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Clinical and immunologic evaluation of three metastatic melanoma patients treated with autologous melanoma-reactive TCR-transduced T cells

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Abstract

Malignant melanoma incidence has been increasing for over 30 years, and despite promising new therapies, metastatic disease remains difficult to treat. We describe preliminary results from a Phase I clinical trial (NCT01586403) of adoptive cell therapy in which three patients received autologous CD4⁺ and CD8⁺ T cells transduced with a lentivirus carrying a tyrosinase-specific TCR and a marker protein, truncated CD34 (CD34t). This unusual MHC Class I-restricted TCR produces functional responses in both CD4⁺ and CD8⁺ T cells. Parameters monitored on

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Conflict of Interest:

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transduced T cells included activation (CD25, CD69), inhibitory (PD-1, TIM-3, CTLA-4), costimulatory (OX40), and memory (CCR7) markers. For the clinical trial, T cells were activated, transduced, selected for CD34t⁺ cells, then re-activated and expanded in IL-2 and IL-15. After lymphodepleting chemotherapy, patients were given transduced T cells and IL-2 and were followed for clinical and biological responses. Transduced T cells were detected in the circulation of three treated patients for the duration of observation (42, 523, and 255 days). Patient 1 tolerated the infusion well but died from progressive disease after six weeks. Patient 2 had a partial response by RECIST criteria then progressed. After progressing, Patient 2 was given high-dose IL-2 and subsequently achieved complete remission, coinciding with the development of vitiligo. Patient 3 had a mixed response that did not meet RECIST criteria for a clinical response and developed vitiligo. In two of these three patients, adoptive transfer of tyrosinase-reactive TCR-transduced T cells into metastatic melanoma patients had clinical and/or biological activity without serious adverse events.

Keywords

adoptive transfer; metastatic melanoma; clinical trial; transduced T cells; immunotherapy; vitiligo

Introduction

Melanoma incidence has risen steadily to over 80,000 new cases a year in the United States alone (approximately 232,000 worldwide) [1, 2]. Despite promising new therapies, metastatic melanoma is difficult to treat, with a 5-year survival rate of 17.9%, leading to over 10,000 mortalities in the United States last year (approximately 55,000 deaths worldwide). Conventional therapies are rarely curative for metastatic melanoma, yet results are encouraging for immunotherapies. Several new immunotherapies have been approved by the FDA, including anti-PD-1, anti-PD-L1, and anti-CTLA-4 monoclonal antibodies, which block checkpoint pathways that suppress T cell responses, thereby releasing inhibited T cells to attack the tumor [3–5]. However, these therapies do not induce tumor regression in all patients [6, 7]. There is evidence that responders to PD-1 blockade may have pre-existing tumor-reactive T cells in the tumor margins [8, 9] and that responders to CTLA-4 blockade change their T cell repertoire in ways consistent with expansion of novel tumor-reactive T cells and/or expansion of pre-existing anti-tumor T cells [10, 11]. Evidence is mounting that these therapies require the presence of tumor-reactive T cells, and patients having insufficient tumor-reactive T cells may not respond to these checkpoint inhibitors. We and others are developing strategies to introduce anti-tumor T cell receptors (TCRs) into T cells, redirecting large numbers of T cells to be tumor-reactive [12–17]. Previous studies have found that delivering anti-tumor T cells to patients can induce durable regression of tumors [18]. Such studies found objective clinical responses (13–56%) when treating with T cells targeted to both melanocyte-differentiation antigens and cancer-germline antigens [19–23]. Melanocyte-differentiation antigen-specific T cell studies demonstrated on-target off-tumor toxicities to melanocytes in the skin, eye, and ear (vitiligo, uveitis, and hearing loss) while targeting cancer-germline antigens has had off- and on-target off-tumor effects including neurological toxicities and colitis. Further studies following the transduced T cells over time

in responding versus non-responding patients will help identify factors that contribute to the safety, success or failure of TCR-transduced T cell therapy.

This report describes the preliminary results of monitoring transduced T cells in three patients in a Phase I clinical trial treating metastatic melanoma patients with autologous T cells transduced with the tyrosinase-reactive TIL1383I TCR. The three patients treated had three distinct clinical courses: one with rapid progression of disease, a second with an objective clinical response, and a third with a biological response in the form of vitiligo. Patient 2, with the observed clinical response, had greater numbers of TCR-transduced T cells, increased activation of TCR-transduced T cells, and increased expression of some inhibitory receptors. In Patient 2, upregulation of inhibitory receptors on T cells in the blood did not preclude anti-tumor responses of TCR-transduced T cells. Although more patients are needed to definitively demonstrate safety and identify response rates, these preliminary results suggest that patients can have clinical and biological responses to TIL1383I TCR-transduced T cell therapy in the absence of serious adverse events.

