




Immunotherapy resistance driven by loss of NY-ESO-1 expression in response to transgenic adoptive cellular therapy with PD-1 blockade

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ABSTRACT

Background The tumor antigen NY-ESO-1 has been shown to be an effective target for transgenic adoptive cell therapy (ACT) for the treatment of sarcoma and melanoma. However, despite frequent early clinical responses, many patients ultimately develop progressive disease. Understanding the mechanisms underlying treatment resistance is crucial to improve future ACT protocols. Here, we describe a novel mechanism of treatment resistance in sarcoma involving loss of expression of NY-ESO-1 in response to transgenic ACT with dendritic cell (DC) vaccination and programmed cell death protein-1 (PD-1) blockade.

Methods A HLA-A*02:01-positive patient with an NY-ESO-1-positive undifferentiated pleomorphic sarcoma was treated with autologous NY-ESO-1-specific T-cell receptor (TCR) transgenic lymphocytes, NY-ESO-1 peptide-pulsed DC vaccination, and nivolumab-mediated PD-1 blockade.

Results Peripheral blood reconstitution with NY-ESO-1-specific T cells peaked within 2 weeks of ACT, indicating rapid in vivo expansion. There was initial tumor regression, and immunophenotyping of the peripheral transgenic T cells showed a predominantly effector memory phenotype over time. Tracking of transgenic T cells to the tumor sites was demonstrated in on-treatment biopsy via both TCR sequencing-based and RNA sequencing-based immune reconstitution, and nivolumab binding to PD-1 on transgenic T cells was confirmed at the tumor site. At the time of disease progression, the promoter region of NY-ESO-1 was found to be extensively methylated, and tumor NY-ESO-1 expression was completely lost as measured by RNA sequencing and immunohistochemistry.

Conclusions ACT of NY-ESO-1 transgenic T cells given with DC vaccination and anti-PD-1 therapy resulted in transient antitumor activity. NY-ESO-1 expression was lost in the post-treatment sample in the setting of extensive methylation of the NY-ESO-1 promoter region.

Biological/clinical insight Antigen loss represents a novel mechanism of immune escape in sarcoma and a new point of improvement in cellular therapy approaches.

Trial registration number NCT02775292.

INTRODUCTION

The last decade has demonstrated that immunotherapies can be robust and effective treatments for several previously untreatable cancers. Adoptive cell therapy (ACT) of T cells engineered to express a tumor antigen-specific T-cell receptor (TCR) allows for the generation of large numbers of T cells that target a uniform antigen and has shown clear evidence of antitumor efficacy in patients with metastatic solid tumors.^{1,2} Initial work used this approach against melanoma-specific antigens like MART-1 and gp100 to induce tumor regression,¹⁻³ though more recently, the cancer testis antigen NY-ESO-1 has been shown to be an effective target for a variety of solid tumors.³⁻⁷

Given the dismal overall survival in patients with advanced and metastatic sarcomas in response to conventional therapies, a number of recent studies have attempted to use immunotherapies to improve treatment responses.⁶⁻⁹ ACT targeting of NY-ESO-1 has been one of the most promising avenues as multiple studies have shown that it is possible to induce tumor regression in a large fraction of patients.⁶⁻⁸ However, the antitumor activity induced tends to be transient, with most patients progressing within months of initiation of treatment. This underscores the need to better understand the mechanisms driving treatment resistance to inform the development of treatment approaches that can lead to more durable responses.

Here we show for the first-time disease relapse in the setting of loss of NY-ESO-1 expression in sarcoma. The patient described was treated with NY-ESO-1-targeted transgenic ACT with dendritic cell (DC) vaccination and nivolumab. The addition of antigen



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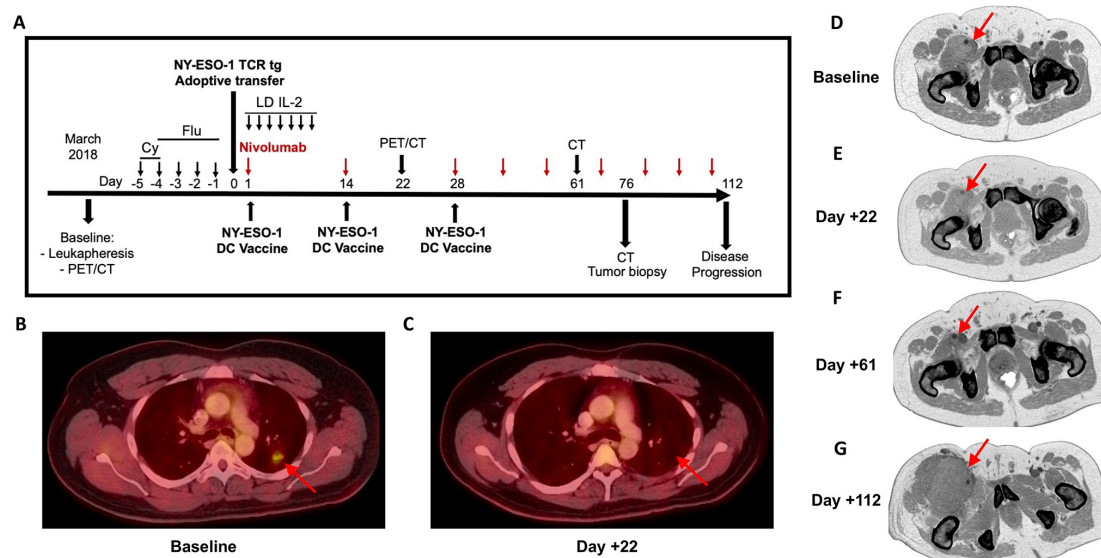


Figure 1 ACT of NY-ESO-1 transgenic T cells and given with DC vaccination and concurrent anti-PD-1 therapy resulted in transient antitumor activity. (A) Overview of the clinical and sample collection timeline for the analysis performed in this study. Dosing schedule for nivolumab was every 2 weeks and is represented by red arrows. (B) Pretreatment and (C) post-treatment PET-CT images from the patient showing evidence of initial antitumor activity (decreased 18F-FDG uptake). Arrows point to the pulmonary nodule. (D–G) CT scans of the inguinal mass (arrows point to the mass) are shown at (D) baseline before ACT, (E,F) during tumor regression on days +22 and +61 of ACT, and (G) during tumor progression on day +112 of ACT. ACT, adoptive cell therapy; Cy, cytarabine; DC, dendritic cell; Flu, fludarabine; IL, interleukin; PET, positron emission tomography; TCR, T-cell receptor; 18F-fluorodeoxyglucose, 18F-FDG.

in the form of a vaccine has been shown in animal models to stimulate T-cell expansion *in vivo*, in turn, enhancing the effectiveness of ACT.^{10–11} Indeed, our group has shown that, for both MART-1 and NY-ESO-1, addition of a peptide-pulsed DC vaccine to ACT is both safe and feasible in humans.^{3,6} PD-1 blockade with nivolumab was added to our dual-cell therapy clinical protocol⁶ in an attempt to increase duration of the antitumor response, as preclinical models have shown PD-1 blockade enhances antitumor efficacy of transgenic ACT.^{12–13} The patient had an initial response to therapy but later developed disease progression. This tumor growth coincided with loss of NY-ESO-1 expression in tumor tissue obtained at time of relapse. This novel mechanism of immune escape in sarcoma demonstrates a novel vulnerability in cellular therapy approaches.

METHODS

Conduct and sample acquisition

For trial eligibility, see online supplemental methods.

