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ORIGINAL RESEARCH

T cells targeting NY-ESO-1 demonstrate efficacy against disseminated neuroblastoma

Nathan Singh[a], Irina Kulikovskaya[b], David M. Barrett[c], Gwendolyn Binder-Scholl[d], Bent Jakobsen[d], Daniel Martinez[e], Bruce Pawel[e], Carl H. June[b], Michael D. Kalos[f], and Stephan A. Grupp[c,e]

[a]Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA; [b]Abramson Cancer Center and Department of Pathology, University of Pennsylvania, Philadelphia, PA, USA; [c]Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, PA, USA; [d]Adaptimmune, Abingdon, UK; [e]Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Philadelphia, PA, USA; [f]Lilly Research Laboratories, Eli Lilly and Company, New York, NY, USA

ABSTRACT

The cancer-testis antigen NY-ESO-1 is expressed by many solid tumors and has limited expression by mature somatic tissues, making it a highly attractive target for tumor immunotherapy. Targeting NY-ESO-1 using engineered T cells has demonstrated clinical efficacy in the treatment of some adult tumors. Neuroblastoma is a significant cause of cancer mortality in children, and is a tumor type shown to be responsive to immunotherapies. We evaluated a large panel of primarily resected neuroblastoma samples and demonstrated that 23% express NY-ESO-1. After confirming antigen-specific activity of T cells genetically engineered to express an NY-ESO-1 directed high-affinity transgenic T cell receptor *in vitro*, we performed xenograft mouse studies assessing the efficacy of NY-ESO-1-targeted T cells in both localized and disseminated models of neuroblastoma. Disease responses were monitored by tumor volume measurement and *in vivo* bioluminescence. After delivery of NY-ESO-1 transgenic TCR T cells, we observed significant delay of tumor progression in mice bearing localized and disseminated neuroblastoma, as well as enhanced animal survival. These data demonstrate that NY-ESO-1 is an antigen target in neuroblastoma and that targeted T cells represent a potential therapeutic option for patients with neuroblastoma.

Abbreviations: CAR, chimeric antigen receptor; CT, cancer testes; tTCR, transgenic T cell receptor

Introduction

A significant barrier to the development and application of immune-based therapies targeting cancer is the identification of appropriate tumor antigens. Both CD20 and CD19 have proven to be successful antigen targets for B cell malignancies,[1,2] however, one of the primary side effects of these therapies is B cell aplasia. While this toxicity can be managed medically, many solid tumor antigens are expressed by normal and indispensible somatic tissues, making the search for appropriate antigen targets difficult. In the late 1990s, analysis of esophageal squamous cell carcinoma led to the identification of NY-ESO-1, a protein normally expressed only in fetal and testicular tissue but aberrantly expressed by some solid malignancies.[3] This added NY-ESO-1 to a growing list of molecules expressed appropriately in the germ line and abnormally by some cancers. These molecules were termed "cancer/testis" antigens,[4] and are capable of serving as targets for antigen-directed immune therapies.[5] Despite initial optimism, trials of vaccination against NY-ESO-1 to induce antitumor immunity have proven largely unsuccessful, and to date have not yet resulted in significant antitumor efficacy.[6] Deletion of high-affinity clones directed against NY-ESO-1 during thymic selection and suppression of auto-reactive T cells by peripheral tolerance mechanisms may account for the poor antitumor activity observed in these trials.

One method to overcome the limited antitumor activity of endogenous T cells is infusion of genetically-engineered high-affinity T cells re-directed to tumor antigens. The two primary antigen receptors used to re-target T cells are chimeric antigen receptors (CARs) and transgenic T cell receptors (tTCRs). CAR therapy[7] has recently demonstrated significant success in targeting B cell leukemias,[2,8-11] and trials targeting solid tumors are underway.[12-14] While CARs have great potential as therapeutic agents in cancer immunotherapy, they are limited in their ability to only recognize cell-surface molecules. On the contrary, tTCRs have the ability to identify any processed antigen that is presented by the major histocompatibility complex (MHC), thus greatly expanding the list of possible targets. *In vitro* studies have demonstrated that cells engineered with endogenously-occurring NY-ESO-1 TCRs have activity against NY-ESO-1-expressing melanoma and non-melanoma cell lines.[15] In a recent clinical trial employing NY-ESO-1-directed T cells, engineered cells bearing high-affinity tTCRs were delivered to patients with melanoma and synovial cell carcinoma. Nearly half of patients in this study demonstrated objective clinical responses, highlighting the potential of tTCR T cells in treating established solid tumors.[16]

Neuroblastoma is the most common extra-cranial pediatric solid tumor. Derived from neuro-endocrine tissue of the sympathetic nervous system, it accounts for 9% of cancer diagnoses and 15% of cancer deaths in children.[17] Current standard of care for high-risk disease consists of chemotherapy, surgery, consolidation chemotherapy, stem-cell transplant, tumor-directed radiation, and finally antibody-based therapy. This exhaustive regimen yields a three year event-free survival from diagnosis of only ~45% of patients.[18,19] In addition, the outcome for relapsed neuroblastoma is very poor, with a current achievable goal of short-term disease control and very few patients who achieve longer-term remissions. Improved outcomes for this disease will require incorporation of further innovative therapeutic strategies.

In this study, we established that NY-ESO-1 is a potential antigenic target in neuroblastoma. Our recent clinical experiences[2,9] using engineered T cells to target CD19+ tumors have been successful in large part due to a robust, bead-based cell manufacturing process which produces highly effective anti-tumor T cells capable of significant *in vivo* expansion and persistence for as long as three years.[20] Previous data have demonstrated the superiority of high-affinity TCRs in targeting NY-ESO-1,[21] and combining our *ex vivo* cell manufacturing process and a high-affinity HLA-A*02 restricted TCR recognizing the peptide NY-ESO-1$_{157\text{-}165}$ (SLLMWITQC), we demonstrated antigen-specific T-cell activity against NY-ESO-1+ neuroblastomas *in vitro*. Using a well-characterized bioluminescent xenograft animal model system,[22,23] we evaluated the efficacy of these tTCR cells in two *in vivo* models of neuroblastoma. We demonstrated that these cells were able to slow the progression of both local and disseminated disease, and significantly enhanced animal survival. Together, these data suggest that cells engineered to express tTCRs targeting NY-ESO-1 are a viable therapeutic option for patients with neuroblastoma.

Results

NY-ESO-1 is an antigenic target in neuroblastoma

We first sought to assess NY-ESO-1 expression in tumor biopsies from our patient population at the Children's Hospital of Philadelphia to evaluate this molecule as a relevant immunotherapy target in neuroblastoma. We evaluated a panel of 187 neuroblastoma tumor samples from 165 patients, and of 124 evaluable tumors we found that ~23% stained positively for NY-ESO-1, with positivity defined as ≥10% of cells expressing target based on immunohistochemical staining, and overall intensity of staining quantified as ≥1 on a 0-3 scale (Table 1) (NY-ESO-1 score was calculated by multiplying % positive with the intensity score). Using immunohistochemical staining, expression of NY-ESO-1 was observed in both the nucleus and cytoplasm. Examining the pathological characteristics of these tumors, 23/28 (82%) NY-ESO-1+ samples were found to be poorly differentiated and/or of unfavorable histology. Additionally, the samples varied from low to high-risk tumors based on the International Neuroblastoma Staging System (INSS) risk score. Similarly, these tumors were found to vary in MYCN amplification status, disease location and patient age at diagnosis.

NY-ESO-1+ neuroblastomas stimulate T cell cytotoxicity

With a validated antigenic target in our pediatric cancer population, we then assessed the efficacy of NY-ESO-1-directed T

Table 1. Profile of NY-ESO-1+ neuroblastoma patient tumors. Resected specimens from the Center for Childhood Cancer Research at the Children's Hospital of Philadelphia were examined histologically, and NY-ESO-1-expressing tumor profiles are represented. These tumors vary widely in histology, grade and overall risk score, and represent ~23% of all neuroblastomas in our cancer center tumor bank. NY-ESO intensity was graded on a scale of 0-3, and overall score was calculated my multiplying % positive with intensity score.

