD2 CAR T cells (on day 7), there was a minor accumulation of T cells around the periphery of the tumor, which has again largely dissipated by day 10. This pattern is also observed after the third cell infusion, with a peripheral accumulation around the edge of the tumor within 24 hours, which was quickly lost.
(Figure 6A). As expected, control RNA CD19 CAR T cells did not demonstrate any significant tumor-infiltration (Figure 6B).

Discussion

High-risk neuroblastoma remains a significant cause of cancer death in children. While we have seen dramatic improvement in the treatment of both solid and liquid pediatric tumors, survival rates for both have plateaued since the mid-1990s (30). Patients with relapsed neuroblastoma are largely incurable, although temporary control of disease can be achieved for some patients. Thus a new therapeutic paradigm is needed to improve survival. Not until recently has engineered T-cell therapy begun to meet its potential. Integration of the powerful cytotoxicity, significant in vivo proliferation and surveillance mechanisms of memory T cells with the antigen-specificity endowed by CARs has created a platform for the success of cell therapy. As we have suggested, the recent successes of CAR therapy (8-10) may primarily be attributed to improved cell-manufacturing processes that generate T cells armed with rapid and potent in vivo effector and proliferative capacity (31).

Previous reports have demonstrated efficacy of GD2 CAR T cells against disseminated GD2+ melanoma using two infusions of $10^7$ CAR T cells each (32). In the only other study to our knowledge of CAR T cells in a model of systemic solid malignancy, $2\times10^7$ prostate-specific membrane antigen (PSMA)-directed T cells demonstrated efficacy in a disseminated model of prostate cancer (33). In this study, we were able to demonstrate disease eradication in more than half of the animals and disease suppression in the rest of the animals treated using a single infusion of only $10^6$ cells, ~5% of the dose used in the previous studies of CAR T cells in solid tumors. Not only were these cells able to control disease, we also observed massive cell expansion driven by antigen-specific activation and expansion. Indeed, in some cases the T-cell
expansion was high enough to cause death of some animals from xenogeneic graft-versus-host disease. T cells that could not recognize tumor (CD19 CAR T cells) did not proliferate to the same degree, as evidenced by the lack of GVHD in these animals. Both of these observations highlight the significant proliferative capacity of our CAR T cells, an attribute endowed by a combination of cell manufacturing processes and CAR signaling constructs. The ability to use fewer cells to achieve clinical efficacy is of particular importance in pediatric patients, who undergo extensive chemotherapy which creates significant lymphodepletion as part of standard treatment for many malignancies. Reducing the number of cells needed for treatment would enhance the number of patients eligible for this therapy.

GD2 serves as an exciting target in neuroblastoma given its narrow expression profile. However, as is the case with most solid-tumor antigens, on-target off-tumor toxicity remains a persistent issue, as GD2 is also expressed on sensory nerves. Clinical experience has demonstrated that antibody therapy directed at GD2 can result in significant neuropathic pain (34, 35). This toxicity is managed clinically during infusion of the antibody, and when the infusion ends the toxicity resolves. Previous clinical studies with the first generation GD2/ζ CAR T cells found that of the 19 patients followed for more than 5 years, only two had local pain at site of tumor necrosis and one had unexplained local pain (36), with no patient experiencing pain greater than grade 3. While suggestive that the physiology of this toxicity may be distinct when using an antibody versus a cell-based therapy comprising a single chain Fv without an Fc domain, this remains a potential on-target off-tumor toxicity of highly active GD2 CAR T cells. Our cell production techniques, structure of CAR signaling domains, in vivo expansion and persistence all differ from those described in previous clinical trials (31). For this reason we have been very interested in an RNA-based approach.
Having demonstrated cure of leukemic mice using multiple infusions of RNA-electroporated CAR T cells in ALL, we sought to assess the efficacy of this engineering platform in treating solid tumors. In the traditional flank tumor xenograft model, our RNA GD2 CAR T cells demonstrated rapid and potent antitumor activity when delivered locally. While this demonstrated cytolytic activity against neuroblastoma in vivo, this model is more difficult to translate clinically, as local tumor injections may not be feasible in some patients. Moreover, late-stage cancer patients may have many sites of disease. To more closely mimic systemic disease, we developed a sensitive in vivo bioluminescent model that enabled the establishment and monitoring of disseminated disease, primarily manifested in the liver and bone marrow. We found that a single small dose of permanently-modified second generation GD2/4-1BB/ζ CAR T cells could produce long-term disease control, while multiple infusions of RNA GD2 CAR T cells were able to delay disease progression and enhance animal survival, but not control disease long-term.