Study overview

The schedule of events for the patient receiving the NY-ESO-1-specific TCR transgenic lymphocytes with NY-ESO-1 peptide-pulsed DC vaccination and concurrent PD-1 blockade is outlined in [figure 1A](#). Briefly, a pretreatment biopsy was obtained, and on day 0, the patient underwent ACT of 10^9 transgenic TCR lymphocytes as an intravenous infusion of freshly prepared cells. The patient received intravenous nivolumab (3mg/kg) over 60 min on day 1 with treatment repeating every 2

weeks, along with intradermal administration of NY-ESO-1_{157–165} peptide-pulsed DCs and low-dose interleukin (IL)-2 therapy (500 000 IU/m², subcutaneous) two times a day for up to 14 doses. A repeat biopsy was scheduled for ~3 months following treatment initiation. Standard supportive care included antibiotics and filgrastim, and blood product transfusions were administered as needed and adverse events were analyzed following NCI CTCAE V.3.0.

MHC dextramer immunological monitoring

Detection of NY-ESO-1 TCR expression using fluorescent major histocompatibility complex (MHC) dextramer analysis for NY-ESO-1 (Immudex; HLA-A*0201, SLLM-WITQV) was performed on cryopreserved peripheral blood mononuclear cells (PBMCs) collected at different time points, as described previously.^{3,6}

Flow cytometry phenotyping analysis

PBMCs were centrifuged (500×g for 5 min), resuspended in 100 µL of adult bovine serum (Omega Scientific, Tarzana, California, USA) and stained with pre-conjugated fluorescent antibodies for flow cytometry and acquired on an Attune flow cytometer (Thermo Fisher). Detailed description of the antibodies and staining is described in our previously published work¹⁴ and can be found in the online supplemental methods. Gating strategy can be found in online supplemental figure 1A.

Deep sequencing of TCRβ alleles

Genomic DNA for TCRβ sequence analysis was isolated, and productive TCRβ sequences were identified from

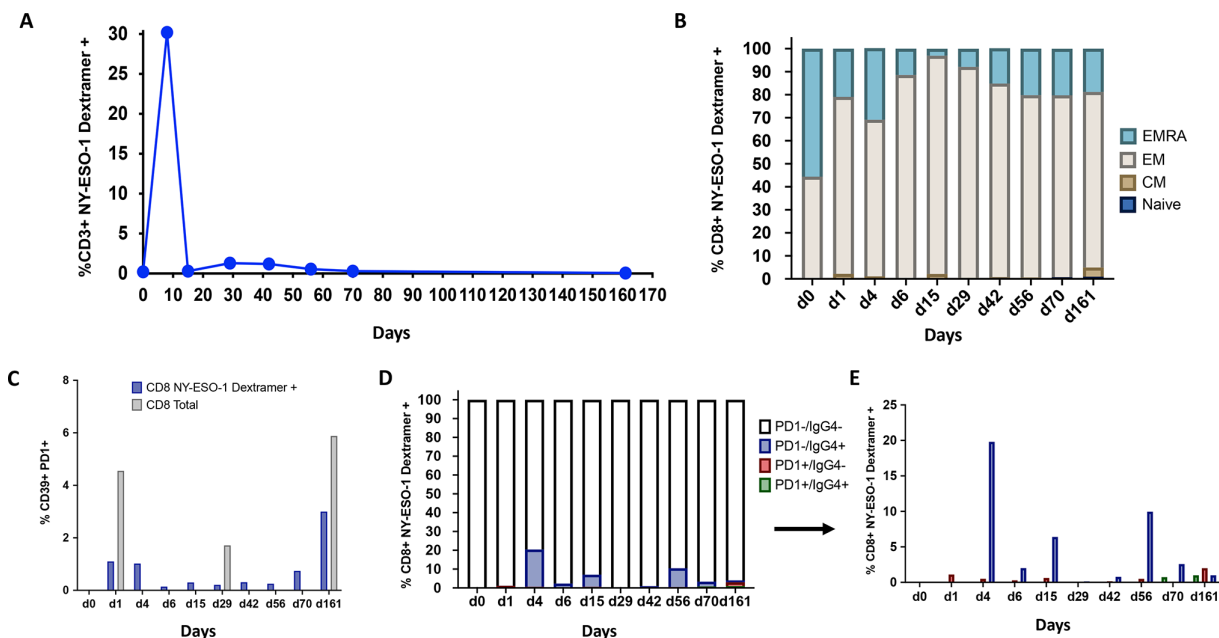


Figure 2 Immunophenotyping of peripheral T cells shows a predominantly EM phenotype over time and confirms nivolumab-mediated PD-1 blockade. (A) Postinfusion peripheral blood levels of NY-ESO-1 TCR transgenic CD3⁺ cells over time. (B,C) Immunophenotyping of adoptively transferred TCRs overtime the CD8⁺ population. T-cell compartments analyzed include naïve, CM, EM, and EMRA. (C) analysis of T cell exhaustion among total CD8⁺ and NY-ESO-1 TCR transgenic CD8⁺ populations. exhaustion determined by CD39/PD1 positivity. (D) PD-1-receptor occupancy analysis. IgG4 positivity serves as a measure of nivolumab binding. PD1 positivity represents residual PD1 not bound by nivolumab. (E) same as in (D) excluding PD1-/IgG4- population. CM, central memory; EM, effector memory; EMRA, effector memory cells re-expressing CD45RA; TCR, T-cell receptor.

formalin-fixed, paraffin-embedded tumor biopsies, patient-matched infusion products, and postinfusion PBMCs as previously described.⁶

RNA sequencing and analysis

mRNA libraries were generated using the KAPA Stranded mRNA Kit and were sequenced on the Illumina HiSeq 3000 platform (2×150bp). Sequencing data were aligned to the human reference genome (GRCh38) using HISAT2, and gene expression quantification was performed using Stringtie and the Ensembl reference transcriptome V.84. Differential gene expression was performed with GFold, and TRUST4 was employed for immune repertoire analysis.

Immunofluorescence experiments

Multiplex immunofluorescence analysis was conducted on baseline and on-treatment biopsies. Full details of antibodies used, antigen retrieval techniques, antibody dilutions and incubation times can be found in online supplemental table 1 and in the online supplemental methods.

Promoter methylation

Post-treatment tumor DNA (350 ng) was used for bisulfite conversion (Zymo D5001). Bisulfite converted DNA was used as template for PCR amplification of the NY-ESO-1 promoter region (for primers, see online supplemental methods). The PCR product was cloned into a pCR4-TOPO linearized vector via the TOPO TA Cloning kit (Thermo K4575J10) and transformed into TOP1

chemically competent bacteria. Plasmid DNA was isolated from 20 colonies, 19 of which had an identity of >25% to the target sequence and were thus used for analysis.