Patient ID	Age (days)	Histology	Grade	INSS Risk	% of cells NY-ESO-1+	NY-ESO-1 intensity	NY-ESO-1 score
UPN 6	1747	Unfavorable	Poorly differentiated	Low	20	1	20
UPN 7	154	Unfavorable	Poorly differentiated	Low	20	1	20
UPN 9	804	Unfavorable	Poorly differentiated	Low	50	3	150
UPN 10	355	Unfavorable	Poorly differentiated	Low	60	3	180
UPN 16	317	Favorable	Poorly differentiated	Low	20	1	20
UPN 30	532	Favorable	Differentiating	Low	10	1	10
UPN 31	124	Favorable	Poorly differentiated	Intermediate	20	2	40
UPN 41	573	Favorable	Differentiating	Low	20	1	20
UPN 120	401	Unfavorable	Poorly differentiated	High	50	1	50
UPN 121	1936	Unfavorable	N/A	Low	70	3	210
UPN 123	709	Unfavorable	N/A	Low	60	2	120
UPN 129	10	Unfavorable	Undifferentiated	High	20	1	20
UPN 131	1467	Unfavorable	Poorly differentiated	High	20	1	20
UPN 133	3624	Unfavorable	Differentiating	High	20	1	20
UPN 153	3871	Unfavorable	Differentiating	High	40	1	40
UPN 169	4233	Unfavorable	Differentiating	High	90	2	180
UPN 170	342	Favorable	Poorly differentiated	Low	40	2	80
UPN 173	19	Favorable	Poorly differentiated	Low	10	1	10
UPN 185	101	Favorable	Poorly differentiated	Intermediate	80	1	80
UPN 187	1155	Favorable	Differentiating	Low	70	2	140
UPN 241	75	Favorable	Poorly differentiated	Low	50	1	50
UPN 244	5229	N/A	Differentiating	Intermediate	60	1	60
UPN 245	173	Favorable	Poorly differentiated	Intermediate	10	1	10
UPN 247	611	Favorable	Differentiating	Low	80	1	80
UPN 267	40	Favorable	Poorly differentiated	Low	10	1	10
UPN 288	1064	Unfavorable	Poorly differentiated	High	20	1	20
UPN 292	533	Unfavorable	Poorly differentiated	Low	10	1	10
UPN 299	32	Favorable	Poorly differentiated	Intermediate	10	1	10

Table 2. NY-ESO-1 expression profile of neuroblastoma cell lines. Quantitative RT-PCR evaluation of NY-ESO-1 expression in neuroblastoma cell lines is demonstrated. Harvested mRNA was evaluated by RT-PCR, and relative expression quotients (RQ) calculated using an internal control gene (GUS B) and the $\Delta\Delta$Ct method for expression standardization. As shown, SY5Y exhibit undetectable (ND, not detected) NY-ESO-1 transcript levels, whereas SKNAS and NB16 cell lines have progressively higher levels of transcript

Sample	GUS B Ct	NY-ESO-1 Ct	NY-ESO-1 RQ
SY5Y	22.91	ND	ND
SKNAS	23.10	23.50	0.3839
NB16	22.57	22.28	0.6217

cells against neuroblastoma *in vitro*. While transgenic expression of NY-ESO-1 by genetic engineering of neuroblastoma cell lines would create viable antigen targets for assay development, this method is likely to result in supra-physiologic target expression, and thus risk potential over-presentation of antigen as compared to endogenous presentation seen in actual tumor cells. To more closely capture physiologic expression and presentation, we examined the endogenous expression of NY-ESO-1 in our neuroblastoma cancer cell lines. Evaluation of several cell lines as assessed by qRT-PCR revealed one with robust NY-ESO-1 expression (NB16), one with moderate

expression (SK-NAS), and one with undetectable NY-ESO-1 levels (SY5Y) (Table 2), thus giving us neuroblastoma lines expressing a range of NY-ESO-1 to test. Our tTCR targeting NY-ESO-1 is HLA-A*02-restricted. Thus, to allow for TCR recognition of presented antigen, these neuroblastoma cell lines were engineered to express HLA-A2 using lentiviral vector engineering, creating sublines NB16-A2, SK-NAS-A2 and SY5Y-A2. This engineering strategy allowed unhindered antigen presentation, without driving over-presentation.

T cells were engineered to express tTCRs and were combined *in vitro* with HLA-A2+ neuroblastoma cell lines. T-cell activation and degranulation was assessed by expression of CD107a, a marker of cytotoxic T-cell function,[24] after 4 hours in co-culture with target cells (Fig. 1). Not all T cells in the co-culture expressed the tTCR, and thus the tTCR-negative cells served as controls to evaluate antigen-driven degranulation. To quantify the degree of antigen-specific degranulation, we developed a metric to assess the specificity of surface CD107a expression, termed the "degranulation ratio." This is a ratio of cells expressing the transgenic receptor that demonstrated degranulation (tTCR+CD107a+, antigen-dependent degranulation) compared to cells that didn't express the transgenic receptor that demonstrated degranulation (tTCR−CD107a+,
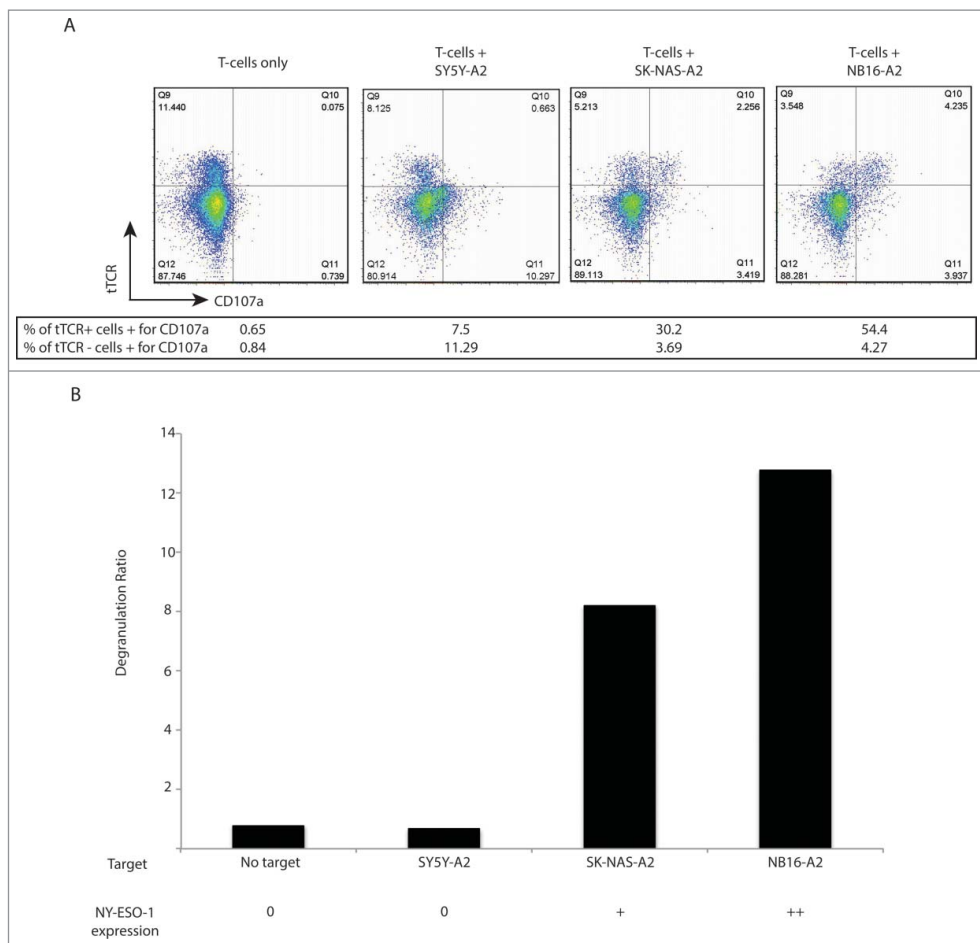