Based on the fundamental differences in tumor biology, we have hypothesized that the success observed using RNA CAR T cells for liquid tumors (20) might not directly translate to solid tumors. T cells targeting leukemic cells immediately traffic to sites of disease, as disease reservoirs are the natural sites of T-cell residence and surveillance. This all but removes the necessity of antigen-driven T-cell trafficking and retention. Trafficking in solid tumors is further complicated given the disorganized nature of the tumor vasculature, another issue which is averted when targeting liquid tumors. Thus, while creating a proliferative niche and optimal dosing strategy were the primary hurdles in successful targeting of liquid tumors using RNA CAR T cells (19), solid tumors present the additional barriers of trafficking, penetration and efficient antigen engagement within a six day CAR expression period.

Our histologic data support these hypotheses, elucidating the dynamics of T cell and tumor interaction in vivo. Within 72 hours of infusion of lentiviral CAR T cells, there is a large burden
of T cells within the tumor site, and by day 5 all tumor sites are heavily infiltrated with CAR T cells. The dynamics of RNA CAR T-cell activity are quite different: 72 hours after the first cell infusion a few cells have penetrated into the tumor site, providing a potentially favorable effector:target ratio on day 3, but these cells are lost by day 6. After a second infusion, the T cells localize to site of these expanding tumors, but are unable to penetrate and are lost quickly. The third infusion results in similar kinetics of T-cell localization to the periphery of enlarging tumor masses. These observations correlate well with our bioluminescent data, which show a transient decline in tumor burden after the first cell infusion, followed by modest disease stabilization, likely reflecting a short-lived antitumor response at the periphery after the second and third T-cell infusions (Figure 3B). The diminishing returns seen with each additional T-cell infusion reflect the fact that each infusion faces the same structural and vascular barriers as the first, in addition to an expanding tumor burden.

The rapid increase in number of permanently-modified GD2 CAR T cells present within the tumor seen over days 1-5 after T-cell infusion raises the question of whether this occurs as a result of continued cell recruitment, in situ expansion of recruited cells, or both. If indeed this is continued cell recruitment of circulating CAR T cells to the site of tumor antigen over days 1-5, it would suggest that RNA CAR T cells do not have the same antigen-driven migratory capabilities, as this is within the window of RNA CAR expression. If the increased presence of T-cell infiltration is a result of intra-tumoral proliferation in response to antigen, this suggests a difference in proliferative capacity of RNA and permanently-modified CAR T cells. While we cannot formally distinguish between these two possibilities, the latter seems likely to be the dominant mechanism, given the importance of antigen-driven proliferation to CAR T cell efficacy.
The observation that 3 infusions of RNA GD2 CAR T cells were not able to control systemic disease led us to assess the efficacy of repeated infusions. In another iteration of the multiple infusion strategy, we injected a total of $10^8$ RNA CAR T cells over the course of 8 weeks and found that while 9 infusions further slowed disease progression as compared to three infusions, disease continued to progress (data not shown). This, along with the poor tumor infiltration of RNA CAR T cells, suggests that increasing cell dose will not increase efficacy of this therapy, as it did with ALL (20).