RESULTS

Clinical course

The patient was in their 40s and diagnosed with a high-grade undifferentiated pleomorphic sarcoma (HGUPS) of the right thigh with pulmonary metastases. The sarcoma was refractory to standard-of-care chemotherapy (ifosfamide and doxorubicin), radiation therapy as well as radical resection of the right thigh mass. The patient was HLA-A*0201-positive, and the tumor was NY-ESO-1-positive (figure 1A). At the time of enrollment in the clinical trial, the baseline positron emission tomography (PET)-CT showed disease progression in the lungs and thigh. Adoptive cell transfer of NY-ESO-1 TCR-transduced T cells occurred on April 11, 2018. Following ACT, the patient received a combination of low-dose IL-2, nivolumab, and DC vaccine loaded with NY ESO-1 peptide per study protocol (figure 1A). IL-2 administration was held two times for sinus tachycardia, but treatment was otherwise well tolerated. PET-CT at day 22 showed partial response by RECIST V.1.1. Notably, there was uniform improvement in pulmonary disease burden (figure 1B,C) and significant improvement in the size of the right lateral thigh and inguinal mass (figure 1D,E). Repeat CT scan in June showed continued response at

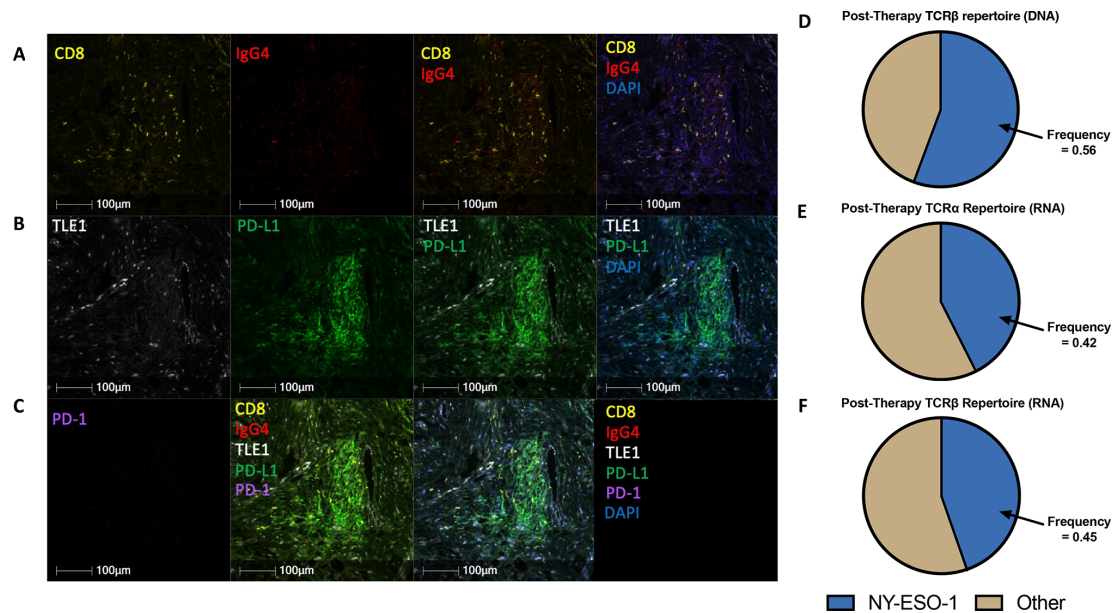


Figure 3 Nivolumab and NY-ESO-1 transgenic TCR track to the site of the tumor. (A) IHC of the patient's tumor at the time of biopsy for CD8, IgG4, and 4',6-diamidino-2-phenylindole (DAPI). (B) Same as in (A) but for sarcoma marker TLE1, PD-L1, and DAPI. (C) Same as in (A) but for sarcoma marker PD-1 alone, a combination of CD8, IgG4, TLE1, PD-L1, PD-1, or the same combination with DAPI. (D) Post-therapy TCRβ repertoire as determined by deep sequencing of TCRβ alleles using genomic DNA from post-therapy tumor biopsy. Blue represents the frequency of NY-ESO-1 TCRβ alleles; beige represents the frequency of all other sequenced TCRβ alleles. (E) Post-therapy TCRα repertoire as determined by repertoire reconstruction using bulk RNA-sequencing from post-therapy tumor biopsy. Blue represents the frequency of NY-ESO-1 TCRα alleles; beige represents the frequency of all other sequenced TCRα alleles. (F) Same as in (E) except for TCRβ repertoire. IHC, immunohistochemistry; TCR, T-cell receptor.

the right proximal thigh/inguinal mass (figure 1F), and the patient was able to return to work. A post-treatment biopsy was performed on day +76. Unfortunately, the patient developed new right thigh pain near the end of July, at which point a CT scan showed progressive disease (figure 1G). The patient continued nivolumab through August, though they were removed from the trial and started on pazopanib following continued progression. Pazopanib failed to stop further disease progression, and the patient passed away in early 2019.

Immunophenotyping of the peripheral T cells shows predominantly effector memory (EM) phenotype over time

Consistent with previous work from our laboratory for patients on different ACT protocols,^{3,6} TCR transgenic cell frequency was determined to peak within 2 weeks of ACT, after which the percentage and absolute number of TCR transgenic cells in peripheral blood decreased (figure 2A). Less differentiated memory T cells are the preferred population, given the fact they have superior in vivo expansion, persistence, and antitumor activity.^{15,16} The infusion products for the patient contained large proportions of effector memory (EM; CCR7⁺/CD45RA⁻) and effector memory re-expressing CD45RA (EMRA; CCR7⁺/CD45RA⁺) (figure 2B) cells. However, in contrast to prior work from our laboratory,⁶ the proportion of these less differentiated phenotypes persisted throughout treatment for this patient instead of progressing to a more terminally differentiated effector phenotype (figure 2B).

This persistence of less differentiated phenotypes was also noted when analyzing the total CD8⁺ T-cell population (online supplemental figure 2A).

Peripheral T cells showed no evidence of exhaustion and confirmed nivolumab-mediated PD-1 blockade

A challenge associated with ACT involves exhaustion of the adoptively transferred T-cell population due to chronic antigen stimulation. The adoptively transferred T cells in this work showed no evidence of significant exhaustion throughout treatment (figure 2C). To confirm PD-1 blockade on adoptively transferred lymphocytes, PD-1-receptor occupancy was estimated by analyzing the percentage of PD-1/IgG4 on CD8⁺ T cells (figure 2D) by means of flow cytometry. Nivolumab is a human IgG4 monoclonal antibody, and as such, surface IgG4 binding can be used as a measure of nivolumab saturating the PD-1 receptor.¹⁷ By day 4 of treatment, a significant percentage (~20%) of NY-ESO-1 CD8⁺ T cells (figure 2D,E) and the overall CD8⁺ T-cell population (online supplemental figure 2B,C) had evidence of nivolumab binding as measured by IgG4 positivity (figure 2D,E). The PD-1⁻/IgG4⁺ population was significantly greater than the PD-1⁺/IgG4⁺ population throughout the course of treatment. Additionally, the PD-1⁻/IgG4⁺ population was significantly greater than the PD-1⁺/IgG4⁻ population at all time points while the patient was enrolled on the trial except for days 1 and 161 (figure 2D,E). The patient received the first nivolumab dose on day 1, and thus, at