Methods

Study Design

This study describes preliminary results from the first three patients in a 3×3 Phase I dose escalation trial introducing gene-modified autologous T cells into metastatic melanoma patients. Patients received 2.5×10^6 /kg lentivirally-transduced T cells after non-myeloablative lymphodepletion and were given low-dose IL-2 for one week following the T cell infusion. The study was performed at Loyola University Chicago, and registered with Clinicaltrials.gov (NCT01586403). Informed consent was obtained prior to enrolling patients. This study was approved by the Institutional Review Board at Loyola Medical University Center (LU 203732), the Recombinant DNA Advisory Committee (RAC Protocol 1101–1086), the Institutional Biosafety Committee (LU 203732), the Cancer Therapy Evaluation Program (CTEP 9358), and the United States Food and Drug Administration (IND 14971).

See full eligibility and exclusion criteria in Supplementary Table 1. Briefly, patients must have a diagnosis of metastatic melanoma, which must test positive for HLA-A2 and the tyrosinase antigen by immunohistochemistry (Supplementary Figure 1). Patients may not have metastatic lesions in the brain unless controlled and may not have previously received non-myeloablative chemotherapy or immunotherapy specifically targeting tyrosinase. Patient descriptions are found in Table 1.

T cell production

For each patient, PBMCs (isolated from an apheresis) were activated for two days with anti-CD3 antibody (CD3 Pure, 50ng/mL, Miltenyi Biotec GMP grade), recombinant human IL-2 (rhIL-2, Prometheus Therapeutics & Diagnostics, 300IU/mL), and recombinant human IL-15 (rhIL-15, Biologic Resources Branch of the National Cancer Institute, 100ng/mL). Activated T cells were transduced by spinoculation for 2 hours at 2000xg with a GMP-grade replication-deficient lentivirus carrying the TIL1383I TCR and CD34t genes

(Supplementary Figure 2). Transduced T cells were cultured four days then isolated by CD34-selection using the CliniMACS cell selector. After culturing four more days, CD34⁺ T cells were tested for viral copy number, then expanded by culturing with CD3 Pure (30ng/mL), rhIL-2 (300IU/mL), rhIL-15 (100ng/mL), and irradiated non-autologous PBMCs at a 1:200 T:PBMC ratio for ten days (fourteen for Patient 1). During the last five days of culture, patients were treated with cyclophosphamide (60mg/kg) and fludarabine (25mg/m²) to induce non-myeloablative lymphodepletion [24]. Twenty-four hours after the end of lymphodepletion, expanded transduced T cells were collected, and 2.5×10⁶/kg (1.67×10⁶/kg for Patient 1) CD34⁺ T cells were administered to the patient via continuous i.v. infusion. Patients were treated with a reduced dose IL-2 regimen [25, 26] of 72,000IU/kg, delivered i.v. three times daily for seven days. Cell counts during the production process are described in Supplementary Table 2.

Flow cytometry

Each set of patient samples was stained with the following antibodies: CD3-Brilliant Violet 785 (OKT3, Biolegend), CD4-APC/Cy7 (OKT4, Biolegend), CD8-Alexafluor 700 (SK1, Biolegend), CD11b-Brilliant Violet 570 (M1/70, Biolegend), CD19-Brilliant Violet 570 (HIB19, Biolegend), CD34-Brilliant Violet 421 (561, Biolegend), TCR $\alpha\beta$ 12-PE (VER2.32.1, Beckman Coulter), CD25-Brilliant Violet 711 (BC96, Biolegend), CD69-PE/Cy7 (FN50, Biolegend), OX40-FITC (Ber-ACT35, Biolegend), PD-1-PerCP/Cy5.5 (EH12.2H7, Biolegend), T cell immunoglobulin and mucin-domain containing-3 (TIM-3)-APC (F38-2E2, Biolegend), CTLA-4-PE-CF594 (BNI3, BD Biosciences), C-C motif chemokine receptor 7 (CCR7, CD197)-Brilliant Violet 650 (G043H7, Biolegend), and a viability dye (Live/Dead Aqua, Invitrogen). Representative stains are shown in Supplementary Figure 3. All samples were analyzed on the LSRFortessa in the Loyola University Chicago Flow Cytometry Core. The absolute number of TCR-transduced T cells per milliliter (mL) of study blood was estimated by combining percentages of TCR-transduced T cells with counts of total white blood cells from known volumes of whole blood.