The mechanisms underlying this observed difference in T-cell retention and infiltration are unclear. We have evaluated CAR molecule expression after RNA electroporation in vitro and demonstrated a progressive decay over the course of 6 days, but we have not assessed expression kinetics in vivo. It is certainly possible that infusion into and circulation within a living organism alters CAR expression. It is known that upon engagement with target antigen, the T-cell receptor is internalized as more TCRs are integrated into the cell membrane. This receptor recycling may also apply to CAR molecules, and while lentivirally-modified CAR T cells are able to continually present new CARs on the surface, RNA-modified CARs are more limited. Another manner in which RNA CAR signal may be diluted is during in vivo expansion. As antigen engagement results in T-cell activation and proliferation, permanently-modified CAR T cells produce daughter cells that also have permanent CAR expression. RNA-modified CAR T cell proliferation, however, may produce daughter cells with a fraction of the originally delivered CAR RNA. This kinetics may play an essential role in limiting RNA CAR T-cell retention and infiltration at tumor sites.

Mechanisms independent of CAR expression kinetics also may account partially for the differences observed. While the majority of cell production is identical for both RNA and lentivirus CAR T cells, it is unclear if the process of electroporation has an effect on T-cell
function. Data from intra-tumoral injection studies suggest that these RNA CAR T cells are functional and able to mount effective antitumor responses, however subtle differences in T-cell activity may be present that are unmasked when delivering cells systemically. These differences may occur in the form of variations in cell signaling or variability in expression of surface molecules that impact T-cell trafficking, retention and infiltration, such as chemokine receptors, adhesion molecules and T-cell inhibitory receptors. It is possible that a combination of these factors accounts for the differences observed, and further characterization of these mechanisms will allow identification of the processes that can be targeted to enhance efficacy.

In order to translate this therapy, which shows greater efficacy with local delivery, into a clinically-viable treatment modality, solving the problem T-cell recruitment and expansion in metastatic tumors is key. One likely option is the prolongation of RNA CAR expression. One such method under study is the biochemical stabilization of transferred RNA molecules (37). This strategy will produce CAR T cells with more sustained receptor expression that retain the self-inactivating qualities of RNA electroporation, prolonging the critical time period of T-cell infiltration.

While the RNA CAR T cell approach will minimize toxicities that occur over days, such as seen with permanently-modified CD19 CARs (10), this may not be the case for adverse events reported in previous clinical trials (12, 13) that occur within hours of cell infusion. We can predict, however, that the toxicities observed would have been self-limited and thus potentially less severe, as antigen-driven cell division would result in restriction and temporally-restricted expression of CAR molecules. Our recent report of cytokine release syndrome in children treated with CD19 CAR T cells (10) is an example of toxicity that may have been self-limited had these T cells been modified with RNA CARs as opposed to permanently-modified with lentiviral CARs. Similarly, long-term B-cell aplasia, which we have observed as a necessary complication
of CD19 CAR T cell persistence, would not occur using this approach. Another approach to limit CAR T cell persistence is to utilize suicide gene systems that may eliminate 1-3 logs of cells. However, T cells have robust proliferative capacity and are capable of repopulating from a single cell, and thus a guaranteed “off” system such as RNA electroporation remains an attractive approach.

In conclusion, we demonstrate the efficacy of permanently-modified GD2 CAR T cells in controlling disseminated neuroblastoma, and that a lack of longer-term control of metastatic disease by temporarily-modified GD2 CAR T cells results from reduced tumor infiltration in the setting of large tumor burdens.

Acknowledgements

We thank Daniel Martinez and Dr. Bruce Pawel for their assistance with immunohistochemistry, and Dr. Curt Lind for assistance with HLA phenotyping.
References


Figure Legends

Figure 1. RNA GD2 CAR T cells mediate rapid destruction of neuroblastoma cells and control tumor growth. NSG mice were implanted with 2x10^7 SY5Y neuroblastoma cells subcutaneously in the flank and given 3 weeks to establish disease. Mice were then injected intra-tumorally with 5x10^6 (A) RNA CD19 CAR T cells or (B) RNA GD2 CAR T cells, and tumors were excised 5 days later and immuno-stained for human CD3. RNA CD19 CAR T cells do not affect the tumor architecture and passively filter out of the tumor to the periphery of the tissue, while RNA GD2 CAR T cells mediate rapid destruction of SY5Y cells and disperse throughout the tumor. (Inlay 2x magnification, main image 6x magnification). (C) RNA GD2 CAR T cells mediate long-term disease suppression as compared to RNA CD19 CAR T cells (black arrows represent cell infusions, 5x10^6 on day 15, 1.5x10^7 on days 29 and 36).

