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Authors

Nowicki, Theodore S
Berent-Maoz, Beata
Cheung-Lau, Gardenia
et al.

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A Pilot Trial of the Combination of Transgenic NY-ESO-1-reactive Adoptive Cellular Therapy with Dendritic Cell Vaccination With or Without Ipilimumab

Theodore S. Nowicki[1], **Beata Berent-Maoz**[2], **Gardenia Cheung-Lau**[2], **Rong Rong Huang**[3], **Xiaoyan Wang**[4], **Jennifer Tsoi**[2], **Paula Kaplan-Lefko**[2], **Paula Cabrera**[2], **Justin Tran**[2], **Jia Pang**[2], **Mignonette Macabali**[2], **Ivan Perez Garcilazo**[2], **Ignacio Baselga Carretero**[2], **Anusha Kalbasi**[5,6,7], **Alistair J. Cochran**[3], **Catherine S. Grasso**[2], **Siwen Hu-Lieskovan**[2], **Bartosz Chmielowski**[2], **Begoña Comin-Anduix**[6,7,*], **Arun Singh**[2,*], and **Antoni Ribas**[2,6,7,8,9]

[1.] Division of Pediatric Hematology-Oncology, Department of Pediatrics, University of California Los Angeles, Los Angeles, California.

[2.] Division of Hematology-Oncology, Department of Medicine, University of California Los Angeles, Los Angeles, California.

[3.] Department of Pathology, University of California Los Angeles, Los Angeles, California

[4.] Department of General Internal Medicine and Health Services Research, University of California Los Angeles, Los Angeles, California.

[5.] Division of Molecular and Cellular Oncology, Department of Radiation Oncology, University of California Los Angeles, Los Angeles, California

[6.] Jonsson Comprehensive Cancer Center, Los Angeles, California.

[7.] Division of Surgical-Oncology, Department of Surgery, University of California Los Angeles, Los Angeles, California.

[8.] Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, University of California Los Angeles, Los Angeles, California.

[*]Co-corresponding authors. **Corresponding authors:** Arun Singh, M.D.; Jonsson Comprehensive Cancer Center (JCCC) at the University of California Los Angeles (UCLA); 2020 Santa Monica Blvd Suite 580, Santa Monica, California 90404 Phone: 310-829-5471; Fax: 310-829-6192; asingh@mednet.ucla.edu, Antoni Ribas, M.D., Ph.D.; Jonsson Comprehensive Cancer Center (JCCC) at the University of California Los Angeles (UCLA); 9-954 Factor Building, 10833 Le Conte Avenue, Los Angeles, CA, 90095. Phone: 310-206-3928; Fax: 310-825-2493; aribas@mednet.ucla.edu.

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[9.] Department of Molecular and Medical Pharmacology, University of California Los Angeles, Los Angeles, California.

Abstract

Background: Transgenic adoptive cell therapy (ACT) targeting the tumor antigen NY-ESO-1 can be effective for the treatment of sarcoma and melanoma. Preclinical models have shown that this therapy can be improved with the addition of dendritic cell (DC) vaccination and immune checkpoint blockade. We studied the safety, feasibility, and antitumor efficacy of transgenic ACT with DC vaccination, with and without CTLA-4 blockade with ipilimumab.

Methods: Freshly prepared autologous NY-ESO-1-specific T-cell receptor (TCR) transgenic lymphocytes were adoptively transferred together with NY-ESO-1 peptide-pulsed DC vaccination in HLA-A2.1-positive subjects alone (ESO, NCT02070406) or with ipilimumab (INY, NCT01697527) in patients with advanced sarcoma or melanoma.

Results: Six patients were enrolled in the ESO cohort, and four were enrolled in the INY cohort. Four out of six patients treated per ESO (66%), and two out of four patients treated per INY (50%) displayed evidence of tumor regression. Peripheral blood reconstitution with NY-ESO-1-specific T cells peaked within 2 weeks of ACT, indicating rapid *in vivo* expansion. Tracking of transgenic T cells to the tumor sites was demonstrated in on-treatment biopsies via TCR sequencing. Multiparametric mass cytometry of transgenic cells demonstrated shifting of transgenic cells from memory phenotypes to more terminally differentiated effector phenotypes over time.

Conclusions: ACT of fresh NY-ESO-1 transgenic T cells prepared via a short *ex vivo* protocol and given with DC vaccination, with or without ipilimumab, is feasible and results in transient antitumor activity, with no apparent clinical benefit of the addition of ipilimumab. Improvements are needed to maintain tumor responses.

Keywords

adoptive cell therapy; dendritic cell vaccine; ipilimumab; NY-ESO-1; melanoma; sarcoma

INTRODUCTION

Adoptive cell therapy (ACT) with genetically engineered T cells directed against tumor antigens utilizes the *ex vivo* gene transfer of a cancer antigen-specific T cell receptor (TCR) into a patient's T cells, which are then re-infused into the individual (1, 2). Studies pioneered by Rosenberg and colleagues at the National Cancer Institute (NCI) Surgery Branch demonstrated the utility of this approach against a variety of tumor antigens (3–5). While early efforts were directed against melanoma-specific antigens such as MART-1 and gp100 (3, 4, 6), later efforts have been directed against the cancer testis antigen NY-ESO-1 (7, 8), which is expressed in melanomas and various sarcoma subtypes, but not in normal somatic tissue (other than the testes) (9). These therapeutic approaches have been shown to induce objective tumor regression in a large proportion of patients, but these initial responses are often not sustainable, and patients frequently relapse. This underscores the need for better ACT approaches to sustain the antitumor efficacy.

Previous studies have shown that tumor antigen-specific dendritic cell (DC) vaccines can enhance the effectiveness of ACT in animal models by stimulating T cell expansion *in vivo* (10–13). Our group's previous clinical experience utilizing MART-1 TCR transgenic T cells co-administered with MART-1 peptide-pulsed DC vaccine (6) was both safe and feasible. Furthermore, we noted that patients receiving freshly infused transgenic cells displayed superior *in vivo* persistence of the cells and responsiveness to DC vaccine boost compared to those who received cryopreserved cell products (6, 14). We sought to further improve our ACT protocols in several key areas, including the use of NY-ESO-1 as a target antigen to reduce side effects associated with MART-1 as a target, as well as to expand to patients with other solid tumors. Furthermore, as an alternative to the high-dose IL-2 regimens routinely used by our group and the NCI following ACT, we utilized low-dose IL-2, given evidence that this is also effective at augmenting the persistence of adoptively transferred T cells with an improved toxicity profile (15, 16). Additionally, we developed a new protocol in parallel which combined NY-ESO-1 transgenic T cells and DC vaccines with the CTLA-4 immune checkpoint inhibitor ipilimumab, based on preclinical data that CTLA-4 blockade can augment ACT effectiveness in animal models (17, 18). Here we report the safety, feasibility, antitumor efficacy, and the *in vivo* cellular characteristics of dual cell therapy with transgenic NY-ESO-1 TCR T cells administered with NY-ESO-1 peptide-pulsed DC vaccine, with and without ipilimumab.

PATIENTS AND METHODS

Study Ethics and Conduct

Patients were non-randomly enrolled in one of two clinical protocols after signing a written informed consent approved by the UCLA Institutional Review Board (#12–000153 and #13–001624) under an investigational new drug (IND#15167) for the NY-ESO-1 TCR. The study was conducted in accordance with local regulations, the guidelines for Good Clinical Practice, and the principles of the Declaration of Helsinki. The studies had the clinical trial registration numbers NCT02070406 and NCT01697527.