Affinity Measurements

The solution (three dimensional, 3D) binding affinity of TIL1383I was determined by surface plasmon resonance (SPR). Soluble TIL1383I TCR and HLA-A2-tyrosinase complexes were refolded from bacterial inclusion bodies and purified chromatographically [27]. Solution (3D) binding affinity was measured at 25°C using a Biacore T200 instrument [27, 28]. HLA-A2-tyrosinase complexes were tethered to a CM5 sensor surface using standard amine coupling. Soluble TIL1383I TCR complexes were injected over the sensor surface at increasing concentrations, and the response due to binding recorded. After subtracting the responses from a mock surface, the data were fit to a 1:1 binding model using Biaevaluation 4.1.

Two-dimensional (2D) affinity measurements were taken using the micropipette adhesion frequency assay [29, 30]. Briefly, frozen patient T cell samples were thawed and permitted to recover for three days in AIM V medium in the presence of rhIL-2 (300IU/mL) and rhIL-15 (100ng/mL). Human RBCs coated with HLA-A2-tyrosinase-peptide (pMHC, using

HLA-A2 molecules mutated to abrogate CD8 binding) and patient T cells were aspirated onto opposing pipettes and brought into contact 50 times with the same area (A_c) and time, using an electronically-controlled piezoelectric actuator. Upon retraction of the T cell, adhesion was observed as a distention of the RBC membrane, allowing for quantification of adhesion frequency (P_a) at equilibrium. Surface pMHC (m_l) and TCR β (m_r) densities were determined by flow cytometry using BD QuantiBRITE PE Beads for standardization (BD Biosciences). Relative 2D affinities were calculated using the following equation: $A_c K_a = -\ln [1 - P_a(\infty)] / m_r m_l$ [29].

Results

Clinical responses

All three patients tolerated the infusion well. Some toxicities were observed (Supplementary Table 3), which were similar to previously observed toxicities seen in patients receiving non-myeloablative lymphodepletion (lymphopenia, neutropenia, thrombocytopenia, rash). Considering toxicities previously observed in patients given melanoma/melanocyte-specific T cells [20, 31], our toxicities did not include uveitis or hearing loss but did include vitiligo. Patient 3 developed vitiligo after treatment with transduced T cells and Patient 2 developed vitiligo after progressing and receiving treatment with high-dose IL-2 (Figure 1c).

In Patient 1, there was neither tumor nor biological response attributable to the TCR-transduced T cells. Patient 1 received two-thirds the dose of TCR-transduced T cells given to the other two patients, following U.S. Food and Drug Administration (FDA) recommendations in response to a high viral copy number in the final T cell product. Patient 1 had 7.5 copies per cell, instead of 5 or fewer copies per FDA guidelines, and the recommendation was to reduce the T cell dose proportionally. Patient 1 also had the highest tumor burden, with tumors over 1cm in size in at least 8 sites, with the largest tumor being over 3cm in diameter at time of treatment. Patient 1 passed away 47 days post-transfer without responding to treatment.

Patient 2 had a partial response with tumors shrinking over 30% for 5 months post-treatment (Figure 1a). He began the trial with tumors at six locations, each measuring less than 1.5cm in diameter. Tumors at all locations regressed or stabilized for over two months. After his disease progressed, Patient 2 received PD-1 blockade with pembrolizumab for six weeks (days 214–256). After progressing again, Patient 2 received high dose IL-2 therapy (600,000 IU/m² given i.v. every 8 hours for a maximum of 14 doses on days 284, 298, and 368 post-T cell infusion), which preceded tumor regression and remission persisting over two years. On two separate occasions of receiving high-dose IL-2 treatment, he developed rhabdomyolysis. This rhabdomyolysis was not considered a treatment-related event from the transduced T cells, as histology of biopsy samples of inflamed muscle tissue stained for CD3 and CD34t did not reveal a significant population of CD34t⁺ T cells. Patient 2 developed progressive vitiligo after high-dose IL-2 and has very little normal skin pigmentation remaining.

Patient 3 had an observed biologic response to the T cells, in the form of progressive vitiligo (Figure 1b). Histology demonstrated that CD3⁺vβ12⁺CD34t⁺ T cells were present at the margins of the vitiligo but not normal skin in both Patient 2 and Patient 3 (Figure 1c). There

were substantially higher percentages of these in the skin at the margin of the vitiligo than in the blood, suggesting that patients' transduced T cells were functional and targeting melanocytes. Patient 3 began the trial with a 3cm tumor at one location and another tumor less than 1cm in diameter. Although his tumors had a small reduction in size (cumulatively about 10%) for five months, other tumors progressed; therefore Patient 3 was nonresponsive by RECIST criteria. He went on to receive PD-1 blockade, but passed away from progressive disease shortly thereafter.