Figure 1. NY-ESO-1 tTCR cells degranulate in response to NY-ESO-1+ tumors. T cells transduced with engineered transgenic T cell receptor (tTCR) targeting NY-ESO-1 were incubated with target neuroblastoma cells and activation was measured by CD107a upregulation. (A) Flow cytometry plots demonstrating increasing degranulation with increasing NY-ESO-1 expression. Percentages shown are percent of tTCR+ cells expressing CD107a / total tTCR+ cells (upper number, reflecting antigen-specific degranulation) and percent of tTCR− cells expressing CD107a / total tTCR− cells (bottom number, reflecting non-specific degranulation). (B) Bar graph representing the data shown in Figure 1A. The degranulation ratio represents degranulation of tTCR+ cells relative to tTCR− cells after incubation with neuroblastoma cell lines, thus controlling for non-specific degranulation.

antigen-independent degranulation), and provides a method to assess tTCR-dependent T-cell activation while controlling for non-specific activation across groups.

T cells incubated without target cells demonstrated <1% CD107a expression, independent of tTCR expression (degranulation ratio 0.774). $tTCR^+$ cells incubated with SY5Y-A2 (no NY-ESO-1) showed 7.5% degranulation, with a degranulation ratio of 0.664. $tTCR^+$ cells incubated with SK-NAS-A2 (moderate NY-ESO-1) showed 30.2% degranulation, and a degranulation ratio of 8.18. Finally, $tTCR^+$ cells incubated with NB16-A2 (high NY-ESO-1) showed 54.4% degranulation, with a degranulation ratio of 12.74 (Fig. 1). The results of this sensitive assay demonstrate that varying levels of NY-ESO-1 expression may result in varying levels of T-cell activation in this short time period. Additionally, we demonstrate that tTCR T cells exhibit cytolytic activity against only NY-ESO-1-positive targets, and not against NY-ESO-1-negative $HLA-A2^+$ targets, confirming selective antigen-driven T-cell activity.

NY-ESO-1 directed T cells control localized tumors in mice

After confirming *in vitro* activity, we next assessed the immunologic activity of these anticancer immune cells *in vivo*. We first assessed antigen expression in SY5Y-A2 and NB16-A2 cells using immunohistochemistry (as described previously) and found that SY5Y-A2 tumors had an NY-ESO-1 score of 0 (0% cells positive) and NB16-A2 cells had an NY-ESO-1 score of 270-285 (90-95% cells positive, intensity grading 3 − data not shown), consistent with our RT-PCR findings. We next injected 2×10^7 neuroblastoma cells, either SY5Y-A2 or NB16-A2, subcutaneously into immunodeficient NOD/SCID/c$\gamma^{-/-}$ (NSG) mice. When large tumors were established and reached a mean volume of 500 mm^3, we injected 10^7 T cells, either $tTCR^+$ (73% $tTCR^+$) or untransduced (NTD) directly into tumor sites. In order to control for MHC-restriction of the T cells injected, all T cells were harvested from $HLA-A2^+$ normal human donors. To control for the non-specific activity of untransduced T cells (derived from $HLA-A2^+$ donors) in response to $HLA-A2^+$ tumors, each study included a group of animals injected with saline only (PBS). Animals bearing SY5Y-A2 (i.e., NY-ESO-1 negative) tumors were injected with T cells on day 22 after tumor injection, and demonstrated rapid tumor growth independent of treatment modality (Fig. 2A, $p = 0.75$). Animals with NB16-A2 (NY-ESO-1 positive) tumors were injected on day 10 after tumor injection, and those animals that were treated with untransduced cells or PBS also demonstrated rapid growth. In contrast, those animals injected with a single dose of NY-ESO-1-directed tTCR T cells ($\sim7.3 \times 10^6$ tTCR+ cells) demonstrated significant control of disease and delay of disease progression (Fig. 2B, $P < 0.001$). NB16-A2 tumors were excised from several mice at day 7 and day 14 after T-cell injection and examined histologically. Tumors injected with tTCR cells demonstrated extensive T-cell infiltration and expansion within tumor sites by day 7, while tumors treated with untransduced T cells lacked significant T-cell infiltration. Examination on day 14 revealed necrosis of tumors treated with tTCR cells, while untransduced cells did not alter tumor architecture, and T cells were found at the periphery of tumor tissue (Fig. S1).

Survival of animals harboring SY5Y-A2 tumors was similar among all treatment groups, with median survival ranging from 28-31 days (Fig. 3A, $p = 0.84$). In contrast, survival of animals with NB16-A2 tumors treated with tTCR T cells was significantly enhanced (median survival 42 days) as compared to those treated with untransduced T cells (median survival 20 days) or PBS (median survival 17 days) (Fig. 3B, $p = 0.0019$). Of note, comparison of animals treated with PBS demonstrates an impressive difference in median survival of animals with SY5Y-A2 (28-31 days) compared with NB16-A2 (17-20 days), highlighting the aggressive nature of this tumor cell line.

NY-ESO-1 tTCR T cells significantly delay progression of a rapidly progressive disseminated neuroblastoma in vivo

After demonstrating efficacy of tTCR T cells *in vitro* and in a localized model of neuroblastoma *in vivo*, we next sought to investigate their potential in a model that more closely mimicked the clinical scenario in which genetically engineered T cells are likely to be used. We have developed a disseminated model of neuroblastoma in which neuroblastoma cells are genetically engineered to express click beetle green (CBG) luciferase and then delivered systemically to NSG mice.[25] Using an *in vivo* imaging bioluminescent system, we were able to monitor disease progression over time. We have previously demonstrated that neuroblastoma delivered in this manner establishes significant disease burdens in the liver and lymph nodes, both relevant sites of neuroblastoma metastasis.[25]

We engineered both SY5Y-A2 cells and NB16-A2 cells to express CBG, and injected 2×10^6 cells i.v. via tail vein. We monitored the hosts for the establishment of systemic disease as demonstrated by bioluminescent signal of >0.5 log$_{10}$ over background. Animals were then injected with 10^7 T cells (again, engineered cells were 73% $tTCR^+$). Animals with disseminated SY5Y-A2 tumors demonstrated rapid disease progression (Fig. 4A, $p = 0.99$), again independent of therapy delivered. Infusion with tTCR transduced T cells significantly delayed disease progression in animals with disseminated NB16-A2 tumor cells as compared to those animals treated with untransduced cells or PBS (Fig. 4B, $P < 0.001$), and a ~2.5 log$_{10}$ difference in radiance (reflecting tumor burden) at day 30. These therapeutic effects again translated to animal survival, with no difference in survival of animals with SY5Y-A2 tumors (median survival of 63 days in all groups, Fig. 5A, $p = 0.31$) and significantly enhanced survival in animals with NB16-A2 tumors receiving tTCR cells (median survival of animals treated with PBS 50.5 days, untransduced cells 47 days and tTCR cells 92 days, Fig. 5B, $p = 0.0077$). As in the localized model, all animals in this disseminated model eventually succumbed to disease.

Visual representation of animals over time (Fig. 6) highlights the effects of these tTCR cells. Initial disease suppression is followed by extended tumor growth delay. Several animals demonstrated transient disease progression (day 29), which was temporarily controlled (Day 47) and likely serves as a harbor site of eventual disease progression (day 63).