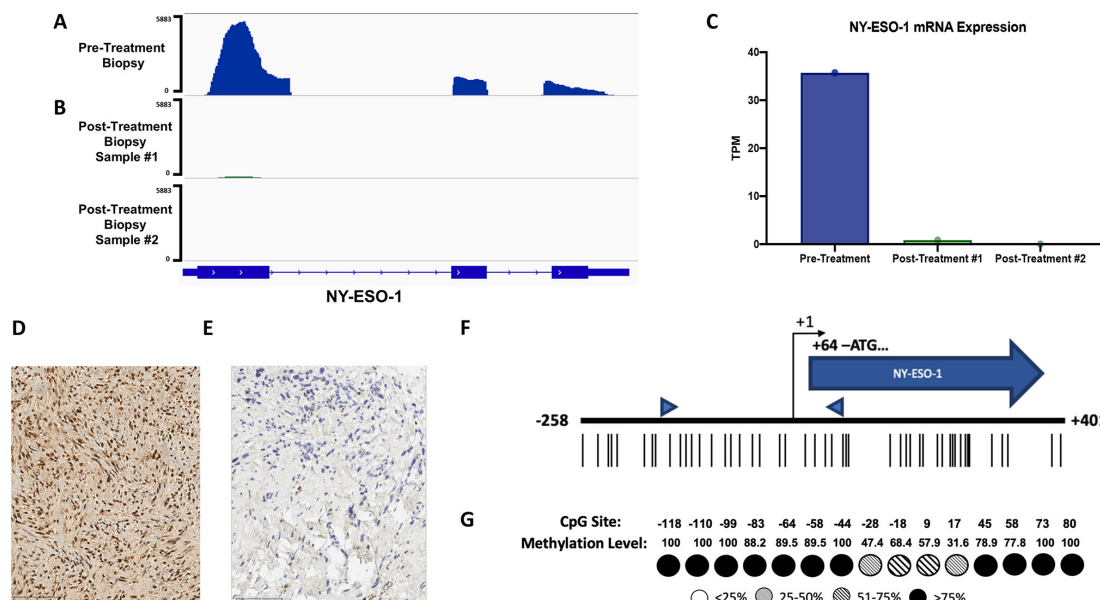


Figure 4 NY-ESO-1 antigen expression is lost following ACT treatment. (A) Histogram of mapped reads corresponding to NY-ESO-1 expression in the pretreatment biopsy sample. (B) Same as in (A) but for two post-treatment biological replicates. Scale is normalized to pretreatment maximum. (C) NY-ESO-1 expression (TPM) from the pretreatment biopsy and both post-treatment biological replicates. (D) IHC for NY-ESO-1 using pretreatment tumor biopsy. (E) Same as in (D) but for post-treatment tissue. (F) Genomic structure of the NY-ESO-1 promoter region and transcriptional start site. Bent arrow represents the +1 TSS; blue arrow indicates CDS; blue arrowheads represent bisulfite primer sites; upright lines represent CpG dinucleotides. (G) Analysis of methylation status at CpG dinucleotides in the NY-ESO-1 promoter region. CpG sites assayed for methylation are depicted (top) and numbered relative to the TSS. Methylation level for the aggregate experiments quantified for each site (middle). Schematic depiction of the aggregate methylation level depicted (bottom). ACT, adoptive cell therapy; CDS, coding sequence; CpG, cytosine-phosphate-guanine; IHC, immunohistochemistry; TPM, transcript per million; TSS, transcription start site.

this time, there were presumably low levels of circulating antibody. By day 161, the patient had been removed from the trial and had not received a nivolumab dose in over 3 weeks. As such, our data indicate limited exhaustion and effective and efficient binding of PD-1 by nivolumab on adoptively transferred lymphocytes.

Nivolumab and NY-ESO-1 transgenic TCR track to the site of the tumor

We next used immunohistochemistry (IHC) to confirm the presence of tumor-infiltrating CD8⁺ T cells at the tumor site in the post-treatment biopsy. We found that CD8⁺ T cells were present at the site of the tumor, and further, IgG4 partially colocalized with CD8 staining, indicating nivolumab binds CD8 T cells at the tumor (figure 3A). We then stained for PD-L1 with TLE1 serving as our tumor marker to confirm strong expression of PD-L1 by tumor cells (figure 3B). Finally, we stained for PD-1, which was negative, allowing us to conclude effective and efficient binding of PD-1 by nivolumab at the tumor site (figure 3C). To confirm the presence of the transgenic TCR at the tumor site, we performed TCRseq of the on-treatment biopsy sample. TCRseq revealed that the major clone in the on-treatment biopsy is the NY-ESO-1 transgenic TCR (figure 3D). Analysis of clinical transgenic ACT products in vivo has shown that while the transgenic TCR may exist at the DNA level, epigenetic suppression can lead to profound decreases in the levels of TCR RNA and protein expression.¹⁸ To confirm RNA

expression of the NY-ESO-1 TCR, we analyzed on-treatment bulk RNA sequencing of the tumor and employed the TRUST4 algorithm¹⁹ to reconstruct the immune repertoire from bulk RNA-seq samples. Both the α -chain and β -chain of the NY-ESO-1 transgenic TCR were the most abundant in the sample, making up nearly half of the TCR repertoire (figure 3E,F). Of note, no NY-ESO-1 TCR was detected in the pretreatment biopsy (online supplemental figure 3AB). Further, repertoire clonality dramatically increased (online supplemental figure 3CD) and repertoire entropy significantly decreased in the post-treatment sample (online supplemental figure 3EF). Thus, we conclude that adoptively transferred NY-ESO-1 T cells localized to the tumor and continued to express their transgenic TCR.

NY-ESO-1 antigen expression is lost following ACT treatment in the setting of promoter methylation

The patient's tumor was noted to be NY-ESO-1-positive on enrollment for the trial, and indeed, our pretreatment RNA-sequencing sample confirmed NY-ESO-1 was significantly expressed (figure 4A). However, gene expression analysis of the post-treatment biopsy showed a transcript per million value less than 1, indicating a complete loss of NY ESO-1 expression (figure 4B,C and online supplemental figure S4A). In fact, NY-ESO-1 was one of the most downregulated genes when comparing expression both pretreatment and post treatment (online supplemental figure 4B). This loss of NY-ESO-1 was confirmed at the

protein level as IHC was positive in the tissue from the pretreatment biopsy (figure 4D) and negative in the tissue from the post-treatment biopsy (figure 4E). Given that DNA demethylation is a primary mechanism underlying aberrant re-expression of cancer-testis antigens like NY-ESO-1,²⁰ we hypothesized that promoter methylation might play a role in the post-treatment loss of NY-ESO-1 expression. To probe for this, we performed bisulfite sequencing on a region of the NY-ESO-1 promoter (figure 4F). We found that the NY-ESO-1 promoter was extensively methylated in the post-therapy sample (figure 4G). This strongly implies that disease relapse occurred in the setting of loss of NY-ESO-1 expression driven by extensive methylation of its promoter region during therapy.

DISCUSSION

While transgenic ACT has been demonstrated to be a potent form of cancer immunotherapy, clinical responses are often transient with patients frequently developing progressive disease despite an initial clinical response to treatment. Here, we describe novel mechanism of immune escape in sarcoma whereby NY-ESO-1 expression was lost in response to TCR transgenic ACT with DC vaccination and PD-1 blockade.

With respect to this case, the patient with HGUPS had an initial partial response to therapy with uniform improvement in pulmonary disease burden and significant improvement in size of the right lateral thigh and inguinal mass. The biopsy showing NY-ESO-1 loss was performed at the end of June, a few weeks prior to the development of significant disease progression. The timing of disease progression in relation to the biopsy suggests loss of NY-ESO-1 expression as the primary mechanism for resistance to therapy. While loss of expression has been documented in NY-ESO-1-targeted melanoma trials,²¹ this is a heretofore unexplored mechanism of resistance in sarcoma. It is possible that this loss of expression is driven by evolution of a clone that initially expressed NY-ESO-1. However, it is also possible that the adoptively transferred lymphocytes were very effective at targeting immunogenic, NY-ESO-1-expressing clones, leaving behind a population of non-immunogenic clones lacking NY-ESO-1 expression. As to why this mechanism of resistance has not previously been seen in sarcoma, it is important to note that prior trials have targeted synovial sarcoma (SS).^{6–8} It might be that the characteristic SS18/SSX fusion mediating the epigenetic dysregulation in SS drives more durable expression of NY-ESO-1.²² Alternatively, the oncogenic program in SS has been shown to actively repress immunogenicity,²³ and thus, it is possible adoptively transferred lymphocytes are less effective at completely eliminating NY-ESO-1-expressing populations in SS.