Trial eligibility and screening procedures

Eligible patients were HLA-A*0201 by high-resolution molecular phenotyping, with locally advanced or metastatic solid tumors, and with either no available standard therapeutic options with curative intent, or progression on standard-of-care chemotherapy/radiotherapy regimens. Tumors were NY-ESO-1 positive by immunohistochemistry. Patients were >16 years of age, with a life expectancy of >three months, an ECOG performance status of 0 or 1, adequate organ function required to receive systemic IL-2 (19), and seronegative for Hepatitis B/C, and HIV. Patients with clinically active brain metastases were excluded. More than 4 weeks must have elapsed since any prior systemic steroid use or cancer therapy and associated toxicities.

Treatment Schedule

The treatment schedule is outlined in Figure 1. Patients underwent baseline leukapheresis for the manufacture of the NY-ESO-1 TCR transgenic T cells and the NY-ESO-1 peptide-pulsed DC vaccine (see Supplemental Methods). Patients were then admitted and received

lymphodepleting chemotherapy consisting of cyclophosphamide 60mg/kg/day x 2 days i.v. on days −5 and −4, and fludarabine 25mg/m^2/day x 4 days i.v from day −4 to −1. On day 0, patients received ACT of up to 10^9 transgenic TCR lymphocytes as an i.v. infusion of freshly prepared cells. Patients received their first dose of 1mg/kg ipilimumab (if on the INY protocol), intradermal administration of NY-ESO-$1_{157–165}$ peptide-pulsed DC, and began low-dose IL-2 therapy (500,000 IU/m^2 subcutaneously) the following morning (day +1) twice daily for up to 28 doses (patients ESO-1, ESO-2, ESO-3, ESO-4, INY-1, INY-2) or 14 doses (patients ESO-5, ESO-6, INY-3, and INY-4), as tolerated. Standard supportive care provided post-infusion included filgrastim (Neupogen; Amgen), antibiotics for neutropenic fever, and blood product transfusions as needed. When patients recovered peripheral neutrophil counts, and were no longer transfusion-dependent, they were discharged from the hospital. Patients continued to receive two more doses of DC vaccines at 2-week intervals, and 1mg/kg ipilimumab every three weeks for four total doses (if on the INY protocol). A research [^{18}F]FDG positron emission tomography-computed tomography (PET/CT) scan with IV contrast was performed on day +30, and formal restaging PET/CT scans with IV contrast were performed on day +90. Baseline and on-treatment biopsies were obtained when clinically indicated.

Safety assessments

Adverse events were analyzed following NCI CTCAE v3.0. The known toxicities/side effects of the preparative chemotherapy, IL-2, or filgrastim, as listed in the protocol or package insert, were not considered for the assessment of DLTs. Analysis of patient blood samples for potential replication competent retrovirus and cytokine release syndrome is described in the Supplementary Methods.

Assessment of feasibility

After entering two patients in each cohort, followed for a minimum of one month after the last subject had received the infusion of the NY-ESO-1 TCR transgenic cells, an assessment of protocol feasibility was performed by the study investigators. Both cell therapies, the NY-ESO-1 TCR engineered T cells and the NY-ESO-$1_{157–165}$ peptide-pulsed DC, required strict lot release criteria of the final product before administration (see Supplemental Methods). Feasibility was determined at every cohort, and was based on potential problems in the manufacturing of NY-ESO-1 TCR engineered lymphocytes or the NY-ESO-$1_{157–165}$ peptide-pulsed DC vaccines, or potential problems in the delivery of the combinatorial therapy with the addition of ipilimumab to the clinical protocol. If two patients could not receive the intended cellular therapies or immune checkpoint blockade, further accrual would not be warranted.

Assessment of antitumor activity

Quantification of changes in PET imaging for the intratumoral accumulation of [^{18}F]FDG within tumor sites and secondary lymphoid organs was performed by counting the total number of FDG avid lesions, as well as the maximum standardized uptake value (SUVmax), normalized to the body weight of the patient at baseline, on day +30, and day +90, along with assessment of initial response utilizing the CT portion of the PET/CT scan at day +30. The objective clinical response rate was assessed on day +90 utilizing the CT portion of the

PET/CT scan and recorded following modified Response Evaluation Criteria in Solid Tumors (RECIST1.1, (20)).

MHC dextramer immunologic monitoring

Detection of NY-ESO-1 TCR expression using fluorescent MHC dextramer analysis for NY-ESO-1 (Immudex, Copenhagen, Denmark) was performed on cryopreserved peripheral blood mononuclear cells (PBMCs) collected at different time points, as previously described (6, 21). Our previous definitions for a positive or negative immunologic response using standardized MHC multimer assays were used, which are based on the assay performance specifications by defining changes that were beyond the assay variability with a 95% confidence level (21).

Deep sequencing of TCRβ alleles

Genomic DNA was isolated from FFPE tumor biopsy shavings on an Anaprep 12 nucleic acid extraction platform (BioChain), and from FACS-sorted NY-ESO-1 dextramer-positive T cells from final manufacture products of all patients, and from patient-matched infusion products and post-infusion peripheral PBMCs recovered at day +70 (+/− 10 days) where available, with an AllPrep DNA/RNA isolation kit according to the manufacturer's instructions (Qiagen). TCRβ alleles were sequenced at 100,000 reads by Adaptive Biotechnologies (Seattle, WA). Productive TCRβ sequences, i.e. those that could be translated into open reading frames and did not contain a stop codon, were reported. Productive clonality and Shannon entropy calculations for each sample were generated as previously described (22). The transgenic NY-ESO-1 TCR was identified based on comparison of reads with the known TCRβ sequence for the transgenic product.

Immunohistochemistry

Serial sections from patient tumor samples were stained for NY-ESO-1, PD-L1, PD-1, CD8, and Major Histocompatibility Complex I (MHC I) at the UCLA Anatomic Pathology Immunohistochemistry Laboratory. Slides were deparaffinized and rehydrated with a series of graded ethanol to deionized water. For PD-L1, PD-1, CD8 and MHC-I immunostaining, antigen retrieval was performed in Tris-EDTA at pH 9 and slides were cooked at high pressure at 120ºC for 5 minutes. Immunostaining was performed on a Leica Bond III autostainer using Leica Bond ancillary reagents (Leica Biosystems). Antibodies used included anti-CD8 clone C8/144B (1/100, DAKO), anti–PD-L1 clone SP142 (1/200, Spring-Bio), anti–PD-1 clone NAT105 (1/50, Cell Marque), and anti-MHC-I clone EMR8–5 (1/100, Abcam). Antigen-antibody binding was visualized via the REFINE polymer 3,30 diaminobenzidine (DAB) detection system (Lieca). NY-ESO-1 immunostaining was performed using the anti–NY-ESO-1 clone E978 (1/50, Santa Cruz Biotechnologies). Antigen retrieval was performed in Tris-EDTA buffer at pH 8, slides were boiled at 100 ºC for 25 minutes and stayed in the buffer at room temperature for another 20 minutes. Antigen binding was visualized using Biotinylated Horse Anti-Mouse IgG Antibody, rat adsorbed (1/200, Vector Laboratories), followed by agarose streptavidin (1/1000, Vector Laboratories). DAB and hydrogen peroxide were used as the substrates for the peroxidase enzyme. All slides were counterstained with hematoxylin and cover slipped for subsequent analysis. All slides were scanned at an absolute magnification of 200X (0.5 mm/pixel) on an

Aperio ScanScope XT system and imported for analysis using the Indica Labs Halo platform as previously described (22, 23).

Mass Cytometry Antibodies

Metal conjugated antibodies were purchased from Fluidigm or conjugated to unlabeled antibodies at the UCLA Flow Cytometry Core. All conjugations were performed using X8 polymer as per manufacturer's protocol (Fluidigm) and were performed at 100 μg scale. Appropriate antibody dilution of custom conjugated antibodies was determined by serial dilution staining experiments with replicates of relevant biological samples (healthy donor human PBMCs) to minimize background and optimize detection of positively expressing populations. The mass cytometry staining panel (24) is detailed in Table S1. Surface and intracellular staining cocktail master mixes were prepared prior to each experiment. Transgenic cells identified using an anti-TCRVβ13.1 antibody (BioLegend) that recognizes the β chain of the 1G4-a95:LY anti-NY-ESO-1 transgenic TCR.