Discussion

Identification of clinically-viable tumor antigens is one of the largest hurdles facing rapid development of targeted immunotherapies for cancer, specifically solid tumors. Currently, tumor
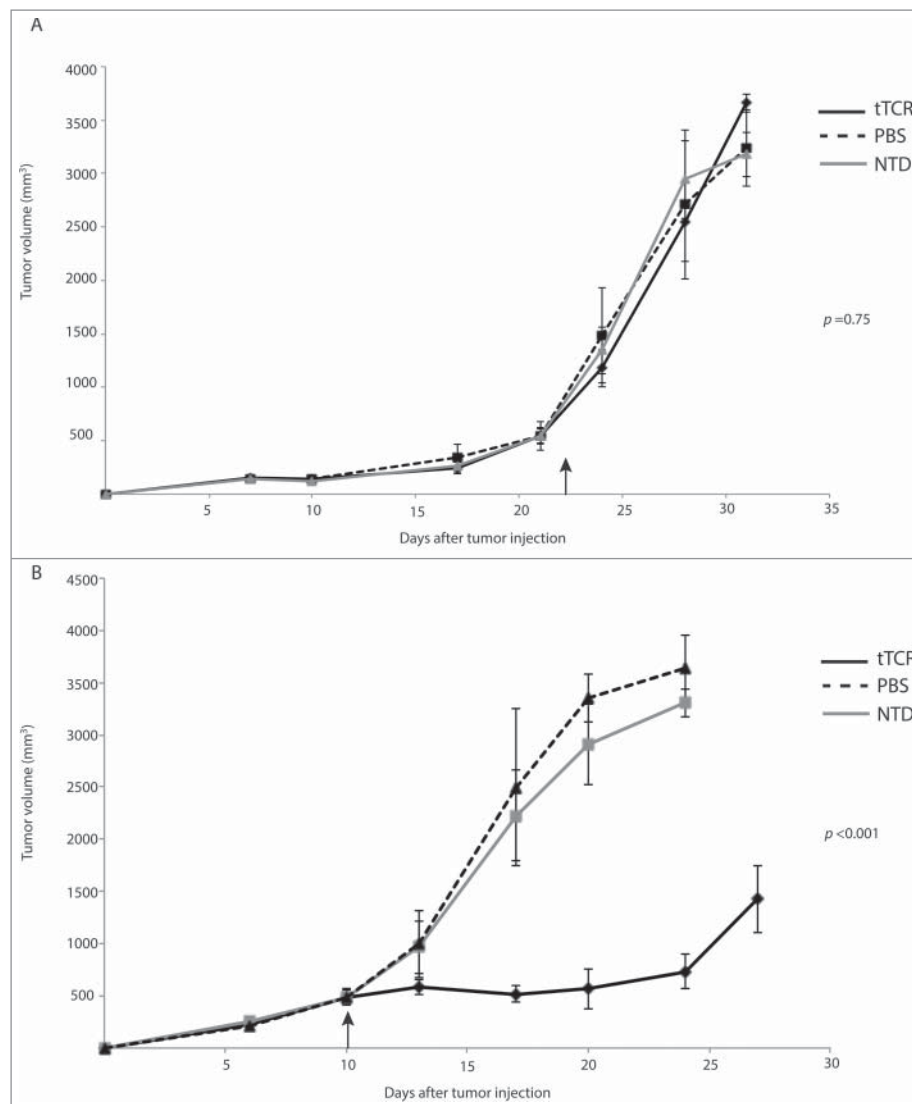
Figure 2. NY-ESO-1 tTCR cells control growth of localized neuroblastoma. Host mice were injected s.c. with 2×10^7 neuroblastoma xenograft cells followed by treatment with human donor-derived T cells. After large tumors were established, 1×10^7 NY-ESO-1 tTCR cells (73% tTCR$^+$) or untransduced cells (NTD) were injected intratumorally (black arrow). (A) NY-ESO-1 tTCR cells have no effect on NY-ESO-1 negative SY5Y-A2 tumor growth. (B) NY-ESO-1 tTCR cells restrict NY-ESO-1-expressing NB16-A2 tumor growth. (n = 6 animals in each group).

antigen identification methods focus on defined groups of targetable antigens: novel peptides produced by somatic mutations,[26] antigens expressed by expendable tissues,[9] and cancer-testes antigens.[16] Several immunotherapy platforms have now demonstrated clinical efficacy in re-targeting immune tissue to cancer cells by manipulating the endogenous immune response, including antibody-based and cytokine-based therapies. Novel therapies like bi-specific antibodies are demonstrating early clinical success,[27,28] and methods that bypass the endogenous immune system, such as genetically-engineered T cells, have also drawn a great deal of attention for the successes demonstrated using chimeric antigen receptors.[2,8,9,29]

While several adult cancer have been evaluated for NY-ESO-1 expression and its ability to serve as an immunotherapeutic target,[15,16,30] pediatric cancers have been largely overlooked. A trial targeting NY-ESO-1$^+$ synovial cell carcinoma using the T cells investigated in this report is currently enrolling; however, this clinical trial is, to our knowledge, the only such pediatric trial targeting NY-ESO-1 (Clinicaltrials.gov Identifier NCT01343043). While

pediatric cancer diagnoses certainly represent a small proportion of the worldwide cancer burden, outcomes have largely plateaued since the mid-1990s[31] and further progress will require alternate therapeutic platforms. In this study we show that T cells engineered to target NY-ESO-1 using a transgenic TCR demonstrate immuno-therapeutic efficacy against NY-ESO-1$^+$ neuroblastoma.

Using the Children's Hospital of Philadelphia tumor bank, we were able to analyze the expression of NY-ESO-1 among a large number of resected primary neuroblastoma samples. We found that ∼23% of our samples express NY-ESO-1, approximately the same percentage of malignant melanomas that have been shown to express NY-ESO-1.[32] When considering potential clinical translation, it is important to remember that this therapy is restricted by expression of the appropriate MHC Class I (HLA-A2). Approximately 40% of the Caucasian population of the United States is positive for HLA-A2, and from this we can predict that 10% of all neuroblastoma patients will be eligible for this therapy. If shown to be promising among HLA-A2$^+$ patients, this will spur the development of NY-ESO-1 targeting TCRs with
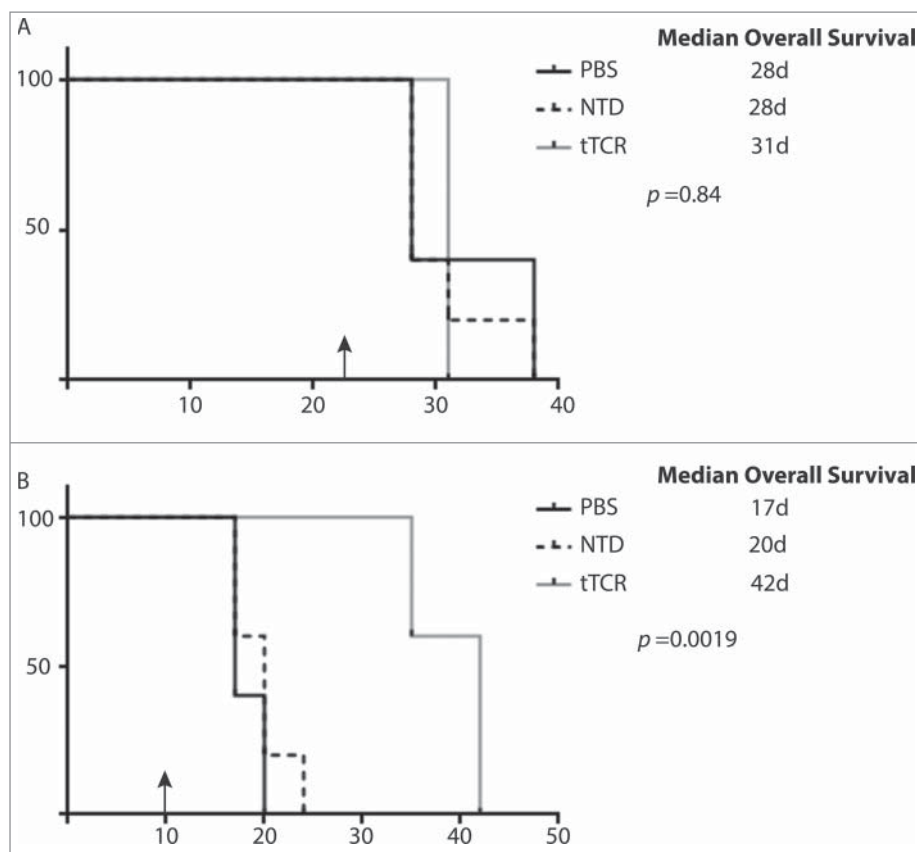
Figure 3. NY-ESO-1 tTCR cells extend survival of mice bearing localized NY-ESO-1+ neuroblastoma. NOD-SCID-yc-/- mice with established flank tumors were injected with T cells or PBS at the time indicated (black arrows). (A) Mice with SY5Y-A2 tumors treated with tTCR cells, untransduced cells (NTD) and PBS had equivalent overall survival. (B) Intratumoral injection of NB16-A2 tumors with tTCR cells resulted in significantly enhanced survival as compared to injection with NTD cells and PBS treated animals. Shown are the median (n D 6 animals per group) survival. Statistical analysis was performed by log-rank test with Bonferroni correction.