The finding that antigen loss can lead to disease progression in sarcoma highlights the importance of developing new technologies that prevent this mechanism of therapy

resistance. One possible approach would be combinatorial targeting of antigens, an approach most notably being performed with chimeric antigen receptor T-cell (CAR-T) therapies in leukemia.²⁴ Another approach involves inducing durable expression of NY-ESO-1. As expression of NY-ESO-1 is known to be regulated by methylation,²⁰ the addition of a hypomethylating agent like the azanucleosides azacytidine and decitabine could induce durable expression of NY-ESO-1 in tumors exposed to NY-ESO-1-targeted therapies and, as such, could prevent or delay disease progression. Similarly, this approach could be pursued by other therapies which rely on a single molecular target to maintain tumor homogeneity.

With respect to the adoptively transferred lymphocytes, less differentiated memory T cells are the preferred population due to the fact they have been shown to have superior in vivo expansion, persistence, and antitumor activity when compared with the more terminally differentiated effector T-cell subsets.^{15 16} In this patient, we found they had a predominantly EM phenotype over time. This contrasts with our previous experiences with transgenic ACT in combination with DC vaccination where adoptively transferred cells displayed a shift toward more terminally differentiated phenotypes.^{3 6} It is possible that integration sites within the host cell's genomes might have affected the differentiation of adoptively transferred lymphocytes, a phenomenon previously reported in the generation of CAR-T cells.²⁵ However, it has previously been shown that there is a marked accumulation of CD8⁺ EM cells in lymphoid organs and tissues of PD-1-deficient mice,²⁶ and further, patients who responded to PD-1 blockade had tumor-infiltrating lymphocytes (TILs) that displayed a predominantly EM phenotype.²⁷ Given the addition of nivolumab to the regimen for the patient in this work, the sustained predominately EM phenotype is consistent with the anticipated mechanism of action of therapy.

In conclusion, ACT of NY-ESO-1 TCR transgenic T cells combined with DC vaccination and anti-PD-1 therapy resulted in transient antitumor activity. Adoptively transferred lymphocytes displayed a predominantly EM phenotype without progression to exhaustion/terminal differentiation. On-treatment biopsy showed complete loss of NY-ESO-1 expression with extensive methylation of the promoter sequence. This work highlights a new mechanism of treatment resistance in sarcoma that must be considered in efforts for ACT to have a durable antitumor response.

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Contributors Conception, design, and study supervision: LF, AS, AR, and TSN. Acquisition of data and writing, review, and/or revision of the manuscript: LF, AS, CP, BC-A, BB-M, MM, KS, CQ, PK-L, IBC, AR, and TSN. Analysis and interpretation of data (eg, statistical analysis, biostatistics, and computational analysis): LF, CP, BC-A, and TSN. Guarantor: TSN.

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Competing interests TSN received honoraria from consulting with Allogene Therapeutics, PACT Pharma, and Adaptive Biotechnologies. AR received honoraria from consulting with Amgen, Bristol-Myers Squibb, Chugai, Genentech, Merck, and Novartis; is or has been a member of the scientific advisory board; and holds stock in Advaxis, Arcus Biosciences, Compugen, CytomX, Five Prime, RAPT, Highlight, ImaginAb, Isoplexis, Kite-Gilead, Lutris Pharma, Merus, Rgenix, and Tango Therapeutics. AS received honoraria from consulting with Daiichi Sankyo, Aadi Biosciences, and Deciphera; is on the board of directors; holds stock in Certis Oncology Solutions; and has provided institutional support for clinical trials for RAIN Therapeutics, Ayala Therapeutics, and Tracoon.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants who gave informed consent to participate in the study before taking part. The patient was non-randomly enrolled in the trial after signing a written informed consent approved by the UCLA Institutional Review Board (#15-01 433) under an investigational new drug (IND#15167) for the NY-ESO-1 T-cell receptor.

Provenance and peer review Not commissioned; externally peer reviewed.

All data relevant to the study are included in the article or uploaded as supplemental information. All sequencing data are deposited in the Database of Genotypes and Phenotypes and are available through dbGaP accession code phs002762.

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Supplementary Methods

Study Ethics

The study was conducted in accordance with local regulations, the guidelines for Good Clinical Practice, and the principles of the current version of the Declaration of Helsinki, and the study protocol and its amendments were approved by the University of California, Los Angeles, Institutional Review Board (UCLA IRB #15-001433).

Trial Eligibility

The patient was had to be HLA-A*0201 by high-resolution molecular phenotyping, with locally advanced or metastatic solid tumor, and with no available standard therapeutic options. The patient's tumor was screened for NY-ESO-1 positivity by IHC. Other important inclusion criteria included the fact the patient had to be >16 years of age, have an ECOG performance status of 0 or 1, have adequate organ function required to receive systemic IL2, be seronegative for Hepatitis B/C and HIV, and the patient must have had at least 1 lesion amenable for outpatient biopsies.

Exclusion criteria included but were not limited to patients with active brain metastases, bone marrow involvement on CT or PET at time of screening, the patient could not have received systemic treatment for cancer within one month prior to initiation of dosing within protocol nor could they have received 3 or more prior myelotoxic treatment regimens, and they could not have a history of, or significant evidence of risk for, chronic inflammatory or autoimmune disease.

Manufacture of NY-ESO-1 TCR transgenic lymphocytes

Transgenic lymphocytes were manufactured as described previously.¹ Briefly, the clinical grade retroviral vector expressing NY-ESO-1 TCR was manufactured at the Indiana University Viral Production Facility based on a master cell bank that was provided by Drs. Steven A. Rosenberg and Paul F. Robbins from the Surgery Branch, National Cancer Institute (NCI). This vector was previously used in a clinical trial run by the NCI² and there have been multiple clinical trials using adoptively transferred T cells specific for NY-ESO-1(HLA-A*0201) in the setting of advanced malignancies (e.g. NCT01343043³, NCT00871481⁴).

A non-mobilized leukapheresis processing two plasma volumes from the study patient was processed in the UCLA Human Gene and Cell Therapy Facility. PBMCs were isolated by Ficoll gradient centrifugation and cultured in AIM V media (Gibco, Invitrogen, Chicago, IL) supplemented with 5% human AB serum (Omega Scientific, Tarzana, CA) in the presence of 50 ng/ml anti-CD3 (OKT3,

Miltenyi Biotec, Auburn, CA) and 300 IU/mL IL-2 to stimulate T-cell growth to prepare for viral vector transduction. Activated PBMC were then infected by the clinical grade MSGV1-A2aB-1G4A-LY3H10 retroviral vector supernatant using retronectin-coated plates (Retronectin, Takara, Otsu, Shiga, Japan) for two consecutive days, maintained in culture for 4 days from the start of transduction in IL-2, and then infused fresh into the trial subject. In-process and final product testing included Gram stain, fungal stain, sterility culture for bacteria and fungus, mycoplasma assay (MycAlert assay, Lonza, Walkersville, MD), and endotoxin assay (Endosafe PTS system, Charles River, Charleston, SC). Transduction efficiency was tested with MHC dextramer analysis for NY-ESO-1 (Immudex, Copenhagen, Denmark) gated in CD3 (BD Bioscience, Franklin Lakes, NJ) positive lymphocytes. Finally, the potency of the NY-ESO-1 TCR transgenic cells was assessed using a co-culture system to detect NY-ESO-1- specific IFN- γ production by ELISA.