Mass Cytometry Data Acquisition

Cryopreserved PBMCs from infusion products and post-infusion peripheral PBMCs recovered at day +70 (+/− 10 days) were thawed, resuspended in pre-warmed RPMI with 10% serum and 1% penicillin/streptomycin fungizone media, and incubated with DNAse for 30 minutes at 37ºC. Cells were then spun down, washed in PBS, and resuspended at a concentration of $1–5\times10^6$ cells/mL in serum-free RPMI and live-dead stained with 5 μM Cisplatin (Fluidigm) for 5 minutes at 37ºC. Cells were washed and stained with a surface protein antibody cocktail for 30 minutes at room temperature, and were then washed and spun for 10 minutes at 4°C. Cells were then fixed using a FoxP3/transcription factor-specific fixation buffer (eBioscience) for 60 minutes at room temperature, followed by addition of the accompanying FoxP3/transcription factor permeabilization buffer, and were spun for 10 minutes at 4°C. Cells were then stained with an intracellular antibody cocktail for 60 minutes at room temperature. Finally, cells were incubated for 60 minutes at room temperature with 250 nM iridium intercalator (Fluidigm) to label cellular DNA. Cells were then washed with PBS, and finally with distilled water. Mass cytometry data acquisition was performed at the UCLA Flow Cytometry Core on a CyTOF2.1 (Helios) mass cytometer (Fluidigm).

Mass Cytometry Data Analysis

See Supplemental Methods.

Statistical Analysis

Graphing, heatmaps, and descriptive statistical analyses were carried out with GraphPad Prism version 7.0 (GraphPad, San Diego, CA). Unless otherwise indicated, two-tailed unpaired Student's t test was used for comparison of two groups. Log-transformation was performed on datasets if normality assumption was violated according to the Shapiro-Wilk test. P-values of <0.05 were considered statistically significant.

RESULTS

Patient characteristics

Between March 2013 and November 2016, six HLA-A*0201–positive patients with NY-ESO-1–positive tumors were accrued on the ESO protocol and four such patients were accrued on the INY protocol. The malignancies treated included synovial sarcoma (n = 5), metastatic melanoma (n = 2), and one each with osteosarcoma, liposarcoma, and malignant peripheral nerve sheath tumor. Patients enrolled had stage IIIc or stage IV disease and had progressed on standard-of-care chemotherapy/radiotherapy regimens, as well as a variety of experimental therapies. Patient demographics and prior treatments are summarized in Table 1. One patient with synovial sarcoma was treated within the ESO protocol (as patient ESO-5) and, following disease progression after an objective clinical response, had the PBMCs re-collected with a new leukapheresis and used to generate the new gene-modified preparation, and was subsequently enrolled in INY (as patient INY-3).

Safety, feasibility, and antitumor activity of NY-ESO-1 TCR transgenic lymphocytes with DC vaccine with and without CLTA-4 inhibition

Adverse events experienced by trial patients in both cohorts are summarized in Table S2. There were no serious toxicities in all ten patients in both protocols beyond known toxicities that were attributed to the conditioning chemotherapy, systemic IL-2 therapy, and ipilimumab therapy, and these serious toxicities were generally reversible. Prior to enrolling, patient ESO-2 had received multiple lines of chemotherapy, including multiple lines of alkylator-based chemotherapy. This patient suffered bone marrow failure from which he never recovered, which was attributed to the conditioning chemotherapy (of note, this patient developed renal insufficiency which may have contributed to poor renal clearance of the conditioning chemotherapy). Following this, we amended both protocols to exclude subjects who had previously received three or more cycles of myelotoxic treatment regimens. Two patients (ESO-3 and INY-4) experienced cytokine storm, which was attributed to systemic IL-2 therapy, as both occurred during the IL-2 treatment period and subsided after IL-2 were no longer given (Figure S1); patient INY-4 also developed significant transaminitis during this period of cytokine storm, which was attributed to IL-2 and ipilimumab. Due to the frequency of adverse events attributed to low-dose IL-2, both protocols were amended to reduce the maximum number of IL-2 doses from 28 to 14. Patients ESO-5, ESO-6, INY-3, and INY-4 were enrolled and treated after these amendments. No serious adverse events (SAEs) in either protocol were attributed to the transgenic-TCR lymphocytes or the DC vaccine doses. TCR transgenic cell preparations and NY-ESO-1 peptide-pulsed DC vaccines that were manufactured for all patients met the lot release criteria of viability >70%, negative for mycoplasma, Gram and fungal stain, endotoxin level of 5 EU/kg body weight; additionally, lot release criteria of >10% NY-ESO-1 dextramer-positive CD3 lymphocytes for transgenic cell preparations and >30% of $CD86^{+}$/$HLA\text{-}DR^{+}$ cells were met for all patients' products (Table S3). There was evidence of initial antitumor activity in four out of six patients treated per ESO (67%), two out of four patients treated per INY (50%) and as determined by day +30 PET scans (Figure 2). However, these initial responses were incomplete and transient in five of the six responding patients. At the formal restaging PET/CT scans on day +90, two of the patients treated per ESO had evidence of a partial

response by RECIST1.1. One patient treated per ESO had a PR which eventually progressed to a CR without additional therapy, and has had an ongoing complete response for over four years (Figure S2). There were no apparent overall differences in progression-free survival or overall survival between the two cohorts, although our small sample sizes were not sufficiently powered to adequately address this comparison.

NY-ESO-1 transgenic TCR integrates randomly across a highly polyclonal TCRβ repertoire

Given that transgenic adoptive cell therapy manufacturing fundamentally involves forcing expression of a transgenic TCR on a population of T cells with pre-existing endogenous TCRs, we sought to determine whether or not the NY-ESO-1 TCR was selectively taken up and expressed by a subset of TCR clonotypes, or if it integrated randomly among clonotypes. To determine this, we sorted NY-ESO-1 dextramer-positive T cells from infusion products of all patients from both clinical trials by flow cytometry, followed by genomic DNA extraction and TCRβ sequencing. Following TCR repertoire generation, the dominant NY-ESO-1 TCR sequence was then removed from the analysis, leaving only endogenous TCR sequences which are co-expressed on the T cells (Figure S3A). We then calculated Shannon entropy and clonality metrics for the co-expressed endogenous TCR repertoires, which are summarized in Figure S3B. Infusion products for all patients displayed a highly polyclonal population (similar to that of a healthy control donor), with no highly dominant clonotypes. Furthermore, we examined the TCR repertoire in the infusion products and recovery products and calculated diversity metrics (clonality and entropy) in the non-NY-ESO-1 TCR specificities over time. While the overall clonality/entropy trended towards a more clonally focused/less diverse TCR repertoire over time, we observed no significant differences between baseline and recovery non-NY-ESO-1 clonality or entropy values or between the two cohorts (Figure S4A, S4B). We also did not observe any significant difference between the change in clonality or change in entropy over time between the two cohorts (Figure S4C). Furthermore, we did not observe any significant association of any of these metrics (in the infusion product or recovery product) with transient clinical response, progression-free survival, or overall survival.