Biology of the T cells: T cell numbers and phenotype pre- and post- transfer

In this study, CD34t is expressed on the surface of transduced cells and provides a powerful marker to uniquely identify transduced T cells, as the transduced TCR is expressed at lower levels due to competition with the endogenous TCR for CD3 components [32, 33]. Over 85% of the final preparation of T cells were CD34t⁺ whereas only a fraction of CD34t⁺ T cells had detectable TCR (vβ12 in Table 1). The clinical responder, Patient 2, had the highest CD34 expression in T cells prior to transfer, but interestingly, there was not a strong correlation between clinical response and the intensity of TCR expression (Supplementary Figure 4). We detected transduced (CD34t⁺) T cells in all patients for the duration of observation (Figure 2). In all patients, the percentage transduced of total T cells peaked within seven days, while the number of transduced T cells per milliliter of blood kept increasing until it peaked between fourteen and twenty-one days. The prolonged increase in the number but not the percentage of transduced T cells is due to a substantial increase in total T cell numbers after day seven (Supplementary Figure 5). Peak numbers of transduced T cells are highest in the clinical responder, Patient 2 (1145 CD8⁺ and 2174 CD4⁺ cells/mL) and lowest in the non-responder, Patient 1 (461 CD8⁺ and 661 CD4⁺ cells/mL).

Treatment of Patient 2 with high-dose IL-2 preceded a dramatic increase in both the percent and number of transduced CD8⁺ T cells and a smaller increase in the percent and number of transduced CD4⁺ T cells, occurring after the third course of high-dose IL-2. Thus, Patient 2 had the highest initial peak number of transduced T cells and a substantial increase in the number of transduced T cells after the third course of high-dose IL-2, potentially contributing to the lasting remission thereafter.

Activation (CD25 and CD69)

In order to determine whether the transduced T cells were responding to tumor or melanocyte antigens in the patients, we utilized two well-characterized activation markers, CD25 (IL-2Rα) and CD69. CD25 and CD69 are both upregulated within hours of T cell activation and decrease back to baseline levels after several days; thus expression of these markers identifies recently activated T cells [34, 35]. Due to prior activation and culture in IL-2 and IL-15, up to 40% of transduced CD4⁺ and CD8⁺ T cells co-express CD25 and CD69 immediately prior to transfer into the patients (Figure 3). The percent of activated transduced CD4⁺ and CD8⁺ T cells is higher than endogenous T cells at almost all points and increases after day one in each patient, suggesting transduced cells were activated in all patients. Comparing patients, Patient 2 had higher percentages of activated transduced CD4⁺ and CD8⁺ T cells than Patient 1 or 3. Thus, while TCR-transduced T cells were somewhat

activated in all patients, the clinical responder (Patient 2) had the highest frequency of activated transduced CD4⁺ T and CD8⁺ T cells in the blood.

PD-1 blockade did not precede any increase in activation markers in Patient 2. In Patient 3, PD-1 blockade preceded a substantial increase (from 15.6 to 37.1% in CD4⁺ cells and 8.2 to 23.1% in CD8⁺ cells) in expression of activation markers in transduced T cells and a slight increase in untransduced T cells. During high-dose IL-2 therapy in Patient 2, activation markers fluctuate on transduced CD4⁺ and CD8⁺ T cells. In these patients, therefore, treatment with immunotherapies after progression coincides with changes in activation but further work is necessary to determine whether and how immunotherapies influence transduced T cell activation.

Inhibitory Receptors (PD-1, TIM-3 and CTLA-4)

In melanoma patients, tumor reactive T cells have been found to up-regulate inhibitory receptors, including PD-1, CTLA-4, and TIM-3, and to be functionally inhibited by interaction of these receptors with their cognate ligands [36–39]. PD-1 and CTLA-4 blockade are promising FDA-approved immunotherapies [40–46] and TIM-3 antibody-mediated blockade is currently in Phase I clinical trials (NCT02817633, NCT02608268) for the treatment of metastatic melanoma. We analyzed PD-1 and TIM-3 co-expression on transduced T cells in the patients over time. At most time-points in all three patients, less than 20% of transduced CD4⁺ and CD8⁺ T cells co-express PD-1 and TIM-3, suggesting that there was not overwhelming exhaustion of transduced T cells in the blood (Figure 4) although tumor-infiltrating transduced T cells might be substantially more exhausted. Patients 1 and 3 did not have consistently higher percentages of CD4⁺ or CD8⁺ CD34⁺ T cells coexpressing PD-1 and TIM-3 than Patient 2. Patient 2 had higher percentages of transduced CD4⁺ and CD8⁺ cells expressing PD-1 than Patients 1 or 3 (Supplementary Figure 6), perhaps because PD-1 is upregulated on activated T cells. These results suggest that absent or reduced anti-tumor responses in Patient 1 and 3 cannot be attributed to higher levels of exhaustion in transduced cells in the blood.