other common HLA restrictions, a key step to broaden application of engineered TCRs. The therapeutic effect combined with the poor outcomes for this disease warrant clinical evaluation of this therapy.

We posit that the successful targeting and inhibition of tumor growth demonstrated in this study primarily relies on two issues: targeting the immunodominant epitope of NY-ESO-1 (peptides 157-165)[33] and constraining the long-term cytotoxicity of these engineered T cells. We have previously demonstrated that *ex vivo* expansion using CD3/CD28 beads produces a T-cell population that is more effective *in vivo*, at least in part due to the phenotype of cells produced using this stimulation method.[20] These cells have a greater proliferative capacity *in vivo*, and in our CD19 CAR model they demonstrated enhanced antitumor activity as compared to cells produced using other methods of expansion.

As assessed by CD107a degranulation, we demonstrated that lower levels of NY-ESO-1 expression are sufficient to induce an antigen-driven response, highlighting the sensitivity of our high-affinity tTCR cells. Interestingly, the level of degranulation seemed to correlate with the level of antigen expression. Whether this finding is an artifact of a 4-hour incubation period such that cells expressing lower levels of NY-ESO-1 would have demonstrated further degranulation over time, or if this is a sustained phenomenon, is unclear. NY-ESO-1 expression was found to be variable among our primary patient

samples, and the observed degranulation with lower-levels of NY-ESO-1 expression suggests that tumors with lower levels of antigen production would also be targetable, although this assumption was not evaluated in our *in vivo* xenograft model. To date, clinical trials targeting NY-ESO-1 have excluded patients with lower levels of NY-ESO-1 expression, limiting enrollment to those with moderate or high levels of expression.[34] We thus have no clinical data to suggest that NY-ESO-1 transgenic TCR expressing T cells are capable of successfully targeting low expressing tumors. These *in vitro* data suggest that tTCR cells can recognize and degranulate in response to lower levels of antigen, but they do not answer the question of what the "threshold" for recognition and resultant cytotoxicity is required *in vivo*. We can hypothesize 2 response models: (1) the degree of antigen production and presentation correlates to T-cell activity, and thus "lower-expressers" will have a more limited T cell-driven anti-tumor response (a gradient-response model), or (2) T-cell activity is driven by a threshold effect, in that a minimal antigen burden is needed to initiate robust T-cell activity that is "on" or "off" (a binary-response model). Further clinical studies are necessary to determine the role of antigen intensity on response and outcome.

In both the subcutaneous model and the disseminated tumor model, we observed that tTCR cells were able to slow disease progression of NY-ESO-1+ neuroblastoma but were unable to eradicate disease. The majority of this effect is
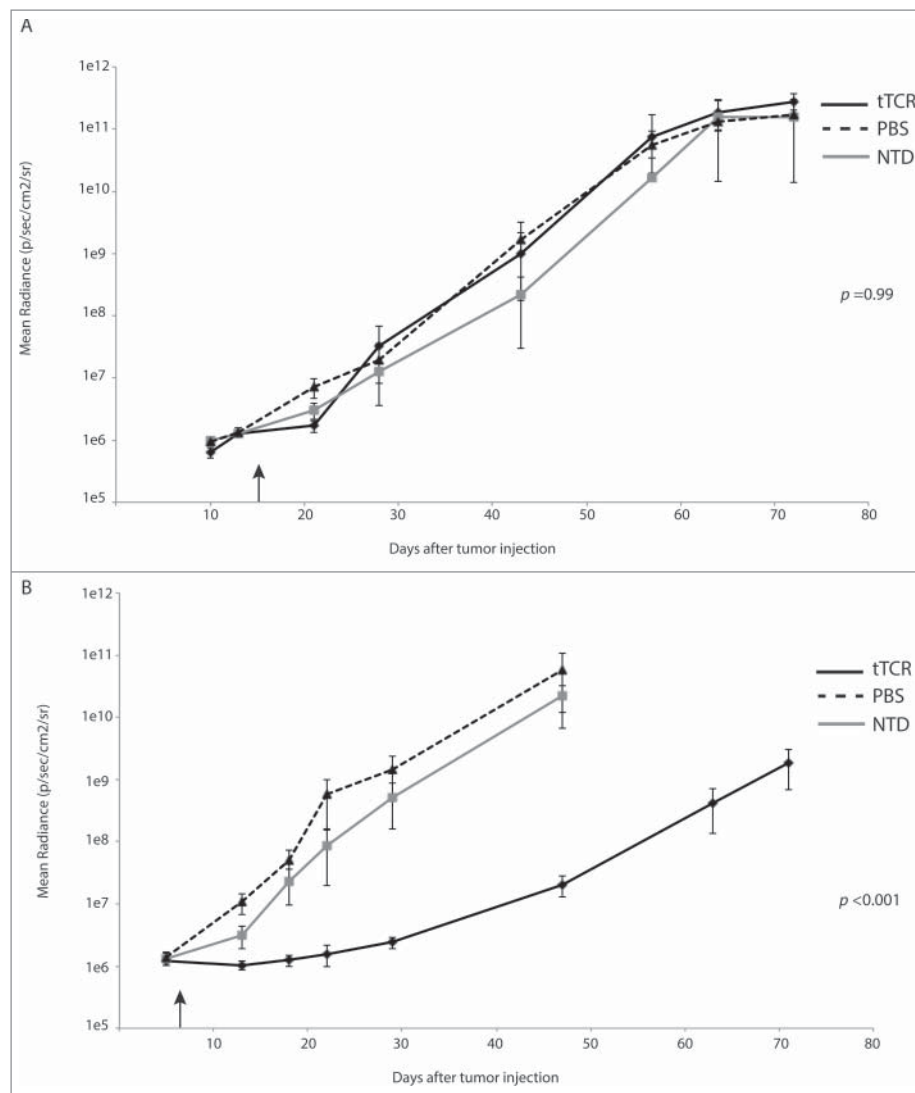
Figure 4. NY-ESO-1 tTCR cells delay progression of disseminated neuroblastoma. To establish disseminated neuroblastoma, 2×10^6 neuroblastoma cells were injected i.v. into immunocompromised host mice. After tumor burdens reached mean radiance of 1×10^6 p/sec/cm^2/sr, animals were injected with 1×10^7 transgenic T cell receptor (tTCR) expressing T cells i.v. (black arrows). (A) NY-ESO-1 tTCR cells have no impact on SY5Y-A2 tumor progression. (B) NY-ESO-1 tTCR cells significantly delayed progression of NY-ESO-1$^+$ NB16-A2 tumors. (n = 7 animals in each group).