Peripheral blood reconstitution and persistence of NY-ESO-1 TCR transgenic lymphocytes with and without CLTA-4 inhibition

On average, median transduction efficiency as measured by NY-ESO-1 dextramer positivity in the infusion product was 47.2% for ESO (range, 28–66.4%), and 67% for INY (range, 61.8–71.2%). However, there were no significant differences in the absolute number of delivered NY-ESO-1 TCR transgenic cells. Despite administering a similar number of cells to these patients, peripheral cell reconstitution varied widely (Figure 3). In the ESO cohort, the median peak blood levels of TCR transgenic cells were 13.6% total CD3+ T lymphocytes (range, 4.25–27%), while in the INY cohort, the median peak blood levels were 1.7% (range, 0.52–21.6%); patient INY-2 was not clinically stable enough to collect PBMCs at day +7 and +15. The peak of TCR transgenic cell frequency was early, within the first 2 weeks after ACT, and the percentage and absolute number of TCR transgenic cells in peripheral blood decreased over time in all patients. Patients ESO-2 and INY-1 suffered early bone marrow failure secondary to conditioning chemotherapy and disease progression, respectively, and we were therefore unable to obtain any longitudinal samples beyond day 0.

There was no clear evidence that ipilimumab enhanced or inhibited the persistence of the transgenic cells. Furthermore, we found no correlation between transgenic cell persistence and clinical outcome.

Addition of ipilimumab to transgenic NY-ESO-1 TCR lymphocytes and DC vaccination increases serum Flt-3L levels over time

We examined patients' serial plasma samples up to day +90 (the formal end of the study) for 38 different human cytokines/chemokines/growth factors. We compared the area under the curve (AUC) measurements for individual serum cytokines between the two patient cohorts, in order to provide the most inclusive and biologically relevant measure of exposure to a cytokine within a given patient. We found that the serum levels of FMS-like tyrosine kinase 3 ligand (Flt-3L) had a significantly higher AUC through day +90 in the patients who received ipilimumab than those who did not ($p<0.05$, Figures S5A, S5B). Given the crucial importance of Flt-3L in DC differentiation and survival, we also explored the differences in peripheral levels of CD14+DCs, myeloid DCs (mDCs), and plasmacytoid DCs (pDCs) between the two cohorts. Although CD14+DC and mDC population proportions at day +70 trended higher in the INY cohort compared to the ESO cohort, they did not achieve statistical significance (Figure S5C).

NY-ESO-1 TCR transgenic lymphocytes track to tumor sites

One patient with synovial sarcoma was treated per ESO (as ESO-5) and, following disease progression after an objective clinical response, was subsequently enrolled in INY (as INY-3), which also resulted in an objective clinical response followed by disease progression. One on-treatment biopsy (a wedge resection of a lung metastasis) was obtained following disease progression after ACT/dendritic cell therapy (ESO protocol), while the second biopsy (a core needle biopsy of the recurrent primary lesion) was obtained following disease progression after ACT/dendritic cell therapy with ipilimumab (INY protocol). NY-ESO-1 tumor antigen expression remained robustly positive throughout all phases of treatment (Figure 4A). Tumor-infiltrating CD8 T cells, which were absent in the baseline biopsy, were demonstrated in both on-treatment biopsies; TCR sequencing of both samples also revealed the transgenic NY-ESO-1 TCR was present within the TCR repertoire (0.5% and 11% of total, respectively) of the tumor-infiltrating lymphocytes (Figure 4B, 4C). While there were no mutational events in HLA genes and the tumor cells maintained expression of MHC I in close proximity to infiltrating T cells in both biopsies (without basal MHC I expression elsewhere in the tumor tissue), the tumor invasive margin in the post-ESO biopsy was also characterized by proximal increases in PD-1 and PD-L1 expression in the lymphocytes and tumor cells in the invasive margin, respectively. PD-1/PD-L1 expression in the post-INY biopsy, which was a core needle biopsy, was not appreciated.

Phenotypic evolution of transgenic TCR-engineered T cells over time

To comprehensively characterize the transgenic T cell phenotypic subpopulations over time, we utilized a mass cytometry staining panel with 33 different surface and intracellular markers, and generated phenotypically similar clusters of transgenic TCR T cells as described above, which we were then able to compare between the two cohorts at infusion and day +70 (Figure 5A-D). Infusion products of both cohorts contained significantly

greater proportions of transgenic regulatory CD4 transgenic T cells (T_{reg}), which were almost completely depleted by day +70. Naïve CD4 transgenic T cell proportions were also higher in the infusion products, while effector CD4 cells with increased expression of the inhibitory markers PD-1 and CTLA-4 were predominant in the recovery products (although these trends did not achieve significance in every cluster for INY, potentially due to low sample size).

Within the CD8 transgenic T cell phenotypes (25), infusion products contained the greatest proportions of central memory (CM; $CCR7^+/CD45RO^+$), effector memory (EM; $CCR7^-/CD45RO^+$), and effector memory RA (EMRA; $CCR7^-/CD45RO^-/CD45RA^+$) phenotypes with high Ki67 expression (although ESO did not achieve statistical significance in all of these clusters). Conversely, the recovery products at day +70 displayed a relative loss of these phenotypes, and instead had significantly greater proportions of more terminally differentiated effector CD8 T cells with relative increases in the transcription factor T-bet, as well as one effector cluster with increases in PD-1 as well. Additionally, the recovery products displayed significant increases ($p<0.05$) in a second EM cluster which was distinguished by relative increases in PD-1 and T-bet expression, which are collectively associated with a more hypofunctional, “exhausted” T cell phenotype. Interestingly, the ESO post-infusion products also displayed a significant increase ($p<0.05$) in a subpopulation of naïve transgenic CD8 T cells characterized by high CCR7 and CD45RA expression (which was non-significant in the INY cohort). Patients’ proportions of individual T cell phenotype clusters did not correlate with individual responses to therapy, PFS, or OS.

Of note, the cluster subpopulations which predominated the infusion products, despite being less terminally differentiated CM, EM, and EMRA phenotypes, all displayed elevated expression of the T cell inhibitory marker TIM3. When we analyzed the TIM3 expression of all transgenic $CD3^+$ T cells manually, we found that the infusion products of both cohorts displayed significantly greater expression of TIM3 than the day +70 post-infusion recovery cells ($p<0.05$, Figure 5E). Additionally, we found that PD-1 expression in all transgenic TCR T cells increased over time in the ESO cohort, which was not observed in the INY cohort. Overall CTLA-4 expression in all transgenic TCR T cells did not achieve statistical difference between infusion and day +70 samples in either cohort (data not shown).

DISCUSSION

Transgenic ACT has emerged as a potent and widely applicable form of cancer immunotherapy. However, despite frequent early objective clinical responses, patients often go on to develop progressive disease. This widespread finding demonstrates the need to understand why these treatments can ultimately fail, and use this knowledge to improve future ACT protocols. The addition of NY-ESO-1 peptide-pulsed DC vaccine and CTLA-4 immune checkpoint blockade to NY-ESO-1 TCR transgenic ACT was feasible, with serious toxicities attributed to conditioning chemotherapy, systemic IL-2 therapy, and ipilimumab therapy (which were generally reversible). Our experience with these protocols was consistent with previously reported studies showing frequent early responders, but few long-term remissions (6, 8, 26). Furthermore, there was no apparent benefit from the addition of ipilimumab, although further study in larger cohorts would be needed to objectively compare

any differences between the two regimens. While the rapid *in vivo* expansion of transgenic cells within the first two weeks may have been aided by the initial DC vaccine in conjunction with the systemic IL-2, we did not observe any subsequent increases in transgenic T cell proportion in response to the subsequent booster doses of DC vaccination. Overall, our transgenic cell persistence in peripheral circulation was short-lived, which is consistent with previously reported transgenic ACT studies (3, 6). It is possible that the peripheral circulation may not be the ideal location to assess the presence of transgenic T cells. However, since these transductions rely on retroviral or lentiviral vectors, which are commonly subject to epigenetic silencing via methylation of the viral promoter (27, 28), it is possible that epigenomic silencing mechanisms can also contribute to this phenomenon. Further studies are needed to explore this widely observed phenomenon.