Manufacture of NY-ESO-1 peptide-pulsed dendritic cells

DC vaccines were manufactured as described previously¹. Briefly, autologous DC were differentiated from adherent peripheral blood monocytes in a one-week *in vitro* culture in media containing 5% heat-inactivated autologous plasma supplemented with GM-CSF and IL-4 as previously^{1,5,6}. Phosphor activation of STAT-6 and STAT-5 was used to measure the functionality of IL-4 and GM-CSF respectively⁷. DC were pulsed with the NY-ESO-1₁₅₇₋₁₆₅ anchor-modified immunodominant peptide (Biosynthesis Lewisville, TX) in the HLA-A2*0201 haplotype and administered intradermally. In-process and final product testing included Gram stain, fungal stain, sterility culture for bacteria and fungus, mycoplasma assay and endotoxin assay.

Flow Cytometry Phenotyping Analysis:

Antibodies against CD3, CD8, CD4, IgG4, CD279(PD-1), CCR7 and CD45RA (Biolegend, San Diego), CD45RA, as well as 7-Aminoactinomycin D (Beckman Coulter) or SYTOXX®AADvanced (Thermo Fisher Scientific, Waltham, MA). NY-ESO-1 HLA-A*0201(SLLMWITQV) dextramers and negative controls were purchased from Immudex.

For T-cell phenotypic classification, we defined naïve T-cells as CCR7⁺/CD45RA⁺, central memory T-cells as CCR7⁺/CD45RA⁻, effector memory T-cells as CCR7⁻/CD45RA⁻, and effector memory RA/effector T-cells as CCR7⁻/CD45RA⁺. Coexpression of CD39⁺ PD1⁺ was used to phenotype exhausted T cells.⁸ All flow data analyses were done with FlowJo (Tree Star Inc., Asland, OR).

Immunofluorescence Experiment:

Multiplex immunofluorescence analysis was conducted on baseline and on-treatment biopsies. Serial sections from patient tumor samples were deparaffinized and rehydrated with a series of graded ethanols to deionized water on a BOND RX platform (Leica Biosystems). Full details of antibodies used, antigen retrieval techniques, and antibody dilutions and incubation times are summarized in Supplemental Table 1. Briefly, antigen retrieval was performed in either an EDTA based pH 9 buffer (ER2) or a citrate-based pH 6 buffer (ER1). Then, slides were then serially stained with anti-CD8 clone C8/144B (1:100, DAKO), anti-PD-1 clone NAT105 (1:50; Cell Marque), anti-PD-L1 clone SP142 (1:100; SpringBio), anti-IgG4 clone HP6205 (1:200, Millipore), which served as a marker of nivolumab, or anti-TLE1 clone ab183742 (1:2,000; Abcam), which served as a specific nuclear marker for undifferentiated pleomorphic sarcoma tissue. TSA-based Opal method was used in this study for multiplex immunofluorescence (mIF) staining (Opal Polaris 7-Color Automation IHC Kit; Akoya Biosciences). Since TSA and DAB oxidation are both peroxidase-mediated reactions, the primary antibody conditions and order of staining determined using DAB detection were directly applied to the fluorescent assays, unlike conventional IHC wherein a chromogenic peroxidase substrate is used for antigen detection, each antibody is paired with an individual Opal fluorophore for visualisation. The Opal fluorophores were used at a 1:150 dilution. As such, a fluorescent singleplex was performed for each biomarker and compared to the appropriate chromogenic singleplex to assess staining performance.

Once each target was optimized in uniplex slides, the Opal multiplexed assay was used to generate multiple staining slides. Primary antibodies were applied to normal human tonsil specimens as controls at optimized concentrations previously determined on the uniplex control tissues. Slides Staining was performed consecutively Leica BOND RX by using the same steps as those used in uniplex IF, and the detection for each marker was completed before application of the next antibody. The sequence of antibodies for multiplex staining was determined for panel combination is: CD8 (opal 480), PDL1 (opal 520), PD1 (opal570), IgG4 (opal 620), TLE1 (opal 690), and spectral DAPI (Invitrogen).

All fluorescently labelled slides were scanned on the Vectra Polaris (Akoya Biosciences) at 40x magnification using appropriate exposure times. Sections that did not have any antibodies or fluorescent labeling were used to capture the background tissue autofluorescence. Prior to analysis, all images were assessed for quality control. Criteria for rejection included poor tissue quality, (e.g. folded tissue, missing sections) or staining artifacts (e.g. air bubbles, signal dropout, or inadequate washing). The data from the multispectral camera were analyzed by the imaging InForm software (Akoya Biosciences) and Halo (Indica Labs).

Immune Repertoire Analysis:

Immune repertoire analysis was performed using the TRUST4 algorithm.⁹ The frequency, or proportion of read count, was used to rank abundancy of the TCR α and TCR β chains. Clonality (1 - Shannon entropy/log(N), where N is the number of unique clones) and Shannon entropy calculations were performed on the reconstructed repertoires.

Promoter Methylation:

The following primers were used to amplify the NY-ESO-1 promoter region from -119 to +84:

Fwd: 5'-ACGTGTTTCGGGGTTTATTCGG

Rev: 5'-CCCCGACCTTCGACCTACATAAC

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A.