observed in the first 30-40 days after T-cell infusion, after which time tumor growth rate accelerates. Several mechanisms may account for this observation. Immune escape resulting from selective pressure may take the form of downregulation of antigen presenting machinery or antigen expression itself. MHC downregulation is a well-described method of tumor immune escape.[35] Indeed, loss of HLA-A*02 in a murine model of multiple myeloma was confirmed as a mechanism of immunoevasion when animals were treated with NY-ESO-1 tTCR cells.[36] While we have not confirmed this either histologically or molecularly, HLA-A2 downregulation is less likely to account for tumor progression in our study. The NB16 neuroblastoma cell line used was genetically engineered to express HLA-A2, and silencing of this engineered locus driven by the *Ef-1α* promoter, while possible, is unlikely and has not been observed in our experience using lentivirally-engineered cells. Deletion or silencing of target antigens is also a well-described mechanism of immune escape, and our group has reported immunoevasion of CD19-negative ALL observed in our trial of

CD19 CAR T cells.[9] Preliminary data have demonstrated that subcutaneous NB16 tumors retain similar levels of NY-ESO-1 expression after treatment with NY-ESO-1 tTCR cells, even in the setting of disease response followed by progression (equivalent NY-ESO-1 score prior to infusion and at time of animal death, data not shown). These data suggest that neither NY-ESO-1 silencing nor outgrowth of endogenously NY-ESO-1-negative cells are contributing mechanisms of disease progression in this setting. Larger studies to compare the expression levels of both HLA-A2 and NY-ESO-1 in tumors from animals treated with control cells and those under selective pressure from NY-ESO-1 tTCR cells should be pursued to confirm this finding.

We have observed that T-cell persistence is an essential component of a maintained antitumor response in our CAR T cell therapy trials[8] and our CAR T cells have been successful in large part due to their maintained persistence *in vivo*. While persistence was not directly evaluated in our current study, previous data from prior studies using the same NY-ESO tTCR T
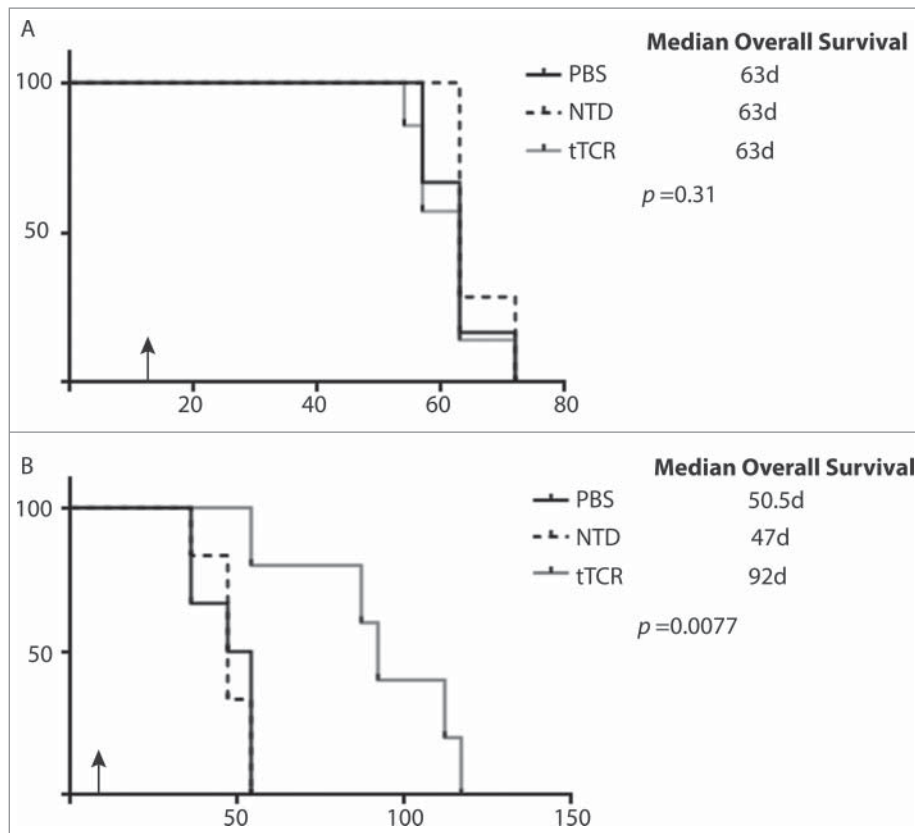
Figure 5. NY-ESO-1 tTCR cells extend survival of mice bearing disseminated NY-ESO-1+ neuroblastoma. NOD-SCID-γc-/- mice with established disseminated tumors were injected with 1 x 107 T cells (black arrows). (A) Animals with SY5Y-A2 tumors have no survival benefit when treated with tTCR cells. (B) tTCR cells significantly enhanced survival of mice with disseminated NB16-A2 tumors.

cells in patients with sarcoma and myeloma demonstrated maintained persistence up to 2 years after cell infusion.[37] Evaluation of persistence will be an essential component of our studies moving forward. If indeed persistence is not maintained, a multiple infusion strategy may be of value to enhance the antitumor responses observed with a single infusion.

We hypothesize that one specific mechanism that greatly contributes to the lack of disease control long-term is the differential rate of cell division between our T cells and the tumor cells. As demonstrated in our study, the NB16 cell line is an extremely aggressive cancer. *In vitro* it is the fastest growing neuroblastoma line that we have evaluated, and this malignancy results in remarkably rapid animal death *in vivo* (as quickly as 17 days after tumor injection in the case of subcutaneous disease). Prior to T-cell infusion, animals with both subcutaneous and disseminated disease were permitted to establish significant disease burdens. While it is clear that an adequate effector:target cell ratio is achieved acutely as reflected by the stabilization of disease growth immediately following T cell infusion, the timing of treatment may have been too late to achieve an adequate effector:target cell ratio over the long-term. Tumor cell division may have simply outpaced T-cell division within the tumor site. It is indeed true that in our clinical experience with acute lymphoblastic leukemia (ALL), an aggressive and rapidly dividing tumor, we do observe the ability of CAR T cells to achieve adequate effector:target cell ratios long term. Solid tumors, however, present different barriers to achieve effective antitumor activity. Leukemia occupies the

same anatomical niche as the transferred T cells, and thus obviates the need for effective tumor infiltration dependent on an unstable vascular supply.[25,38] These barriers may be rate-limiting with regards to achieving successful antitumor responses in large, rapidly dividing solid tumors. When considering clinical translation, both disease burden and rate of tumor growth will be relevant parameters when determining patient eligibility for engineered T-cell therapy for solid tumors.