Previous ACT trials conducted by the NCI and our group administered high-dose IL-2 (720,000 IU/kg every 8 hours) post-adoptive transfer to support the proliferation of adoptively transferred TCR transgenic cells (3, 6–8). Our NY-ESO-1 ACT protocols utilized low dose IL-2 (500,000 IU/m^2 every 12 hours). This modification was based on data that IL-2 receptors are saturated at low doses of IL-2, and that adoptively transferred T cell clones can persist *in vivo*, preferentially localize to tumor sites, and mediate an antigen-specific immune response in response to low-dose IL-2 (15, 16). Furthermore, there is evidence that high-dose IL-2 can impair the generation of memory T cells and compromise overall T cell effectiveness (29, 30). Despite the use of low-dose IL-2, our patients still experienced significant side effects which were attributed to IL-2, including cytokine storm, ventricular tachycardia, and respiratory distress requiring elective intubation. Following these adverse events, we amended both protocols to reduce the maximum number of IL-2 doses given from 28 to 14. There were no apparent differences in transgenic T cell persistence, phenotype, or clinical response between these two dosing regimens, although a larger, more directed study would be required to compare these dosing regimens effectively. Furthermore, the use of two different IL-2 dosing regimens may have had contributed to tumor responses independent of the transgenic T cells, and thus may confound some of our comparative analyses between the two patient cohorts.

One of the questions explored by our study was the effect of ipilimumab on serum cytokine profiles in patients receiving transgenic TCR adoptive cell therapy. We found that patients who received ipilimumab displayed significantly greater longitudinal serum levels of the cytokine Flt-3L than those who did not, as measured by the serum AUC through day +90 (the formal end of study monitoring). Flt-3L is crucial for steady-state plasmacytoid and classical dendritic cell development (31). A lack of Flt-3L results in low levels of dendritic cells (DCs), while administration of Flt-3L generates large numbers of dendritic cells from hematopoietic stem cells and augments immune responses *in vivo* (32, 33). Flt-3L has been explored as an adjuvant to peptide-based and DC-based tumor vaccines (34), and recombinant human Flt-3L has been shown to safely cause effective expansion of peripheral plasmacytoid and myeloid dendritic cells in humans (35). Although patients' peripheral $CD14^+$DC and mDC populations trended higher at day +70 in the INY cohort compared to the ESO cohort, they did not achieve statistical significance. These results imply that, while the addition of ipilimumab was associated with significant increases in the longitudinal serum levels of Flt-3L, this did not appear to lead to increased amounts of DC

subpopulations over time. It may be that Flt-3L is able to function as a biomarker of ipilimumab activity *in vivo*, although further studies with larger and more standardized sample sizes are needed to assess what effects, if any, ipilimumab has on DC expansion and persistence over time.

The phenotype of gene-modified T cells and their associated functionality is critically important to the effectiveness of these therapies *in vivo.* Previous work has demonstrated the role of less differentiated naïve and memory T cell subsets in successful cellular therapies, as they possess superior *in vivo* expansion, persistence, and antitumor activity when compared with the more terminally differentiated effector T-cell subsets (36–38). While our infusion products in both cohorts contained large proportions of less differentiated CM, EM, and EMRA phenotypes, these did not widely persist in the post-infusion recovery products, which were instead predominated by more terminally differentiated effector phenotypes. The late expansion of naïve T cells may possibly be explained by how these cells behave differently than other less differentiated T cell phenotypes (i.e. memory T cell). After entering the antigen-experienced pool, memory T-cells circulate through peripheral lymphatics and accumulate in secondary lymphoid tissues where they can be re-stimulated by their cognate antigen, whereas naïve T-cells continue to circulate through peripheral lymphatics awaiting initial antigenic stimulation (39). This TCR antigenic stimulation has also been shown to lead to expansion of naïve T cells (40, 41), which could explain their increase in proportion over time from only minimal proportions present at infusion, while the memory T cells progressively differentiated into effector T cell populations over time.

While the overall shift of transgenic T cells towards terminally differentiated phenotype is consistent with our previous experiences with transgenic ACT in combination with DC vaccine (6, 14, 42), it contrasts with the recently reported study by D'Angelo et al, which reported that the vast majority of persisting transgenic NY-ESO-1^{c259} TCR T cells consisted of less differentiated memory T cell phenotypes (26), despite an overall lack of persistence of transgenic cells and frequent tumor relapses (consistent with our clinical experiences). These persistent transgenic memory T cells possessed the same co-expressed native TCR repertoire as the infusion product in one patient, implying that long-lived clones which were initially present at infusion persisted throughout treatment. It is possible that transgenic T cell manufacturing differences between our groups' products, such as the vector utilized (their trial used a human lentivirus, while ours used a murine retrovirus) and its integration sites within the host cells' genomes may have been more favorable to the generation of memory T cell phenotypes. Indeed, this phenomenon has been recently reported in CAR-T cells, where lentiviral insertion was shown to disrupt the *TET2* gene, and give rise to a long-lived, highly functional memory phenotype T cell clone (43).

The importance of transgenic T cell phenotype in the effectiveness of ACT is further complemented by the cells' overall functionality and the development of inhibitory marker expression. Infusion products from both cohorts, despite being predominantly less terminally differentiated CM, EM, and EMRA phenotypes, all displayed elevated expression of the T cell inhibitory marker TIM3, which did not persist in circulation. TIM3 expression has been shown to be induced in T cells by IL-2 in an antigen-independent manner *in vitro* (44). Given the prolonged exposure to IL-2 utilized in our transgenic T cell culture and

expansion manufacturing protocols, and the lack of persistent TIM3 expression *in vivo*, this may be a likely mechanism for this phenomenon. Indeed, the high proportion of CD4 T_{regs} seen in our infusion products secondary to prolonged IL-2, which was followed by rapid clearance of this subpopulation *in vivo*, is consistent with our previously published experience with MART-1 TCR transgenic T cell therapy (14). Additionally, we found that PD1 expression in all peripheral and tumor-infiltrating transgenic TCR T cells increased over time in the ESO cohort, which is also consistent with our previous observations in our MART-1 TCR transgenic T cell trial (14). This phenomenon was not observed in the peripheral samples from the INY cohort, nor in the on-treatment biopsy obtained from this cohort. However, the post-INY biopsy was a small core needle biopsy, which prevented us from comprehensively assessing the tumor invasive margin, where tumor-infiltrating lymphocytes and associated PD-1/PD-L1 expression are often more pronounced (22, 23). Unfortunately, we did not have sufficient clinical material to conduct more in-depth studies of transgenic T cell functionality or gene expression over time or within on-treatment biopsies. However, the lack of persistence of the transgenic cells would likely outweigh any retained functionality of the cells several months after infusion. Furthermore, the overall lack of sustained responses also suggest sub-optimal long term functionality, consistent with our previous experiences with transgenic adoptive cell therapy (6).

While the addition of ipilimumab to our dual cell therapy clinical protocol was feasible and not associated with any irreversible toxicities, there was no apparent clinical benefit from this combination (although our small sample size was insufficiently powered to make any formal comparison). However, combining other forms of immune checkpoint blockade with transgenic ACT may ultimately hold great promise. PD1 blockade has been shown in preclinical models to enhance the antitumor efficacy of transgenic ACT (45, 46). Our current and previous experiences demonstrating progressive acquisition of PD1 expression by transgenic adoptively transferred T cells *in vivo* (14) underscore the importance of continued study of such a combination in future clinical trials. Furthermore, given the progressive loss of transgenic cells over time in many transgenic ACT approaches, there may be utility in modalities which provide a continuous supply of transgenic T cells to the patient. Preclinical models have demonstrated that CD34+ peripheral blood stem cells carrying a transgenic TCR can endogenously differentiate into fully functional T cells expressing the TCR (47–49). Such approaches may yield improved clinical outcomes by circumventing many of the short and long-term phenotypic and persistence disadvantages of current ACT practices which have been demonstrated. We have recently opened two phase I clinical trials which utilize this approach against NY-ESO-1 in solid tumors (NCT03240861) and multiple myeloma (NCT03506802).