Treatment with PD-1-blockade did not precede substantial or consistent increases in the percentage of transduced cells expressing PD-1 or co-expressing PD-1 and TIM-3 in Patient 2 or 3, suggesting that PD-1-blockade did not enhance the survival or proliferation of PD-1-expressing transduced T cells in the blood in these patients. High-dose IL-2 therapy preceded a substantial transient increase in the percentage of transduced CD4⁺ T cells co-expressing PD-1 and TIM-3 and an increase in the percentage of PD-1-expressing transduced CD8⁺ T cells. It is possible that in Patient 2, high-dose IL-2 augmented activation and tumor-trafficking of TCR-transduced T cells, thereby increasing PD-1 and TIM-3 expression and mediating some or all of the tumor regression seen after high-dose IL-2. This does not exclude the possibility that endogenous tumor-specific T cells may have been activated and responding as well.

CTLA-4 is an inhibitory receptor upregulated after activation and expressed on tumor-reactive T cells [36, 47]; CTLA-4-blockade has been found to enhance anti-tumor T cell responses [42–46]. In all patients, the percentage of CTLA-4⁺ cells in both transduced and endogenous CD8⁺ and CD4⁺ T cells increased transiently during low-dose IL-2 treatment.

Patient 1 had the highest peak percentage of transduced CD8⁺ T cells expressing CTLA-4, reaching 99% by day 7 (Supplementary Figure 7). Patient 2 had the highest peak percentage of transduced CD4⁺ T cells expressing CTLA-4, reaching 44.2% vs. 18.5% and 25.7% in Patients 1 and 3, respectively. The absence of a cytolytic tumor response in Patient 1 might be related to the very high percentage of CTLA-4-expressing transduced CD8⁺ T cells in this patient's blood immediately post-transfer.

Following CTLA-4 expression after further clinical intervention, we found that PD-1 blockade preceded an increase in CTLA-4-expressing transduced CD4⁺ but not CD8⁺ T cells in both Patient 2 (from 11.3 to 28.6%) and Patient 3 (from 11.9 to 25.7%). High-dose IL-2 therapy did not precede consistent changes in CTLA-4 expression on CD4⁺ or CD8⁺ T cells. Therefore, in Patient 1, PD-1 blockade preceded greater expression of CTLA-4 but not PD-1, while in Patient 2, high-dose IL-2 may have preceded more expression of PD-1 but not CTLA-4 on TCR-transduced T cells.

CD25 and CTLA-4 may be co-expressed on regulatory CD4⁺ T cells (T_{reg}) [48]. We assessed the percentages of CD25⁺CTLA-4⁺ transduced CD4⁺ cells in all three patients, and found that Patient 2 had the highest percentage, reaching 41.9%, versus 16.7% and 21.7% in Patients 1 and 3 (Supplementary Figure 8). Differences in the percentages of potential T_{reg} in the blood do not explain the absence of a clinical or biological response in Patient 1 or clinical response in Patient 3.

Costimulation (OX40)

OX40 is upregulated after activation in both CD4⁺ and CD8⁺ T cells and enhances T cell proliferation, survival and development of memory [49, 50]. Tumor-antigen-specific T cells may express OX40 and OX40 costimulation augments anti-tumor responses [51, 52]. Patients expressing OX40 on a higher percentage of their TIL have a better prognostic outcome [53] so we examined OX40 expression on transduced T cells. Patient 2 had fewer OX40-expressing transduced CD8⁺ T cells at most timepoints (Supplementary Figure 9). However, Patient 2 had the highest peak percentage of transduced CD4⁺ T cells expressing OX40 (81% in Patient 2 vs. 51.6% and 61.2% in Patients 1 and 3). After PD-1 blockade in Patients 2 and 3, there was an increase in the percent of transduced CD4⁺ T cells expressing OX40, although neither patient had tumor regression. Overall, OX40 expression on transduced CD4⁺ or CD8⁺ T cells in the blood does not clearly correlate with clinical responses and future studies with more patients are needed to determine whether PD-1 blockade regulates OX40 on tumor-reactive CD4⁺ T cells.

Memory Differentiation (CCR7)

To develop long-term protection from tumor recurrence, transduced T cells must differentiate into memory T cells. CCR7 is a chemokine receptor regulating entry into lymphoid structures which identifies central memory T cells [54]. We examined CCR7 expression on transduced vs. endogenous CD4⁺ and CD8⁺ T cells in all three patients. Prior to transfer, a fraction of transduced CD4⁺ and CD8⁺ T cells expressed CCR7. As expected by the accumulation of memory cells, the percentage of transduced and endogenous cells expressing CCR7 increased over time in all patients (Supplementary Figure 10). Patient 2

had a higher mean percentage of CCR7-expressing transduced CD4⁺ and CD8⁺ cells than Patients 3 and 1. Collectively, these results indicate that a substantial fraction (over 50%) of transduced CD4⁺ and CD8⁺ cells eventually expressed memory markers in the blood in Patient 2.