We have previously reported long-term control of disseminated neuroblastoma using CAR T cells targeting the antigen GD2.[25] In this prior study, we observed disease eradication in animals treated with GD2 CAR T cells whereas the animals in this study demonstrated attenuation of disease progression, but with animals eventually died of disease. Although several factors may contribute to the observed difference in outcome, a key component is likely the role of integrated vs accessory co-stimulatory signals. Initial T-cell activity may be enhanced with the integrated 4-1BB activation that is present in CAR-mediated activity, as may long-term T-cell persistence, and further studies are necessary to illuminate this and other potential differences in signaling that may result from disintegrated vs integrated co-stimulation. These distinct outcomes highlight an opportunity to establish different therapeutic goals for patients receiving engineered T-cell therapy based on their unique clinical characteristics. Many neuroblastoma patients are ineligible for autologous stem cell transplant due to large disease burdens, rapidly progressive disease or other co-morbidities, and the goal for these patients may be simply disease stabilization prior
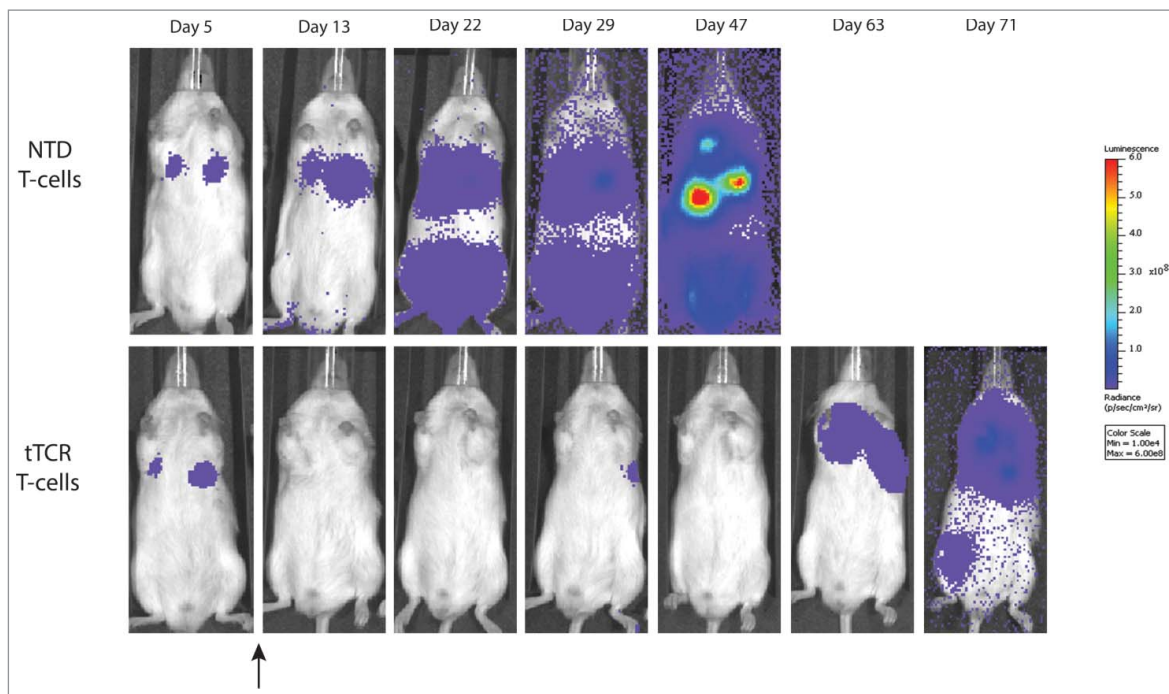
Figure 6. NY-ESO-1+ tTCR cells delay progression of neuroblastoma. NY-ESO-1+ NB16-A2 disseminated tumors were established, followed by delivery to 1 x 107 T-cells on day 6 after tumor injection (black arrow). Untransduced T-cells (NTD) have no impact on tumor progression, while tTCR T-cells suppress and control established neuroblastoma long-term. At shown, several animals had disease progression (here present at day 29), which was subsequently controlled, but then reappeared after day 60.

to proceeding with standard therapies. Recent advances and a large influx of clinical trial data now allow us to identify the different clinical settings in which these novel therapies may be applied, and that the goals for all diseases may not be uniform.

A potential hurdle to the application of this therapy is the fact that neuroblastoma is known to have low levels of MHC Class I expression *in vivo*.[39] This low level of Class I expression has been correlated with a low degree of T-cell infiltration, and thus it has been proposed that reduced MHC expression levels may confer "protection" from cell-mediated immunity in some tumors. In contradistinction, CAR T cells targeting neuroblastoma via an MHC-independent mechanism demonstrate robust tumor infiltration and antitumor responses.[25,38] The cell lines used in our *in vitro* and *in vivo* experiments were engineered to express high levels of MHC Class I, thus removing the potential for low levels of antigen presentation at the time of tTCR T-cell infusion. Previous work from our group and others has demonstrated that exogenous interferon γ (IFNγ) results in upregulation of MHC Class I on previously MHC Class I-low/negative tumors, and that this upregulation can result in increased T-cell infiltration and enhanced tumor killing *in vivo*.[39,40] Combining this observation with the robust antitumor activity of the cells described in this study, one could envision a clinical trial design in which high-risk neuroblastoma patients are treated with IFNγ surrounding the time of tTCR T-cell infusion to enhance tumor infiltration and antitumor activity. There is also the possibility that not all tumor cells will have undetectable levels of Class I, and some tTCR T cells will be able to engage and activate. As mentioned, these cells are cultured and engineered in the same manner as our CAR T cells, which we have recently reported secrete large amounts of IFNγ.[8] This alone may be sufficient to induce MHC Class I

upregulation within the tumor microenvironment, without the need for exogenous IFNγ.

Low MHC Class I expression is most often associated with high-grade neuroblastomas, and has been shown to be biochemically driven by MYCN expression.[41] Examination of our neuroblastoma tissue microarray (TMA) demonstrates that approximately 31% of these all resected neuroblastomas are classified as high-risk, as assessed by INSS staging. Of these high-risk tumors, 47% are *MYCN* amplified, reflecting the poor prognosis of this genetic amplification. Interestingly, while 30% of the NY-ESO-1+ tumors are high-risk (a similar to the proportion seen in the TMA as a whole) only 14% were found to display *MYCN* amplification. Similarly, whereas ~16% of all neuroblastomas were found to express MYCN (independent of INSS risk), only 3.7% of all NY-ESO-1+ tumors were *MYCN* amplified. While this may simply represent sampling error from our group of tumors, NY-ESO-1+ tumors may represent a subgroup of neuroblastomas less likely to express MYCN, and thus perhaps be less likely to downregulate MHC molecules. Further evaluation is necessary to confirm this correlation.

Previously published data has suggested that the hypo-methylating agent decitabine may induce increased CT antigen expression.[42] Although we were not able to reproduce this finding in our own cell lines (data not shown), it is possible that this effect may be observed in some primary neuroblastomas, thus increasing the number of patients eligible for this T-cell immunotherapy. Mechanisms to enhance antigen expression certainly warrant further investigation. Another approach to improve efficacy of engineered T cells against solid malignancies is combination with checkpoint blockade inhibitors. The recent clinical success of these molecules in the treatment of a variety of solid tumors[43,44] has led to investigation of combining this immunotherapy with adoptive cellular therapy and some initial success has been

demonstrated.[45] Addition of PD-1 or CTLA4 inhibition may result in enhanced antitumor responses using our engineered tTCR cells.

To date, successful trials of engineered T cells have largely targeted B cell malignancies using CD19. The on-target off-tissue loss of normal B cells is a clinically tolerable side effect. Previous studies targeting solid tumors using CAR T cells have resulted in numerous adverse events mediated by multiple mechanisms.[46-48] These events further drive the search for improved antigen selection. Previously published data using tTCR cells targeted to NY-ESO-1 demonstrated efficacy and, importantly, did not demonstrate on-target toxicity.[16] The limited expression restricted to germ-line tissues that do not express MHC Class I molecules likely accounts for this limited toxicity and confirms that CT antigens are promising immunotherapeutic targets.

To our knowledge, this is the first assessment of a transgenic TCR targeting pediatric neuroblastoma. We have found that NY-ESO-1 is a relevant immunotherapy target in this disease, and T cells engineered to target this molecule using engineered tTCRs demonstrate antitumor efficacy and significantly improve animal survival. Given these findings, clinical translation to investigate the efficacy of these cells in patients with refractory neuroblastoma is both justified and needed.