In conclusion, the manufacture and administration of transgenic NY-ESO-1 TCR T cells with NY-ESO-1 peptide-pulsed DC vaccine with or without ipilimumab is generally safe, feasible, and results in frequent initial antitumor activity. Addition of ipilimumab, while safe and feasible, had no apparent effect on transgenic cell persistence, transgenic cell phenotype, or overall clinical response. Further improvements in ACT protocols are needed to maintain TCR transgenic cell functionality and perpetuate antitumor responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

This article describes an investigator-initiated clinical trial using dual cell immunotherapy, consisting of genetically modified lymphocytes with NY-ESO-1-specific T-cell receptor adoptively transferred together with NY-ESO-1 peptide-pulsed dendritic cell vaccination, with or without CTLA-4 immune checkpoint blockade with ipilimumab. We report that these protocols are generally safe, feasible, and result in high anti-tumor activity in patients with advanced sarcoma and melanoma. However, further improvements in durability of these cellular therapies are needed, as patients frequently relapse after an initially vigorous anti-tumor response. With evidence that transgenic adoptive cell therapy (ACT) is a viable treatment option for certain patients with advanced cancers, there is an increasing need to establish viable ACT programs at other institutions, further optimize responses with addition of immune checkpoint blockade agents, and characterize these responses at the cellular level.

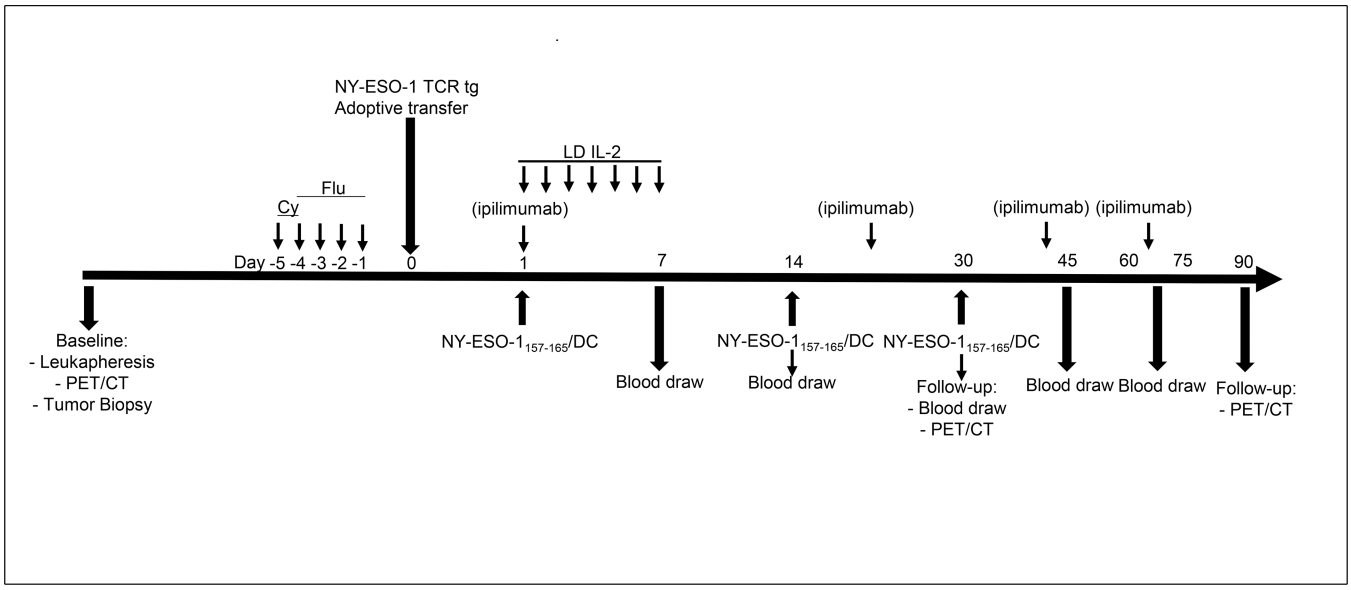

Figure 1. Study overview.

Schedule of events for patients who received NY-ESO-1 TCR transgenic adoptive cell therapy and NY-ESO-1 peptide-pulsed DC vaccination. For patients who received ipilimumab, their dosing schedule (every three weeks) is indicated in parentheses.

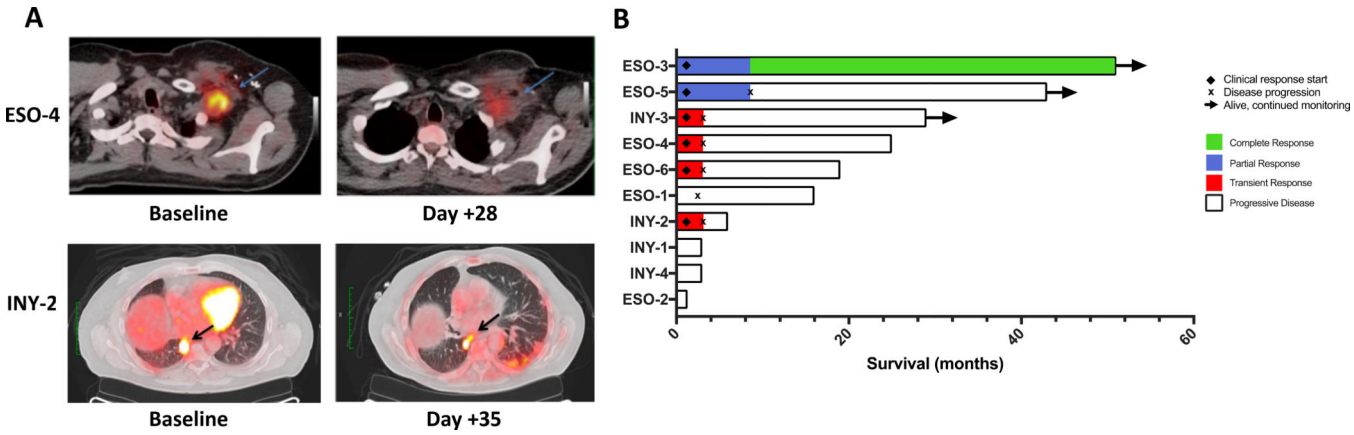

Figure 2. NY-ESO-1 transgenic TCR ACT + DC vaccination with or without ipilimumab produces frequent antitumor responses, but these are generally transient and incomplete.

(A) Pre- and post-treatment PET/CT images from patients showing evidence of initial antitumor activity (decreased FDG uptake) in both ESO and INY cohorts. (B) Swimmer plot showing onset and duration of antitumor activity, as well as progression-free survival and overall survival in all patients in both groups.