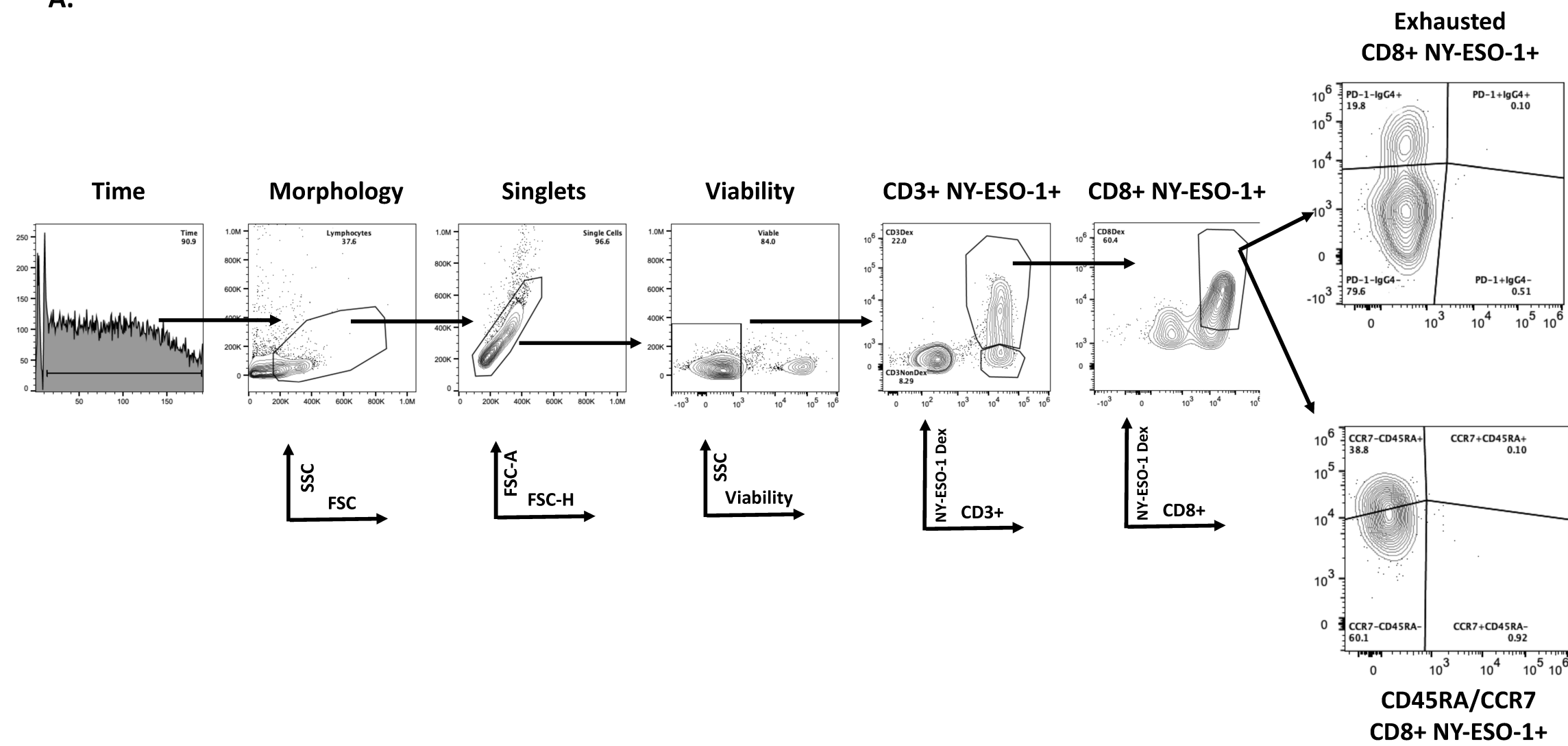


Figure S1

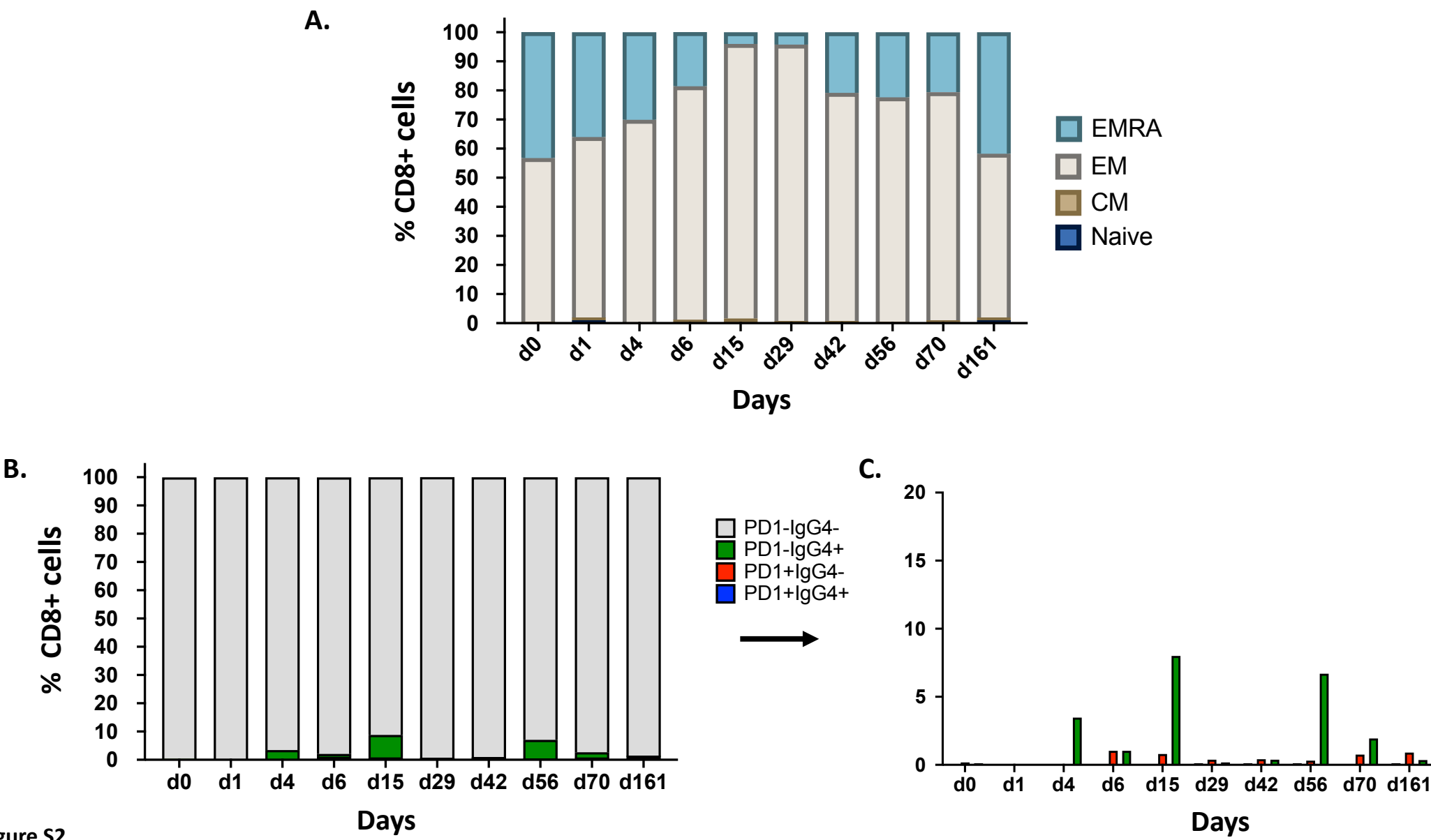
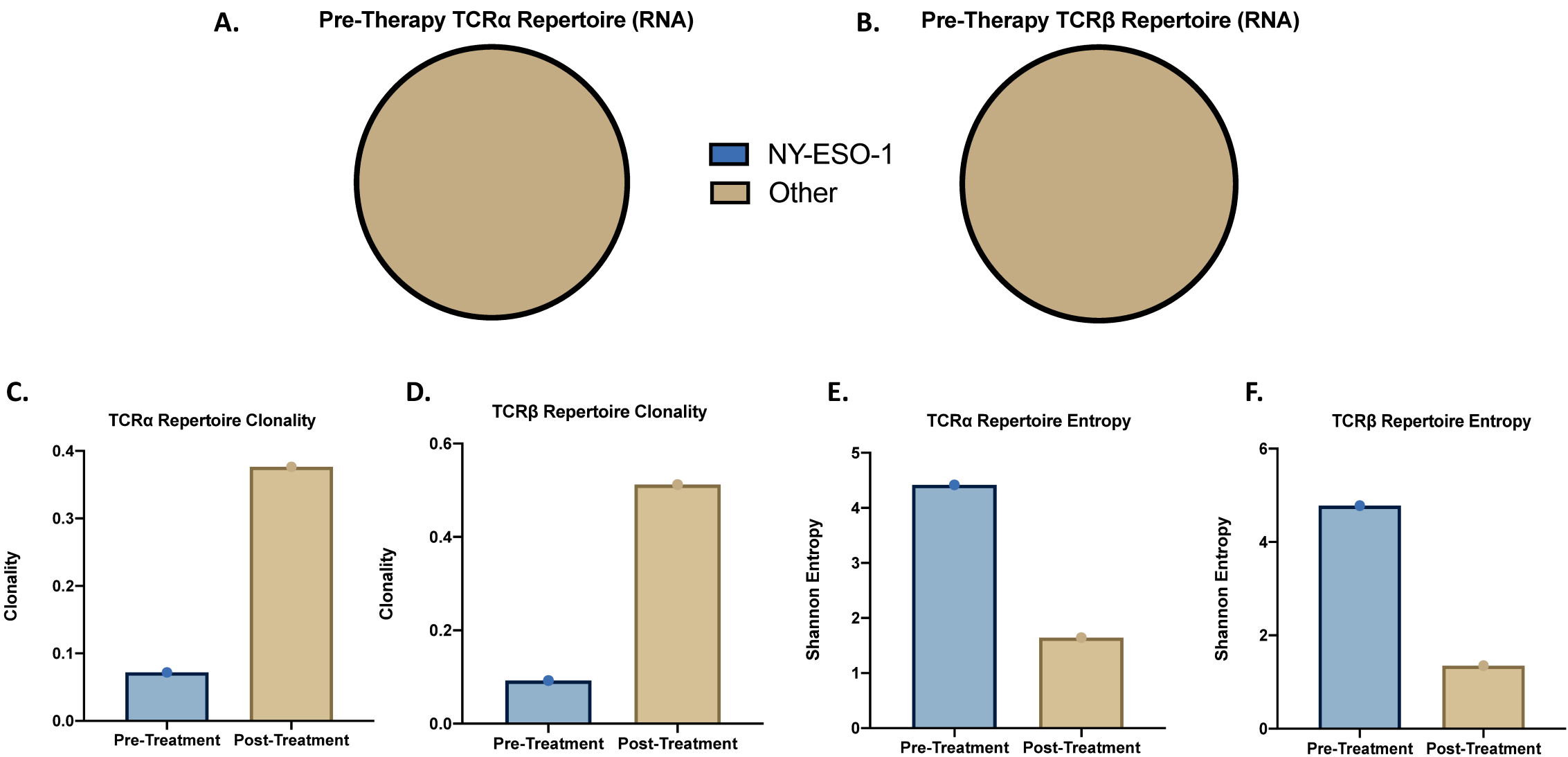