Figure 2. Lentivirally-modified GD2 CAR T cells control disseminated neuroblastoma long-term and prolong animal survival. Mice with disseminated neuroblastoma were given a single dose of 1x10^7 CAR T cells (day 14, black arrow; CD19 cells 56% CAR+, GD2 CAR cells 10.9% CAR+). (A) One dose of GD2 CAR T cells mediated sustained disease control long-term. (B) Overall survival of animals treated with either CD19 or GD2 CAR T cells. All animals treated with GD2 CAR T cells died of xenogeneic graft-versus-host disease, with no animals succumbing to cancer. This is further illustrated in Figure 2(C), which demonstrates mortality attributed to malignancy in animals treated with either CD19 or GD2 CAR T cells. All animals treated with CD19 CAR T cells died of malignancy. 5/8 animals treated with GD2 CAR T cells died in disease remission and 3/8 died with controlled minimal disease burdens.

Figure 3. Multiple infusions of RNA GD2 CAR T cells delay disease progression and enhance animal survival. Mice with disseminated neuroblastoma were given an initial infusion of 5x10^6 CAR T cells on day 8, followed by two infusions of 1.5x10^7 CAR T-cells on days 22 and 29 (black arrows). (A) An initial dose of GD2 CAR T cells demonstrates a modest reduction in disease burden, and the next two infusions mediated transient disease stabilization, resulting in a cumulative delay in disease progression over time. (B) Three infusions of RNA GD2 CAR T cells enhance overall animal survival.

Figure 4. Effect of GD2 CAR T cells on disseminated neuroblastoma. (A) An initial infusion of RNA GD2 CAR T cells reduces disease burden, while the subsequent infusions modestly stabilize disease. (B) Lentiviral GD2 CAR T cells mediate long-term disease control. On day 28 a transient disease recurrence appears but is eliminated, highlighting the memory response of permanently-modified CAR T cells.

Figure 5. Permanently-modified GD2 CAR T cells demonstrate rapid tumor infiltration. Animals were sacrificed on the days indicated and livers examined histologically after staining for human CD3. Tumor sites boxed in the 1x magnification are enlarged in the 10x magnification. (A) Within 3 days of T-cell infusion, GD2 CAR T cells have infiltrated into tumor sites. By day 5, gross examination reveals significant T-cell infiltration and activity within tumors. (B) CD19 CAR T cells do not penetrate tumor sites.

Figure 6. RNA GD2 CAR T cells localize to but do not infiltrate disseminated tumors. Animals with established disseminated tumors were sacrificed on the days indicated and examined histologically after staining for human CD3. Black arrows indicate infusion of 1x10^7 T cells. (A) With small tumors, some RNA GD2 CAR T cells are able to penetrate and remain within tumors sites in the first 3 days of injection, however subsequent injections demonstrate...
(B) Initial passive infiltration by CD19 CAR T cells is lost by day 3, and subsequent injections do not result in localization to tumor sites.
Figure 3

(A) Mean Radiance (cpm/sec/cm²/µm²) vs. Days after tumor injection

- Saline
- CD19 CAR T-cells
- GD2 CAR T-cells

p = 0.0032

(B) Percent survival vs. Days of survival

- CD19 CAR
- GD2 CAR
- Saline

p = 0.0128
Figure 4

A

Day 7  Day 11  Day 16  Day 24  Day 31  Day 38  Day 45  Day 52

CD19 RNA

GD2 RNA

B

Day 13  Day 15  Day 18  Day 28  Day 35  Day 42  Day 49  Day 56  Day 63  Day 75

CD19 Lenti

GD2 Lenti
Cancer Immunology Research

Nature of tumor control by permanently and transiently-modified GD2 chimeric antigen receptor T cells in xenograft models of neuroblastoma

Nathan Singh, Xiaojun Liu, Jessica Hulitt, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/2326-6066.CIR-14-0051

Supplementary Material  Access the most recent supplemental material at: http://cancerimmunolres.aacrjournals.org/content/suppl/2014/08/07/2326-6066.CIR-14-0051.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.