TCR affinity

All three patients received autologous T cells transduced with the same high-affinity (CD8-independent) TIL1383I TCR. The solution (3D) affinity of the TIL1383I TCR was measured at 9 μ M, consistent with a high affinity TCR/pMHC interaction (Figure 5a). However, TCR affinities measured in solution do not account for all of the interactions that lead to T cell activation and function, and accordingly do not always correlate with functional outcomes [55]. Therefore, we performed 2D affinity measurements on the TIL1383I TCR-transduced T cells delivered to the patients to determine whether an assay that measures TCR/pMHC affinity in the context of other cell surface interactions correlates with clinical outcome. Interestingly, the 2D affinity was notably lower in cells from Patient 1 (nonresponder) than Patients 2 (partial responder) or 3 (vitiligo), as was adhesion frequency (Figure 5b, c). Although based on just three patients, these results suggest that 2D affinity and adhesion frequency should be explored as markers for predicting clinical outcomes.

Discussion

While melanoma-specific T cells are present in most patients [56], self-reactive T cells have low affinity for target antigens [57, 58] and tumor-reactive T cells may have poor viability and function after sustained growth in the immunosuppressive tumor microenvironment [58, 59]. Specialized protocols that expand tumor-infiltrating cells with antigen and IL-2 *ex vivo* have had some success inducing complete remission in some patients but these protocols depend on expanding a small number of cells substantially to generate sufficient cells to treat the tumor—a process that requires an accessible tumor with tumor-infiltrating lymphocytes as well as successful expansion of these TIL—which is not always feasible [60, 61]. In this clinical trial, we are utilizing a viral vector to introduce a high-affinity antigen-specific TCR into metastatic melanoma patients' CD4⁺ and CD8⁺ T cells to redirect them to attack the melanoma. Following the fate of the TCR-transduced T cells after transfer, we found that, in these three patients, TCR-transduced T cells had a phenotype distinct from endogenous CD34⁺ T cells, including higher expression of both activation and inhibition-related receptors, reminiscent of tumor-reactive T cells visualized by tetramer staining. Certain characteristics of the T cell response were distinct in Patient 2, who had a clinical response, compared to nonresponding Patient 1 and Patient 3, who developed vitiligo. In Patient 2, there were substantially more transduced CD8⁺ T cells present at later time points, with a greater expansion of transduced CD8⁺ and CD4⁺ T cells in the blood. This was associated with higher expression of activation markers and of activation-associated inhibitory receptor PD-1 on transduced CD4⁺ and CD8⁺ T cells. Collectively these results indicate that, in these three patients, there were higher numbers of TCR-transduced T cells that expressed more activation markers in a clinical responder. The mechanisms behind increased activation of the transduced T cells are more elusive, and further elucidation of

mechanisms that enhance T cell anti-tumor efficacy *in vivo* would greatly help develop more effective strategies to target melanoma.

The affinity measurements gave some indication about underlying cellular differences that might have led to a better response in Patient 2. Despite being treated with T cells expressing the same TCR, different 2D affinity measurements on the final product suggested that transduced T cells given to Patient 2 might have had higher binding affinity. Parameters such as membrane composition, TCR clustering, and cooperative binding will influence 2D but not 3D TCR/pMHC affinity measurements. While 3D TCR/pMHC affinity measurements are the gold standard for selecting TCRs for cell therapy, preliminary results from our three patients indicate that 2D affinity measurements might be predictive of the potency of T cell products for patient treatment. Further experiments are necessary to see if this observation is repeatable in a larger cohort of patients.

In the field of immunotherapy of cancer, there has been a great deal of research about generating the most effective T cell response for treatment of patients. However, clinical responses depend on many patient and tumor-specific factors such as tumor mutational load [62–66], expression of immune-inhibitory receptors and molecules [67], recruitment of immunosuppressive cells [68–70], loss of antigen or antigen expression [71–73], and total tumor burden [74, 75]. In this study, Patient 1 had the highest tumor burden and had no response to the therapy, while Patients 2 and 3 with lower tumor burdens had a clinical and a biological response respectively.