Figure S2



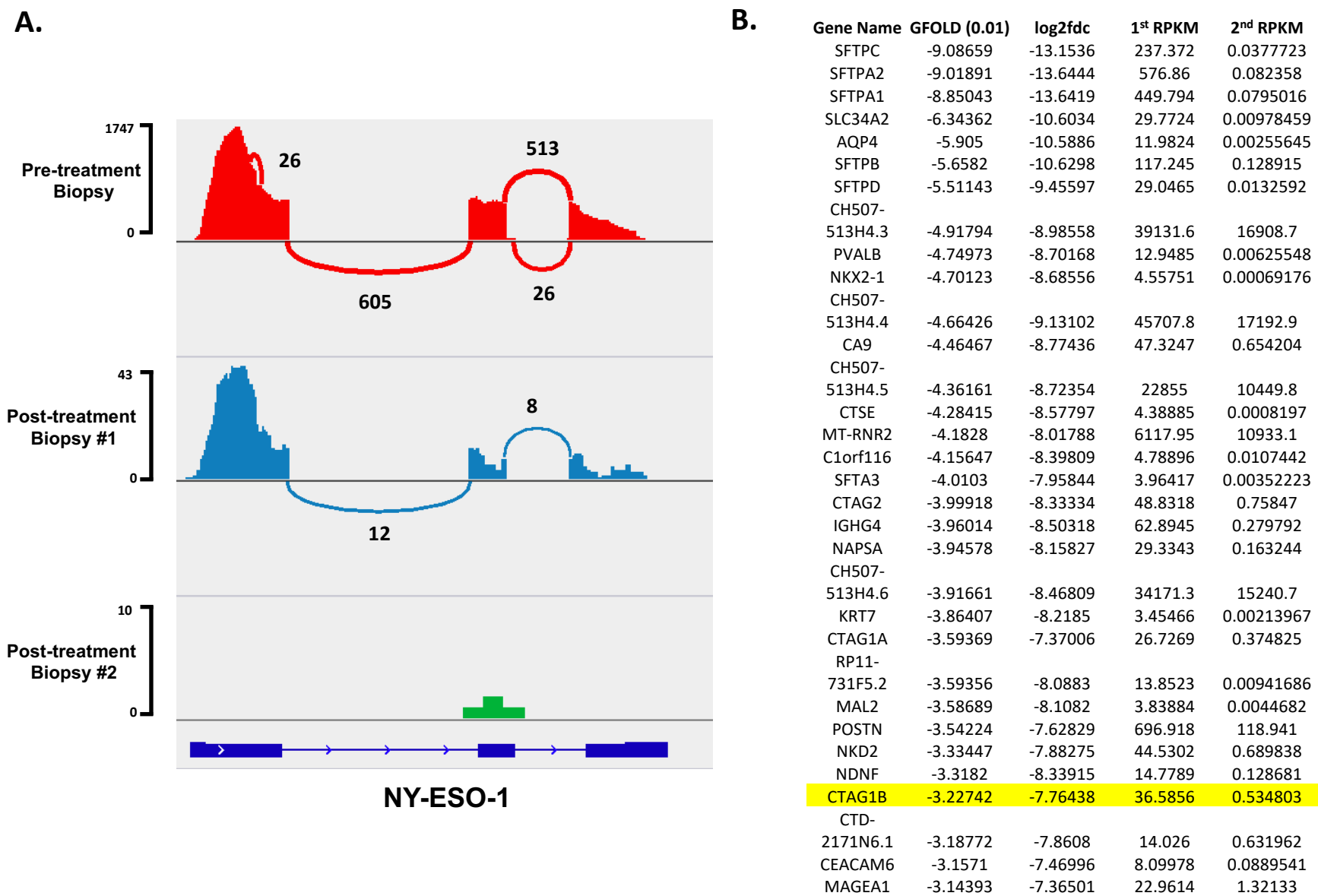


Figure S4.

Reagent	Fluorochrome	Clone	Vendor
CD3	AlexaFluor 488	UCHT1	Biolegend
Negative Dextramer HLA-*0201	PE	N/A	Immudex
NYESO-1 (HLA-A-*201) (SLLMWITQV) Dextramer	PE	N/A	Immudex
CD19	PE/Cy5	HIB19	Biolegend
CD19	PE	HIB19	Biolegend
OneComp eBeads Compensation Beads	N/A	N/A	ThermoFisher
SYTOX AADVANCED DEAD CELL	N/A	N/A	ThermoFisher
IgG4	AlexaFluor 647	HP6025	Southern Biotech
Mouse IgG1- Isotypec control for IgG4	AlexaFluor 647	15H6	Southern Biotech
CD45RA	APC-FCy7	HI100	Biolegend
CD279 (PD-1)	Brilliant Violet 650	MIH4	BDBiosience
CD197(CCR7)	Brilliant Violet 711	G043H7	Biolegend
CD8	Brilliant Violet 785	RPA-T8	Biolegend
CD39	Brilliant Violet 421	A1	Biolegend

APC – Allophycocyanin; PE-phycoerythrin

Supplementary Table 1

<u>Antibody</u>	<u>Vendor</u>	<u>Cat</u>	<u>Clone</u>	<u>Antibody Dilution</u>	<u>Antigen retrieval</u>	<u>Incubation time</u>	<u>Opal dye</u>
IgG4	Millipore	411492	HP6025	1:200	ER2 20min	30 mins	opal 620
CD8	DAKO	M7103	C8/144B	1:100	ER2 20min	30 mins	opal 480
PD-1	Cell Marque	NAT105 315m-96	NAT105	1:50	ER2 30min	30 mins	opal 570
PDL-1	Spring/	M4420/	SP142	1:100	ER2 30min	30 mins	opal 520
	Abcam	ab228462					
TLE1	Abcam	ab183742	EPR9386(2)	1:2000	ER1 20min	30 mins	opal 690

Supplementary Table 2