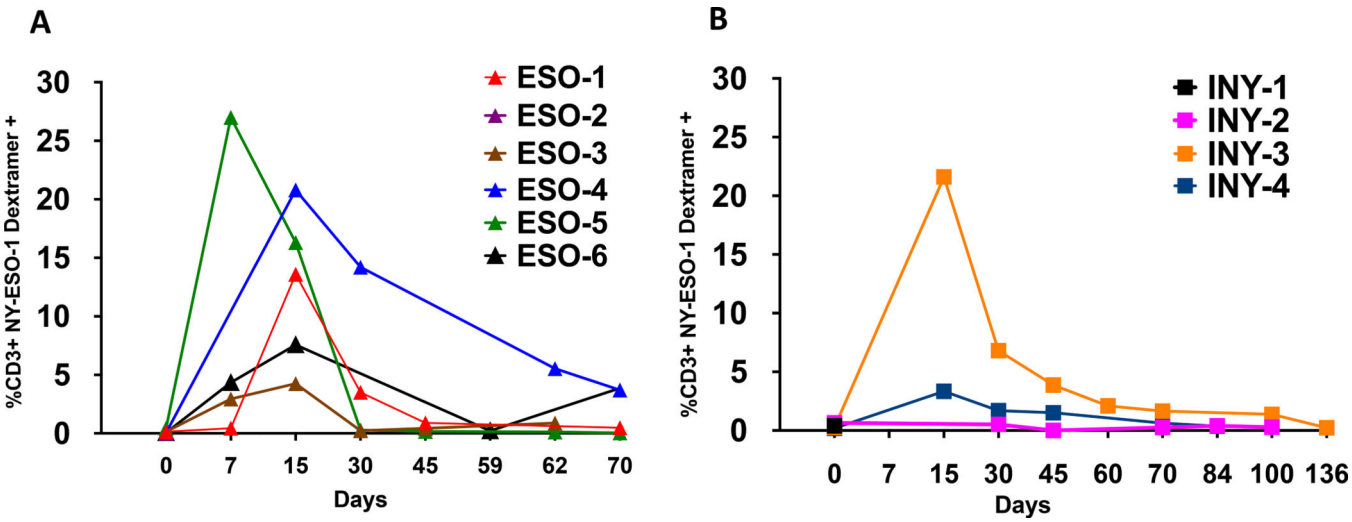

Figure 3. NY-ESO-1 TCR transgenic T cells display peripheral expansion and contraction over time.

Postinfusion peripheral blood levels of NY-ESO-1 TCR transgenic CD3+ cells over time in patients receiving transgenic cells and DC vaccinations alone (ESO, A), or in combination with ipilimumab (INY, B).

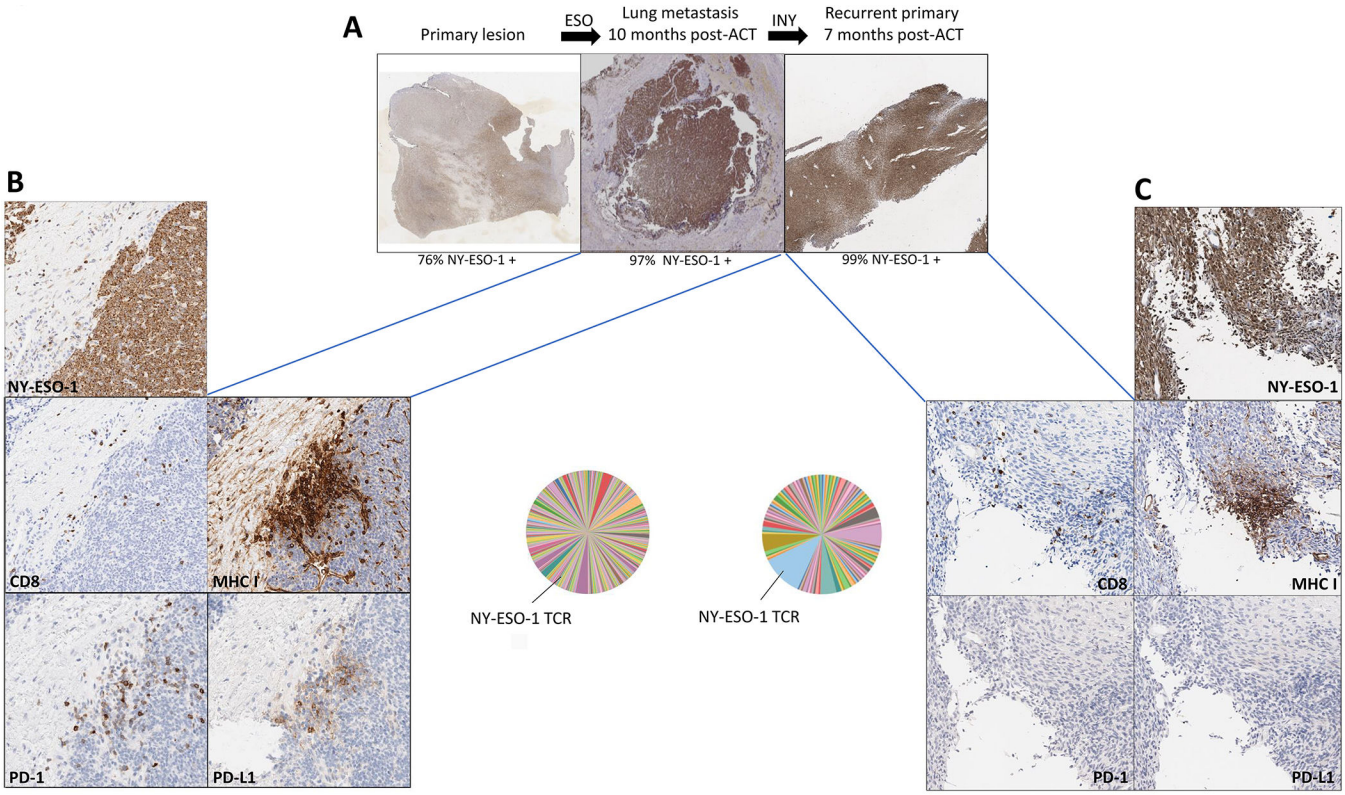

Figure 4. NY-ESO-1 transgenic TCR T cells track to tumor sites.

(A) NY-ESO-1 IHC staining of biopsies from a patient with synovial sarcoma who enrolled on ESO (ESO-5) and, following a partial response, developed progressive disease and enrolled to the INY protocol (INY-3) and experienced a transient response, followed by disease progression. (B,C) Representative IHC sections demonstrating CD8 T cell infiltration and co-localized MHC I expression in the tumor tissue obtained after ESO (B) and INY (C) protocols, both of which had been absent in the baseline tumor tissue. In the progression biopsy obtained after ESO protocol (B), PD-1 and PD-L1 expression was also increased in areas with significant CD8 T cell infiltration in the tumor invasive margin. TCR sequencing showing the relative clonotype frequencies in the TCR repertoire within both biopsies is indicated by the accompanying pie charts, with each slice representing a different TCR clonotype and its frequency. NY-ESO-1 transgenic TCR is highlighted within each chart in cyan.