Novel immunotherapies, most notably checkpoint blockades such as PD-1 blockade or CTLA-4 blockade, offer promises of helping suppress inhibitory mechanisms that may vary between patients. A question that has come up is whether combination of these therapies with T cell adoptive transfer would be beneficial. Given the expression levels of PD-1 on adoptively transferred T cells in the blood, it appeared that PD-1 blockade should have enhanced the T cell responses in Patient 2, but it had no effect on tumor burden or T cell activation. Conversely, PD-1 blockade in Patient 3 immediately preceded an increase in the activation and number of adoptively transferred CD4⁺ T cells, in spite of the observation that only a small fraction of adoptively transferred T cells in Patient 3 express PD-1. It is possible that PD-1-expressing T cells in Patient 3 are suppressed by high expression of PD-L1 on the tumor but the tumor was not tested for PD-L1 expression. While PD-1 blockade had no effect on Patient 2, a second immunotherapy, high dose IL-2, immediately preceded a lasting remission. It is not possible to dissect what part of the remission and autoimmune response seen in Patient 2 is due to transduced T cells, PD-1 blockade, high-dose IL-2, or the interactions of these three therapies. Notably, while PD-1 blockade had neither a clinical effect nor an effect on transduced T cell numbers or phenotype in Patient 2, high-dose IL-2 therapy preceded both an increase in the number of TCR-transduced T cells in Patient 2 and the progression of vitiligo associated with the presence of transduced T cells in the skin. Therefore, in one of two patients given further immunotherapies after progressing on TCR-transduced T cells, the immunotherapies led to tumor regression. Further experiments will help find biomarkers identifying which patients will benefit from immunotherapies after transduced T cell delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
CCR7	C-C motif chemokine receptor 7
CD34t	Truncated CD34
FDA	U.S. Food and Drug Administration
pMHC	Peptide-loaded MHC complexes, in this study, tyrosinase peptide-loaded HLA-A2 MHC molecules
rhIL-2	Recombinant human IL-2
rhIL-15	Recombinant human IL-15
TIM-3	T cell immunoglobulin and mucin-domain containing-3
T_{reg}	Regulatory CD4 ⁺ T cells

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Fig. 1.

Clinical responses of patients

a. Lesions in Patient 2 before and after T cell transfer. (Top) Tumor in the right axial lymph node. (Bottom) Tumor in the right apical lung nodule. **b.** Vitiligo in Patient 3, with boundaries approximately shown by white outline. **c.** Transduced T cells in the margins of the vitiligo in Patients 2 and 3. (i) vitiligo in Patient 2 with location of punch biopsy (arrow); (ii) vitiligo in Patient 3 with locations of first punch biopsy (1, taken at 3 weeks post-transfer, had neither melanocytes nor T cells present) and second punch biopsy (2, taken at 4 weeks post-transfer); (iii–viii): Second punch biopsy from Patient 3, scale bars are 10µm (iii) Remaining TRP-1⁺ melanocytes (black arrows, blue staining) in second biopsy taken from patient 3; (iv) CD3 expression in violet; (v) Vβ12 expression in red; (vi) CD34 expression in green; (vii) DAPI nuclear staining in blue (E = epidermis, D = dermis); (viii) Colocalized CD3⁺Vβ12⁺CD34⁺ staining after DAPI; (ix) Percent of CD3⁺Vβ12⁺ cells (grey bar), percent of CD3⁺CD34⁺ cells (black bar), and percent of CD3⁺Vβ12⁺CD34⁺ cells (white, striped bar) in biopsy from patient 2 and biopsy 2 from patient 3.

Fig. 2.

Transduced T cell percentages and numbers in PBL samples

Patient blood samples were collected at indicated time-points post-infusion. PBMC were isolated from these blood samples by density gradient centrifugation and cryopreserved for later batch analysis. At least a year after each patient was treated, or after the patient passed away, the collection of samples for that patient was thawed and collectively analyzed by flow cytometry. All patients were treated with low-dose IL-2 (72,000IU/kg, i.v., three times daily) for seven days after T cell infusion (green line). Two patients went on to receive pembrolizumab, each course (2mg/kg, given i.v. every 3 weeks) indicated by closed purple arrows, and one patient further received high-dose IL-2, with each course (600,000 IU/m², given i.v. every 8 hours for a maximum of 14 doses on days 284, 298, and 368 post-T cell infusion) indicated by open green arrows. **a.** The percent transduced (CD34⁺, green

triangles), transduced CD4⁺ (CD34t⁺CD4⁺, blue diamonds) and transduced CD8⁺ (CD34t⁺CD8⁺, red squares) of CD3⁺ T cells in patient blood samples drawn at indicated times post-T-cell transfer. **b.** The number of CD34t⁺CD3⁺ (green triangles), CD34t⁺CD3⁺CD4⁺ (blue diamonds), and CD34t⁺CD3⁺CD8⁺ (red squares) T cells per milliliter patient sample blood at indicated times post-transfer. Bar graphs show the average percentages of indicated cells across all time points with error bars representing the standard error.