Methods

Neuroblastoma tissue microarray and immunohistochemistry

The Center for Childhood Cancer Research at the Children's Hospital of Philadelphia has developed a tissue microarray of 187 neuroblastoma tumor cores collected from 165 patients. Flank tumors from our xenograft animal studies were excised and preserved in 4% paraformaldehyde. Evaluation of antigen expression, tumor grade, histologic profile, *Mycn* amplification status, and International Neuroblastoma Staging System (INSS) stage was done for each sample. Staining was performed on a Bond Max automated staining system, using the Bond Refine polymer staining kit (Leica Microsystems, Buffalo Grove, IL). Standard staining protocol was followed, with the exception of the primary antibody incubation, which was extended to 1 h at room temperature. NY-ESO-1 antibody (Life Technologies, Grand Island, NY, Catalog #35-6200) and anti-human CD3 antibody (Dako, Carpinteria, CA, Catalog #M7254) were used at 1:50 dilution and antigen retrieval was performed with E1 retrieval solution for 20 min (Leica Microsystems). 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 and analyzed using ImageScope Software (both Aperio,Vista, CA). NY-ESO-1 expression was evaluated by both percentage of cells staining positively for NY-ESO-1 (graded 0-100%), as well as intensity of staining (graded 0-3). These two numbers were multiplied to calculate a combined "NY-ESO-1 score," ranging from 0-300. Only samples that had >10% of cells stain positively and had a combined NY-ESO-1 score of >10 were considered positive.

NY-ESO-1 mRNA expression in neuroblastoma cell lines

Neuroblastoma cell lines were evaluated for expression of NY-ESO-1 using quantitative reverse-transcription polymerase chain reaction (RT-PCR). RNA extraction was performed using Ambion RNAqueous or RiboPure kits (Life Technologies, Grand Island, NY), and cDNA was produced using iScript DNA Synthesis kits (Bio-Rad Laboratories, Life Technologies, Grand Island, NY). Quantitation was performed on an ABI 7500 Fast Real-Time PCR System (Life Technologies, Grand Island, NY). Relative quantities (RQs) were calculated using the comparative Ct method on ABI Data Assist software.

Lentiviral vector production

High-titer, replication-defective lentiviral vectors were produced using 293T human embryonic kidney cells as previously described.[49] Briefly, HEK293T cells were seeded at 10^7 cells per T150 tissue culture flask 24 h before transfection. On the day of transfection, cells were treated with 7 μg of pMDG.1, 18 μg of pRSV.rev, 18 μg of pMDLg/p.RRE packaging plasmids and 15 μg of transfer plasmid in the presence of Lipofectamine 2000 transfection reagent (Life Technologies, Grand Island, NY, Catalog #11668019). Transfer plasmids containing TCR constructs were modified so that expression of the TCR was under control of the *EF-1α* promoter as previously described.[50] Viral supernatants were harvested 24 h and 48 h after transfection and concentrated by ultracentrifugation overnight at 10,500x*g*.

T cell engineering

Primary human T cells from normal donors were procured through the University of Pennsylvania Human Immunology Core. T cells were combined at a ratio of 1:1 CD4:CD8 cells at a concentration of 10^6 cells/mL in T cell culture media with stimulatory microbeads coated with antibodies directed against CD3 and CD28 (Life Technologies, Grand Island, NY, Catalog #111.32D) at a concentration of 3 beads/cell, as reported previously.[51] For each experiment, cells were derived from the same donor and split into 2 large cultures. Following 24 h after initial stimulation, one culture was exposed to lentiviral vector at a multiplicity of infection (MOI) of 5-10 particles/cell, and the other culture was left undisturbed. Cells were counted and volumes measured serially until growth and size trends indicated cells were rested down, at which time they were frozen. Cells were then thawed 12-18 hours prior to *in vivo* injection.

T cells were transduced with a lentiviral vector encoding a high-affinity NY-ESO-1$_{157-165}$-directed transgenic T cell receptor (AdaptImmune, Abington, United Kingdom) at a concentration of 7 infectious particles per T cell using protocols previously described.[52] Using an HLA-A*0201-restricted MHC molecule loaded with a dextramer specific for this tTCR (SLLMWITQV) conjugated to either phycoerythrin (PE, Catalog #WB2696-PE) or allophycocyanin (APC, Catalog #WB2696-APC) fluorophores (Immudex USA, Fairfax, Virginia), transduced cells were stained to assess tTCR expression. Fluorescence was assessed using an Accuri C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ).

Modification of neuroblastoma cell lines

Lentiviral vectors encoding HLA-A2-restricted Class I MHC molecules and Click Beetle Luciferase − GFP (CBG/GFP) were manufactured as described above. Neuroblastoma cell lines were plated and given 24 h to adhere to culture vessels, and then exposed to lentiviral vectors encoding HLA-A2 at multiplicity of infection (MOI) of 8-10. HLA-A2 expression was then assessed by flow cytometry after incubation of neuroblastoma cells with antibodies directed against HLA-A2 (BD Biosciences, Franklin Lakes, NJ). Both SY5Y and NB16 cell lines had >95% HLA-A2 expression. Cells were then re-plated and exposed to vectors encoding CBG/GFP, after which expression of GFP was assessed by flow cytometry. Again, all cells demonstrated >95% CBG/GFP expression.

CD107a degranulation assay

T cells modified to express the NY-ESO-1 tTCR were co-incubated with target neuroblastoma cells engineered to express HLA-A2 at a ratio of 5:1 target:T cell. This co-culture was combined with an antibody cocktail consisting of anti-CD107a-e660 (eBiosciences, San Diego, CA, Catalog #50-1079), and stimulatory antibodies directed against CD28 (clone 9.3, generously provided by Dr. Bruce Levine, University of Pennsylvania) and CD49d (BD Biosciences, Franklin Lakes, NJ, Catalog #555051) for one hour. Intracellular protein transport was halted by addition of GolgiStop (BD Biosciences, Franklin Lakes, NJ, Catalog #554724) and cells were incubated for an additional three hours. Cells were then harvested and stained for CD3 (BD Biosciences, Franklin Lakes, NJ, Catalog #554832) and tTCR as described and analyzed on an Accuri C6 Flow Cytometer. Specificity of degranulation was controlled for by calculation of the Degranulation Ratio. This number was calculated using the formula $((\%tTCR+CD107a+)/(\%tTCR+))/((\%tTCR-CD107a+)/(\%tTCR-))$, which controlled for non-specific degranulation of tTCR-negative cells.

Mouse xenograft studies

6-16 week old NOD-SCID-γc$^{-/-}$ (NSG) 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 with 2×10^7 neuroblastoma cells suspended in 0.2 mL Matrigel (BD Biosciences, San Jose, CA, Catalog #354234) subcutaneously. For disseminated tumor studies, animals were injected via tail vein with 2×10^6 cells in 0.1 mL sterile PBS. T cells were injected in 0.1 mL sterile PBS either directly intratumoral for flank tumor studies or intravenously for disseminated studies at the indicated times. Animals were monitored for signs of disease progression and overt toxicity, such as xenogeneic graft-vs.-host disease, as evinced by >20% loss in body weight, loss of fur, diarrhea, conjunctivitis and disease-related hind limb paralysis. Histological studies presented in **Figure S1** were performed on flank tumors excised from animals sacrificed at the indicated times.

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 $((\text{width} + \text{length})/2)^3)/2$. Mice were sacrificed when tumors reached 3 cm^3, or when tumor burden inhibited activity.

Bioluminescent imaging

Disease burdens were monitored over time using the Xenogen sensitive bioluminescent imaging system, as described previously.[22,23] Animals were sacrificed when bioluminescent signal reached $> 10^{11}$ photons/sec/cm^2/steridian.

Cell line identity testing

Parent cell lines were genotyped by short tandem repeat (STR) analysis.[53] Cell lines and samples were verified every 6 months, or after any genetic modification to ensure identity.

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.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

N.S., M.K., G.B.S., B.J., D.M.B., C.H.J, and S.A.G. designed the research. N.S., I.K., D.M., B.P. performed the research.

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