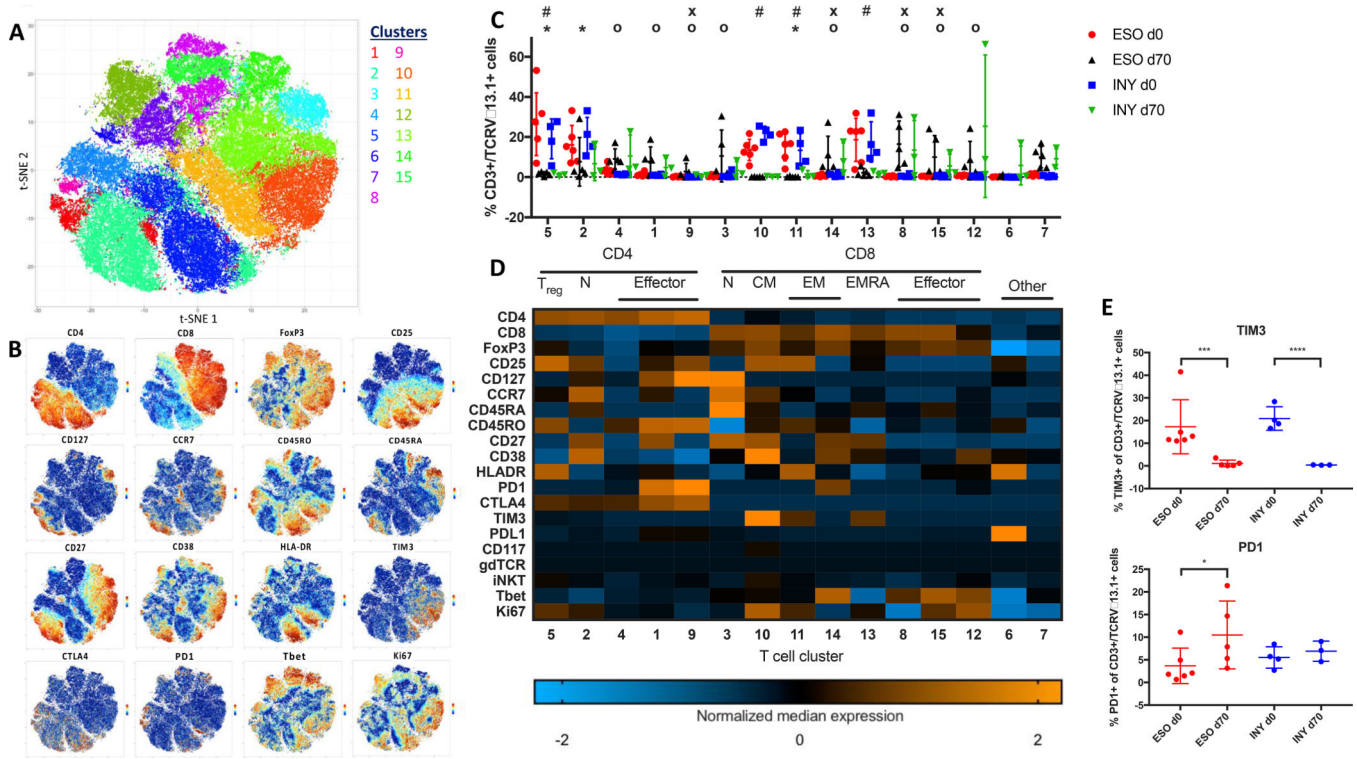

Figure 5. Post-infusion phenotypic evolution of NY-ESO-1 transgenic TCR T cells over time.

(A) t-SNE plot of total lineage−/CD3+/TCRVβ13.1+ T cells from all samples overlaid with color-coded clusters. (B) t-SNE plots of total lineage−/CD3+/TCRVβ13.1+ T cells from all samples overlaid with the expression of selected markers. (C) Frequency of T cell clusters displayed on a per-sample basis with mean ± SD (*, ESO d0>d70; o, ESO d70>d0; #, INY d0 > d70; x, INY d70>d0; $p < 0.05$). T cell compartments are denoted below including CD4 T_{reg}, CD4 Naïve (N), and CD4 effector, as well as CD8 Naïve (N), Central Memory (CM), Effector Memory (EM), Effector Memory re-expressing CD45RA (EMRA), and Effector. (D) Heat map of median arcsinh-transformed marker expression normalized to a mean of 0 and a SD of 1. Orange indicates relative marker over-expression, blue indicates relative under-expression. The median expression was calculated on single, live, CD45+/CD33−/CD56−/CD19−/CD3+/TCRVβ13.1+ cells from thawed PBMC samples from patients. (E) Relative expression of TIM3 and PD1 on CD3+/TCRVβ13.1+ infusion product (day 0) cells and recovery (day +70) cells in both ESO and INY treatment groups (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$).

Table 1.

Patient demographics and outcomes

Patient study number	Sex (M/F)	Ethnicity	Age	Type of Cancer	Prior Treatments	Active Disease Sites	Stage	Number of NY-ESO-1 TCR transgenic cells	IL-2 doses	DC doses	Evidence of transient tumor response	Response at EOS (day 90)	PFS (mo)	OS (mo)	Sites of Progression	Post-Study Treatments	Current Status
ESO-1	M	Hispanic	47	Liposarcoma	Surgery; Radiation; Gemcitabine +docetaxel +ontuxizumab; doxorubicin +ifosfamide	Right Renal Fossa; Liver left Lobe; Hepatic Segment; Peritoneal, Perihepatic Nodule	IV	7.7×10^8	28/28	3/3	No	PD	2.6	16	Liver	Trabectedin, pazopanib	Died of disease
ESO-2	M	Hispanic	37	Malignant Peripheral Nerve Sheath Tumor	Surgery; Radiation; Gemcitabine +docetaxel; doxorubicin +ifosfamide; trabectedin; navelbine +avastin +cytoxan; pexidartinib	Right Iliopsoas; Peritoneal; Retroperitoneal	IV	1×10^9	28/28	3/3	No	NA	NA	1.3	NA	NA	Died of disease
ESO-3	F	Caucasian	24	Synovial Sarcoma	Surgery; Radiation; doxorubicin +ifosfamide +gemcitabine +doxetaxel	Right Lung; Multiple pulmonary Nodules	IV	1×10^9	19/28	1/3	Yes by PET/CT	PR	51	51	NA	NA	Alive with CR
ESO-4	M	Caucasian	41	Synovial Sarcoma	Surgery; Radiation; Doxorubicin +ifosfamide; Gemcitabine +docetaxel; pazopanib	Left Infraclavicular Mass; Left Pectoralis Mass	III	1×10^9	18/28	3/3	Yes by PET/CT	PD	3	25	Left pectoralis mass	Ifosfamide, nivolumab, Yondelis	Died of disease
ESO-5	F	Caucasian	43	Synovial Sarcoma	Surgery; doxorubicin +ifosfamide; radiation	Right popliteal fossa; Lung	IV	1×10^9	14/14	3/3	Yes by PET/CT	PR	9	43	Intrathoracic LN and pulmonary nodules	INY	Alive with disease
ESO-6	M	Caucasian	26	Osteosarcoma	Surgery; doxorubicin +cisplatin +methotrexate +ifosfamide +etoposide; gemcitabine +docetaxel; MAGE-A3 transgenic TCR ACT; Nivolumab +ipilimumab	Lung	IV	1×10^9	14/14	3/3	Yes by PET/CT	PD	2.5	19	Right Upper Lobe Lung, Left Lower Lobe Lung	NA	Died of disease

Patient study number	Sex (M/F)	Ethnicity	Age	Type of Cancer	Prior Treatments	Active Disease Sites	Stage	Number of NY-ESO-1 TCR transgenic cells	IL-2 doses	DC doses	Evidence of transient tumor response	Response at EOS (day 90)	PFS (mo)	OS (mo)	Sites of Progression	Post-Study Treatments	Current Status
INY-1	M	Caucasian	51	Synovial Sarcoma	Surgery, doxorubicin +ifosfamide; Pazopanib	Lung, bone marrow	IV	1×10^9	18/28	3/3	No	NA	NA	2	NA	NA	Died of disease
INY-2	M	Caucasian	66	Melanoma	Vemurafenib, Ipilimumab	LN, Liver	IV	1×10^9	21/28	2/3	Yes by PET/CT	PD	3	6	Thigh, Abdominal mesentery, parenchymal nodules, mediastinal nodes, brain, right orbit, chest wall	NA	Died of disease
INY-3	F	Caucasian	44	Synovial Sarcoma	Surgery; doxorubicin +ifosfamide; radiation	Right popliteal fossa; Lung	IV	1×10^9	14/14	3/3	Yes by PET/CT	PD	3	29	Right popliteal fossa, soft tissue posterior to the right femur, intrathoracic LN and pulmonary nodules	Nivolumab, pazopanib	Alive with disease
INY-4	M	Hispanic	24	Melanoma	Nivolumab +ipilimumab; dabrafenib +trametinib +nivolumab	Lung, LN, adrenal gland, liver, trachea, brain	IV	1×10^9	10/14	3/3	No	PD	NA	3	Lung, adrenal gland, bone marrow	Nivolumab, anti-CD137	Died of disease

Abbreviations: F: female; LN: lymph nodes; M: male; NA: not available; PFS: progression-free survival; OS: overall survival; EOS: end of study; DC: dendritic cells; TCR: T cell receptor; IL-2: interleukin-2; mo: months