Fig. 3.

Expression of activation markers, CD25 and CD69, in transduced and endogenous T cells. Patients were treated and samples collected and stained as described in Figures 1 and 2. Cells were analyzed by flow cytometry to identify the percentage of transduced (CD34t⁺) and endogenous (CD34t⁻) CD4⁺ and CD8⁺ T cells co-expressing CD25 and CD69. **a.** The percent CD25⁺CD69⁺ of transduced (CD34t⁺, solid red line) vs. endogenous (CD34t⁻, dotted black line) CD8⁺CD3⁺ T cells. **b.** The percent CD25⁺CD69⁺ of transduced (CD34t⁺, solid blue line) vs. endogenous (CD34t⁻, dotted black line) CD4⁺CD3⁺ T cells. Bar graphs show the average percentages of indicated cells across all time points with error bars representing the standard error.

Fig. 4.

Transduced and endogenous T cell expression of exhaustion-related receptors PD-1 and TIM-3 post-transfer

Patients were treated and blood collected as described in Figures 1 and 2. Cells were analyzed by flow cytometry to identify the percentage of transduced (CD34t⁺) and endogenous (CD34t⁻) CD4⁺ and CD8⁺ T cells co-expressing PD-1 and TIM-3. **a.** The percent PD-1⁺TIM-3⁺ of transduced (CD34t⁺, solid red line) vs. endogenous (CD34t⁻, dotted black line) CD8⁺CD3⁺ T cells. **b.** The percent PD-1⁺TIM-3⁺ of transduced (CD34t⁺, solid blue line) vs. endogenous (CD34t⁻, dotted black line) CD4⁺CD3⁺ T cells. Bar graphs show the average percentages of indicated cells across all time points with error bars representing the standard error.

Fig. 5.**Affinity measurements**

a. Solution (3D) affinity measurements were performed by surface plasmon resonance, analyzing the binding of soluble TIL1383I-TCR to surface-bound HLA-A2-tyrosinase at 25°C using a Biacore T200 instrument. The data were fit to a 1:1 binding model using Biaevaluation 4.1. RU: Response Units **b.** Adhesion frequencies for transduced T cells were determined using a two-dimensional micropipette adhesion frequency assay with transduced patient T cells from each patient and tyrosinase-peptide-HLA-A2-coated RBCs. Shown are the frequencies of binding events in each of the 21 T cell-pMHC-RBC pairs that were tested. **c.** Relative 2D affinities were calculated using the specific adhesion frequency (P_a) along with the surface pMHC (m_i) and TCR β (m_r) densities, as determined by flow cytometry. For each patient, 21 T cell-RBC pairs were used to obtain P_a values from which the affinity was calculated as: $A_c K_a = -\ln [1 - P_a(\infty)] / m_r m_i$. Statistics shown were done by Student's t-test. * $p < 0.05$

Table 1**Patient and Product Descriptions**

	Patient 1	Patient 2	Patient 3
Age (years)	56	41	66
Gender	Male	Male	Male
Stage of melanoma at diagnosis ^a	IIIA (T2aN2aM0)	Not Determined ^b (TxN2bMx)	IIIB (T1aN2bM0)
Prior therapies received	High-dose IFN- α , High-dose IL-2, Ipilimumab, Palliative intensity-modulated radiation therapy to right upper lobe of lung, Paclitaxel/Carboplatin	Ipilimumab	High-dose Vemurafenib, IL-2, Ipilimumab, Dabrafenib
Tissue involvement	LNs, Right Upper Lobe Lung, Colonic Mass	LNs, Subcutaneous tissue	LNs, Right Adrenal
Number of transduced (and total) T cells given	2.00×10^8 (2.27×10^8)	2.72×10^8 (2.87×10^8)	2.06×10^8 (2.22×10^8)
Transduced T cells/kg	1.67×10^6	2.5×10^6	2.5×10^6
Final product percent CD34 ⁺ of CD3 ⁺	88.1	95.8	92.9
Final product percent v β 12 ⁺ of CD34 ⁺	27.7	46.5	43.7
Final product IFN γ : T2+Tyro (T2 alone) [pg/mL]	879 (32)	1492 (449)	2738 (176)
IFN γ : 624-Mel (A2- 624-28) [pg/mL]	356 (10)	494 (18)	908 (77)
Treatments received post-T cell therapy	None	Pembrolizumab High-dose IL-2	Pembrolizumab

^a All patients Stage IV at time of treatment

^b In Patient 2, the primary site of melanoma was not identified